Transport mechanisms of D-amino acids and their physiological implications 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

Claudia Hener

aus Tettnang

Tübingen

2020

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

Tag der mündlichen Qualifikation:	29.05.2020
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Klaus Harter
2. Berichterstatter:	Prof. Dr. Uwe Ludewig

Zusammenfassung

Die meisten proteinogenen L-Aminosäuren (L-AS) haben Enantiomere, die D-Aminosäuren (D-AS) genannt werden. Diese unterscheiden sich nicht nur in ihrer molekularen räumlichen Anordnung, sondern auch in ihrer biologischen Funktion. Da D-AS Bausteine der bakteriellen Zellwand sind, sind sie allgegenwärtig und kommen besonders häufig im Boden vor. Pflanzen sind in der Lage D-AS aktiv über ihre Wurzeln aufzunehmen. In mehreren Studien wurde die Toxizität von D-AS für Pflanzen gezeigt. Eine Erklärung für die Toxizität und den Umgang von Pflanzen mit D-AS ist jedoch noch nicht bekannt.

Im Zuge dieser Arbeit stellte sich heraus, dass die Wachstumshemmung von *Arabidopsis thaliana* Keimlingen, die mit D-Met behandelt werden, mit einer erhöhten Ethylen-Produktion verbunden ist. Der Zusammenhang von D-Met und Ethylen wurde bereits gezeigt, da D-Met ein ACC (1-Aminocyclopropan-1-Carbonsäure) metabolisierendes (Malonylat-konjugierendes) Enzym hemmt und dadurch die Ethylen-Produktion erhöht wird. Zusätzlich konnten wir jedoch den Zusammenhang von der Ethylen-Zunahme und der Aktivität der D-Aminosäure-Transferase (DAT1) herstellen. Die resultierenden Hauptprodukte der DAT1 sind D-Ala und D-Glu, wobei die Enzymaktivität mit D-Met als Substrat am höchsten ist. Somit trägt das DAT1 Protein maßgeblich zur Reduktion der D-Met-konzentration in den Pflanzen bei. Infolgedessen war beim Col-0-Wildtyp die Ethylen- und Malonyl-Methionin-Produktion und damit die Wachstumsreduktion nur in geringem Maße erhöht. Im Vergleich dazu wurden die DAT1-Funktionsverlustmutanten und der Landsberg erecta Öko-Typ, der natürliche DAT1 Funktionsdefizite besitzt, bei einer Behandlung mit 0,5 mM D-Met stark im Wachstum beeinträchtigt.

Darüber hinaus konnten wir nachweisen, dass eine passive Exsudation von D-AS erfolgt, die in Menge und Zeit vergleichbar mit den L-AS auftritt. Die Umwandlung von D-AS durch Pflanzen in D-Ala und D-Glu, die für bakterielles Peptidoglykan essentiell sind, könnte in Verbindung mit deren Exsudation nützliche Bakterien anziehen. Es wurde auch berichtet, dass bakterielle Biofilme auf Pflanzenwurzeln durch D-AS reguliert werden. Daher wäre ein Einfluss auf die Bakterien-Vergesellschaftung durch D-AS vorstellbar, die von der Pflanze freigesetzt oder kontrolliert wird. Der Mechanismus der Efflux-Regulierung ist jedoch unklar.

Die hier vorgestellte Studie zeigt die zentrale Rolle des Enzyms AtDAT1 im D-AS-Stoffwechsel. Darüber hinaus zeigt sie die Beziehung zwischen diesem Stoffwechselweg und der Wachstumsreduktion der Pflanze aufgrund der erhöhten Ethylen-Synthese. Ein weiteres Ergebnis ist die passive Exsudation von D-AS durch Pflanzenwurzeln, was eine Strategie zur Bewältigung des D-AS-Überschusses in der Pflanzenzelle ist und eine mutmaßliche Pflanze-Mikroben-Interaktion nahelegt.

Summary

Almost all proteinogenic L-amino acids (L-AA) have enantiomers called D-amino acids (D-AA) which do not differ just in their molecular spatial arrangement but also in their biological function. Since D-AA are part of the bacterial cell wall, they can be found in all kinds of tissues and materials, which are associated with bacteria. This is the reason why D-AA are found in soil and plants are able to actively take up D-AA by their roots. There have been several studies stated D-AA toxicity for plants. However, an explanation for the toxicity and plants handling with ubiquitous D-AA are still unknown.

Here we show that growth inhibition of *Arabidopsis thaliana* seedlings treated with D-Met is connected to increased ethylene production. This ethylene related phenomenon has been described before as it was shown that D-Met interferes with ACC (1-aminocyclopropane-1-carboxylic acid) metabolizing (malonyl conjugating) enzymes resulting in an increased ethylene production. But additionally, we could establish the link between the ethylene magnitude and the activity of the main D-AA metabolizing enzyme the D-amino acid transferase (DAT1). We could reveal that the DAT1 protein has a great impact on the plants D-Met concentration since it transforms all tested D-AA, but preferably D-Met, directly into D-Ala and D-Glu and into their respective L-enantiomer indirectly. As a result, in the Col-0 wild-type the increased ethylene and malonyl-methionine production and thus growth reduction was almost negligible. By comparison, the dat1 loss of function mutants and the DAT1 defective Arabidopsis ecotype Landsberg erecta were severely affected upon 0.5 mM D-Met treatment.

In addition, we could proof that exudation of D-AA occurs passively and comparable to their L-forms in amount and time. The rhizosphere is harbouring a great number of beneficial as well as detrimental bacteria. Plants transformation of D-AA into D-Ala and D-Glu, which are essential for bacterial peptidoglycan combined with their exudation could attract beneficial bacteria. On the other hand, biofilms on plant roots have also been reported to be regulated by D-AA. Thus, an influence on the bacterial rhizosphere community by D-AA released or controlled by the plant can be envisioned. However, the efflux regulation mechanism remains unclear.

The here presented study displays the central role of the DAT1 enzyme in D-AA metabolism. Moreover, it reveals the relationship of this metabolic pathway and plants reduction in growth due to increased ethylene synthesis. Another outcome is the passive exudation of D-AA through plant roots suggesting a strategy to cope with D-AA overflow and a putative plant-microbe interaction.

Table of Contents

List of Abbreviations

D-AA	D-amino acids
L-AA	L-amino acids
DAT1	D-amino transferase 1
DAO	D-amino acid oxidase
LHT1	Lysine histidine transporter 1
ABC	ATP binding cassette
At	*Arabidopsis thaliana*
Col-0	Columbia 0 (Arabidopsis accession)
Ler	Landsberg erecta (Arabidopsis accession)
PG	Peptidoglycan
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazine
OV	Na-Orthovanadate
ATP	Adenosine triphosphate
SAM	S-Ado Met / S-adenosylmethionine
SAMS	SAM-synthetase
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC-synthase
MTA	5′-methylthioadenosine
NMT	N-malonyl transferase
MACC	Malonyl-ACC
ACO	ACC oxidase
JAR1	Jasmonic acid resistance 1
JACC	Jasmonyl-ACC
GGT	γ-glutamyl transpeptidase
GACC	γ-glutamyl-ACC
ACCD	ACC deaminase
LC/MS	Liquid chromatography–mass spectrometry
HPLC	High-performance liquid chromatography
GC	Gas chromatography

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Gln	Glutamine
Glu	Glutamic acid
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

1. Introduction

1.1 L-and D-amino acids

Amino acids are low molecular weight, non-volatile, crystalline organic compounds. All proteinogenic α-amino acids, except glycine, contain a chiral center (*) surrounded by a carboxyl group (-COOH), an amino group (-NH_2), and a side chain (-R). Glycine has two (indistinguishable) hydrogen atoms attached to its alpha carbon atom and thus is achiral. There are two stereoisomers of each amino acid naturally occurring, which resemble the mirror image of one another, called enantiomers. They do not differ in their molecular weight or formula, but in their three-dimensional structure (see Fig. 1). The relative configuration of enantiomers is referred to the symbols D and L, which implies the ability to rotate plane-polarized light either to the right "dexter" D, or to the left "laevis" L (Sharma 2008).

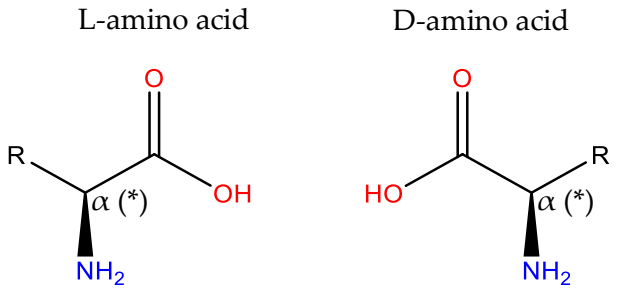


Figure 1: Structures of L- and D-amino acids, which are superimposable on each other.

The enantiomers are not only distinguished by their structure, but also by their biological activity. The 20 canonical L-α-amino acids (L-AA) are metabolic intermediates and building blocks of proteins and are therefore indispensable for all kingdoms of life. On the contrary, D-amino acids (D-AA) have different functions like the cross linking of peptidoglycan in bacteria (Rogers, Perkins, and Ward 1980), as neuromodulators in mammals (D-Ser) (Miller 2004) or as part of natural antibiotic peptides (Jack and Jung 1998). Interestingly, there is not much known about D-AA in plants and their functional and ecological significance is the focus of my research.

1.2 Occurrence of D-amino acids in plants and soil

D-AA were found in different parts of various plant species, where they were detected in abundances of up to 34% relative to the respective L-AA, amongst which D-Ala was the most frequent one (Pätzold and Brückner 2005).

The major source of D-AA for plants is certainly soil. The occurrence of D-AA is mostly related to the presence of bacteria in which the most abundant D-AA are D-alanine and D-glutamic acid. Since bacteria are one of the main clades of soil-dwelling organisms, a huge D-AA abundance is present there. Their D-AA incorporation into their cell wall is advantageous for several reasons: D-AA peptide bindings are almost not susceptible to enzymatic cleavage of certain proteases (Miller et al. 1995). Furthermore, some bacteria integrate, besides D-Ala and D-Glu, other D-AA like D-Ser to gain resistance to certain antibiotics like vancomycin (Reynolds and Courvalin 2005). Certain bacteria produce D-Met, D-Leu or D-Tyr and D-Phe at millimolar concentrations to modulate their peptidoglycan compositions, amounts and strengths (Lam et al. 2009). Not only the cell wall physiology of bacteria is affected by different D-AA, but biofilm development is considerably influenced, too (Kolodkin-Gal et al. 2010). This results in dispersal of D-Met, D-Tyr, D-Leu, and D-Trp in significant amounts.

Therefore, it is not surprising that D-AA are found ubiquitously and to a great extend in bacterial rich environments like soils (Radkov et al. 2016). D-AA are, like their L-form, present in the rhizosphere, which is the region of soil directly surrounding the plant roots. Due to lysis or cellular efflux from plants and microbes and proteolysis of existing peptides this region is particularly substantial for D-AA research. Generally, there are less D-AA in soils compared to their enantiomers. Soils from different geographic regions were studied, which revealed a D/L-ratio of an average of about 10% for alanine and slightly more for glutamic acid (Amelung and Zhang 2001). Wichern et al. (2004) could show an increase in the D/L-ratios by the fallow age of abandoned terrace soils. Another study showed a D/L ratio of 15.5% for alanine and 7,1% for glutamic acid (Brückner and Westhauser 2003). Results for aqueous soil extractions were found at low micromolar concentrations (Jones, Owen, and Farrar 2002) for all amino acids in sum. Among those, the most frequent amino acids were alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, histidine and serine (Werdin-Pfisterer, Kielland, and Boone 2009). However, there is a huge variation in composition of amino acids within the soil types, which differed in land-use history, soil qualities, and climatic regime. (Amelung and Zhang 2001). However, one big problem in amino acid research in soil is, that amino acids are not always detected separately due to their stereoisomeric properties (for review see (Moe 2013)).

1.3 Methods for D-amino acid analysis

A major prerequisite for studies on D-AA in plants is its qualitative and quantitative analysis from plant material. There are two main hurdles for this task: extraction and chemical analysis of D-AA. The extraction procedure needs to be performed on ice to avoid enzymatic activities in the plant samples. These cold conditions lead to a lower solubility of the amino acids. In contrast, extractions at high temperatures are unsuitable and could lead to a loss or damage of compounds or even to chemical racemisations, which are explained later on.

Another challenge involves plants, which are grown in soil. Since there are endophytic and epiphytic bacteria present (Bacon and White 2016), it is impossible to distinguish between D-AA from plants and D-AA produced by bacteria associated with plants. Therefore, most of our experiments were based on sterile growing plants to avoid this problem.

Not only the extraction techniques are challenging, but also the methods for chemical analysis of the samples are quite specific. First of all, the enantiomers of the amino acids have to be separated. As mentioned before, enantiomers have the same molecular weight, the same chemical formula and the same polarity. This is one challenging aspect in the separation of chiral molecules. Moreover, L-AA are found in high levels in biological tissues, while their D-enantiomers are usually only present in lower concentrations (Szökő, Vincze, and Tábi 2016). Furthermore, most amino acids are rather hydrophilic and lack a chromophore or fluorophore moiety. To overcome these challenges, either special chromatographic columns are applied (direct chiral GC/HPLC analysis) or more commonly used, the amino acid solution is derivatized before analysis. Derivatizing is considered as an indirect chiral approach for the chromatographic separation of enantiomers (Bhushan and Brückner 2004). This means, the enantiomers are reacting with a chiral reagent prior to the chromatographic analysis, forming diastereomers, which can be separated by chromatography due to different retention times. There are different ways to perform this procedure (for review see Ilisz et al. (2008)). Here, we decided to use a variant of Marfey´s reagent (N^{α}-(2, 4-dinitro-5-fluorophenyl)-L-valine amide) like it was described by Gördes, Kolukisaoglu, and Thurow (2011). They replaced the L-alanine amide (Brückner and Keller-Hoehl 1990) by L-valine amide in the synthesis of Marfey´s reagent for derivatization, followed by LC/MS analysis. With this method, they were able to quantify 16 of the 20 canonical amino acids including their enantiomers in Arabidopsis seedlings in a single analysis.

1.4 Import and export of D-amino acids by plant roots

Since plants grow in a bacterial rich environment, their ability to regulate the uptake of D-AA by their roots was subject of different studies (Svennerstam et al. 2007; Forsum et al. 2008). In these studies, the Lysin Histidine Transporter 1 (AtLHT1) for facilitated root amino acid uptake got into focus. Gördes, Kolukisaoglu, and Thurow (2011) tested the uptake of different D-AA in a *lht1* knock out mutant of Arabidopsis. Seedlings of this mutant revealed to be more tolerant against high concentrations of several D-AA through a decreased transport activity. They already hypothesized that the active import of D-AA is needed to utilize these amino acids as a nitrogen source. Shortly thereafter, it has been shown that wheat plants actually use D-AA as a nitrogen source and therefore take up D-AA actively at rates comparable to L-AA and inorganic N (Hill et al. 2011).

While LHT1 belongs to the ATF (amino acid transport family) superfamily of amino acid transporters, there are several other amino acid transporter families present in plants (Ortiz-Lopez, Chang, and Bush 2000; Fischer et al. 1998). Membrane transport systems can be categorized as pumps, channels, or cotransporters (Buchanan, Gruissem, and Jones 2015). Active transport is facilitated by certain membrane-spanning proteins and requires cellular energy to achieve molecular movement. Pumps catalyse active transport, coupling the energy liberated from hydrolysis of ATP (H^+-translocating ATPases). Carrier proteins facilitate active transport by transporting another molecule for example H^+ in either the same direction (symport) or to the opposite direction (antiport). Besides the active transport, passive transport does not require an input of cellular energy. It occurs spontaneously by diffusing through a decreased concentration gradient until equilibrium is reached. Since the plants cell membranes consist of lipid bilayers, lipophilic compounds are passing more easily. For hydrophilic and ionic compounds, such as amino acids, aqueous channels facilitate their transport. In previous experiments, Arabidopsis mutants lacking LHT1 showed just a decreased import rate of D-AA but not a complete elimination of it (Gördes, Kolukisaoglu, and Thurow 2011). This means that other transporters or transport mechanisms apart from LHT1 contribute to D-AA influx into plants that await to be unravelled.

Since particular D-AA inhibit plant growth, not only the import of D-AA is of great interest. Additionally, the amino acid root efflux seems to play a big role in the amino acid homeostasis of the root (Jones and Darrah 1994). The authors used *Zea mays* (maize) seedlings in a hydroponic environment and measured rates of influx and efflux as well as the effects of various enzyme inhibitors predicted to impact these processes. In conclusion, they argued that amino acid loss from plant cells was due to passive diffusion and the

majority of AA are released by the root tip. Moreover, a study showed an amino acid exporter, which is involved in phloem unloading of amino acids in roots that could play a putative role in D-AA exudation (Besnard et al. 2016). Root efflux thus can be regarded as being selective for different amino acids (Lesuffleur and Cliquet 2010). On the contrary, mere passive diffusion was observed by Jones and Darrah (1994). This strategy can have various reasons like detoxification or attraction of beneficial soil-dwelling microorganisms, but these hypotheses await to be tested.

1.5 Metabolization of D-amino acids

Besides regulating import and export of D-AA, plants can also cope with high D-AA concentrations in other ways, which are summarized in Fig. 2. One major way is the degradation of D-AA, which is not fully understood, yet. However, it is quite remarkable that there are several putatively D-AA metabolizing enzymes encoded in the Arabidopsis genome (for a summary see Kolukisaoglu and Suarez (2017)). One interesting enzyme in this regard, which is well conserved over the plant kingdom, is the D-amino acid transferase 1 (DAT1) (Funakoshi et al. 2008). It catalyses the reaction of a D-AA being deaminated, whereby the amino group is transferred onto either pyruvate or 2-oxoglutarate. The two resulting products are D-alanine and D-glutamic acid, respectively with D-Ala as the main product. Another enzyme that was found in plants and bacteria is the D-amino acid oxidase (DAO). The flavoenzyme catalyses the oxidative deamination of D-AA to the corresponding α-keto acids, ammonia, and hydrogen peroxide (Curti, Ronchi, and Pilone Simonetta 1992). This enzyme is a potential agent to further transform the D-alanine, which is the most widespread D-amino acid of higher plants (Robinson 1976). Another known enzyme for D-alanine turnover is a racemase, which was found as the first amino acid racemase in plants, more precisely in *Medicago sativa* L. seedlings (Ono et al. 2006). Followed by a serine racemase, which was characterized in *Arabidopsis thaliana* (Fujitani et al. 2006). There is currently no evidence for enzymatical racemic activities for the other D-AA, although quite recently there was a new Isoleucine racemase gene family identified (Strauch et al. 2015). Furthermore, the presence of racemases suggests that plants are able to produce D-AA *de novo*.

Besides the enzymatic reactions in plants, there is chemical racemization occurring through Maillard reaction. In this process, D-AA can be generated from their enantiomers and vice-versa. It can occur in tissues like fruits in which both, amino acids and saccharides are present (Pätzold and Brückner 2005). Chemical racemisation can

especially occur under harsh alkaline or acidic treatment, together with increased heat and pressure. The racemization reaction starts with the removal of the proton of the α-carbon to form a planar carbanion. This is followed by the rapid re-addition of a proton from either side (non-stereospecifically) with a 50 % probability of forming either the D- or the L-enantiomer (Neuberger 1948). In nature, this reaction also can occur under normal conditions, but in it needs much more time until a 1:1 racemate will develop (Bada 1971).

There is another strategy for metabolizing D-AA, namely inactivation of D-AA through formation of conjugates (Kawasaki, Ogawa, and Sasaoka 1982; Fukuda et al. 1973). The authors showed two systems of D-AA conjugate forming in pea seedlings (*Pisum sativum* L.): first the N-malonylation of common D-AA; second the γ-L-glutamylation, which was highly specific and active for D-alanine. Another example revealed the presence of D-Ala-D-Ala in tobacco leaves (Noma, Noguchi, and Tamaki 1973) as well as in grass leaves (Frahn and Illman 1975). The N-malonylation as a main metabolic pathway besides the N-acetylation for D-methionine was already described before they actually knew the involved enzyme catalysing the reaction (Pokorny, Marčenko, and Keglević 1970).

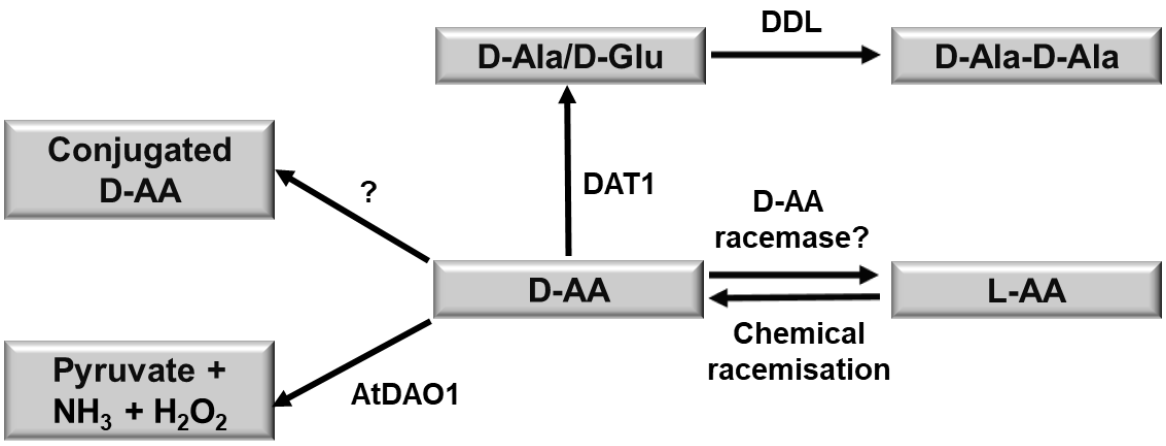


Figure 2: Model of D-AA fate and possible metabolization pathways in plants

1.6 Physiological effects of D-amino acids

The functions of D-AA in plants are currently largely unexplored. In the past, D-AA were only regarded as toxic agents since they were observed to inhibit seedling growth. Bollard (1966) investigated amino acids and derivatives in their function as nitrogen source in duckweed (*Spirodela polyrhiza*) and found that all plants, which were grown on selected D-AA showed reduced growth capability compared to low nitrogen media. However, also some L-AA were responsible for growth inhibition. A more recent study tested Arabidopsis growth on D-Ala, D-Ser, D-Arg, D-Val and D-Ile in 3 mM concentrations.

They found, unlike L-AA, all tested D-AA having an inhibitory effect, except for D-Ile, which was comparable to the control. At the same time they investigated the uptake rate of those compounds, which revealed that those being less toxic are taken up in lower quantity (Forsum et al. 2008). As a next step Gördes, Kolukisaoglu, and Thurow (2011) explored the maximum inhibitory concentrations of 17 D-AA in *Arabidopsis thaliana*. They concluded that some D-AA such as D-Ala, D-Tyr or D-Ser inhibit seedling growth in micromolar concentrations while others, like D-Asn or D-Gln have such an effect upon 10 mM treatment.

Besides the growth inhibitory impact, there are other studies reflecting growth enhancing effects by adding D-AA. This was shown for D-tryptophan, since it is a precursor of the plant growth hormone indol-3-acetic acid it was found to act growth promoting in *Pisum sativum* L. (Law 1987). Furthermore, it was shown for wheat (*Triticum aestivum* L.) that it is able to utilise D-Ala nitrogen at rates comparable to L-AA and inorganic nitrogen (Hill et al. 2011). Besides, Hirano et al. (2016) revealed D-Ala to be built into moss (*Physcomitrella patens*) plastids since they possess a peptidoglycan envelope. In Arabidopsis and tobacco (*Nicotiana benthamiana*), D-serine was found to play a central role in pollen tube growth and morphogenesis through modulation of apical $[Ca^{2+}]_{cyt}$ gradients (Michard et al. 2011).

As also described in the previous chapter, D-AA can be conjugated *via* N-malonylation to make them compatible for vacuolar storage (Bouzayen et al. 1989). The preferable targets being malonylated are D-phenylalanine, D-valine, D-leucine, D-threonine, D-methionine and D-eithionine, with D-Met and D-Phe inducing the highest activity, while the L-isomers are not forming such N-malonyl conjugates (Satoh and Esashi 1980). However, this conjugation seems to be catalysed by the ACC N-malonyltransferase. This means in effect that the reaction entails a change in ethylene synthesis. The mechanism can be explained as followed (Fig 3): ethylene is a volatile plant hormone that is regulating plant growth, defence, and development in many regards. The starting material is L-methionine, which is converted into S-adenosylmethionine (SAM). 1-aminocyclopropane-1-carboxylic acid (ACC) is one reaction product of SAM. The backbone as second reaction product is recycled again. Ethylene is then finally produced by oxidizing the ACC (Adams and Yang 1979).

Since ethylene is involved in seed germination, seedling growth, organ development and senescence, leaf and petal abscission, fruit ripening, stress and pathogen responses, the significance of the ACC regulatory mechanism is obvious (Abeles, Morgan, and Saltveit Jr 2012). To avoid overproduction of ethylene, there is a regulatory aspect at the end of this reaction chain, in which ACC can additionally be inactivated by conjugation instead of

being oxidised to form ethylene. Most interestingly, D-AA such as D-Met are involved in this process, because they compete with the ACC for the N-malonyltransferase (Liu, Hoffman, and Yang 1983; Ling-Yuan, Yu, and Shang 1985). Besides the N-malonyltransferase, there are more enzymes to regulate the ACC content (Le Deunff and Lecourt 2016; Van de Poel and Van Der Straeten 2014). Jasmonic acid resistance 1 (JAR1) is forming jasmonyl-ACC (JACC), the γ-glutamyl transpeptidase (GGT) is generating γ-glutamyl-ACC and finally the ACC-deaminase (ACCD) catalyses the reaction to produce α-ketobutyrate and ammonia (Fig 3). The influence of these different ways to conjugate ACC on the homeostasis of ACC and ethylene are subject of the present research.

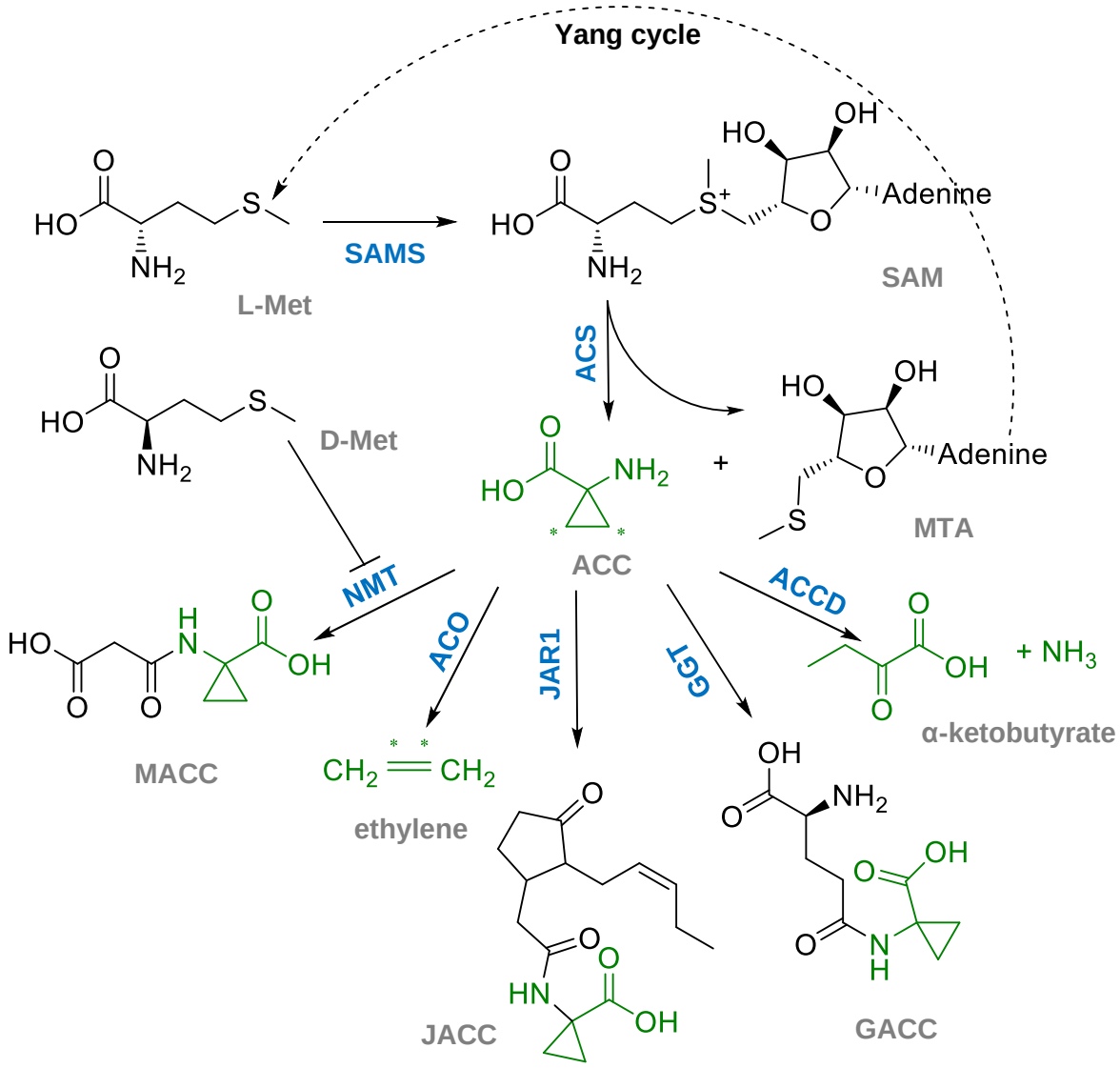


Figure 3: Scheme of ethylene biosynthesis and ACC regulation mechanism (conjugation/metabolism). L-Met is converted into SAM (S-adenosyl-methionine). The SAMS (SAM synthetase) catalyses the reaction forming ACC and MTA (5'-methylthioadenosine). The MTA is recycled back to L-Met via several enzymatic steps (Yang cycle). The ACC is here depicted in green for being traceable in its products. For producing ethylene, the ACC has to be oxidized (ACO). Conjugation of the ACC can occur via NMT (N-malonyl transferase) to generate N-malonyl-ACC (MACC), JAR1 (jasmonic acid resistance 1) to generate jasmonyl-ACC, and γ-glutamyl transpeptidase to generate γ-glutamyl-ACC. In presence of D-Met the formation of MACC is inhibited. The last way to metabolize ACC is the deamination via ACCD (ACC deaminase) to produce α-ketobutyrate and ammonia.

2 Aims of the Study

It has been implied that D-AA have only limited relevance in the plants and there were few physiological effects found for D-AA on plants in the past. Considering the abundance of D-AA in nature leads to the question what the plants mechanisms and strategies are to deal with D-AA? Are the D-AA only "toxic" for plants or are there undiscovered processes or connections between uptake, metabolism and physiological effects in *Arabidopsis thaliana*?

In the beginning of my study, it was already investigated that plants are capable of D-AA uptake. But when we fed plants with D-AA and transferred them to media without these compounds, we observed a decrease of D-AA contents in the plants after a certain time. With that observation, the question arose: what causes the decrease? There were two possible scenarios explaining this phenomenon: One was the metabolization of D-AA by plants. The other one was the exudation of the D-AA by the plants. Both hypotheses should be tested in the course of my studies.

A second observation attracted my attention, too. After germinating Arabidopsis seedlings on D-methionine, mutants of the DAT1 gene, which are not able to metabolize D-AA showed growth disorders. It is my aim to find out which processes are going on in those plants and why the DAT1 enzyme is crucial for the plants.

3 Results and Discussion

There are two major findings of my studies, which I would like to present and discuss in the following. It is widely thought that D-AA have no or little functions in plants, since most of the amino acid studies do not distinguish between the two enantiomers (for review see Moe (2013)). However, I could confirm that the D-AA are imported actively into plants. Moreover, it turned out that they are exuded as well as their stereoisomers. This process revealed to occur passively without ATP utilisation. But besides exudation, there are alternative ways to decrease D-AA concentrations in plants, which are discussed in the following chapter.

In the second part of this chapter I will focus on the physiological effect and metabolic mechanisms upon long term D-Met treatment. It turned out that AtDAT1 has specialized on D-AA catabolism and therefore plays a central role in D-AA metabolization process. Furthermore, I was able to explain the phenotypic appearance of the *dat1* defective plants, since ethylene production was increased in those mutants. In addition, I found the responsible mechanisms for this increase.

3.1 Plant D-amino acid efflux

In our experiment, we treated plants with D-Ala and D-Glu, the two major metabolites of D-AA in *Arabidopsis thaliana*. 1 mM of each compound was added to 2 weeks old Arabidopsis Col-0 wild-type seedlings for 24 h. Afterwards, all seedlings were washed in MilliQ water and put into fresh medium without any D-AA additives. At different time points, the seedlings were harvested, washed, frozen in liquid nitrogen, and stored at -18 °C until sample analyses. With this experimental setup we were able to investigate both, the seedlings and the liquid media in which they were grown. We observed a relatively fast decrease; after 2 h there was only 41 % of D-Ala and 32 % of D-Glu left, compared to the initial level of D-AA in the plants (Paper I, Fig. 1). However, the initial levels already varied considerably between D-Ala (127,3 nmol per seedling) and D-Glu (26,7 nmol per seedling). This phenomenon was previously shown by Gördes, Kolukisaoglu, and Thurow (2011). They determined the different D-AA uptake levels and found that D-Ala is taken up in higher amounts than D-Glu. Furthermore, they still found D-Ala and D-Glu in plant tissue of the *lht1* knock out transporter mutant. They argued with the presence of at least 53 additional putative amino acid transporters in the Arabidopsis genome. Thus, it is not a single transport mechanism by which the varying import quantities can be explained as they might differ e.g. in their affinities.

Besides metabolization, efflux had to be considered as a cause for the decrease of D-AA concentration in plants (for review see Canarini et al. (2019)). Our experiments provide evidence for a D-AA release, since they were detected in the fresh medium after the plants have been transferred in there (Paper I, Fig. 3). To answer the question if the efflux occurs actively or passively, two chemicals, inhibiting different mechanisms in the ATP dependent energized transport could provide clarification. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was used as a protonophore preventing the H^+ gradient formation and consequently inhibiting the electrochemical pumps that require an H^+ gradient (Phillips et al. 2004). The other inhibitor was Na-Orthovanadate (OV), which inhibits plasma membrane-ATPases rather specifically (Rroço, Kosegarten, and Mengel 2002) and is a suitable inhibitor of ABC transporters (Sugiyama, Shitan, and Yazaki 2007). Each of these inhibitors were added to the fresh medium in which the seedlings were transferred into. The result was apparent after 24 h for the OV: D-Ala as well as L-Ala increased in the medium (Paper I, Fig. 4). This result indicated an active transport, energized by ATP and a passive efflux. The plants treated with CCCP responded similarly since increased D- and L-AA were quantified in the medium in comparable concentrations (< 10 nmol per seedling). The effect occurred already after 12 h (Paper I, Fig. 5). This observation is in accordance to Lesuffleur and Cliquet (2010) who discovered a reduction of glycine uptake and only marginal effects in the efflux after inhibitor application. The active import of D-Ala by LHT1 was already described by Gördes, Kolukisaoglu, and Thurow (2011). Moreover, it is not surprising that the uptake is facilitated, since amino acids are valuable compounds, which occur in rather low concentrations in the rhizosphere. Our observed rise of amino acids in the media, which comes along with inhibitor treatment implies the dysfunction of the re-uptake of those compounds. This means that the plants are not capable anymore to take up again the leaked amino acids from the medium, which means that the regulation of the import is disrupted. In contrast to the import, roots keep on exuding, which is still possible since the export is not energized by the proton gradient across the membrane (for a graphical summary see Fig. 4). More evidence is given by the steady state level, which can be observed after 24 h. Passive diffusion works as long as there is a concentration gradient. If the medium has reached the same level compared to the plant cells, diffusion stops. This indicates that plants are able to decrease their D-AA level, but only in terms of a D-AA overflow.

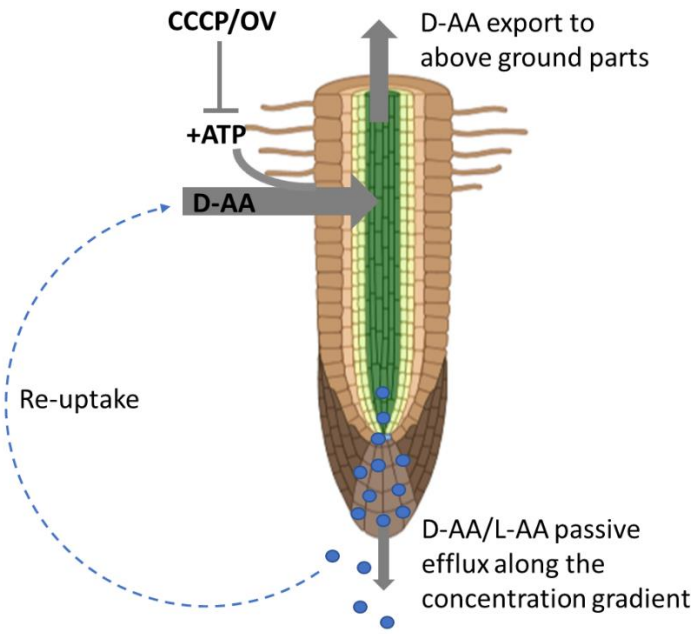


Figure 4: Scheme of amino acid transport in roots

With our experiments we could show that D-AA are leaking out in the same manner as their respective L-forms. Moreover, we tested other D-AA in this regard (Paper I, table 1). We treated the *dat1-1* mutant with D-Asp, D-Leu, D-Lys, D-Phe or D-Pro for 24 h, transferred them into fresh medium and took samples after 12 and 72 h. We used the mutants in this experiment since there would be none of those D-AA left after 72 h in presence of the DAT1 enzyme, which converts D-AA into D-Ala and D-Glu. It was also interesting in this respect to compare the treated plants with the control plants. Most of the D-AA are not present in the control plants and the L-AA concentration of the treated plants are quite similar to the control. The constant level of L-AA after D-AA treatment can only be observed in the *dat1* mutant. This is another evidence for the DAT1 protein being part of a pseudo racemization process, since it creates the precondition for L-aminating enzymes by D-AA deamination. The media of the D-AA treated plants were also investigated to determine the concentrations of the treated D- and their respective L-AA. In all samples, efflux was observed. However, after 72 h some of the L-AA were below the detection limit due to either re-uptake or absent efflux. D-Asp is the only exception, being exuded in even higher amounts after 72 h. The fact that there is a re-uptake reveals that the plants are not using this mechanism to decrease harmful compounds. In case of a detoxification event, plants would disable the re-uptake. Moreover, there are variances in the exudation amounts between the different D-AA. But those differences do not correlate with the toxicity of the compounds. In former experiments (Kolukisaoglu and Suarez

2017), different lethal D-AA concentrations for Arabidopsis seedlings were determined. By comparing the toxicity with the amount of exuded D-AA found in the media, there is no coincidence. Additionally, the quantification of the D-AA as well as their L-enantiomers did not reveal any correlation with the concentration measured in the plants. This suggests rather a selective (passive) export of the amino acids from the plant roots. Possibly, there are specific transporters involved, which are mediating non-energized amino acid efflux. AtCAT8 could be a suitable candidate as it was identified as a bidirectional H^+-independent glutamine transporter (Yang et al. 2010).

However, those conclusions are quite difficult since there is re-uptake involved, and in addition, amino acid export still needs to be further investigated (Okumoto and Pilot 2011). Further studies have to include D-AA uptake using more different D-AA combined with energy-dependent inhibitors like CCCP and OV. Moreover, mechanistically different inhibitors could be applied for example like p-chloromercuribenzenesulfonic acid (PCMBS), which inhibits carrier-mediated transport. This was shown for amino acid export from pea seeds (De Jong and Wolswinkel 1995).

In nature, measurements of efflux and re-uptake is not as simple as in *in vitro* cultures though. There are some challenges that have to be faced. First, the microbiome in the rhizosphere ingests exudates from solution. Second, exudates can be absorbed by the soil solid phase and third, there is a large proportion of metabolites, released due to organic matter degradation (Oburger and Jones 2018). However, the presence of soil-dwelling microorganisms leads to new questions. For example, what is the impact of competition for nutrients, i.e. D-AA, between plants and the rhizobiome? This question cannot be answered unambiguously, since there are again crucial aspects like nitrogen limitation or overall concentration of these compounds playing a role (Nasholm, Kielland, and Ganeteg 2009; Zhu, Riley, and Tang 2017). However, it was discovered by Harrison, Bol, and Bardgett (2008) that plants are inferior to microbes in the uptake of organic nitrogen, irrespective of the nitrogen form. But in the long run, plants are assumed to be winners for nitrogen acquisition because of the unidirectional flow of nutrients from soil to roots (Kuzyakov and Xu 2013). The work of Broughton et al. (2015) was more focused in a way that this phenomenon was analysed by distinguishing the D- and L-enantiomers. They also addressed the fact that bacteria might prefer to take up D-AA due to a self-cycling mechanism like it was already described by Lam et al. (2009). In the end, however, they could not prove this hypothesis as the tested phospholipid fatty acids (PLFAs) from the soils representing gram-positive and -negative bacteria and fungi, incorporated more ^{13}C of the L-form than of the D-form. Nevertheless, a more recent study found a D-alanine

transporter (DatA) in *Bacillus subtilis*, which has a higher affinity towards D-Ala than to L-Ala for re-assimilation of D-Ala released from the cell wall as the bacterium grows (Sidiq et al. 2019).

Another question in this regard is whether plants are able to control their metabolite efflux, which was also addressed by Farrar et al. (2003). They found different factors influencing the efflux of plants like concentration gradients and growth state but they also concluded that the plant exerts little direct control. A major problem in this regard is that a concentration gradient in nature soil is not trivial to measure.

Amino acid profiles of soil extracts (extracted with H_2O or K_2SO_4 or KCl) are not strictly comparable to amino acid profiles of actual soil solution (Warren 2014). There are several different methods how to extract soil compounds (for review see Vranova et al. (2012)). By using more efficient extraction methods, there is a risk of overestimating the amino acid quantities. The primary aim is to extract "free" amino acids, which are bioavailable for plants. Besides these free compounds, there are non-bioavailable amino acids, which are covalently bound to chemical entities for example sugars, integrated in proteins or absorbed to soil solids (Vranova et al. 2012). The second aim is to be careful in choosing a gentle way of extraction to avoid a chemical racemization processes, which can give a misleading picture of the D-AA quantity. Other factors are soil sampling strategy (number of samples over space and time), sample preparation (sieving and drying), soil storage, extraction temperature, shaking time, and soil-to-extractant volume ratio (Jones and Willett 2006).

As we showed before, plants are able to take up large amounts of D-AA within a quite short time (Paper I, Fig. 1). This might be useful regarding to the re-uptake in presence of competitive bacteria. Additionally, in terms of a microbial population interference, for example through freeze-thaw events (Schimel and Clein 1996) a rapid uptake of D-AA can also be beneficial. Therefore, plants might be able to take an advantage on amino acid import from soil due to both, reduced numbers of microbial competitors and due to a flush of amino acids from decomposing microbial cells (Jones et al. 2005). However, the uptake of amino acids released from soil organic matter (especially after those rather rare freeze-thaw events) cannot be seen as the main role of the root´s amino acid transporter, though. The major function is suggested to recapture amino acids lost through exudation (Jones et al. 2005).

All these studies were, unless otherwise stated, performed detecting both enantiomers of amino acids without separating D- and L-form. Since the phenomenon of exudation and

re-uptake has never been shown for D-AA separately, literature for amino acids in general has to be used. This should be justifiable due to the fact that Hill et al. (2011) provided evidence for D-AA being assimilated equally compared to their L-form. In fact, they had discrepancies in the uptake rate between D- and L-Ala, but they did not take into account that the D-Ala is partially converted into L-Ala as we demonstrated in our results. Additionally, we proved evidence that both enantiomers are taken up actively and exuded passively as it was already described for L-AA (for review see (Jones, Nguyen, and Finlay 2009).

Bacteria in soils are not competitors of plants *per se* and can be also beneficial for them. D-AA might be exuded by plants to attract valuable bacteria forming mutualistic associations in the rhizosphere (Badri et al. 2009). This phenomenon is quite well known in the mycorrhizae or rhizobacteria symbiosis context. Besides, there is one example known of specific attraction and root colonization of the biocontrol agent *Bacillus subtilis* by selective Arabidopsis L-malic acid exudation through an increased ALMT1 expression (Rudrappa et al. 2008). However, there is no evidence to date that there is such an effect between plants and bacteria considering D-AA exchange or even an alteration of the rhizosphere microbial composition due to secretion of D-AA in root exudates. It seems reasonable to attract advantageous bacteria by D-AA exudation, since their cell wall consisting of D-Ala and D-Glu, the two main metabolites of *Arabidopsis thaliana*.

Nevertheless, it was shown that plants are able to shape their soil microbiome community (for review see Sasse, Martinoia, and Northen (2018)). However, it is a lot more to consider in this respect than just the root exudate. There are e.g. physiological factors like the developmental stage of Arabidopsis playing a role in rhizosphere microbiome composition. With increasing age, the amino acid exudation rises (Chaparro, Badri, and Vivanco 2014). On the other hand, with increasing age of the plants diffusion is negligible, since the presence of casparian strips or suberized endodermis in adult roots block apoplastic flow (Sasse, Martinoia, and Northen 2018). Moreover, it is quite challenging to get a good image of the root bacterial communities, since the distinct root segments are colonized differently. The root tip is associated with the highest numbers of active bacteria, the root elongation zone is particularly colonized by *Bacillus subtilis*, and the bacterial communities of the mature root zones distinguish from those of the root tips (Massalha et al. 2017). In the *B. subtilis* genome, 10 chemoreceptors are encoded, which are recognizing a broad variety of amino acids with no preference of those of high nutritional value. This kind of chemotaxis could help to locate plant roots and is therefore crucial for the early steps of root colonization (Allard-Massicotte et al. 2016). Badri et al. (2013)

discovered the correlation between compounds and soil microbes and found that the majority of amino acids related compounds showed a negative correlation with the phyla Actinobacteria. However, in all those studies the D- and L-forms were again not detected separately. With the knowledge generated in this work, it would be crucial to investigate those compounds independently, since their functions were shown to be quite different as well as their impact on plants.

Another central factor for bacterial root colonization investigation is of course the plant species but in addition their respective accessions and genotypes. In Figure 2 (Paper I), we showed that *dat1-1* mutants have compared to the Col-0 wildtype different L-AA levels after D-AA feeding. This results in different D-AA and L-AA efflux compositions between the wild-type and the mutants. Moreover, the presence of the DAT1 enzyme is crucial for D-Ala and D-Glu synthesis from other D-AA. A consequence could be a differential attracting pattern, which would influence the microbiome arrangement. Such microbe modulation by plants was already shown before (Micallef, Shiaris, and Colón-Carmona 2009). The authors discovered a correlation between exudation patterns and rhizobiome variation for eight Arabidopsis accessions by terminal-restriction fragment length polymorphism. Moreover, they found the two *Arabidopsis thaliana* ecotypes Ler and C24 to have the lowest terminal-restriction fragment (T-RF) diversity in their rhizosphere, that was comparable to the bulk soil. This result is not surprising since Ler has a natural mutation in the DAT1 gene (Paper II Fig.1a). However, by using deep 16 rRNA gene profiling, another study (Schlaeppi et al. 2014) revealed that the rhizobiome clustering is varying more through interactions with the environment as within host species. Furthermore, they found more diversity between the different Arabidopsis species than by comparing *A. thaliana* ecotypes.

In conclusion, the D-AA efflux appears not to be controllable for the plants. Exudation can be rather seen as a leakage of small molecules from the roots into the soil. However, the different D-AA vary in their exudation levels. D-AA are partially valuable for the plants, since they can be used as nitrogen source. To regain those compounds, plants have the D-AA re-uptake ability. This could be important for plants due to the presence of soil-dwelling organisms that are shown to compete for those nitrogen containing compounds. On the other hand, there can be also a mutual effect between plants and bacteria in soil. This was shown for amino acids altogether, since most of the studies, dealing with this topic did not use a chiral analysis method. A third hypothesis is the elimination of D-AA through the efflux as reaction of a putative overload of those compounds, since in the past D-AA are generally considered as toxic due to growth inhibitory effects. However, there

is no correlation between the efflux quantity and the level of toxicity. The negative response of plants after a certain D-AA treatment must be regulated in a different way.

3.2 Physiological effects after D-amino acid treatment in DAT1 defective plants

In the previous chapter I focussed on the efflux mechanism of D-AA. In this part of the discussion I want to address the effects within plants caused by D-AA. Furthermore, plant-microbe-interactions are discussed, which are important in this context regarding D-AA circuit in nature. Therefore, the uptake of D-AA is again a crucial step for studying physiological effects of D-AA on plants. Another important factor is the time of D-AA presence in the rhizosphere, and therefore the time of plants being exposed to the D-AA. In contrast to the efflux experiments, in which plants were treated for 24 h, the here presented experiments were performed with plants, germinated on solid media containing certain D-AA. By investigation of plants, which are exposed to D-AA over a long period, we observed a growth inhibition effect (unpublished data) which was also described by Erikson, Hertzberg, and Nasholm (2004). The effective concentrations vary from 0,1 to > 10 mM depending on the respective D-AA. The different harmful concentrations did not correlate with different uptake abilities (Gördes, Kolukisaoglu, and Thurow 2011). Moreover, as we showed before, the efflux rate is also much smaller than the uptake rate. The question thus arises how plants regulate the abundance of D-AA after their uptake besides efflux?

As mentioned before, there are several putative D-AA racemising, oxidising and conjugating enzymes found in plants (Fig.2). But first of all, the AtDAT1, which converts all canonical D-AA into D-Ala, D-Glu and indirectly into their respective L-enantiomer has to be mentioned in this regard. This protein with transaminase activity has turned out to be a key enzyme in D-AA metabolic pathways. By characterization of this enzyme, the preference for D-Met over the other tested D-AA was observed (Paper II, Fig. 3). This means that the DAT1 has the highest conversion rate to D-Ala and D-Glu after D-Met feeding amongst other D-AA. In our genetical and biochemical approaches we found that the *A. thaliana* Ler (Landsberg erecta) ecotype has a reduced AtDAT1 enzyme activity due to a point mutation in the respective active gene site (Paper II, Fig. 1A). Therefore, Ler has only 5 % remaining activity, compared to the Col-0 wild-type. With this observation, experiments followed using solid media, which contained D-Met to grow Arabidopsis seedlings of Col-0 and Ler wild-type as well as the two DAT1 knock out mutants *dat1-1*

and *dat1-2*, which are T-DNA insertion lines in Col-0 background. In these experiments, growth inhibition was observed at concentrations ≥ 200 μM (Paper II, Fig 5). Another significant effect appeared by comparing the wild-type with the mutants, which were more susceptible to D-Met. At concentrations of 500 μM D-Met, the mutants were, compared to the Col-0 wildtype, seriously stunted in their growth. Ler, with its reduced DAT1 activity, showed intermediate growth reduction on these D-Met concentrations and was not as severely affected as the mutants. In this regard the question arose: why is D-Met the favourable substrate of the DAT1 enzyme and how can plants be exposed to D-Met in nature?

Methionine is a sulphur containing canonical amino acid which is important for many metabolic processes. Besides L-Met, which is known to be produced in plastids (Wirtz and Droux 2005), D-Met was never shown to be synthesised *de novo* by plants. There are bacteria, producing D-Met from L-Met like *Streptococcus faecalis* (Shockman and Toennies 1954). Another bacterial species (*Vibrio cholerae*) produces, releases, and incorporates D-Met into its peptidoglycan layer, which could modulate this polymer in its strength and flexibility (Cava et al. 2011) or even help to become penicillin G resistant (Lam et al. 2009). This was also observed for *Escherichia coli* unless they are not capable to produce and release D-Met themselves. Interestingly, the release of extracellular D-amino acids can signal to the whole bacterial community to regulate PG amount, composition, and strength (Cava et al. 2011). A similar event was published by Kolodkin-Gal et al. (2010), as they showed in terms of biofilm formation that a mixture of D-Met, D-Leu, D-Trp, and D-Tyr triggers biofilm disassembly of *Bacillus subtilis*. Moreover, Lam et al. (2009) detected free D-AA concentrations in different bacterial growth stages. As a result, they observed D-Met production for the facultative anaerobic bacteria *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Aeromonas hydophilia* in 0,5 – 1 mM concentrations. Those bacteria could be a putative source for D-Met in plants' environment as they are occurring in rhizospheres and are therefore directly associated with plant roots.

Plants are able to take up D-Met in considerable quantities, which are comparable to D-Ala (Gördes, Kolukisaoglu, and Thurow 2011). D-Met and D-Ala were no competitors in simultaneous L-AA uptake in mustard roots (Wright 1962), suggesting different transport mechanisms. Nevertheless, the natural source of D-Met for plants has not been completely elucidated so far. This becomes clear by examination of D-AA profiles in soil. There are several D-AA discovered in the rhizosphere, but no D-Met or only small quantities are found in soils (Amelung and Zhang 2001) or in plants (Brückner and Westhauser 2003). Additionally, L-Met is only present in relatively low concentrations, too (Hu, Zheng, and

Wanek 2017). As D-AA are only a small percentage of their respective enantiomers, it is quite challenging to find the compound within the limit of quantification. One explanation of the partial absence of D-Met in soils would be that naturally low amounts are taken up immediately by plants and microorganisms. Since it can be regarded as a vital sulphur containing compound, which is utilized by many living organisms (Jones 1999) the presence of the free form can rather be seen as a rare event. Moreover, amino acid influx/efflux ratios were tested in four different plant species by Phillips et al. (2004). Among other amino acids, methionine had the highest influx, compared to the efflux. This implies that methionine in the rhizosphere should always be expected in low quantities. Interestingly, the spectrum of this ratio for the different amino acids varies a lot between species, suggesting species dependent affinities towards amino acids. This leads to another question regarding the AtDAT1 and its substrate affinity: why is there a preferred interaction between AtDAT1 and D-Met and what about homologs of this enzyme in other plant species? This gene is well conserved in different plant species (Paper II, Fig. S5), and only the enzyme from Arabidopsis was characterized. For future studies, it would be interesting to investigate different plant species in order to find correlations between the D-AA preference of the DAT enzyme, their respective influx/efflux ratio as well as the soil properties including their microbial associations.

The DAT1 protein is located in plastids (Paper II, Fig. S3). After uptake, D-Met is assumed to be transported right there. We got first evidence by feeding the plants with a D-Met derivative harbouring an azido group which is able to react with a ethinyl group of a modified fluorophore (click chemistry similar to Hirano et al. (2016)). After this reaction *in planta*, we were able to detect the fluorescent D-Met product in chloroplasts (unpublished data). Kolukisaoglu and Suarez (2017) could already show the localization of D-Ala in plastids. They treated Arabidopsis seedling with HADA (HCC-amino-D-alanine), a D-Ala analogue, attached to a coumarin that has an excitation maximum of 405 nm (Kuru et al. 2015). Consequently, our data suggest that D-AA are transported into plastids and metabolized there. But what happens if the DAT1 is inactive?

If there is no DAT1 to catabolize the D-AA, they therefore accumulate in the cells (as intake of D-AA is always faster than release), resulting in a reduction of growth. By observation of etiolated, D-Met treated seedlings, this stunted development of the seedlings resembled a triple response (Paper II, Fig. 5). This phenomenon is known to occur upon increased ethylene production (Guzman and Ecker 1990). It results into a reduction in elongation, swelling of the hypocotyl, and a change in the direction of growth (Neljubow 1901).

However, as stated before, there are more ways to metabolize D-AA than just by the DAT1. As mentioned earlier, it is known that D-AA and particularly D-Met can be conjugated for inactivation. Furthermore, it was already shown by Liu, Hoffman, and Yang (1983) that there is a coincidence between the malonylation of 1-aminocycloporpane-1-carboxylic acid (ACC) and D-Met. D-Met as well as D-Phe (but not their respective L-AA) were found to inhibit the conjugation of ACC to malonyl-ACC (MACC) in mung-bean hypocotyls. After 50 mM D-AA + 1 mM ACC treatment they observed an increase in ethylene and a decrease in MACC. We were able to confirm this phenomenon in Arabidopsis in three different ways. First, ethylene can be manipulated by different inhibitors, which are distinguished by their mode of action, either inhibiting biosynthesis or signalling of ethylene (for review see Schaller and Binder (2017)). We treated the plants with 500 µM D-Met in combination with α-aminoisobutyric acid (AIB) an ACC oxidase inhibitor. By adding 2 mM AIB, the differences between the DAT1 mutants and the wild-type vanished, suggesting a decrease in ethylene (Paper II, Fig. 6a). As a second approach, we measured the ethylene released by the seedlings using gas chromatography. For this experiment, Col-0, *dat1-1*, *dat1-2* and Ler were grown on 500 µM D-Met followed by ethylene measurements of air samples from the plants' gaseous phase. The results confirmed the outcomes of the previous inhibitor experiments. The mutants produced significantly more ethylene after D-Met treatment than the wild-type plants (Paper II, Fig. 6b). And as a third method we qualified the metabolites after D-Met treatment. It was shown before (Liu, Hoffman, and Yang 1983) that the enzyme, which catalyses the malonylation of ACC also conjugates D-Met. This means, in presence of D-Met there is competition for the malonyl-transferase. Consequently, there would be an increase in malonyl-methionine and a decrease in MACC production with increasing D-Met levels. Moreover, this effect should be more distinct in the DAT1 mutants since they are not capable of metabolizing D-Met and have therefore a higher intercellular D-Met level. By LC-MS/MS analyses, we were able to identify MACC and additionally malonyl-methionine for the first time. In fact, there is an increase of malonyl-methionine upon D-Met treatment, which differs between the wild-types and the mutants (Paper II, Fig. 7). Moreover, we could detect a decrease in malonyl-ACC. However, there was no significant difference between the mutants and the wild-types (Paper II, Fig. 8). This might be explained by different metabolization pathways, in which ACC is conjugated alternatively (Le Deunff and Lecourt 2016; Van de Poel and Van Der Straeten 2014). Consequently, there are more pathways than malonylation involved in regulation of the ACC content (Fig. 3; Paper II, Fig. 9). There is the jasmonyl-ACC derivative, which is formed by jasmonic acid resistance 1 (JAR1). Interestingly, there are already jasmonic acid conjugates with other

amino acids as Ile, Val, Leu and Phe known (Staswick and Tiryaki 2004). The third derivative of ACC is γ-glutamyl-ACC, formed by γ-glutamyl-transpeptidase (GGT) with a requirement of glutathione (GSH). There is even a fourth pathway in which ACC gets catabolized by an ACC deaminase, forming α-ketobutyrate. Therefore, in concurrent presence of D-Met, besides malonylation, alternate ACC metabolization pathways can be promoted.

In this regard it would be interesting to know why growth is decreased in plants upon D-Met treatment if there are other pathways to regulate the ACC? Whether these alternate metabolites have their own mode of action or the system is out of the equilibrium state due to D-Met overload remains unclear. Maybe the ACC quantity is just too huge to compensate. Besides, the affinity of the N-malonyl-transferase for D-Met in comparison with ACC has never been tested. The problem in this regard is that the N-malonyl-transferase enzyme has not been identified, yet. Although the enzyme already has been isolated several times (Guo, Phillips, and Arteca 1993), the gene encoding for this protein is still unknown. Moreover, we have not tested other conjugates after D-Met treatment. Since it is unknown if D-Met can be conjugated with a glutamyl- or jasmonlyl group, it would be interesting to investigate those D-Met metabolites. Potentially, also the other D-AA could interfere in those conjugation processes. This leads to another still remaining question:

What happens with the other D-AA? After we could successfully show that the DAT1 enzyme plays a key role in D-AA metabolization, other D-AA and their effect on growth through increased ethylene production getting interesting to explore. From previous studies it is known that D-Phe causes similar effects like D-Met, since it is also malonylated by the N-malonyl-transferase (Liu, Hoffman, and Yang 1983; Guo, Phillips, and Arteca 1993). Furthermore, we got a first hint when we observed a significant difference in growth and ethylene production between the Col-0 wild-type and the *dat1*-mutants when they were grown on 1 mM D-Phe (unpublished data). Moreover, ethylene contents were measured upon D-Ala, D-Arg, D-His and D-Trp treatment. All of those mentioned D-AA were inducing ethylene production. However, this was not further pursued. Since ethylene is a broad stress response, this could trigger many biochemical pathways. For future studies, it would be worth to elucidate those responsible mechanisms and putative reaction products as well as the investigation of additional D-AA.

The direct and indirect metabolites after DAT1 activity D-Ala, D-Glu and L-Met could also play a role in plants physiological response. It is known that D-Ala can inhibit the ACC oxidase, which leads to a decreased ethylene production (Gibson et al. 1998). Since D-Ala

is only produced in the Col-0 wild-type upon D-AA treatment, it could also downregulate ethylene production. But we were not able to measure any effect by adding D-Ala to our D-Met treatment (unpublished data). Nevertheless, we found that treating the plants with 500 μM D-Met and L-Met simultaneously, even in low concentrations of 10 μM L-Met, there was a rescue of the phenotype. We observed the DAT1 mutants growing almost as big as the Col-0 wild-type (unpublished data). Additionally, we measured reduced ethylene in the mutants when L-Met was present. However, there is no suitable explanation for this phenomenon so far. One hypothesis is that the transport of D-Met is reduced due to the presence of L-Met. This could be possible if D-Met and L-Met would share the same transport mechanisms, resulting in a competitive inhibition of this putative transport protein. Another idea is the disruption of the ethylene pathway through raised L-Met concentrations. Since ethylene synthesis starts with L-Met, an increased intermediate production could be expected. In this case, there might be a feedback regulation occurring as it was found for the ACC synthase, which is inactivated through S-adenosylmethionine (Satoh and Esashi 1986). This means that increased L-Met would slow down the entire ethylene synthesis process. Those two mentioned theories could be tested by using inhibitors, which are available for specific inhibition of either active transport (CCCP, OV) or for ACC synthase (aminoethoxyvinylglycine, AVG). To investigate this observation more in more detail there is another challenging problem. It is quite difficult to detect L-Met, since 80 % of it is converted to S-AdoMet (Ravanel et al. 1998). This fast metabolization is also happening with ACC. It is possible to measure its malonyl-conjugate via LC-MS, but we were not able to detect ACC itself, yet.

As mentioned before, there are beneficial bacteria colonizing the rhizosphere. To decrease the ACC and thus the ethylene level, ACC metabolizing bacteria can be quite advantageous for plants and their root growth. It was shown for canola plants that the presence of ACC deaminase producing bacteria promoted root growth as they act as sink for ACC (Stearns et al. 2005). Furthermore, bacterial ACC deaminase was shown to reduce ethylene levels in plants by decreasing the available ACC. This decrease in ethylene leads to promoted plant growth as a high concentration of ethylene induces root growth inhibition (Glick et al. 2007). Therefore, it is required that plants exudate ACC (Arshad, Saleem, and Hussain 2007). As ACC is an amino acid derivative, it is released most likely through passive diffusion, which is concentration dependent. Release of ACC was confirmed by Penrose, Moffatt, and Glick (2001) as they were able to quantify ACC in root exudates. They also found less ACC in the rhizosphere in the presence of ACC deaminase-containing bacteria species, which additionally promoted growth of plant roots.

Additionally, they suggested that plants might even attract ACC deaminase containing bacteria to establish a rhizosphere interaction. In this regard there is the assumption that maybe there is no growth inhibitory effect in presence of those bacteria. Since ACC was shown to be exuded by plants, soil-dwelling bacteria take up the ACC excess and therefore prevent plants from the re-uptake of this compound. Another argument in this regard is that there is growing evidence of ACC not only being a precursor of ethylene, but additionally acting as a signalling molecule in plant development and beyond (Kieber and Polko 2019).

However, regarding the interconnection between D-Met uptake and ACC release in a beneficial way for plants is already rather complicated. There must be a co-existence of D-Met releasing bacteria on the one side (*Bacillus subtilis*) and an ACC reducing organism on the other side (*Pseudomonas putida* GR 12-2 (Jacobson, Pasternak, and Glick 1994)). However, both genus, *Pseudomonas* and *Bacillus* belong to the growth promoting bacteria (Andrews and Harris 2000; Bais et al. 2006). In this case the conclusion for D-Met would be that on the first glance the effect on plants looks detrimental but could help plants in nature to enhance growth since this compound and/or other D-AA are part of a plant-microbial-interaction, which has to be investigated further.

Another interesting aspect is the concurrent efflux of ACC and the direct (D-Ala, D-Glu) as well as the indirect (respective L-Form of the D-AA) DAT1 metabolites. There might be a coincidence between the re-uptake of those metabolites, which can inhibit the re-uptake of ACC due to their shared transport system, which is LHT1 (Shin et al. 2014). This could be at least true for the metabolite D-Ala, which was found to be transported by LHT1 (Svennerstam et al. 2007). As methionine was not part of the transport studies, this still has to be figured out.

4 Conclusion

This thesis provides the first evidence for the main D-AA metabolization pathway *via* the AtDAT1 enzyme. By transamination, all tested D-AA are converted directly into D-Ala and D-Glu and indirectly into their respective L-form (also of the initial D-AA). This work provides insights in AtDAT1 transamination activity, substrate affinity, and conservation. Experiments using T-DNA insertion lines and the Ler ecotype, which has a natural deficiency in the respective gene site, revealed phenotypic alterations upon D-AA treatment. In our experiments, we used D-Met, since it has turned out to be the preferred substrate for the DAT1 enzyme, probably due to its valuable sulphur containing backbone. The resulting growth decrease upon D-Met treatment was only observed in the *dat1* loss of function mutants and Ler but not in the Col-0 wild-type. Finally, we were able to connect our findings to previous publications by confirming that the reduced growth is linked to an increased ethylene production. This was proven by determination of the resulting metabolites (malonyl-methionine and malonyl-ACC) after D-Met treatment. In the end, we could prove the central metabolic role of the DAT1 enzyme and its connection to growth inhibitory effects of D-AA due to increased ethylene production.

Another part of the study addressed D-AA transport systems. We found energy-driven import of D-AA by plants as it was previously shown for their L-enantiomers. Furthermore, it was demonstrated for the first time that D-AA are exuded passively by *Arabidopsis thaliana* roots. However, the efflux regulation requires further research.

Considering those observations into a greater biological context, it appears to be connected to plant-microbe-interactions. Besides microbial degradation, soil-dwelling bacteria like *Bacillus subtilis* are capable to release D-AA and particularly D-Met in physiologically relevant amounts and can be therefore seen as adequate D-AA source. Plants take up D-AA actively and metabolize them via the DAT1 enzyme. A fraction of the resulting metabolites, if they are not used for protein structures etc., can be found in the rhizosphere again through passive exudation. Since D-Ala and D-Glu are essential building blocks of the bacterial peptidoglycan cell wall, it is possible that plants use this mechanism to attract beneficial bacterial in their rhizosphere. A beneficial microbiome can prevent the root assembly of pathogenic microorganisms and therefore increase the plants health. Ecotypes like Ler would be disadvantaged compared to the Col-0 wild-type since Ler only possesses 5 % of the DAT1 activity. However, their origin is different and it can be assumed that there are different selection pressures in the different growth environments. Since the DAT1 protein is well conserved among different plant species, there could be also

variations in substrate affinities depending on origin or other factors. But those hypotheses still need to be tested.

Literature

Abeles, Frederick B, Page W Morgan, and Mikal E Saltveit Jr. 2012. *Ethylene in plant biology* (Academic press).

Adams, D.O., and S.F. Yang. 1979. 'Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene', *Proceedings of the National Academy of Sciences*, 76: 170-74.

Allard-Massicotte, Rosalie, Laurence Tessier, Frédéric Lécuyer, Venkatachalam Lakshmanan, Jean-François Lucier, Daniel Garneau, Larissa Caudwell, Hera Vlamakis, Harsh P Bais, and Pascale B Beauregard. 2016. 'Bacillus subtilis early colonization of *Arabidopsis thaliana* roots involves multiple chemotaxis receptors', *MBio*, 7: e01664-16.

Amelung, W, and X Zhang. 2001. 'Determination of amino acid enantiomers in soils', *Soil Biology and Biochemistry*, 33: 553-62.

Andrews, John H, and Robin F Harris. 2000. 'The ecology and biogeography of microorganisms on plant surfaces', *Annual review of phytopathology*, 38: 145-80.

Arshad, Muhammad, Muhammad Saleem, and Sarfraz Hussain. 2007. 'Perspectives of bacterial ACC deaminase in phytoremediation', *TRENDS in Biotechnology*, 25: 356-62.

Bacon, Charles W, and James F White. 2016. 'Functions, mechanisms and regulation of endophytic and epiphytic microbial communities of plants', *Symbiosis*, 68: 87-98.

Bada, Jeffrey L. 1971. 'Kinetics of the nonbiological decomposition and racemization of amino acids in natural waters.' in (ACS Publications).

Badri, Dayakar V, Jacqueline M Chaparro, Ruifu Zhang, Qirong Shen, and Jorge M Vivanco. 2013. 'Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome', *Journal of Biological Chemistry*, 288: 4502-12.

Badri, Dayakar V, Tiffany L Weir, Daniel Van der Lelie, and Jorge M Vivanco. 2009. 'Rhizosphere chemical dialogues: plant–microbe interactions', *Current opinion in biotechnology*, 20: 642-50.

Bais, H. P., T. L. Weir, L. G. Perry, S. Gilroy, and J. M. Vivanco. 2006. 'The role of root exudates in rhizosphere interations with plants and other organisms', *Annual Review of Plant Biology*, 57: 233-66.

Besnard, Julien, Réjane Pratelli, Chengsong Zhao, Unnati Sonawala, Eva Collakova, Guillaume Pilot, and Sakiko Okumoto. 2016. 'UMAMIT14 is an amino acid exporter involved in phloem unloading in Arabidopsis roots', *Journal of experimental botany*, 67: 6385-97.

Bhushan, R, and H %J Amino acids Brückner. 2004. 'Marfey's reagent for chiral amino acid analysis: a review', 27: 231-47.

Bollard, E.G. 1966. 'A comparative study of the ability of organic nitrogenous compounds to serve as sole sources of nitrogen for the growth of plants', *Plant and soil*, 25: 153-66.

Bouzayen, Mondher, Alain Latche, Jean-Claude Pech, and Gerard Marigo. 1989. 'Carrier-mediated uptake of 1-(malonylamino) cyclopropane-1-carboxylic acid in vacuoles isolated from Catharanthus roseus cells', *Plant physiology*, 91: 1317-22.

Broughton, RCI, KK Newsham, PW Hill, A Stott, and DL Jones. 2015. 'Differential acquisition of amino acid and peptide enantiomers within the soil microbial community and its implications for carbon and nitrogen cycling in soil', *Soil Biology and Biochemistry*, 88: 83-89.

Brückner, H, and C Keller-Hoehl. 1990. 'HPLC separation of DL-amino acids derivatized withN 2-(5-fluoro-2, 4-dinitrophenyl)-L-amino acid amides', *Chromatographia*, 30: 621-29.

Brückner, H., and T. Westhauser. 2003. 'Chromatographic determination of L- and D-amino acids in plants', *Amino Acids*, 24: 43-55.

Buchanan, Bob B, Wilhelm Gruissem, and Russell L Jones. 2015. *Biochemistry and molecular biology of plants* (John Wiley & Sons).

Canarini, Alberto, Christina Kaiser, Andrew Merchant, Andreas Richter, and Wolfgang Wanek. 2019. 'Root exudation of primary metabolites: mechanisms and their roles in plant responses to environmental stimuli', *Frontiers in plant science*, 10.

Cava, Felipe, Hubert Lam, Miguel A De Pedro, and Matthew K Waldor. 2011. 'Emerging knowledge of regulatory roles of D-amino acids in bacteria', *Cellular and Molecular Life Sciences*, 68: 817-31.

Chaparro, Jacqueline M, Dayakar V Badri, and Jorge M Vivanco. 2014. 'Rhizosphere microbiome assemblage is affected by plant development', *The ISME journal*, 8: 790.

Curti, B, S Ronchi, and M Pilone Simonetta. 1992. "D-and l-amino acid oxidases. Chemistry and Biochemistry of Flavoenzymes (MuÈller, F., ed.)." In.: CRC Press, Boca Raton, FL.

De Jong, A, and P Wolswinkel. 1995. 'Differences in release of endogenous sugars and amino acids from attached and detached seed coats of developing pea seeds', *Physiologia Plantarum*, 94: 78-86.

Erikson, O., M. Hertzberg, and T. Nasholm. 2004. 'A conditional marker gene allowing both positive and negative selection in plants', *Nat Biotechnol*, 22: 455-8.

Farrar, John, Martha Hawes, Davey Jones, and Steven Lindow. 2003. 'How roots control the flux of carbon to the rhizosphere', *Ecology*, 84: 827-37.

Fischer, Wolf-Nicolas, Bruno André, Doris Rentsch, Sylvia Krolkiewicz, Mechthild Tegeder, Kevin Breitkreuz, and Wolf B Frommer. 1998. 'Amino acid transport in plants', *Trends in plant science*, 3: 188-95.

Forsum, Oskar, Henrik Svennerstam, Ulrika Ganeteg, and Torgny Näsholm. 2008. 'Capacities and constraints of amino acid utilization in Arabidopsis', *New Phytologist*, 179: 1058-69.

Frahn, John L, and Richard J Illman. 1975. 'The occurrence of D-alanine and D-alanyl-D-alanine in Phalaris tuberosa', *Phytochemistry (UK)*.

Fujitani, Y., N. Nakajima, K. Ishihara, T. Oikawa, K. Ito, and M. Sugimoto. 2006. 'Molecular and biochemical characterization of a serine racemase from *Arabidopsis thaliana*', *Phytochemistry*, 67: 668-74.

Fukuda, Mitsuri, Akira Tokumura, Tadashi Ogawa, and Kei Sasaoka. 1973. 'D-alanine in germinating Pisum sativum seedlings', *Phytochemistry*, 12: 2593-95.

Funakoshi, Miya, Masae Sekine, Masumi Katane, Takemitsu Furuchi, Masafumi Yohda, Takafumi Yoshikawa, and Hiroshi Homma. 2008. 'Cloning and functional characterization of *Arabidopsis thaliana* d-amino acid aminotransferase–d-aspartate behavior during germination', *The FEBS journal*, 275: 1188-200.

Gibson, Edward J, Zhihong Zhang, Jack E Baldwin, and Christopher J Schofield. 1998. 'Substrate analogues and inhibition of ACC oxidase: conversion of D-valine to iso-butanal', *Phytochemistry*, 48: 619-24.

Glick, Bernard R, Zhenyu Cheng, Jennifer Czarny, and Jin Duan. 2007. 'Promotion of plant growth by ACC deaminase-producing soil bacteria.' in, *New perspectives and approaches in plant growth-promoting Rhizobacteria research* (Springer).

Gördes, D., U. Kolukisaoglu, and K. Thurow. 2011. 'Uptake and conversion of D-amino acids in *Arabidopsis thaliana*', *Amino Acids*, 40: 553-63.

Guo, Lining, AT Phillips, and Richard N Arteca. 1993. 'Amino acid N-malonyltransferases from mung beans. Action on 1-aminocyclopropane-1-carboxylic acid and D-phenylalanine', *Journal of Biological Chemistry*, 268: 25389-94.

Guzman, Plinio, and Joseph R Ecker. 1990. 'Exploiting the triple response of Arabidopsis to identify ethylene-related mutants', *The Plant Cell*, 2: 513-23.

Harrison, Kathryn A, Roland Bol, and Richard D Bardgett. 2008. 'Do plant species with different growth strategies vary in their ability to compete with soil microbes for chemical forms of nitrogen?', *Soil Biology and Biochemistry*, 40: 228-37.

Hill, P. W., R. S. Quilliam, T. H. DeLuca, J. Farrar, M. Farrell, P. Roberts, K. K. Newsham, D. W. Hopkins, R. D. Bardgett, and D. L. Jones. 2011. 'Acquisition and assimilation of nitrogen as peptide-bound and D-enantiomers of amino acids by wheat', *PLoS One*, 6: e19220.

Hirano, T., K. Tanidokoro, Y. Shimizu, Y. Kawarabayasi, T. Ohshima, M. Sato, S. Tadano, H. Ishikawa, S. Takio, K. Takechi, and H. Takano. 2016. 'Moss Chloroplasts Are Surrounded by a Peptidoglycan Wall Containing D-Amino Acids', *Plant Cell*, 28: 1521-32.

Hu, Yuntao, Qing Zheng, and Wolfgang Wanek. 2017. 'Flux analysis of free amino sugars and amino acids in soils by isotope tracing with a novel liquid chromatography/high resolution mass spectrometry platform', *Analytical chemistry*, 89: 9192-200.

Ilisz, István, Robert Berkecz, Antal %J Journal of pharmaceutical Péter, and biomedical analysis. 2008. 'Application of chiral derivatizing agents in the high-performance liquid chromatographic separation of amino acid enantiomers: a review', 47: 1-15.

Jack, Ralph W, and Günther Jung. 1998. 'Natural peptides with antimicrobial activity', *CHIMIA International Journal for Chemistry*, 52: 48-55.

Jacobson, Christian B, JJ Pasternak, and Bernard R Glick. 1994. 'Partial purification and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2', *Canadian Journal of Microbiology*, 40: 1019-25.

Jones, Davey L, Christopher Nguyen, and Roger D Finlay. 2009. 'Carbon flow in the rhizosphere: carbon trading at the soil–root interface', *Plant and soil*, 321: 5-33.

Jones, David L, John R Healey, Victoria B Willett, John F Farrar, and Angela Hodge. 2005. 'Dissolved organic nitrogen uptake by plants—an important N uptake pathway?', *Soil Biology and Biochemistry*, 37: 413-23.

Jones, David L, Andrew G Owen, and John F Farrar. 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: 1893-902.

Jones, DL 1999. 'Amino acid biodegradation and its potential effects on organic nitrogen capture by plants', *Soil Biology and Biochemistry*, 31: 613-22.

Jones, DL, and PR Darrah. 1994. 'Amino-acid influx at the soil-root interface of *Zea mays L.* and its implications in the rhizosphere', *Plant and soil*, 163: 1-12.

Jones, DL, and VB Willett. 2006. 'Experimental evaluation of methods to quantify dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) in soil', *Soil Biology and Biochemistry*, 38: 991-99.

Kawasaki, Yoshiko, Tadashi Ogawa, and Kei Sasaoka. 1982. 'Two pathways for formation of D-amino acid conjugates in pea seedlings', *Agricultural and Biological Chemistry*, 46: 1-5.

Kieber, Joseph J, and Joanna K Polko. 2019. '1-aminocyclopropane 1-carboxylic acid and its emerging role as an ethylene-independent growth regulator', *Frontiers in plant science*, 10: 1602.

Kolodkin-Gal, Ilana, Diego Romero, Shugeng Cao, Jon Clardy, Roberto Kolter, and Richard Losick. 2010. 'D-amino acids trigger biofilm disassembly', *Science*, 328: 627-29.

Kolukisaoglu, Üner, and Juan Suarez. 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, Erkin, Srinivas Tekkam, Edward Hall, Yves V Brun, and Michael S Van Nieuwenhze. 2015. 'Synthesis of fluorescent D-amino acids and their use for probing peptidoglycan synthesis and bacterial growth in situ', *Nature protocols*, 10: 33.

Kuzyakov, Yakov, and Xingliang Xu. 2013. 'Competition between roots and microorganisms for nitrogen: mechanisms and ecological relevance', *New Phytologist*, 198: 656-69.

Lam, Hubert, Dong-Chan Oh, Felipe Cava, Constantin N Takacs, Jon Clardy, Miguel A de Pedro, and Matthew K Waldor. 2009. 'D-amino acids govern stationary phase cell wall remodeling in bacteria', *Science*, 325: 1552-55.

Law, David M. 1987. 'Gibberellin-enhanced indole-3-acetic acid biosynthesis: D-Tryptophan as the precursor of indole-3-acetic acid', *Physiologia Plantarum*, 70: 626-32.

Le Deunff, E, and J Lecourt. 2016. 'Non-specificity of ethylene inhibitors:'double-edged'tools to find out new targets involved in the root morphogenetic programme', *Plant Biology*, 18: 353-61.

Lesuffleur, F., and J. B. Cliquet. 2010. 'Characterisation of root amino acid exudation in white clover (Trifolium repens L.)', *Plant and soil*, 333: 191-201.

Ling-Yuan, Su, LIU Yu, and Fa Yang Shang. 1985. 'Relationship between 1-aminocyclopropanecarboxylate malonyltransferase and D-amino acid malonyltransferase', *Phytochemistry*, 24: 1141-45.

Liu, Yu, Neil E Hoffman, and Shang Fa Yang. 1983. 'Relationship between the malonylation of 1-aminocyclopropane-1-carboxylic acid and D-amino acids in mung-bean hypocotyls', *Planta*, 158: 437-41.

Massalha, Hassan, Elisa Korenblum, Sergey Malitsky, Orr H Shapiro, and Asaph Aharoni. 2017. 'Live imaging of root–bacteria interactions in a microfluidics setup', *Proceedings of the National Academy of Sciences*, 114: 4549-54.

Micallef, Shirley A, Michael P Shiaris, and Adán Colón-Carmona. 2009. 'Influence of Arabidopsis thaliana accessions on rhizobacterial communities and natural variation in root exudates', *Journal of experimental botany*, 60: 1729-42.

Michard, E., P. T. Lima, F. Borges, A. C. Silva, M. T. Portes, J. E. Carvalho, M. Gilliham, L. H. Liu, G. Obermeyer, and J. A. Feijo. 2011. 'Glutamate receptor-like genes form Ca2+ channels in pollen tubes and are regulated by pistil D-serine', *Science*, 332: 434-7.

Miller, Robert F. 2004. 'D-Serine as a glial modulator of nerve cells', *Glia*, 47: 275-83.

Miller, Susan M, Reyna J Simon, Simon Ng, Ronald N Zuckermann, Janice M Kerr, and Walter H Moos. 1995. 'Comparison of the proteolytic susceptibilities of homologous L-amino acid, D-amino acid, and N-substituted glycine peptide and peptoid oligomers', *Drug Development Research*, 35: 20-32.

Moe, Luke A. 2013. 'Amino acids in the rhizosphere: from plants to microbes', *American journal of botany*, 100: 1692-705.

Nasholm, T., K. Kielland, and U. Ganeteg. 2009. 'Uptake of organic nitrogen by plants', *New Phytol*, 182: 31-48.

Neljubow, D. 1901. 'Uber die horizontale Nutation der Stengel von Pisum Sativum und einiger anderen Planzen', *Bot. Centralbl. Beih.*, 10: 128-39.

Neuberger, Albert. 1948. 'Stereochemistry of amino acids.' in, *Advances in protein chemistry* (Elsevier).

Noma, Masana, Masao Noguchi, and Einosuke Tamaki. 1973. 'Isolation and characterization of D-alanyl-D-alanine from tobacco leaves', *Agricultural and Biological Chemistry*, 37: 2439-39.

Oburger, Eva, and David L Jones. 2018. 'Sampling root exudates–mission impossible?', *Rhizosphere*, 6: 116-33.

Okumoto, Sakiko, and Guillaume Pilot. 2011. 'Amino acid export in plants: a missing link in nitrogen cycling', *Molecular Plant*, 4: 453-63.

Ono, Kazutoshi, Kazuki Yanagida, Tadao Oikawa, Tadashi Ogawa, and Kenji Soda. 2006. 'Alanine racemase of alfalfa seedlings (Medicago sativa L.): first evidence for the presence of an amino acid racemase in plants', *Phytochemistry*, 67: 856-60.

Ortiz-Lopez, Adriana, H-C Chang, and D.R. Bush. 2000. 'Amino acid transporters in plants', *Biochimica et Biophysica Acta -Biomembranes*, 1465: 275-80.

Pätzold, Ralf, and Hans Brückner. 2005. 'Mass spectrometric detection and formation of D-amino acids in processed plant saps, syrups, and fruit juice concentrates', *Journal of agricultural and food chemistry*, 53: 9722-29.

Penrose, Donna M, Barbara A Moffatt, and Bernard R Glick. 2001. 'Determination of 1-aminocycopropane-1-carboxylic acid (ACC) to assess the effects of ACC deaminase-containing bacteria on roots of canola seedlings', *Canadian Journal of Microbiology*, 47: 77-80.

Phillips, Donald A., Tama C. Fox, Maria D. King, T.V. Bhuvaneswari, and Larry R. Teuber. 2004. 'Microbial Products Trigger Amino Acid Exudation from Plant Roots', *Plant physiology*, 136: 2887-94.

Pokorny, M, E Marčenko, and D Keglević. 1970. 'Comparative studies of L-and D-methionine metabolism in lower and higher plants', *Phytochemistry*, 9: 2175-88.

Radkov, Atanas D, Katlyn McNeill, Koji Uda, and Luke A Moe. 2016. 'D-Amino acid catabolism is common among soil-dwelling bacteria', *Microbes and environments*, 31: 165-68.

Ravanel, Stéphane, Bertrand Gakière, Dominique Job, and Roland Douce. 1998. 'The specific features of methionine biosynthesis and metabolism in plants', *Proceedings of the National Academy of Sciences*, 95: 7805-12.

Reynolds, Peter E, and Patrice Courvalin. 2005. 'Vancomycin resistance in enterococci due to synthesis of precursors terminating in D-alanyl-D-serine', *Antimicrobial agents and chemotherapy*, 49: 21-25.

Robinson, Trevor 1976. 'D-amino acids in higher plants', *Life sciences*, 19: 1097-102.

Rogers, HJ, HR Perkins, and JB Ward. 1980. 'Biosynthesis of peptidoglycan.' in, *Microbial cell walls and membranes* (Springer).

Rroço, Evan, Harald Kosegarten, and Konrad Mengel. 2002. 'Importance of plasmalemma H+-ATPase activity for N losses from intact roots of spring wheat (Triticum aestivum L.)', *European journal of agronomy*, 16: 187-96.

Rudrappa, Thimmaraju, Kirk J Czymmek, Paul W Paré, and Harsh P Bais. 2008. 'Root-secreted malic acid recruits beneficial soil bacteria', *Plant physiology*, 148: 1547-56.

Sasse, Joelle, Enrico Martinoia, and Trent Northen. 2018. 'Feed your friends: do plant exudates shape the root microbiome?', *Trends in plant science*, 23: 25-41.

Satoh, Shigeru, and Yohji Esashi. 1980. 'D-Amino-acid-stimulated ethylene production in seed tissues', *Planta*, 149: 64-68.

Satoh, Shigeru, and Yohji Esashi. 1986. 'Inactivation of 1-aminocyclopropane-1-carboxylic acid synthase of etiolated mung bean hypocotyl segments by its substrate, S-adenosyl-L-methionine', *Plant and cell physiology*, 27: 285-91.

Schaller, G Eric, and Brad M Binder. 2017. 'Inhibitors of ethylene biosynthesis and signaling.' in, *Ethylene Signaling* (Springer).

Schimel, Joshua P, and Joy S Clein. 1996. 'Microbial response to freeze-thaw cycles in tundra and taiga soils', *Soil Biology and Biochemistry*, 28: 1061-66.

Schlaeppi, Klaus, Nina Dombrowski, Ruben Garrido Oter, Emiel Ver Loren van Themaat, and Paul Schulze-Lefert. 2014. 'Quantitative divergence of the bacterial root microbiota in *Arabidopsis thaliana* relatives', *Proceedings of the National Academy of Sciences*, 111: 585-92.

Sharma, RK. 2008. *Stereochemistry* (Discovery Publishing House).

Shin, Kihye, Sumin Lee, Won-Yong Song, Rin-A Lee, Inhye Lee, Kyungsun Ha, Ja-Choon Koo, Soon-Ki Park, Hong-Gil Nam, and Youngsook Lee. 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 Cell Physiology*, 56: 572-82.

Shockman, Gerald D, and Gerrit Toennies. 1954. 'Formation of D-methionine from L-by Streptococcus faecalis', *Archives of biochemistry and biophysics*, 50: 9-17.

Sidiq, Karzan R, Man Chow, Zhao Zhao, and Richard Daniel. 2019. 'Alanine Metabolism in *Bacillus subtilis*', *bioRxiv*: 562850.

Staswick, Paul E, and Iskender Tiryaki. 2004. 'The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis', *The Plant Cell*, 16: 2117-27.

Stearns, Jennifer C, Saleh Shah, Bruce M Greenberg, D George Dixon, and Bernard R Glick. 2005. 'Tolerance of transgenic canola expressing 1-aminocyclopropane-1-carboxylic acid deaminase to growth inhibition by nickel', *Plant Physiology and Biochemistry*, 43: 701-08.

Strauch, Renee C, Elisabeth Svedin, Brian Dilkes, Clint Chapple, and Xu Li. 2015. 'Discovery of a novel amino acid racemase through exploration of natural variation in *Arabidopsis thaliana*', *Proceedings of the National Academy of Sciences*, 112: 11726-31.

Sugiyama, A., N. Shitan, and K. Yazaki. 2007. 'Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-Rhizobium symbiosis', *Plant Physiol*, 144: 2000-8.

Svennerstam, H., U. Ganeteg, C. Bellini, and T. Nasholm. 2007. 'Comprehensive screening of Arabidopsis mutants suggests the lysine histidine transporter 1 to be involved in plant uptake of amino acids', *Plant Physiol*, 143: 1853-60.

Szökő, Éva, István Vincze, and Tamás Tábi. 2016. 'Chiral separations for d-amino acid analysis in biological samples', *Journal of pharmaceutical and biomedical analysis*, 130: 100-09.

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

Vranova, Valerie, Helena Zahradnickova, Dalibor Janous, Keith R Skene, Avtar S Matharu, Klement Rejsek, and Pavel Formanek. 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.

Warren, Charles R. 2014. 'Organic N molecules in the soil solution: what is known, what is unknown and the path forwards', *Plant and soil*, 375: 1-19.

Werdin-Pfisterer, Nancy R, Knut Kielland, and Richard D Boone. 2009. 'Soil amino acid composition across a boreal forest successional sequence', *Soil Biology and Biochemistry*, 41: 1210-20.

Wichern, Florian, Ingo Lobe, Wulf Amelung, Torsten Müller, Rainer Georg Joergensen, and Andreas Buerkert. 2004. 'Changes in amino acid enantiomers and microbial performance in soils from a subtropical mountain oasis in Oman abandoned for different periods', *Biology and Fertility of Soils*, 39: 398-406.

Wirtz, Markus, and Michel Droux. 2005. 'Synthesis of the sulfur amino acids: cysteine and methionine', *Photosynthesis Research*, 86: 345-62.

Wright, DE 1962. 'Amino acid uptake by plant roots', *Archives of biochemistry and biophysics*, 97: 174-80.

Yang, Huaiyu, Martin Bogner, York-Dieter Stierhof, and Uwe Ludewig. 2010. 'H+-independent glutamine transport in plant root tips', *PLoS One*, 5: e8917.

Zhu, Qing, William J Riley, and Jinyun Tang. 2017. 'A new theory of plant–microbe nutrient competition resolves inconsistencies between observations and model predictions', *Ecological applications*, 27: 875-86.

Acknowlegement

An dieser Stelle möchte ich allen Leuten danken, die mich während dieser drei Jahre unterstützt haben, mir mit Rat und Tat zur Seite gestanden sind und die mir viele Dinge ermöglicht haben, die ich alleine nicht geschafft hätte. Ein besonderes Dankeschön geht an Üner, der mich in sein tolles Team aufgenommen hat, mir immer mit seinem Rat zur Seite stand und immer für mich da war, wenn ich nicht weiterwusste. Gleichzeitig konnte mich seine wissenschaftliche Begeisterung immer wieder aufs Neue motivieren. Auch besonders danken möchte ich Mark, ohne den ich den Schritt zur Promotion nicht gewagt hätte. Durch sein entgegengebrachtes Vertrauen wurde ich zum einen ermutigt zu promovieren, zum anderen ermöglichte er es mir, sehr selbständig an super teuren Geräten arbeiten zu können. Der gesamten Analytik mit Bettina, Joachim und Edda möchte ich auch für die Unterstützung, das gute Arbeitsumfeld und die guten Gespräche danken. Danke auch an Klaus, mit Hilfe seiner Unterstützung, meine Finanzierung immer irgendwie geklappt hat. Danke für deine Zeit und konstruktiven Beiträge beim Committee-Meeting, auch an Mark, Üner und Prof. Ludewig, der extra immer aus Hohenheim gekommen ist. Prof. Felix, Louis, Max und Volker möchte ich für die Unterstützung bei den Ethylen-Messung danken. Der Ag Schnepf für die Hilfe bei der chemischen Synthese vom HADA. Der gesamten Ag Harter möchte ich nochmal ein extra Dank aussprechen! Juan, Sabine, Xuan, Nata, Thomas, Stefan, Anne, Angela, Nina, Fredi, Leander, ihr seid super! Ihr wart immer sehr geduldig mit mir und dank eurer Hilfe konnte ich mich gut im Labor einarbeiten auch wenn ich mich vor der ein oder anderen molekularbiologischen Standardmethode drücken konnte. Danke auch an die ehemaligen Bachelor StudentInnen Jonas, Vivien und Katja die mir viele zeitaufwändige Experimente abgenommen haben. Ich danke der Happy Hour die immer einen guten Ausgleich zur Arbeit war und mir viele schöne Bier-Stunden mit den ZMBPlern und PClern bescherte. Den größten Ausgleich und liebevollen Beistand hat jedoch mein Freund Matthias geschaffen. Danke dafür und danke, dass es dich gibt.

List of Publications and Contributions

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

II. Suarez, J., Hener, C., Lehnhardt, V., Hummel, S., Stahl, M., & Kolukisaoglu, Ü. 2019. AtDAT1 Is a Key Enzyme of D-Amino Acid Stimulated Ethylene Production in *Arabidopsis thaliana*, *Frontiers in plant science*, 10, 1609.

Contributions:

	Publication I	**Publication II**
Status:	Published	Published
Author position:	1st author	shared 1st author
Scientific ideas:	40%	40%
Data generation:	80%	50%
Analysis & interpretation:	50%	50%
Paper writing:	30%	30%

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.

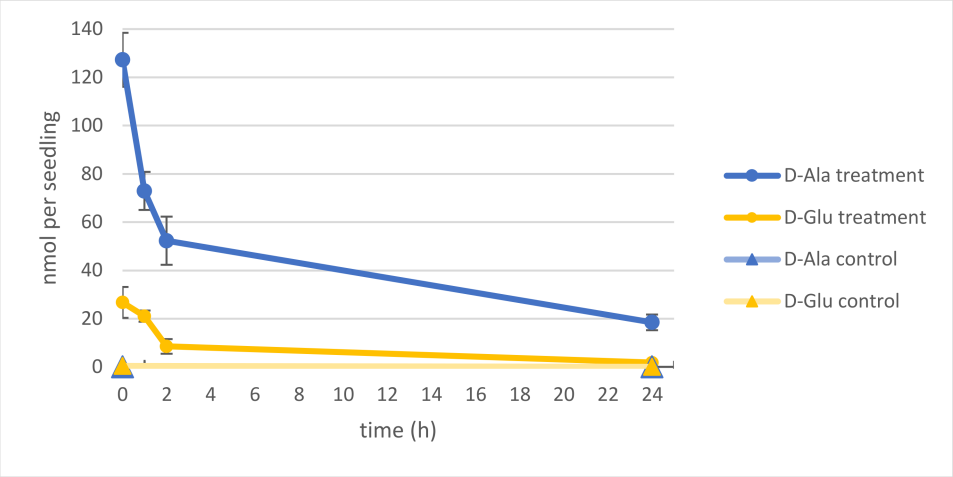
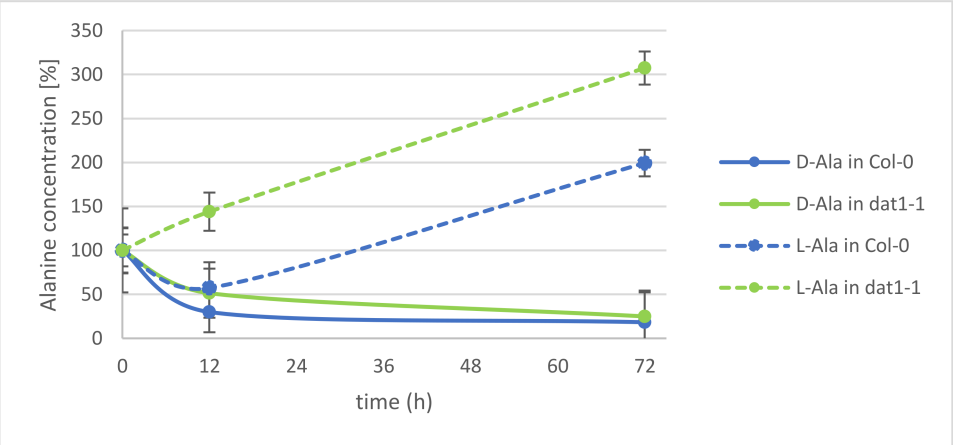


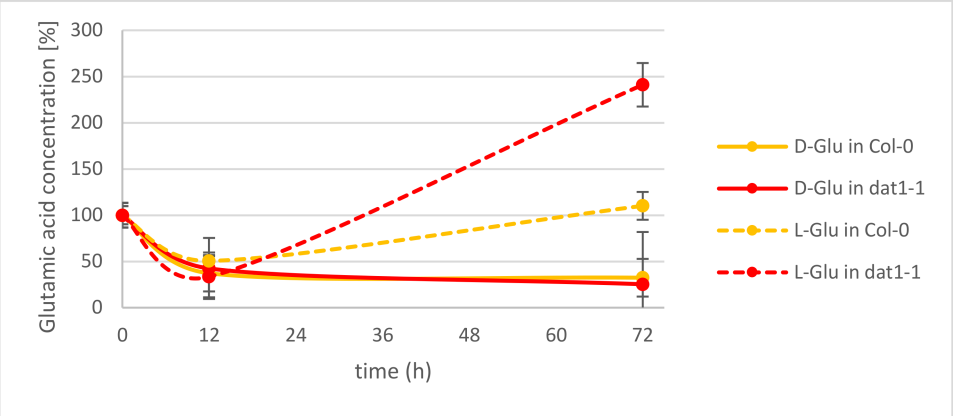
Figure 1. Decrease of D-Ala and D-Glu levels in *Arabidopsis* seedlings within 24 h. Dark blue lines represent the D-Ala and dark yellow lines the D-Glu contents of seedlings, respectively. Measurements from D-amino acid (AA)-treated plants are marked with circles; untreated control plants with lighter 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.

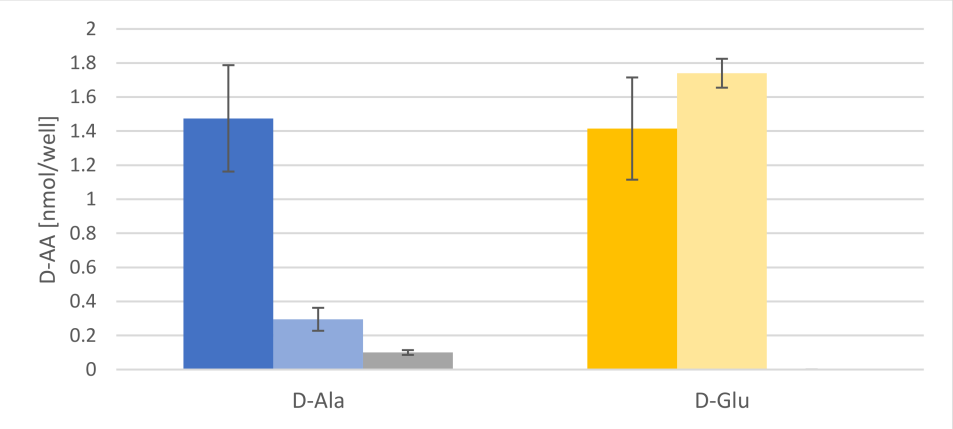
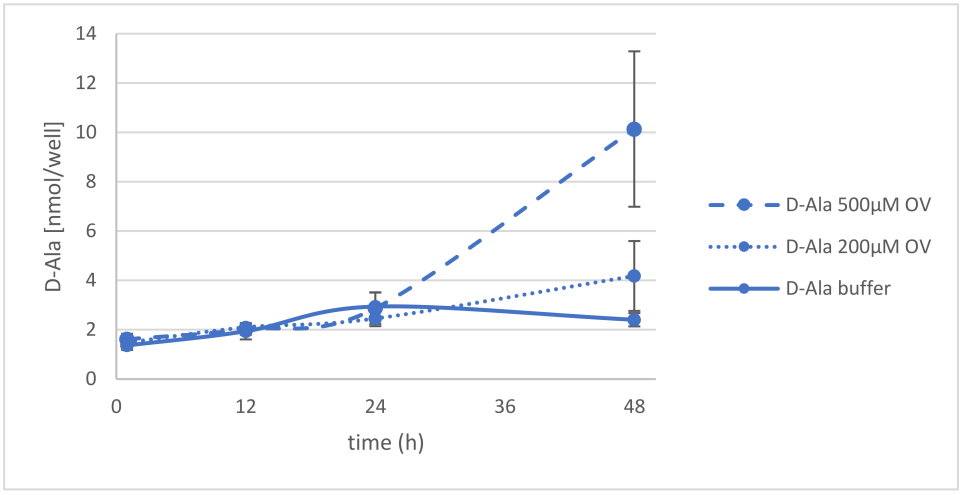


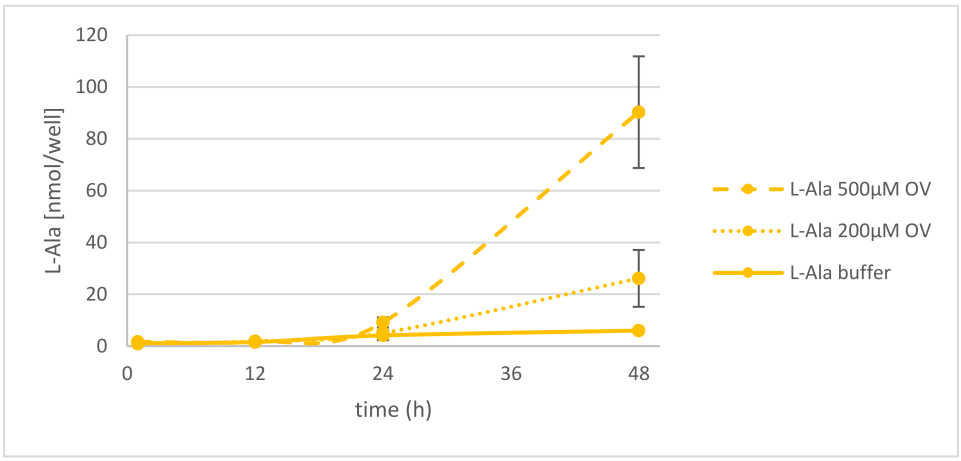
Figure 3. Contents of D-Ala and D-Glu in media released by *Arabidopsis* seedlings treated with both D-AAs. Dark blue and light blue bars represent D-Ala content per well 12 and 72 h after transfer from the D-Ala application medium to fresh medium, respectively. The grey bar represents the 72 h values 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**)

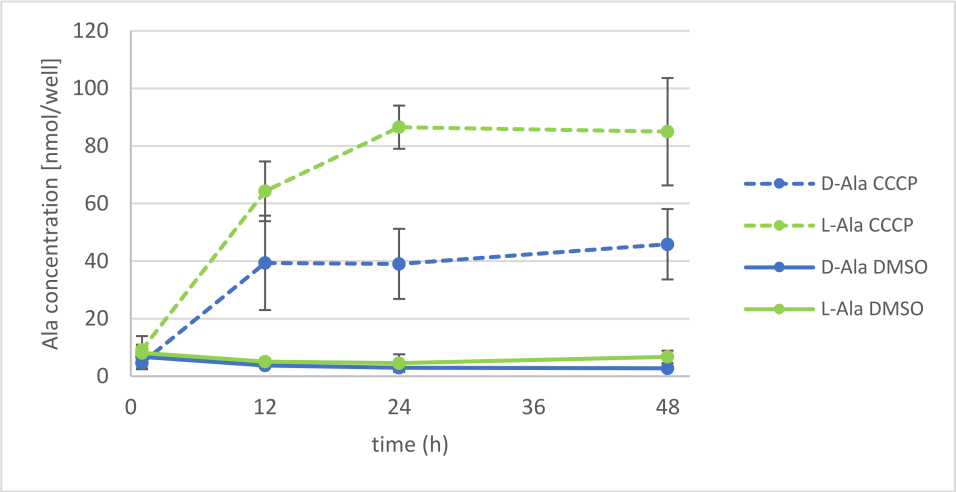


(**B**)

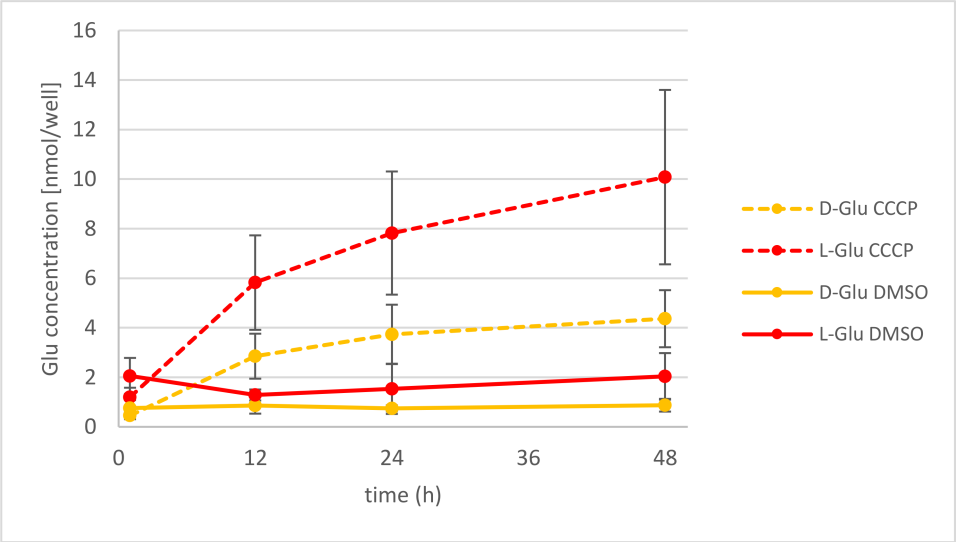
Figure 4. Release of D- and L-Ala into the medium under Na-orthovanadate (OV) treatment. Seedlings were treated with D-Ala and then transferred to fresh medium without OV (solid line), with 200 µM OV (dotted line), and with 500 µM OV (dashed line). Then, the D-Ala (**A**) and L-Ala (**B**) contents in 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).



(**A**)



(**B**)

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

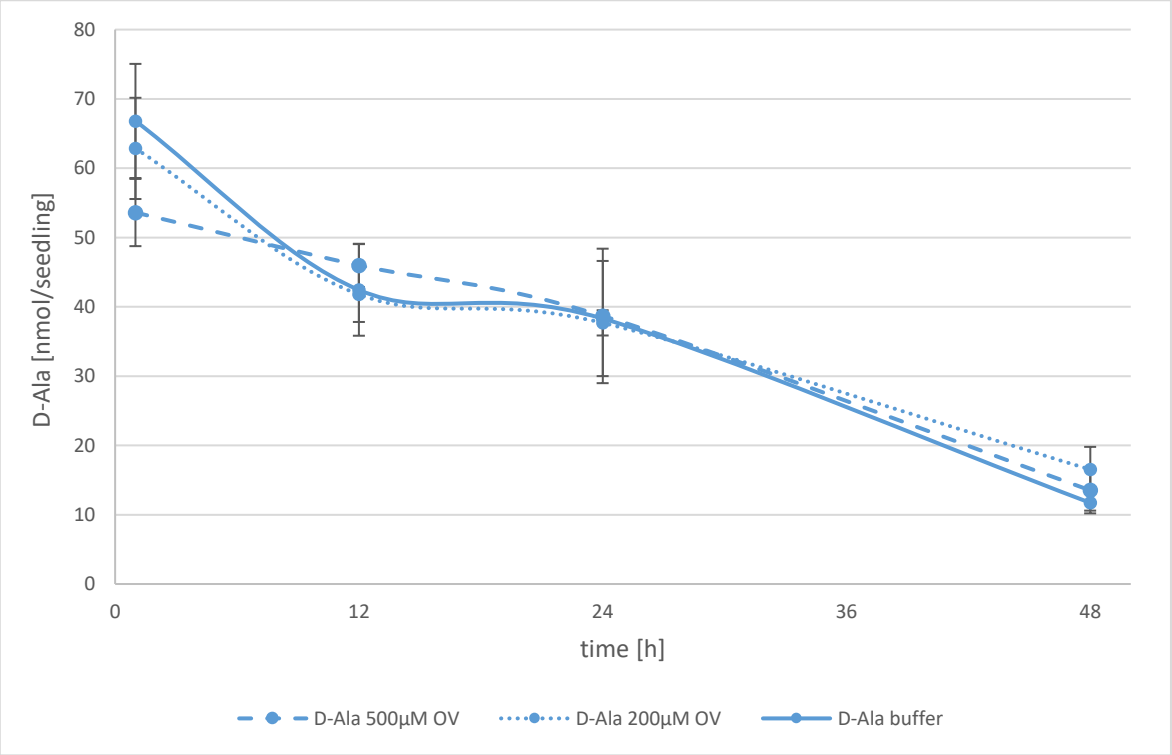
1. Typas, A.; Banzhaf, M.; Gross, C.A.; Vollmer, W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat. Rev. Microbiol.* **2011**, *10*, 123–136. [CrossRef] [PubMed]
2. Ollivaux, C.; Soyez, D.; Toullec, J.Y. Biogenesis of D-amino acid containing peptides/proteins: 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]

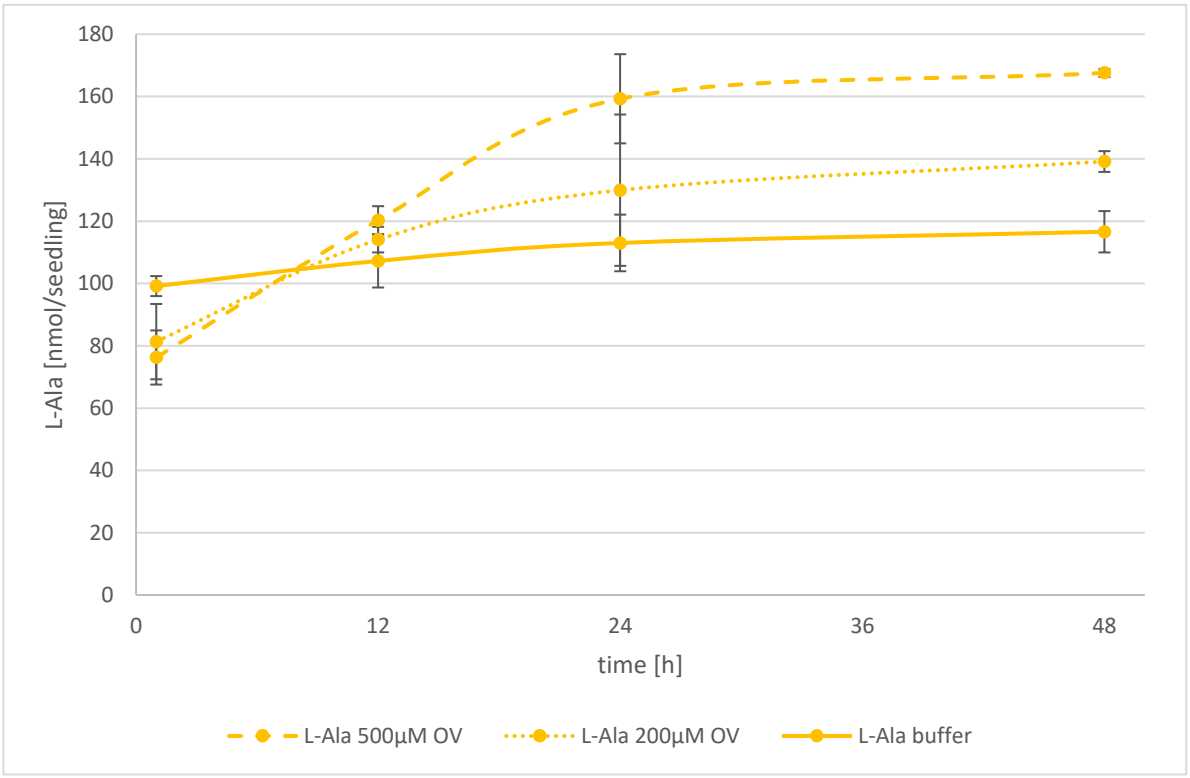
40. Bais, H.P.; Weir, T.L.; Perry, L.G.; Gilroy, S.; Vivanco, J.M. The role of root exudates in rhizosphere interations with plants and other organisms. *Annu. Rev. Plant Biol.* **2006**, *57*, 233–266. [CrossRef] [PubMed]
41. Mendes, R.; Garbeva, P.; Raaijmakers, J.M. The rhizosphere microbiome: Significance 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]

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

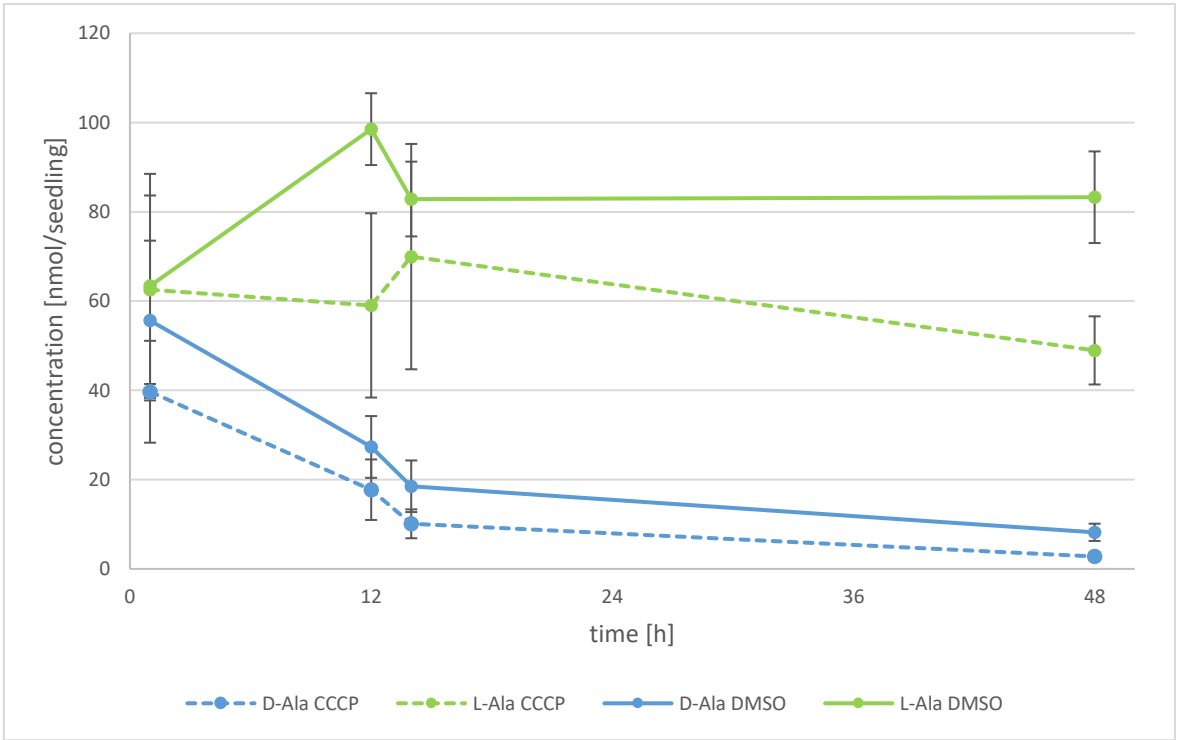


(**A**)

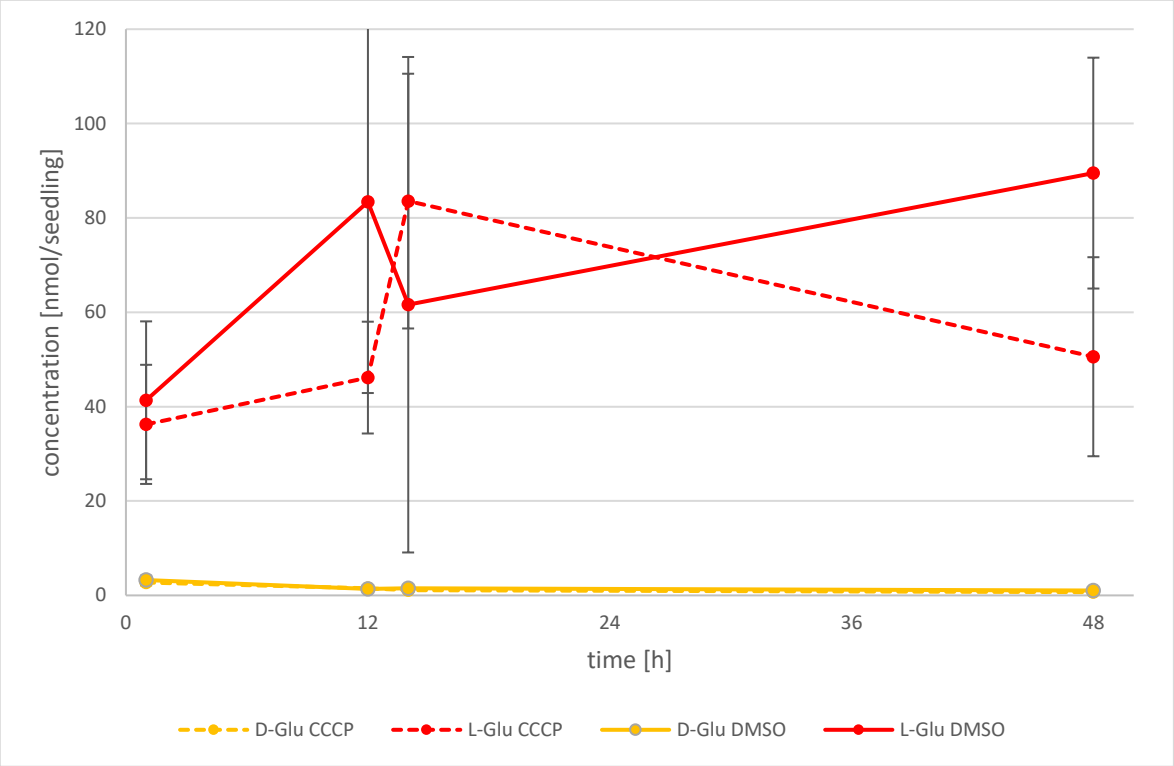


(**B**)

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 December 2019
doi: 10.3389/fpls.2019.01609

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 and Üner Kolukisaoglu*[*]

Center for Plant Molecular Biology (ZMBP), University of Tübingen, Tübingen, Germany

OPEN ACCESS

Edited by:
Anna N. Stepanova, North Carolina State University, United States

Reviewed by:
Jin-Song Zhang, Institute of Genetics and Developmental Biology (CAS), China
Bram Van De Poel, KU Leuven, Belgium

***Correspondence:**
Üner Kolukisaoglu
uener.kolukisaoglu@zmbp.uni-tuebingen.de

[†]*These authors share first authorship*

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *26 July 2019*
Accepted: *15 November 2019*
Published: *12 December 2019*

Citation:
Suarez J, Hener C, Lehnhardt V-A, Hummel S, Stahl M and Kolukisaoglu Ü (2019) AtDAT1 Is a Key Enzyme of D-Amino Acid Stimulated Ethylene Production in Arabidopsis thaliana. Front. Plant Sci. 10:1609. doi: 10.3389/fpls.2019.01609

D-Enantiomers of proteinogenic amino acids (D-AAs) are found ubiquitously, but the knowledge about their metabolism and functions in plants is scarce. A long forgotten phenomenon in this regard is the D-AA-stimulated ethylene production in plants. As a starting point to investigate this effect, the *Arabidopsis* accession Landsberg *erecta* (*Ler*) got into focus as it was found defective in metabolizing D-AAs. Combining genetics and molecular biology of T-DNA insertion lines and natural variants together with biochemical and physiological approaches, we could identify AtDAT1 as a major D-AA transaminase in *Arabidopsis*. *Atdat1* loss-of-function mutants and *Arabidopsis* accessions with defective *AtDAT1* alleles were unable to produce the metabolites of D-Met, D-Ala, D-Glu, and L-Met. This result corroborates the biochemical characterization, which showed highest activity of AtDAT1 using D-Met as a substrate. Germination of seedlings in light and dark led to enhanced growth inhibition of *atdat1* mutants on D-Met. Ethylene measurements revealed an increased D-AA stimulated ethylene production in these mutants. According to initial working models of this phenomenon, D-Met is preferentially malonylated instead of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). This decrease of ACC degradation should then lead to the increase of ethylene production. We could observe a reciprocal relation of malonylated methionine and ACC upon D-Met application and significantly more malonyl-methionine in *atdat1* mutants. Unexpectedly, the malonyl-ACC levels did not differ between mutants and wild type. With AtDAT1, the first central enzyme of plant D-AA metabolism was characterized biochemically and physiologically. The specific effects of D-Met on ACC metabolism, ethylene production, and plant development of *dat1* mutants unraveled the impact of AtDAT1 on these processes; however, they are not in full accordance to previous working models. Instead, our results imply the influence of additional factors or processes on D-AA-stimulated ethylene production, which await to be uncovered.

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

INTRODUCTION

It is widely accepted that proteinogenic L-amino acids (L-AAs) 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 amino acids (D-AAs) are far less clear. Major reasons for this discrepancy 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 that 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 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, mainly 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). However, several reports suggested that D-AAs take up a similarly crucial position in plants as in microbes and animals [for further readings about D-AAs in microbes and animals, see Konno et al. (2007) and Brückner (2011)]. For instance, the D-Ala amount in duckweed (*Landoltia punctata*) was demonstrated to increase during UV light stress (Monselise et al., 2015). Furthermore, D-Ser is involved in pollen 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 (*Physcomitrella patens*), D-Ala and D-Glu were detected in the plastidial envelope, similar 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 implies much more functions for these AAs than currently known (Naranjo-Ortíz et al., 2016). However, it also raises the question about their metabolism in plants, especially how the abundance of different D-AAs is regulated. On the one hand, their content has 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 facts that the rhizosphere is the major natural source of D-AAs for plants (Vranova et al., 2012) and that D-AAs are taken up by roots in considerable amounts (Hill et al., 2011; Gördes et al., 2013). In this respect, the question arises which processes facilitate the catabolism of D-AAs in plants.

In the course of our previous studies, D-Met got into our focus because of its highest conversion rates in almost all tested accessions of *Arabidopsis thaliana* except in L*er* (Gördes et al., 2013), although 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 that 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 and other D-AAs to seedlings of cocklebur (*Xanthium pennsylvanicum*), pumpkin (*Cucurbita moschata*), sunflower (*Helianthus annuus*), mung bean (*Vigna radiata*), water melon (*Citrullus vulgaris*), and pea (*Pisum sativum*) leads to increased ethylene production (Satoh and Esashi, 1980; Liu et al., 1983; Kionka and Amrhein, 1984). This phenomenon was characterized as "D-amino-acid-stimulated ethylene production" (Satoh and Esashi, 1980). The authors tried to explain the effect by competitive malonylation of D-Met and 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene. According to this hypothesis, D-Met would compete with ACC for the same 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 ethylene production (Yang and Hoffman, 1984). However, this hypothesis could not be verified because the corresponding malonyl transferase has not been identified to date.

As shown previously, *Arabidopsis* plants are able to convert particular D-AAs like D-Met, D-Trp, D-Phe, and D-His to their respective L-enantiomers (Gördes et al., 2011). Additionally, the feeding of almost all tested D-AAs led mainly to the formation of D-Ala and D-Glu. In contrast, the *Arabidopsis* accession Landsberg *erecta* (L*er*) is incapable of both the D-AA to L-AA and the D-AA to D-Ala/D-Glu conversion (Gördes 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; Gördes et al., 2013).

Here, 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 D-AAT (Funakoshi et al., 2008), which we named AtDAT1. This enzyme has been shown before to have a second

enzymatic function as an aminodeoxychorismate lyase (ADCL) in the synthesis of p-aminobenzoate, a folate precursor (Basset et al., 2004). Nevertheless, a physiological role could not be assigned to the AtDAT1 encoding gene in plants to date. Most interestingly, the homolog of *AtDAT1* in *Plasmodium falciparum* also displays such a dual function and the ADCL activity is repressed by D-AAs (Magnani et al., 2013). Loss-of-function mutants of *AtDAT1* showed almost identical defects as L*er* in D-AA metabolism, with D-Met as strongest effector. Indeed, we could show that the affected gene in L*er* encodes for an almost non-functional AtDAT1 isoform. Biochemical analyses revealed that this enzyme prefers D-Met as amino donor and pyruvate over 2-oxoglutarate as amino acceptor, confirming the preferential production of D-Ala in Col-0. The discovery of *AtDAT1* and its mutants gave us also the opportunity to verify the working model of D-AA-stimulated ethylene production in plants. We found that D-Met application causes significantly higher ethylene production and growth inhibition in *atdat1* seedlings compared to wild type. According to the current working model, the increase in ethylene should be caused by a decrease in malonylation of ACC due to the increase of malonyl-D-Met, leading to a higher ACC oxidation. Although we found higher malonyl-methionine concentrations in *atdat1* seedlings after D-Met application, the malonyl-ACC levels decreased equally in mutants and their respective wild type. This points to an additional, yet unraveled, mechanism regulating D-AA-stimulated ethylene production in plants. Nevertheless, our findings indicate functions of D-Met in defined plant processes beyond unspecific growth inhibition.

MATERIALS AND METHODS

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).

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 6 days in darkness or 10 days in light (all at 22 °C). As solid growth media ½ MS basal 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.

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 **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 products were sequenced directly by GATC (Konstanz, Germany). For cDNA synthesis RNA of 14 days old seedlings germinated in liquid media under long day conditions 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 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 L*er,* 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 (**Table S1**). PCR products 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 from Invitrogen (Karlsruhe, Germany) according to manufacturer's protocol into pGEX-2TM-GW (kindly provided by 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-terminal GFP tag. pENTR-AtDAT1$_{(Col\text{-}0)}$ was used for Gateway-based cloning into pUB-DEST (Grefen et al., 2010) for complementing *AtDAT1* defective plants.

Arabidopsis Transformation and Tobacco Leaf Infiltration

All plant transformation vectors were transformed into *Agrobacterium tumefaciens* cv. pMP90-RK GV3101. Plant transformation was performed by floral dipping (Clough and Bent, 1998). For selection of transformants, seeds were either germinated on ½ MS-Agar with 1% sucrose containing hygromycin or germinated on soil and sprayed with 2%

BASTA from AgrEvo (Düsseldorf, Germany) depending on the used vector.

For tobacco leaf infiltration transformed *Agrobacterium* containing pUB10-GFP::DAT1 was mixed with a strain of transformed *Agrobacterium* for expression of the mCherry plastid marker (CD3-999 pt-rk; Nelson et al., 2007) and P19 *A. tumefaciens* cells into infiltration media [10 mM MES-KOH (pH 5.7), 10 mM, $MgCl_2$, 0.2 mM Acetosyringone]. Using a syringe 1 ml of infiltration media with the mix of the three types of cells was infiltrated in the abaxial side of *Nicotiana benthamiana* leaves. Plants were then watered and kept on the lab bench for 2 days. Afterwards, single leaf discs were excised for confocal fluorescence microscopy.

Fluorescence Microscopy

Imaging was performed using a Leica laser scanning microscope SP8 with the corresponding software LCS or LASAF X (Leica Microsystems, Wetzlar, Germany). For excitation of GFP-fusion proteins, the Argon laser was used at 488 nm and the detection range was from 500 to 550 nm. For m-RFP excitation was set to 561 nm and detection was from 600 to 650 nm. All autofluorescence of chloroplasts was detected in the range from 670 to 725 nm.

Promoter::GUS Transgenic Analysis

The promoter region from -677 to +11 of the genomic locus of *AtDAT1* from Col-0 and L*er* were amplified by PCR with the primer pair ProDAT1-SGW/ProDAT1-AGW (for sequences, see **Table S1**). The respective fragment was cloned into 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 × 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

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 3,000 V and a resolution of 20,000. Separation of the amino acids was carried out on a Waters Acquity C_{18} HSS T3, 1.0 × 150 mm, 1.8 µm column with a flow rate of 50 µl/min and a 22 min gradient from 70% water to 99% methanol (both with 0.1 % formic acid). For quantification, 3 µl of sample was injected and a 5-point calibration from 0.125 to 1,250 µM was used.

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 a Waters Acquity C_{18} HSS T3, 2.1 × 100 mm, 1.8 µm column, and a flow rate of 0.2 ml/min with a 15 min gradient from 99% water to 99% methanol (both with 0.1% formic acid) was used for separation. The malonylated compounds were identified by the exact mass of their molecular ion followed by a MS/MS fragmentation.

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 6 days. The vials were closed with rubber septa and opened once 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 were analyzed with IBM SPSS Statistics 24. Significance levels were analyzed using an independent two-sided Student's t-test. For further analyses between and within genotypes, we used an ANOVA followed by *post hoc* tests, Gabriel, or Games-Howell, depending on the equality of variances. For testing the homogeneity of variances, a Levene test was applied.

RESULTS

AtDAT1 as a Candidate Gene for D-AAs Metabolism

Initially, we observed the strong decrease of both D-AA to L-AA and especially D-AA to D-Ala/D-Glu conversion rates in L*er* in comparison to other ecotypes (Gördes et al., 2013). According to the transamination hypothesis, the mutation of at least one D-AA specific transaminase could be responsible for this metabolic phenotype. One candidate protein had been previously identified biochemically to be such an enzyme, named AtDAAT1 (Funakoshi et al., 2008). To investigate its role *in planta* we started to analyze T-DNA insertion lines of the corresponding gene (At5g57850; afterwards designated as *AtDAT1*) regarding their D-AA metabolism.

Homozygous plants of such insertion lines, SALK_011686 and SALK_111981 (denoted as *dat1-1* and *dat1-2,* respectively; **Figure 1A**), were isolated and propagated for further analyses (see **Table S1** for primer sequences). RT-PCR analysis of *AtDAT1* expression displayed no transcripts with the given primer combination in *dat1-1* and *dat1-2* mutants compared to the corresponding wild type (Col-0) (**Figure 1B**). As observed previously (Lempe et al., 2005), the *AtDAT1* transcript level in L*er* seedlings was similar to that of wild-type Col-0. Feeding with D-Met caused the highest accumulation of D-Ala, D-Glu, and its respective L-enantiomer in Col-0 seedlings of all tested D-AAs. Therefore, seedlings of the *dat1-1* and *dat1-2* mutants, Col-0 and Ler were grown for 14 days on liquid ½ MS medium in light, then supplemented with D-Met and subsequently analyzed for their AA contents. In sharp contrast to Col-0, both *AtDAT1* insertion mutants were neither able to produce D-Ala, D-Glu, nor additional L-Met after application of D-Met. This AA profile was similar to that found in seedlings of the L*er* accession (**Figure 1C**).

Further *in silico* analyses of public transcriptomic data (Lempe et al., 2005) revealed that the accession M7323S displayed a strongly reduced *AtDAT1* transcript level, which could be confirmed by RT-PCR (**Figure 1B**). When this accession was grown on D-Met supplemented medium, defects in AA metabolism were observed (**Figure S1**) similar to those found in L*er* and the *dat1* mutant seedlings. This defect was not just due to the reduced transcription of *AtDAT1* in M7323S. Sequencing of the genomic locus and the cDNA of *AtDAT1* from M7323S revealed that this gene contains a T→A mutation at genomic position +1259. This leads to a nonsense mutation at the third position of a cysteine codon (TGT) to a stop codon (TGA) at position 248 of the AA sequence (C248STOP) (**Figure 1A**). In contrast, sequencing of the genomic locus and the cDNA of *AtDAT1* from L*er* revealed two missense mutations leading to AA exchanges of the protein sequence (A77T and T303S) (**Figure 1A**).

To examine whether these mutations in the *AtDAT1* L*er* allele are responsible for the metabolic aberrations in this accession, we performed different genetic approaches. First, ubiquitin promoter-driven expression of the *AtDAT1* Col-0 allele in transgenic L*er* plants led to the reconstitution of the D-Met metabolism in L*er* and its complementation in the *dat1-2* mutant (**Figure 2A**). Second, F1 seedlings derived from crosses between Col-0 and L*er* and between Col-0 and *dat1-2* displayed no defects in D-Met metabolism as observed in L*er* and *dat1-2*, irrespective of the maternal origin, whereas the offspring of the L*er* x *dat1-2* crossing did (**Figure 2B**). These data prove the defect of *AtDAT1* function in the L*er* accession and the *dat1-2* insertion mutant.

To answer the remaining question about the reason for this defect in L*er*, the expression of *AtDAT1* was analyzed. As mentioned before, the *AtDAT1* transcript levels appeared similar in Col-0 and L*er* (**Figure 1B**). This observation was supported by analysis of transgenic plants containing the *uidA* reporter gene (GUS) under the control of the *AtDAT1* promoter either from the Col-0 or L*er* allele (**Figures S2A**, **B**). There, it can be seen that the reporter constructs are active in seedlings and

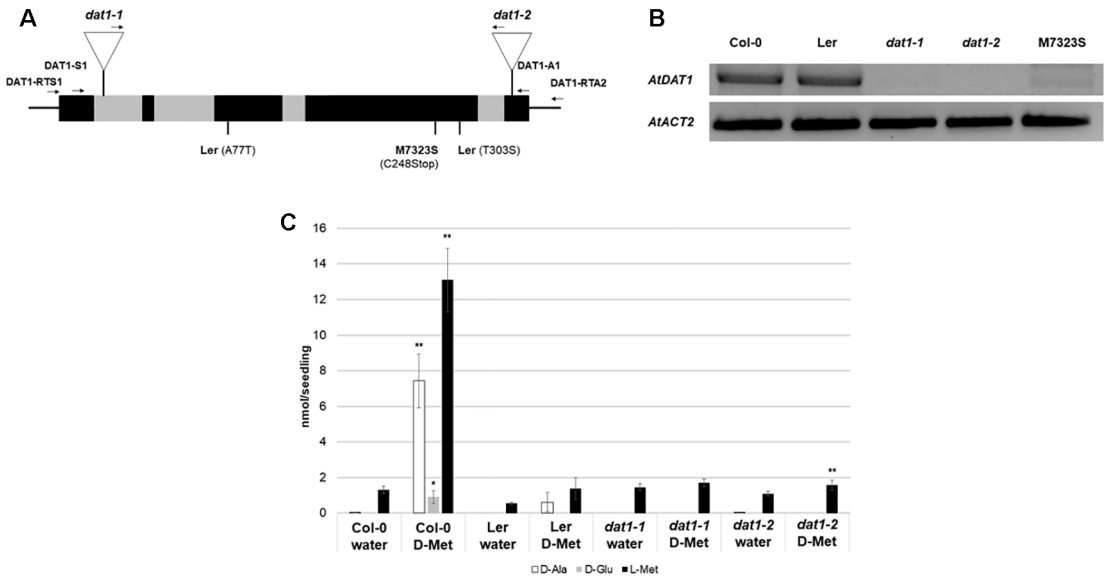


FIGURE 1 | AtDAT1 as a candidate protein for the metabolism of D-AAs in *Arabidopsis.* **(A)** Scheme of the genomic structure of *AtDAT1* (exons and introns in black and grey, respectively) with the positions of T-DNA insertions in *dat1-1* and *dat1-2* as well as the mutations found in L*er* and M7323S. 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, L*er*, *dat1-1, dat1-2*, and M7323S (top: *AtDAT1*; bottom: *AtACT2*). **(C)** Contents of D-Ala (white), D-Glu (gray), and L-Met (black) in seedlings of Col-0, L*er*, *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. The asterisks indicate the significance level (t-test) of differences of all measurements to the respective line without D-Met treatment (*p < 0.05; **p < 0.01).

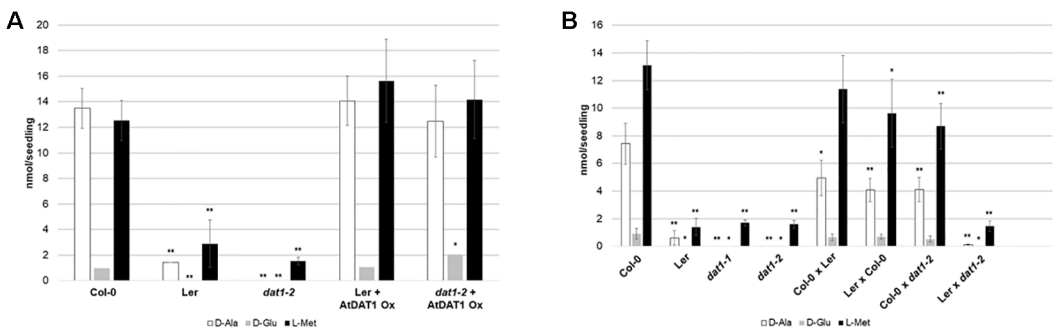


FIGURE 2 | D-Met metabolism in lines overexpressing *AtDAT1* and in F1 seedlings from crosses of Col-0, L*er* and *dat1-2*. Contents of D-Ala, D-Glu, and L-Met after overnight exposure to D-Met **(A)** in L*er* and *dat1-2* seedlings overexpressing *AtDAT1* (AtDAT1 Ox) and their corresponding background lines and **(B)** in seedlings of F1 progeny of crosses of Col-0, L*er*, and *dat1-2* and their corresponding parental lines; for further information, see **Figure 1C** (*p < 0.05; **p < 0.01).

adult plants and with less GUS staining in late floral stages and seeds (**Figure S2A**), corresponding to expression patterns displayed in the eFP browser (Winter et al., 2007). The activity of the *AtDAT1* promoters derived from Col-0 and L*er* showed no apparent differences, irrespective of the presence of L-Met or D-Met in the media (**Figure S2B**). Subcellular mislocalization would have been another explanation for affected *AtDAT1* function in L*er*. Therefore, GFP-tagged *AtDAT1* gene variants derived from cDNA of both ecotypes expressed under the control of the ubiquitin 10 promoter were transiently transformed into tobacco leaves (**Figure S3**). The Col-0 as well as the Ler cDNA derived AtDAT1 fusion proteins localized to the chloroplasts, as it had been shown before for GFP-tagged $AtDAT1_{(Col\text{-}0)}$ (Basset et al., 2004). Therefore, a possible mis-expression of *AtDAT1* or its mis-localization of AtDAT1-GFP in L*er* does not cause the aberrant D-Met metabolism in this accession.

A Missense Mutation of the *AtDAT1* L*er* Allele Leads to an Almost Complete Loss of the Enzymatic Activity

To clarify if the enzyme encoded by the L*er AtDAT1* allele is able to transaminate D-AAs, the L*er* ($AtDAT1_{(Ler)}$) and Col-0 ($AtDAT1_{(Col\text{-}0)}$) versions of AtDAT1 were expressed with an

N-terminal GST-tag in *E. coli*. After purification by affinity chromatography (for purification results, see **Figure S4**), their enzymatic activities were tested according to Funakoshi et al. (2008).

We first tested $AtDAT1_{(Col\text{-}0)}$ for its capability to transaminate 2-oxoglutarate (2-OG) or pyruvate using 16 different D-AAs as amino group donors. With 2-OG used as amino group acceptor, a transaminase reaction was only detectable for the donors D-Met, D-Trp, and D-Ala (**Table S2**), whereas with pyruvate as acceptor, almost all D-AAs, with the exception of D-Pro, led to the formation of 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 applied 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 pyruvate and D-Met 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 characterize the activity of $AtDAT1_{(Ler)}$ in comparison to $AtDAT1_{(Col\text{-}0)}$, enzymatic assays were performed with two substrate combinations: first, with D-Met as amino group donor and pyruvate as acceptor, respectively, as the best substrate combination for $AtDAT1_{(Col\text{-}0)}$ and, second, with D-Ala as amino group donor and 2-OG as acceptor. As shown in **Figures 4A** and B for both substrate combinations, the activity of $AtDAT1_{(Ler)}$ dropped to 0–5% compared to that of $AtDAT1_{(Col\text{-}0)}$.

We next addressed the question whether only one of the missense mutations in $AtDAT1_{(Ler)}$ (A77T or T303S) is sufficient to cause the activity loss. The alignment of DAT1 amino acid sequences from different plant species revealed that the alanine at position 77 is more conserved than the threonine at position 303 (**Figure S5**). To analyze the impact of the mutations, $AtDAT1_{(Col\text{-}0)}$ derived isoforms harboring single amino acid exchanges of $AtDAT1_{(Ler)}$ were also expressed as N-terminal GST fusions in *E. coli*. The recombinant proteins were affinity-purified and tested for their activity. The enzyme isoform with the T303S amino acid exchange $AtDAT1_{(T303S)}$ showed an activity comparable to $AtDAT1_{(Col\text{-}0)}$ (**Figures 4A**, **B**). In contrast, the mutation A77T led to a strong decrease in the production of D-Glu (**Figure 4A**) and D-Ala (**Figure 4B**) with 2-oxoglutarate or pyruvate as substrates, respectively. Instead, the enzymatic defect of $AtDAT1_{(A77T)}$ was quantitatively similar to that of $AtDAT1_{(Ler)}$. From these data, we conclude that solely the A77T amino acid exchange is responsible for the activity loss of $AtDAT1_{(Ler)}$. Furthermore, the enzymatic data also revealed that the L*er* variant of AtDAT1 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 of D-AA metabolism, the question arose whether the loss of *AtDAT1* gene function leads to defects in *Arabidopsis* growth and development. Under greenhouse conditions in soil growth of *dat1-1* and *dat1-2* mutant plants could not be distinguished from Col-0 (**Figure S6**). We next asked of how the mutant lines and L*er* would grow in presence of D-Met. Growth of *dat1-1* and *dat1-2* seedlings on

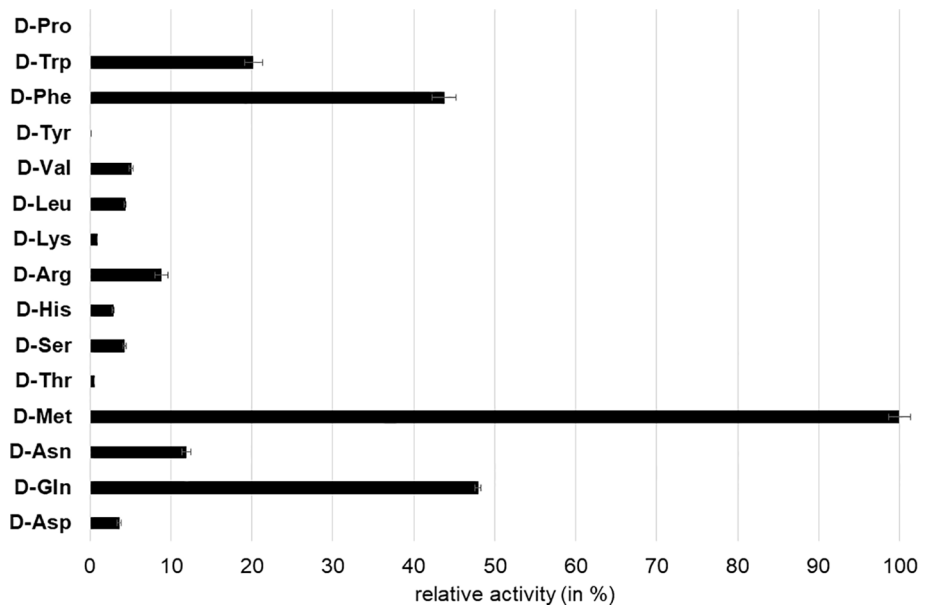


FIGURE 3 | Relative D-Ala producing activity of AtDAT1 with different D-AAs as amino group donor and pyruvate as acceptor. Activity of reaction with D-Met was set to 100% and all other reactions were calculated in relation to it. Each bar represents the mean of measurement of three independent assays.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

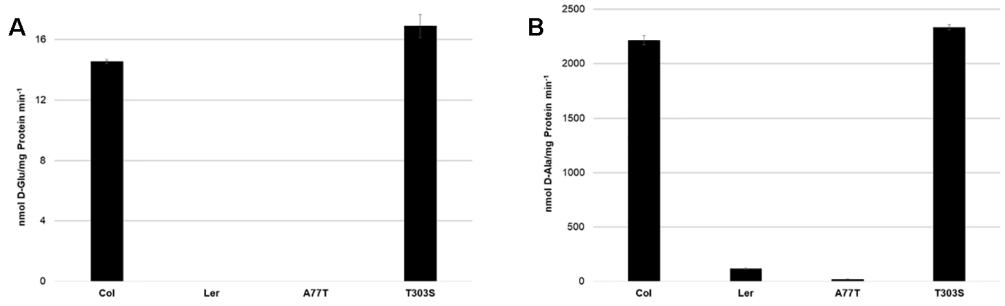


FIGURE 4 | Activities of AtDAT1 variants. Transaminase activities of AtDAT1$_{(Col\text{-}0)}$, AtDAT1$_{(Ler)}$, AtDAT1$_{(A77T)}$, and AtDAT1$_{(T303S)}$ with D-Met as amino group donor and **(A)** 2-oxoglutarate or **(B)** pyruvate as acceptor molecule are displayed; for further information, see **Figure 3**.

media containing 500 µM D-Met resulted in a retardation compared to the corresponding wild type, whereas L*er* took an intermediate response (**Figure 5A**). Testing this growth behavior on the dark-grown etiolated seedlings revealed an even more pronounced growth difference between the *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**). Altogether, D-Met inhibited seedling growth specifically in *AtDAT1* affected lines.

The reduced growth of hypocotyls and roots of *dat1-1* and *dat1-2* seedlings, especially in the dark (**Figure 5A**), reminded of phenotypes caused by the gaseous plant hormone ethylene. This gets even clearer with a look on the hypocotyl length of the four dark grown lines (**Figure 5B**): 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. Although increasing L-Met concentrations also led to shorter hypocotyls, this effect was similar in mutant and wild-type plants. Furthermore, the growth inhibition was by far not as strong as with D-Met (**Figure S7**).

AtDAT1 Mutants Display Enhanced D-AA Stimulated Ethylene Production

To test whether ethylene synthesis is indeed affected in *dat1* mutants by D-Met, we added α-aminoisobutyric acid (AIB) to the growth medium, which leads to the inhibition of ACC oxidase, the enzyme catalyzing the last step of ethylene synthesis (Satoh and Esashi, 1980). As shown in **Figure 6A**, the addition of 2 mM AIB to the growth medium led to a reversion of growth reduction by D-Met of all *dat1* affected lines in the dark. This indicates that the increased ethylene production in these lines is caused by D-Met in the medium.

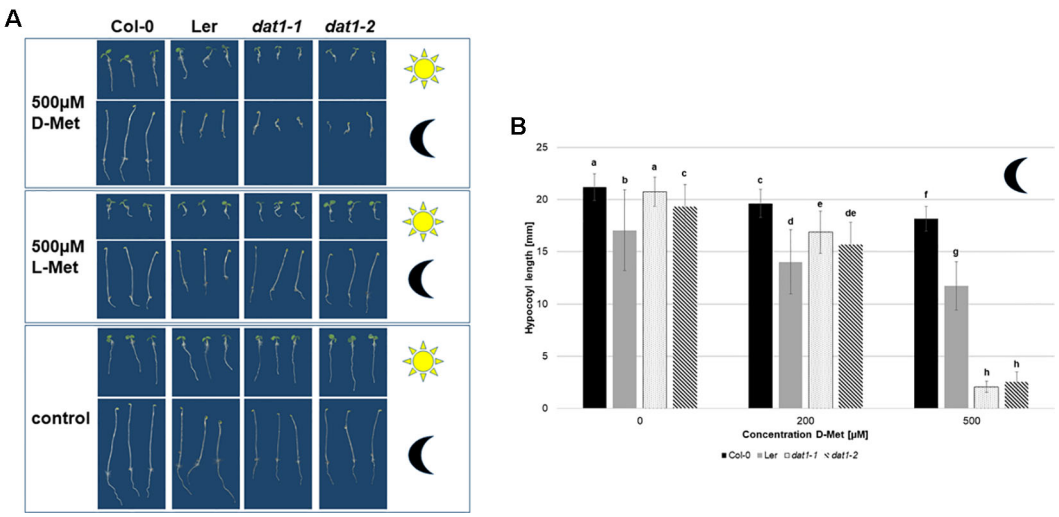


FIGURE 5 | Seedling growth is differentially suppressed by D-Met in *AtDAT1* knock out-lines. **(A)** Seeds of Col-0, *dat1-1*, and *dat1-2*, and L*er* were germinated either in continuous light (sun) or darkness (moon) on different solid growth media (with 500 µM D-Met, with 500 µM L-Met supplemented or without supplementation). **(B)** Hypocotyl growth of the before mentioned dark grown plants. The bars (Col-0: black, L*er*: grey, *dat1-1*: dotted, *dat1-2*: striped; n = 30) represent the average hypocotyl length. Different letters indicate statistically significant differences ($p < 0.05$) tested by an ANOVA. Error bars (± SD).

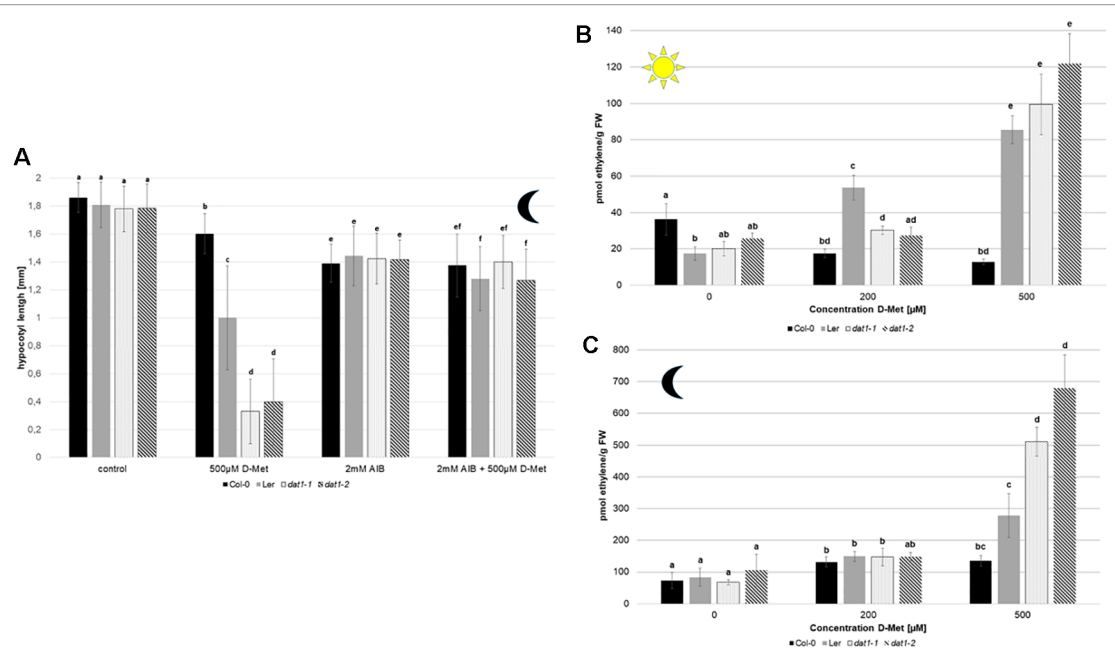


FIGURE 6 | D-Met leads to an increase of ethylene in *AtDAT1* knock out-lines. **(A)** Seeds of Col-0, *dat1-1*, and *dat1-2*, and L*er* were germinated in continuous darkness on solid growth media without any supplementation (control), supplemented just with 500 µM D-Met, supplemented just with 2 mM AIB, and supplemented with both agents together. For further information, see **Figure 5B**. **(B)** Ethylene contents in seedlings of Col-0, L*er*, *dat1-1*, and *dat1-2* were measured after growth in continuous light or **(C)** in darkness in vials with solid growth media supplemented with 200 and 500 µM D-Met, and additionally without supplementation. The bars (Col-0: black, L*er*: gray, *dat1-1*: dotted, *dat1-2*: striped) represent the average values of three biological replicates. Different letters indicate statistically significant differences ($p < 0.05$) tested by an ANOVA. Error bars (± SD).

To elucidate if ethylene production was indeed altered, we measured its content in L*er*, the *dat1* mutants, and Col-0 grown in continuous light and dark. The addition of 500 µM D-Met was sufficient to induce a significant increase of up to threefold of ethylene production in light grown L*er* and *dat1* mutants compared to Col-0 (**Figure 6B**). Even stronger changes in ethylene production could be observed for both *dat1* mutant seedlings grown in the presence of D-Met in the dark, whereas L*er* displayed again an intermediate phenotype (**Figure 6C**).

As mentioned above, the increase of ethylene production by D-AAs was attributed to competitive malonylation of D-AAs instead of ACC, which should lead to enhanced ACC oxidation resulting in higher ethylene concentration (Yang and Hoffman, 1984). To verify this assumption, we measured the contents of malonyl-methionine and malonyl-ACC in D-Met treated seedlings. In these measurements, we detected a significant increase of malonyl-methionine in Col-0, L*er*, and *dat1* seedlings upon D-Met treatment (**Figures 7A**, **B**). This accumulation was far higher (up to fivefold) in the *dat1* mutants compared to the corresponding wild type, irrespective of the light regime (**Figures 7A**, **B**). Furthermore, L*er* also showed a D-Met induced over-accumulation of malonyl-methionine in the light (**Figure 7A**), but not in darkness (**Figure 7B**).

Since the amount of malonyl-ACC in these experiments was below our detection limit, we added 10 µM ACC to the media and measured the malonyl-ACC in the seedlings. In this case, we were able to detect large amounts of malonyl-ACC in the seedlings of all genotypes, which decreased drastically upon D-Met addition (**Figures 8A**, **B**). It must be noted that the treatment of seedlings with 200 or 500 µM D-Met together with 10 µM ACC is relatively extreme and probably does not reflect physiological conditions. However, D-Met induced malonyl-ACC reduction was undue to production of malonyl-methionine caused by ACC, which was comparable with and without ACC addition (**Table S3**). Nevertheless, there was no significant difference of malonyl-ACC reduction of L*er* and *dat1* mutants to Col-0 at higher D-Met concentrations (**Figures 8A**, **B**).

DISCUSSION

For several decades, the detrimental, but partially also beneficial, effects of D-AAs on plants have been investigated (Valdovinos and Muir, 1965; Aldag and Young, 1970; Erikson et al., 2004; Erikson et al., 2005; Gördes et al., 2011; Hill et al., 2011). It is noteworthy, that there are reports of some D-AAs synthesized *de novo* by plants (Brückner and Westhauser, 2003; Strauch et al., 2015). However, there is growing evidence in recent years that almost all D-enantiomers of proteinogenic L-AAs are taken up by plants (Aldag and Young, 1970; Forsum et al., 2008; Gördes et al., 2011; Hill et al., 2011) and also metabolized to significant

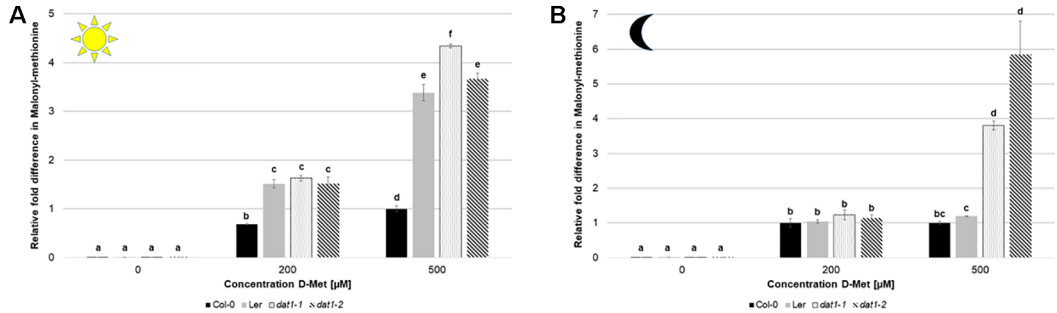


FIGURE 7 | D-Met affects formation of malonyl-methionine differently in Col-0, L*er*, and *dat1* mutants. Malonyl-methionine contents in seedlings of Col-0, L*er*, *dat1-1*, and *dat1-2* were measured after growth **(A)** in continuous light or **(B)** in darkness on agar plates supplemented with 200 and 500 µM D-Met, and additionally without supplementation. The relative values are given in fold changes with the values of Col-0 at 500 µM D-Met set to 1. The bars (Col-0: black, L*er*: gray, *dat1-1*: dotted, *dat1-2*: striped) represent the average values of three biological replicates. Different letters indicate statistically significant differences ($p < 0.05$) tested by an ANOVA. Error bars (± SD).

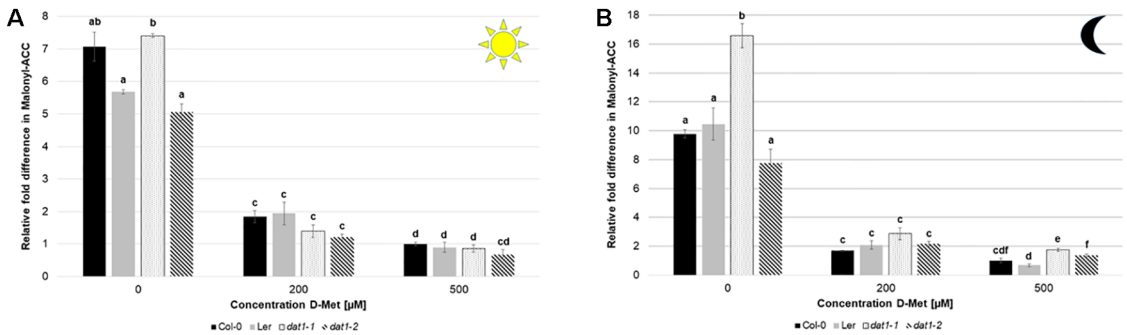


FIGURE 8 | D-Met leads to a decrease of malonyl-ACC levels in all tested lines. Malonyl-ACC contents in seedlings of Col-0, L*er*, *dat1-1*, and *dat1-2* were measured after growth **(A)** in continuous light or **(B)** in darkness on agar plates supplemented with 200 and 500 µM D-Met, and without supplementation. Additionally, all plates contained 10 µM ACC. The relative values are given in fold changes with the values of Col-0 at 500 µM D-Met + 10 µM ACC set to 1. The bars (Col-0: black, L*er*: gray, *dat1-1*: dotted, *dat1-2*: striped) represent the average values of three biological replicates. Different letters indicate statistically significant differences ($p < 0.05$) tested by an ANOVA. Error bars (± SD).

amounts (Aldag and Young, 1970; Gördes et al., 2011). With the proof provided in the actual report, the long standing question was addressed how D-AAs are utilized in plants.

In the light of the observations of Gördes et al. (2011), three possible mechanisms for the metabolism of D-AAs in plants 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*. This is reflected by its broad range of D-AAs transaminated, although its turnover rate and its affinity is low. Furthermore, we showed that the major product of this enzymatic reaction is D-Ala with D-Met as the favored amino group donor. D-Ala was also preferentially produced when plants were fed with other D-AAs. The preferred synthesis of D-Ala is caused by the preference of AtDAT1 on pyruvate over 2-OG as substrate. In comparison to the work of Funakoshi et al. (2008), who used 2-OG as amino group acceptor for their characterization of AtDAT1, our results revealed a higher V_{max} with pyruvate as substrate as compared to 2-OG. Most interestingly, the different enzymatic activities with pyruvate and 2-OG as amino group acceptors with ratios of 100:1 and more were in a comparable range as the D-Ala/D-Glu ratios found in plants after D-AA application (Gördes et al., 2011). Nevertheless, we could approximately substantiate the results from Funakoshi et al. (2008) when we used 2-OG as substrate in our enzymatic assays.

A major question in our studies addressed the role of AtDAT1 in D-AA stimulated ethylene production. As it is demonstrated here, this phenomenon is tightly connected to AtDAT1 (**Figure 9**). The loss of DAT1 leads to a significant increase of ethylene after D-Met application, resulting primarily in shortening of the hypocotyl and root in the *dat1* mutants and L*er* irrespective of the light regime. D-Met application also led to an increased production of malonyl-methionine, especially in the *dat1* mutants and L*er*, and the amount of malonyl-ACC developed reciprocally in all tested lines. The reciprocal accumulation of malonylated D-Met and ACC implies that the loss of *AtDAT* function or enzymatic activity results in over-

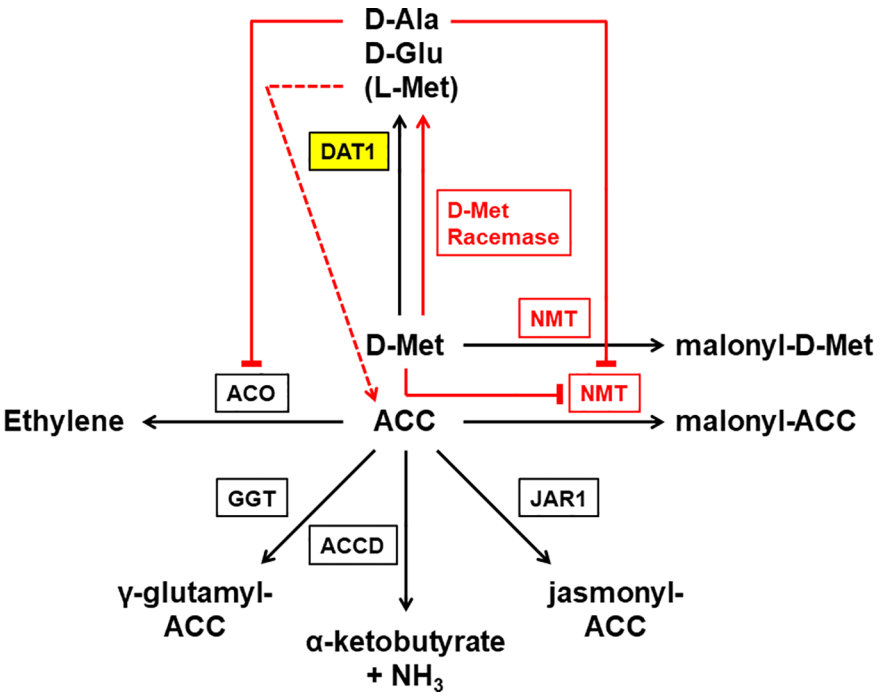


FIGURE 9 | Working model of the different reactions leading to D-Met stimulated ethylene production in plants. This reaction scheme summarizes proven (black) and postulated (red) enzymes and reactions involved in the process of D-Met stimulated ethylene production in plants. As the central enzyme of this study DAT1 is highlighted in yellow. Externally applied D-Met is mainly transaminated by a D-amino acid transaminase (DAT1) to produce D-Ala and D-Glu. Additionally, L-Met is produced that mainly results from a second transamination step after transamination of D-Met. ACC is the precursor of the gaseous hormone ethylene and this reaction is catalyzed by the ACC oxidase (ACO). Alternative to transamination, D-Met is malonylated by a N-malonyl transferase (NMT), which also uses ACC as a substrate. The malonylation of D-Met by NMT leads to the competitive repression of the reaction with ACC. The consequence of DAT1 loss of activity would be an increase of D-Met concentration, which would repress ACC malonylation and lead to increased ethylene production. Although malonylation is thought to be the major route to regulate cellular ACC concentration, there are three additional ways known: the glutamylation of ACC by the γ-glutamyl transpeptidase (GGT), the addition of jasmonic acid to ACC by jasmonic acid resistance 1 (JAR1), and the deamination of ACC by the ACC deaminase (ACCD). But also two other metabolites of D-Met may affect ACC and ethylene levels: D-Ala is able to inhibit ACO but would be missing in case of DAT1 loss. In contrast, L-Met is a precursor of ACC and may also be produced by direct racemization from D-Met. Higher concentrations of L-Met by such a racemization may also lead to an increase of ACC levels even when DAT1 activity is decreased.

accumulation of ACC that is then causing an increased ethylene production and eventually reduced seedling growth. However, this conclusion has to be reviewed critically, because even the *dat1* mutant seedlings did not show the full spectrum of the canonical triple response, as tightening of the apical hook or thickening of the hypocotyl was only partially observed. Furthermore, no differences in levels of malonylated ACC were detected between D-Met treated Col-0 and *dat1* mutants. However, it must be noted that in all previous studies the production of ethylene in response to malonylation of ACC and D-AAs were measured during overnight feeding experiments. The physiological growth responses and the contents of D-Met, ACC, and their malonylated derivatives in the plants over a longer period of the treatment were shown here for the first time.

The increased production of malonyl-methionine and ethylene without a decreased malonyl-ACC production in the *dat1* mutants in comparison to the control raises the question whether the original working model of D-Met stimulated ethylene production needs additional factors or metabolic processes. One explanation may be that malonylation is not the only way to regulate the ACC level in plants (**Figure 9**). There is also the possibility that ACC is conjugated with glutathione to γ-glutamyl-ACC (GACC) and with jasmonic acid to JA-ACC to control the ACC homeostasis. Additionally, plants can irreversibly degrade ACC to α-ketobutyrate by an ACC deaminase [for reviews about ACC content regulation, see Van de Poel and Van Der Straeten (2014); Le Deunff and Lecourt (2016), and Vanderstraeten and Van Der Straeten (2017)]. To date, neither the contribution of each of these ACC catabolic pathways nor their interplay for the control of ACC homeostasis have been studied, yet. It remains to be investigated whether D-Met, its malonylated form, or the loss of this way to degrade D-Met have an impact also on the alternative ACC degradation pathways.

Another explanation would be given by a racemization of D-Met, which has been proposed before but never been proven (Vranova et al., 2012; Gördes et al., 2013). Our results imply that the majority of the increase of L-Met in D-Met fed Col-0 plants arises from the reamination of 4-methylthio-2-oxobutanoate

after removal of the amino group from D-Met by AtDAT1. However, direct racemization of D-Met cannot be excluded. If such a direct racemization really exists, additional L-Met would be produced irrespective of AtDAT1 activity and would be partially converted to ACC in the Yang cycle (**Figure 9**). This additional ACC would contribute to the increased ethylene contents in D-Met treated *dat1* mutants, because ACC malonylation is inhibited competitively by higher D-Met levels.

A third explanation may be given by the effect of the AtDAT1 enzymatic products on the activity of other enzymes. Here we demonstrated that the loss of this enzyme leaves *dat1* mutants without the ability to produce D-Ala, D-Glu and additional L-Met in response to D-Met. Most interestingly, it was shown previously that D-Ala inhibits the ACC oxidase (ACO) (Gibson et al., 1998; Brunhuber et al., 2000; Charng et al., 2001; Thrower et al., 2006). This means that plants with functional DAT1 would malonylate D-Met instead of ACC but the produced D-Ala would partially inhibit the ACO and as one consequence the additional ACC would just be partially converted to ethylene. In the *dat1* mutants this inhibiting effect of D-Ala would be lost and may explain the ethylene increase in these lines in comparison to the corresponding wild type. The same lack of D-Ala accumulation may also contribute to the higher content of malonyl-methionine in the *dat1* mutants and to the comparable amount of malonylated ACC in all tested lines: D-Ala also partially inhibits the putative malonyl transferase (Kionka and Amrhein, 1984; Liu et al., 1985; Chick and Leung, 1997). The malonylation of D-Met would then be limited by D-Ala in Col-0 but not in the *dat1* mutant lines and L*er*. If D-Met is the preferred substrate of the malonyl transferase, the lack of significant differences between the tested lines in their levels of malonylated ACC would not be surprising. To confirm the assumptions that D-Ala influences the D-Met stimulated ethylene production by inhibiting the ACC oxidase, the ACC malonyl transferase or both enzymes, further physiological experiments with D-Ala and structural analogs like D-cycloserine are required. However, the final answer to this question is awaited by the identification of the ACC malonyl transferase and the results of the enzyme´s biochemical and physiological characterization.

Undoubtedly, AtDAT1 affects D-Met stimulated ethylene production and seems to have quite specific effects in this regard. However, as the working model in **Figure 9** implies, the relationship between D-Met and ethylene may be more complex than just the competition for the N-malonyl transferase (NMT). As mentioned above, D-Met may affect the levels of ACC and all its derivatives. It has been shown before that ACC itself acts as a signaling molecule and the same is also discussed for its derivatives (for reviews, see Van de Poel and Van Der Straeten, 2014; Vanderstraeten and Van Der Straeten, 2017; Nascimento et al., 2018). D-Met accumulation leads to an increase of ethylene concentrations, but possibly other compounds like ACC and its derivatives may also contribute to the observed physiological responses of *dat1* affected plants. This would explain why the *dat1* mutants do not show the full spectrum of triple response after treatment in the presence of D-Met. Detailed flux measurements of ACC and its derivatives after D-Met application as well as studies of *dat1* alleles in the background of ethylene synthesis and receptor mutants may shed more light on this aspect.

In this regard also the intracellular localization of these biochemical processes is of interest. The localization of AtDAT1 implies that the transamination takes place in the chloroplast, whereas the ACC oxidation is postulated to happen either in the cytosol or the plasma membrane (Houben and Van De Poel, 2019). The separation of these processes raises the question how D-Met affects malonylation of ACC if it is also located in the cytosol. The most apparent hypothesis would be that chloroplasts have a certain capacity to take up D-Met. Flooding of the chloroplasts with this compound could therefore lead to inhibitory processes in the cytosol. This would be supported by the findings in this study that AtDAT1 is the major enzyme to degrade D-Met but needs further confirmation.

Another remaining question is the source of D-Met in nature, because it was not reported in plants until now. In contrast, it was demonstrated previously that D-Met is released by bacterial biofilms into the environment (Kolodkin-Gal et al., 2010; 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 was reported before (Vlamakis et al. (2013). It is remarkable that D-Met is released by different bacterial species into their growth media to concentrations up to 300–500 μM (Lam et al., 2009), which would match the most effective D-Met concentrations in our study. 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* is dispensable in particular *Arabidopsis* accessions such as L*er* and M7323S. An explanation for the dispensability of the D-Met catabolic function of AtDAT1 would be that in a habitat with only minor D-Met releasing bacteria in the rhizosphere, a recognition system for this compound would be also dispensable for the plant. However, this needs to be tested. The viability of *Arabidopsis dat1* mutants and accessions without functional AtDAT1 also argues against the crucial function of this enzyme in folate biosynthesis. This was implied by the observation that the only known enzyme able to synthesize p-amino benzoic acid (pABA), the substructure of folates, is AtDAT1 (Basset et al., 2004; Hanson and Gregory III, 2011). Consequently, the loss of this enzyme would lead to the inability to produce essential folate which would reduce the plant viability dramatically. Interestingly, pABA is also involved in the regulation of root gravitropism (Nziengui et al., 2018). This implies a modulatory role of AtDAT in differential root growth including gravitropism, which can be tested in future by the analysis of our *dat1* mutants and accessions without or reduced AtDAT1 activity. Interestingly, DAT1 encoding genes seem to be found in all sequenced plant genomes (for a selection, see **Figure S5**), and ethylene production in other plant species than

Arabidopsis is also induced by other D-AAs like D-Leu, D-Thr, D-Val, or D-Phe (Satoh and Esashi, 1980; Satoh and Esashi, 1982; Liu et al., 1983). In this regard, it would be interesting if also other D-AAs than D-Met cause growth defects and ethylene production in *dat1* mutants. Furthermore, it should be tested if the DAT1 enzymes from different species have altered substrate specificities and therefore contribute to the adaptation of plants to changing microbial environments.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

ÜK, JS, and CH designed the study. JS and CH conducted most of the experiments and contributed equally to the study. V-AL and SH conducted another part of the experiments. CH and MS analyzed the biochemical data and ÜK wrote the manuscript.

FUNDING

JS was supported by the Deutscher Akademischer Austauschdienst (DAAD 91567028). We acknowledge support by Open Access Publishing Fund of University of Tübingen.

ACKNOWLEDGMENTS

We would like to acknowledge Prof. Klaus Harter (Center for Plant Molecular Biology, University of Tübingen, Germany) for the provision of research facilities and his invaluable help in the writing of this manuscript. Furthermore, we would like to thank Nina Glöckner and Friederike Wanke for their excellent support in confocal fluorescence microscopy and Georg Felix for his support in ethylene measurements.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01609/full#supplementary-material

REFERENCES

Aldag, R., and Young, J. (1970). D-Amino Acids in Soils. I. Uptake and Metabolism by Seedling Maize and Ryegrass. *Agron. J.* 62, 184–189. doi: 10.2134/agronj1970.00021962006200020002x

Amelung, W., and Zhang, X. (2001). Determination of amino acid enantiomers in soils. *Soil Biol. Biochem.* 33, 553–562. doi: 10.1016/S0038-0717(00)00195-4

Amelung, W., Zhang, X., and Flach, K. (2006). Amino acids in grassland soils: climatic effects on concentrations and chirality. *Geoderma* 130, 207–217. doi: 10.1016/j.geoderma.2005.01.017

Balu, D. T., and Coyle, J. T. (2015). The NMDA receptor 'glycine modulatory site' in schizophrenia: D-serine, glycine, and beyond. *Curr. Opin. Pharmacol.* 20, 109–115. doi: 10.1016/j.coph.2014.12.004

Basset, G. J., Ravanel, S., Quinlivan, E. P., White, R., Giovannoni, J. J., Rébeillé, F., et al. (2004). Folate synthesis in plants: the last step of the p-aminobenzoate branch is catalyzed by a plastidial aminodeoxychorismate lyase. *Plant J.* 40, 453–461. doi: 10.1111/j.1365-313X.2004.02231.x

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. *Physiol. Plant.* 94, 629–634. doi: 10.1111/j.1399-3054.1995.tb00977.x

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

Brückner, H. (2011). *D-amino acids in chemistry, life sciences, and biotechnology* (Zürich, Switzerland: Verlag Helvetica Chimica Acta).

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, 17–42. doi: 10.1007/s10533-005-5733-7

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, 10730–10738. doi: 10.1021/bi0000162

Cava, F., Lam, H., De Pedro, M. A., and Waldor, M. K. (2011). Emerging knowledge of regulatory roles of D-amino acids in bacteria. *Cell. Mol. Life Sci.* 68, 817–831. doi: 10.1007/s00018-010-0571-8

Charng, Y.-Y., Chou, S.-J., Jiaang, W.-T., Chen, S.-T., and Yang, S. F. (2001). The catalytic mechanism of 1-aminocyclopropane-1-carboxylic acid oxidase. *Arch. Biochem. Biophys* 385, 179–185. doi: 10.1006/abbi.2000.2138

Chen, C., Maccready, J. S., Ducat, D. C., and Osteryoung, K. W. (2018). The molecular machinery of chloroplast division. *Plant Physiol.* 176, 138–151. doi: 10.1104/pp.17.01272

Chick, W., and Leung, P. (1997). lmmuno-purification and characterization of a 40 kDa ACC N-malonyl transferase from mungbean seedling hypocotyl. *Plant Physiol.* 113, 119–126. doi: 10.1104/pp.113.1.119

Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743. doi: 10.1046/j.1365-313x.1998.00343.x

Curtis, M. D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* 133, 462–469. doi: 10.1104/pp.103.027979

D'aniello, A. (2007). D-Aspartic acid: an endogenous amino acid with an important neuroendocrine role. *Brain Res. Rev.* 53, 215–234. doi: 10.1016/j.brainresrev.2006.08.005

Dworkin, J. (2014). The medium is the message: interspecies and interkingdom signaling by peptidoglycan and related bacterial glycans. *Annu. Rev. Microbiol.* 68, 137–154. doi: 10.1146/annurev-micro-091213-112844

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* 19, 1349. doi: 10.1093/nar/19.6.1349

Erikson, O., Hertzberg, M., and Nasholm, T. (2004). A conditional marker gene allowing both positive and negative selection in plants. *Nat. Biotechnol.* 22, 455–458. doi: 10.1038/nbt946

Erikson, O., Hertzberg, M., and Näsholm, T. (2005). The dsdA gene from *Escherichia coli* provides a novel selectable marker for plant transformation. *Plant Mol. Biol.* 57, 425–433. doi: 10.1007/s11103-004-7902-9

Felix, G., Grosskopf, D. G., Regenass, M., Basse, C. W., and Boller, T. (1991). Elicitor-induced ethylene biosynthesis in tomato cells: characterization and use as a bioassay for elicitor action. *Plant Physiol.* 97, 19–25. doi: 10.1104/pp.97.1.19

Forde, B. G., and Roberts, M. R. (2014). Glutamate receptor-like channels in plants: a role as amino acid sensors in plant defence? *F1000prime Rep.* 6, 37 doi: 10.12703/P6-37

Forsum, O., Svennerstam, H., Ganeteg, U., and Näsholm, T. (2008). Capacities and constraints of amino acid utilization in Arabidopsis. *New Phytol.* 179, 1058–1069. doi: 10.1111/j.1469-8137.2008.02546.x

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. *Mol. Genet. Metab.* 85, 168–180. doi: 10.1016/j.ymgme.2005.03.003

Fujii, N., Kaji, Y., and Fujii, N. (2011). d-Amino acids in aged proteins: analysis and biological relevance. *J. Chromatogr. B* 879, 3141–3147. doi: 10.1016/j.jchromb.2011.05.051

Fujii, N. (2002). D-amino acids in living higher organisms. *Origins Life Evol. Biosphere* 32, 103–127. doi: 10.1023/A:1016031014871

Funakoshi, M., Sekine, M., Katane, M., Furuchi, T., Yohda, M., Yoshikawa, T., et al. (2008). Cloning and functional characterization of *Arabidopsis thaliana* d-amino acid aminotransferase–d-aspartate behavior during germination. *FEBS J.* 275, 1188–1200. doi: 10.1111/j.1742-4658.2008.06279.x

Gördes, D., Kolukisaoglu, U., and Thurow, K. (2011). Uptake and conversion of D-amino acids in *Arabidopsis thaliana*. *Amino Acids* 40, 553–563. doi: 10.1007/s00726-010-0674-4

Gördes, D., Koch, G., Thurow, K., and Kolukisaoglu, U. (2013). Analyses of Arabidopsis ecotypes reveal metabolic diversity to convert D-amino acids. *Springerplus* 2, 559. doi: 10.1186/2193-1801-2-559

Gibson, E. J., Zhang, Z., Baldwin, J. E., and Schofield, C. J. (1998). Substrate analogues and inhibition of ACC oxidase: conversion of D-valine to isobutanal. *Phytochemistry* 48, 619–624. doi: 10.1016/S0031-9422(97)00965-5

Grefen, C., Donald, N., Hashimoto, K., Kudla, J., Schumacher, K., and Blatt, M. R. (2010). A ubiquitin-10 promoter-based vector set for fluorescent protein tagging facilitates temporal stability and native protein distribution in transient and stable expression studies. *Plant J.* 64, 355–365. doi: 10.1111/j.1365-313X.2010.04322.x

Hamase, K., Morikawa, A., and Zaitsu, K. (2002). D-Amino acids in mammals and their diagnostic value. *J. Chromatogr. B* 781, 73–91. doi: 10.1016/S1570-0232(02)00690-6

Hamase, K. (2007). Sensitive two-dimensional determination of small amounts of D-amino acids in mammals and the study on their functions. *Chem. Pharm. Bull.* 55, 503–510. doi: 10.1248/cpb.55.503

Hanson, A. D., and Gregory, J. F. III. (2011). Folate biosynthesis, turnover, and transport in plants. *Annu. Rev. Plant Biol.* 62, 105–125. doi: 10.1146/annurev-arplant-042110-103819

Hill, P. W., Quilliam, R. S., Deluca, T. H., Farrar, J., Farrell, M., Roberts, P., et al. (2011). Acquisition and assimilation of nitrogen as peptide-bound and D-enantiomers of amino acids by wheat. *PloS One* 6, e19220. doi: 10.1371/journal.pone.0019220

Hirano, T., Tanidokoro, K., Shimizu, Y., Kawarabayasi, Y., Ohshima, T., Sato, M., et al. (2016). Moss chloroplasts are surrounded by a peptidoglycan wall containing D-amino acids. *Plant Cell* 28, 1521–1532. doi: 10.1038/1841296b0

Hofstee, B. (1959). Non-inverted versus inverted plots in enzyme kinetics. *Nature* 184, 1296. doi: 10.1038/1841296b0

Houben, M., and Van De Poel, B. (2019). 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO): the enzyme that makes the plant hormone ethylene. *Front. Plant Sci.* 10, 1–15. doi: 10.3389/fpls.2019.00695

Katane, M., and Homma, H. (2011). d-Aspartate—an important bioactive substance in mammals: a review from an analytical and biological point of view. *J. Chromatogr. B* 879, 3108–3121. doi: 10.1016/j.jchromb.2011.03.062

Kionka, C., and Amrhein, N. (1984). The enzymatic malonylation of 1-aminocyclopropane-1-carboxylic acid in homogenates of mung-bean hypocotyls. *Planta* 162, 226–235. doi: 10.1007/BF00397444

Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010). D-amino acids trigger biofilm disassembly. *Science* 328, 627–629. doi: 10.1126/science.1188628

Konno, R., Brückner, H., D'Aniello, A., Fischer, G., Fujii, N., and Homma, H. (Eds.) (2007). d-Amino acids – a new frontier in d-Amino acids and protein research. *Pract Meth Protocols* (New York, NY, USA: Nova Science Publishers). 151–182.

Lam, H., Oh, D.-C., Cava, F., Takacs, C. N., Clardy, J., De Pedro, M. A., et al. (2009). D-amino acids govern stationary phase cell wall remodeling in bacteria. *Science* 325, 1552–1555. doi: 10.1126/science.1178123

Le Deunff, E., and Lecourt, J. (2016). Non-specificity of ethylene inhibitors: 'double-edged'tools to find out new targets involved in the root morphogenetic programme. *Plant Biol.* 18, 353–361. doi: 10.1111/plb.12405

Lempe, J., Balasubramanian, S., Sureshkumar, S., Singh, A., Schmid, M., and Weigel, D. (2005). Diversity of flowering responses in wild *Arabidopsis thaliana* strains. *PloS Genet.* 1, e6. doi: 10.1371/journal.pgen.0010006

Ling-Yuan, S., Yu, L., and Shang, F. Y. (1985). Relationship between 1-aminocyclopropanecarboxylate malonyltransferase and D-amino acid malonyltransferase. *Phytochemistry* 24, 1141–1145. doi: 10.1016/S0031-9422(00)81090-0

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, 437–441. doi: 10.1007/BF00397737

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 Physiol.* 77, 891–895. doi: 10.1104/pp.77.4.891

Magnani, G., Lomazzi, M., and Peracchi, A. (2013). Completing the folate biosynthesis pathway in Plasmodium falciparum: p-aminobenzoate is produced by a highly divergent promiscuous aminodeoxychorismate lyase. *Biochem. J.* 455, 149–155. doi: 10.1042/BJ20130896

Martínez-Rodríguez, S., Martínez-Gómez, A. I., Rodríguez-Vico, F., Clemente-Jiménez, J. M., and Las Heras-Vázquez, F. J. (2010). Natural occurrence and industrial applications of D-amino acids: an overview. *Chem. Biodivers.* 7, 1531–1548. doi: 10.1002/cbdv.200900245

Michard, E., Lima, P. T., Borges, F., Silva, A. C., Portes, M. T., Carvalho, J. E., et al. (2011). Glutamate receptor-like genes form Ca2+ channels in pollen tubes and are regulated by pistil D-serine. *Science* 332, 434–437. doi: 10.1126/science.1201101

Monselise, E. B., 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 (1)(5)N NMR study. *Plant Biol. (Stuttg)* 17 Suppl 1, 101–107. doi: 10.1111/plb.12198

Naranjo-Ortíz, M. A., Brock, M., Brunke, S., Hube, B., Marcet-Houben, M., and Gabaldón, T. (2016). Widespread inter-and intra-domain horizontal gene transfer of d-amino acid metabolism enzymes in eukaryotes. *Front. Microbiol.* 7, 2001. doi: 10.3389/fmicb.2016.02001

Nascimento, F. X., Rossi, M. J., and Glick, B. R. (2018). Ethylene and 1-Aminocyclopropane-1-carboxylate (ACC) in plant–bacterial interactions. *Front. Plant Sci.* 9, 114. doi: 10.3389/fpls.2018.00114

Nelson, B. K., Cai, X., and Nebenführ, A. (2007). A multicolored set of *in vivo* organelle markers for co-localization studies in Arabidopsis and other plants. *Plant J.* 51, 1126–1136. doi: 10.1111/j.1365-313X.2007.03212.x

Nziengui, H., Lasok, H., Kochersperger, P., Ruperti, B., Rébeillé, F., Palme, K., et al. (2018). Root gravitropism is regulated by a crosstalk between para-aminobenzoic acid, ethylene, and auxin. *Plant Physiol.* 178, 1370–1389. doi: 10.1104/pp.18.00126

Ollivaux, C., Soyez, D., and Toullec, J. Y. (2014). Biogenesis of D-amino acid containing peptides/proteins: where, when and how? *J. Pept. Sci.* 20, 595–612. doi: 10.1002/psc.2637

Radkov, A. D., Mcneill, K., Uda, K., and Moe, L. A. (2016). D-Amino acid catabolism is common among soil-dwelling bacteria. *Microbes Environ.* 31, 165–168. doi: 10.1264/jsme2.ME15126

Satoh, S., and Esashi, Y. (1980). D-Amino-acid-stimulated ethylene production in seed tissues. *Planta* 149, 64–68. doi: 10.1007/BF00386229

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. *Physiol. Plant.* 54, 147–152. doi: 10.1111/j.1399-3054.1982.tb06318.x

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*. *Proc. Natl. Acad. Sci.* 112, 11726–11731. doi: 10.1073/pnas.1503272112

Thrower, J., Mirica, L. M., Mccusker, K. P., and Klinman, J. P. (2006). Mechanistic investigations of 1-aminocyclopropane 1-carboxylic acid oxidase with alternate cyclic and acyclic substrates. *Biochemistry* 45, 13108–13117. doi: 10.1021/bi061097q

Valdovinos, J. G., and Muir, R. M. (1965). Effects of D and L amino acids on foliar abscission. *Plant Physiol.* 40, 335. doi: 10.1104/pp.40.2.335

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!. *Front. Plant Sci.* 5, 1–15. doi: 10.3389/fpls.2014.00640

Vanderstraeten, L., and Van Der Straeten, D. (2017). Accumulation and transport of 1-aminocyclopropane-1-carboxylic acid (ACC) in plants: current status, considerations for future research and agronomic applications. *Front. Plant Sci.* 8, 38. doi: 10.3389/fpls.2017.00038

Vlamakis, H., Chai, Y., Beauregard, P., Losick, R., and Kolter, R. (2013). Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat. Rev. Microbiol.* 11, 157. doi: 10.1038/nrmicro2960

Vranova, V., Zahradnickova, H., Janous, D., Skene, K. R., Matharu, A. S., Rejsek, K., et al. (2012). The significance of D-amino acids in soil, fate and utilization by microbes and plants: review and identification of knowledge gaps. *Plant Soil* 354, 21–39. doi: 10.1007/s11104-011-1059-5

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., and Provart, N. J. (2007). An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PloS One* 2, e718. doi: 10.1371/journal.pone.0000718

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. doi: 10.1016/0031-9422(95)00320-7

Yang, S. F., and Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol.* 35, 155–189. doi: 10.1146/annurev.pp.35.060184.001103

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Tables

Table S1 Primers used in this study

Primer name	**Primer sequence**	**Purpose**
DAT1-S1	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

Supplementary Material

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

The transaminase activity of the *Col-0* variant of AtDAT1 was compared with D-Met, D-Trp and D-Ala (each 10 mM in the assay) as amino donor and 2-OG and pyruvate (each 50 mM in the assay) 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).

		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 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

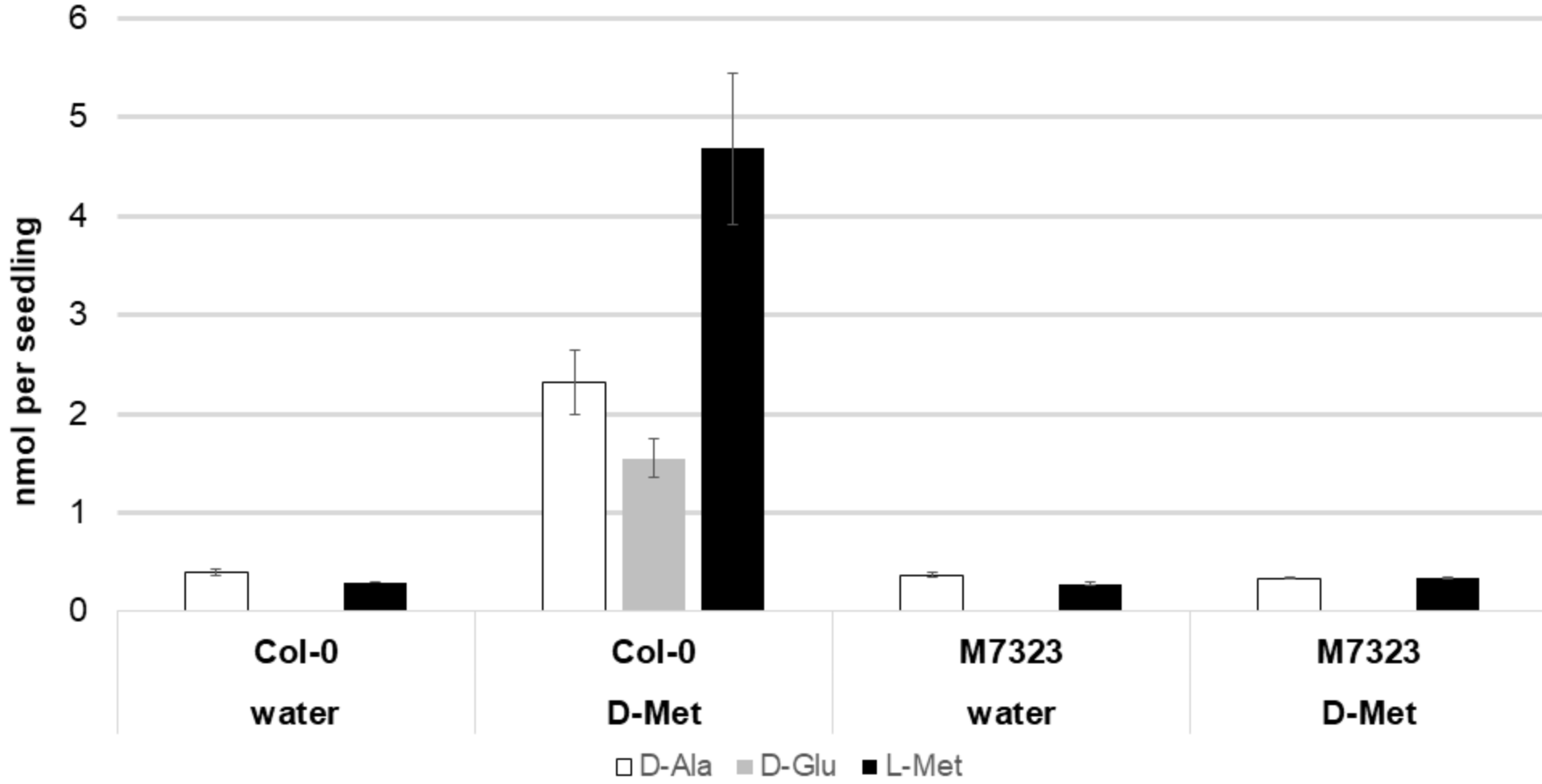


Figure S1: D-Met metabolites in M7323S and Col-0
Contents of D-Ala (black), D-Glu (grey) and L-Met in seedlings of Col-0 and M7323S without (water) and with D-Met treatment (D-Met) for 16 h; for further information see Figure 1c.

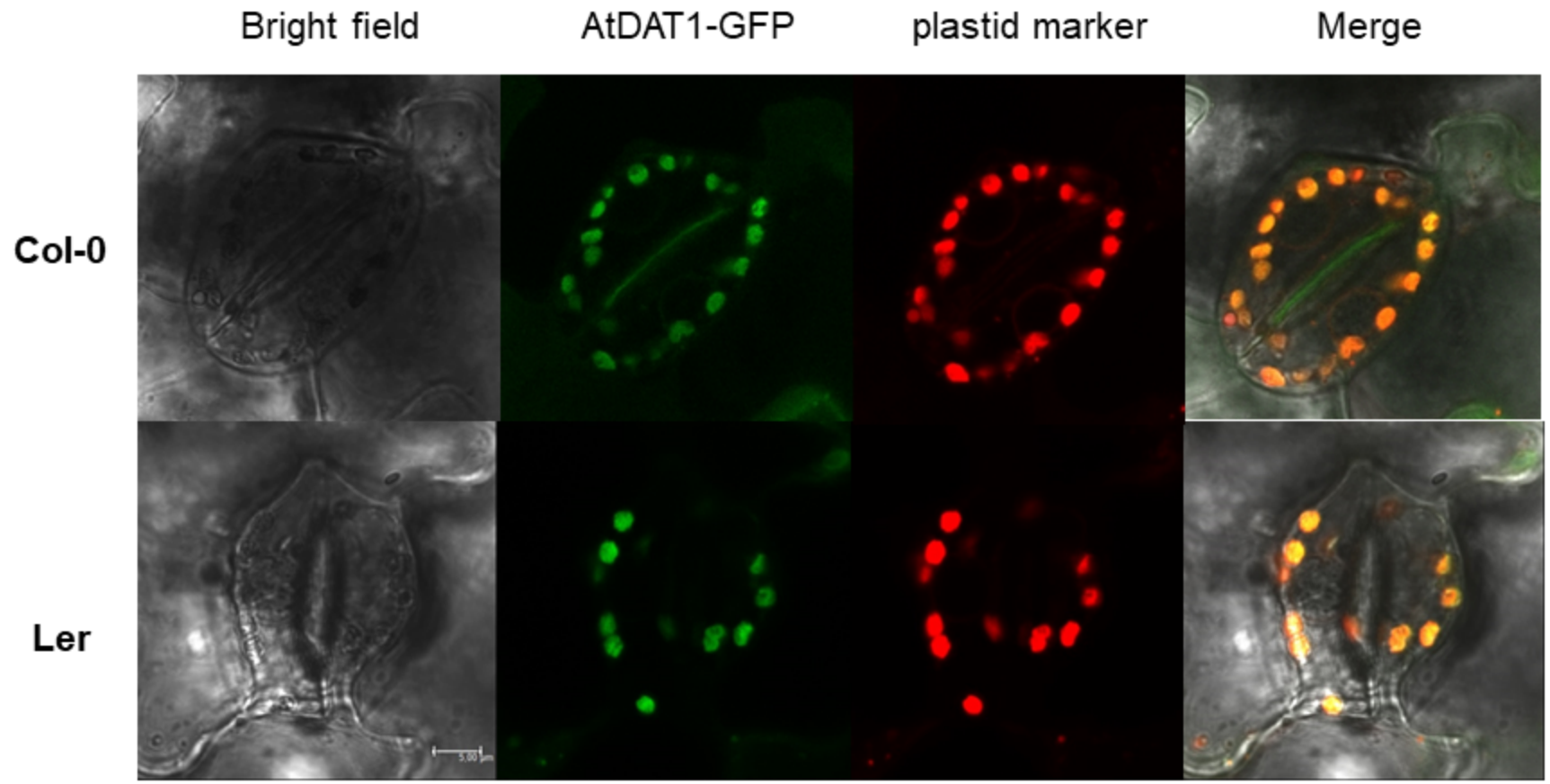


Figure S3: AtDAT1 from Col-0 and Ler localize both in plastids

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

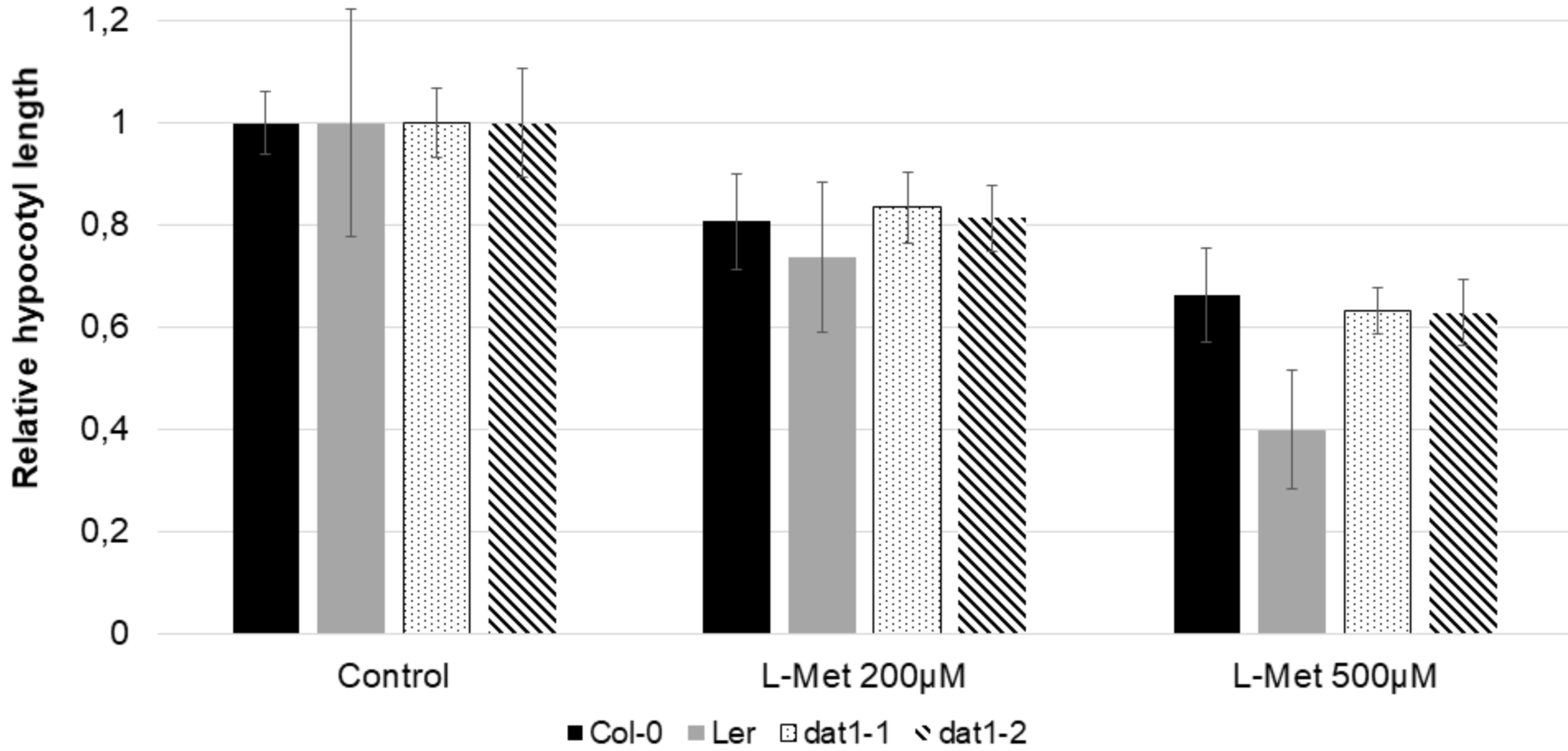


Figure S7: Seedling growth is not differentially suppressed by L-Met in AtDAT1 knock out-lines
For this experiment seedlings were grown as given in Figure 5 with the difference that instead of D-Met growth media was supplemented with L-Met. None of the measurements revealed a significant difference between *dat1* mutants and Col-0 at all tested L-Met concentrations. For further information see Figure 5.

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.

(a)

rosette stage | early floral stage | late floral stage

(b)

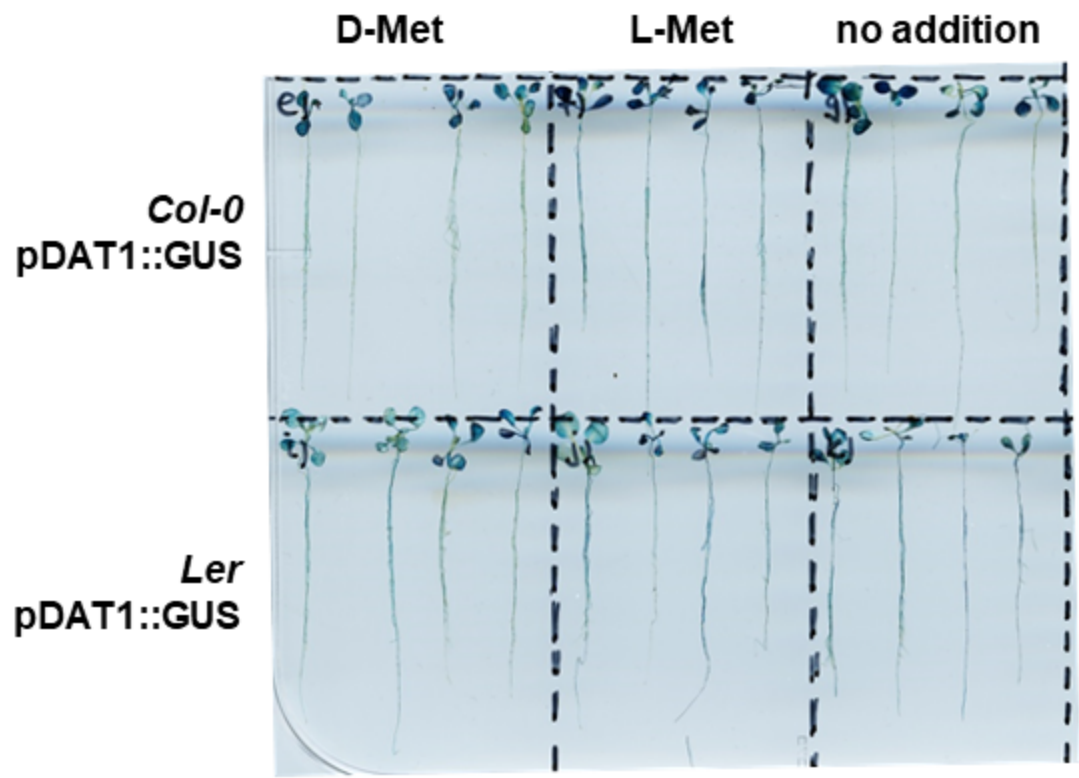
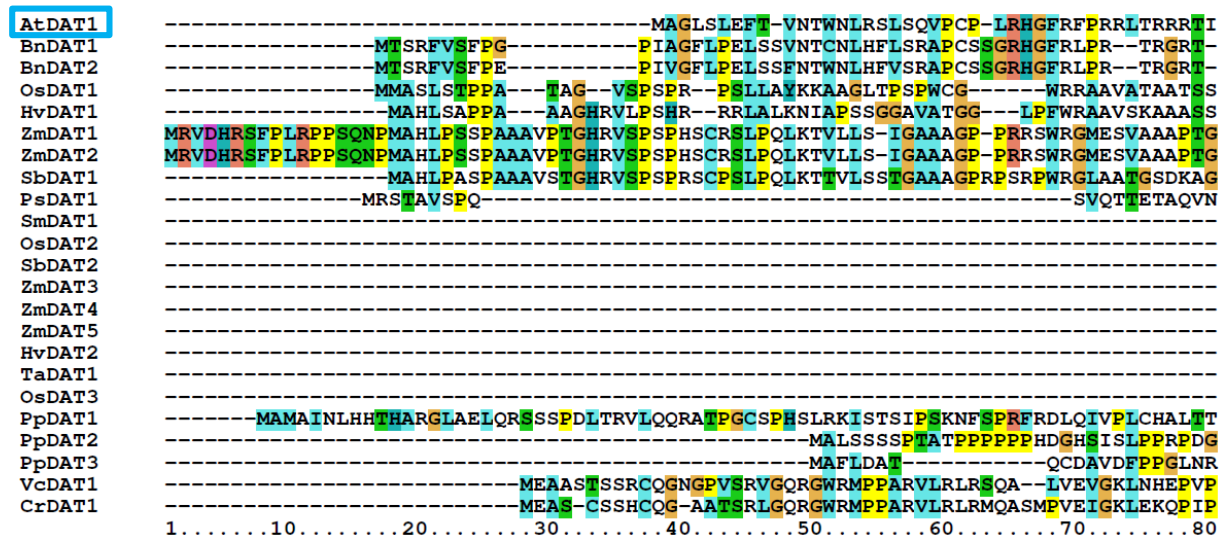
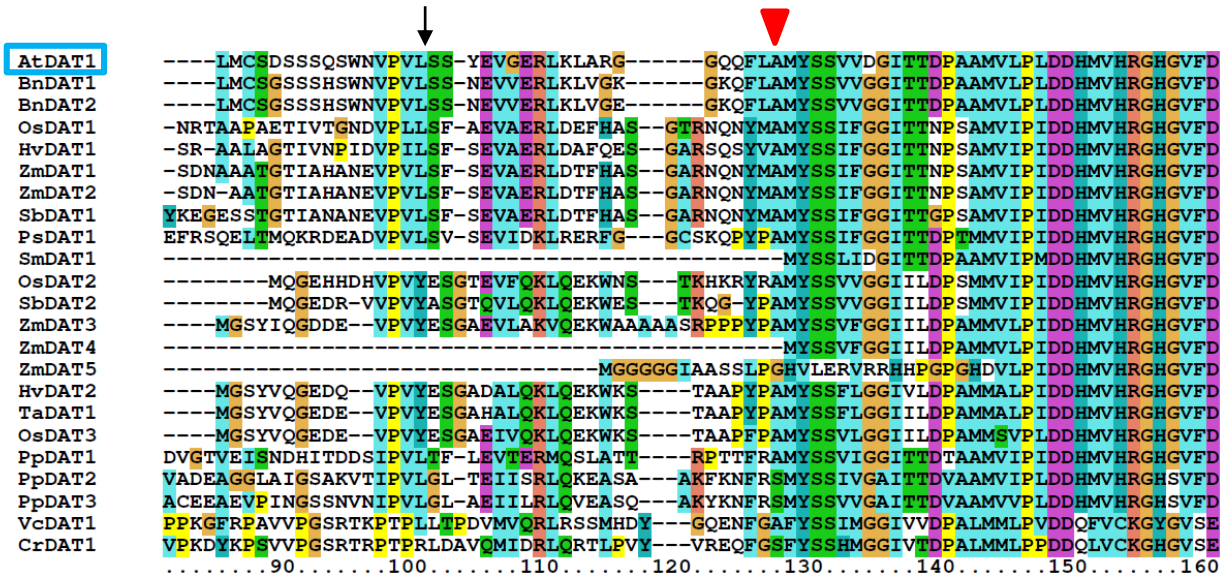
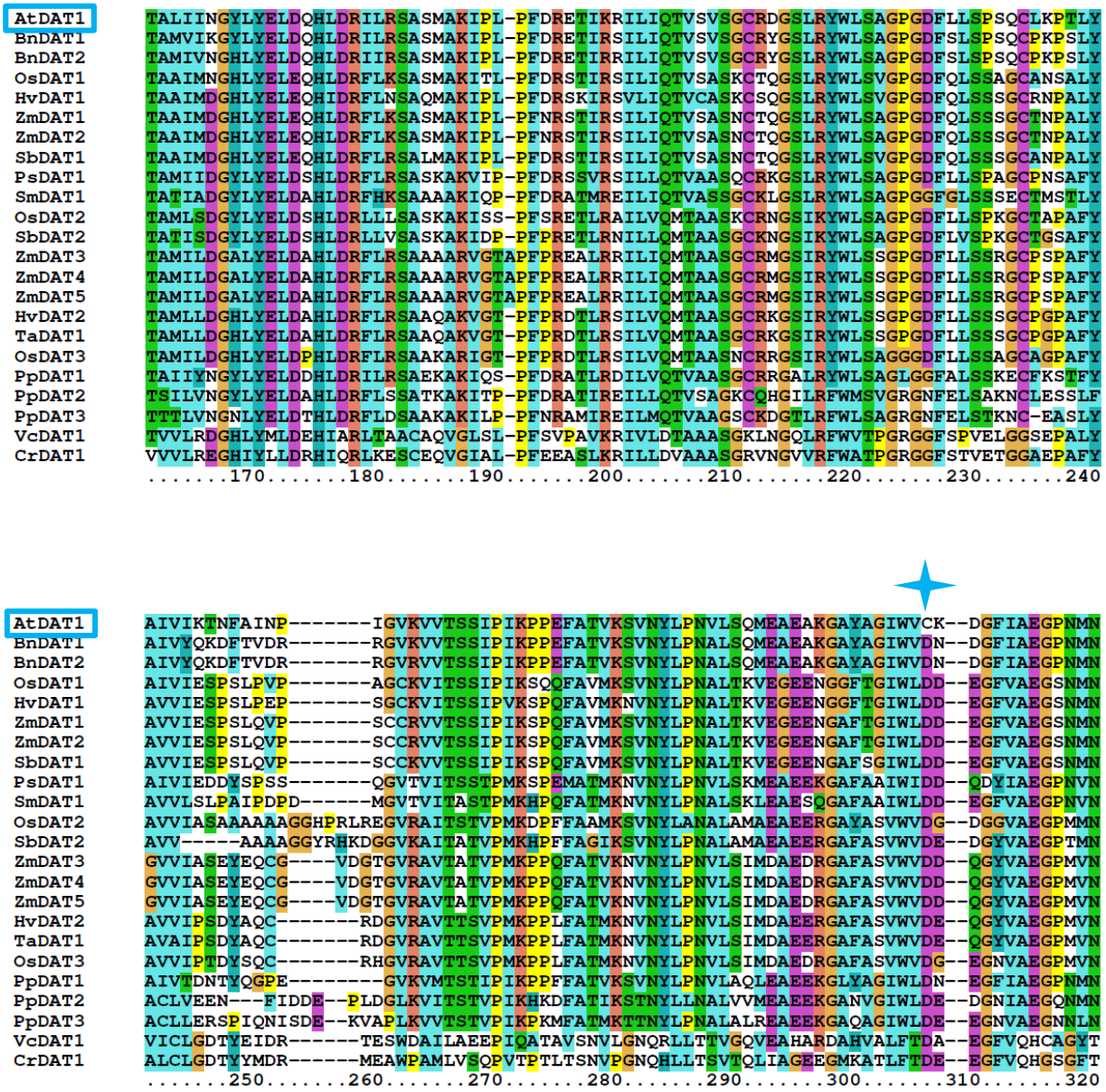


Figure S2: Expression patterns of AtDAT1
(a) GUS staining of rosette leaves (left), early (middle) and late flower stages (right) expressing the ß-Glucuronidase under the control of the AtDAT1 promotor from Col-0
(b) GUS staining of seedlings expressing the ß-Glucuronidase either under the control of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


AtDAT1
BnDAT1
BnDAT2
OsDAT1
HvDAT1
ZmDAT1
ZmDAT2
SbDAT1
PsDAT1
SmDAT1
OsDAT2
SbDAT2
ZmDAT3
ZmDAT4
ZmDAT5
HvDAT2
TaDAT1
OsDAT3
PpDAT1
PpDAT2
PpDAT3
VcDAT1
CrDAT1
1.....10.....20.....30.....40.....50.....60.....70.....80


AtDAT1
BnDAT1
BnDAT2
OsDAT1
HvDAT1
ZmDAT1
ZmDAT2
SbDAT1
PsDAT1
SmDAT1
OsDAT2
SbDAT2
ZmDAT3
ZmDAT4
ZmDAT5
HvDAT2
TaDAT1
OsDAT3
PpDAT1
PpDAT2
PpDAT3
VcDAT1
CrDAT1
.....90.....100.....110.....120.....130.....140.....150.....160


AtDAT1
BnDAT1
BnDAT2
OsDAT1
HvDAT1
ZmDAT1
ZmDAT2
SbDAT1
PsDAT1
SmDAT1
OsDAT2
SbDAT2
ZmDAT3
ZmDAT4
ZmDAT5
HvDAT2
TaDAT1
OsDAT3
PpDAT1
PpDAT2
PpDAT3
VcDAT1
CrDAT1
.....170.....180.....190.....200.....210.....220.....230.....240
.....250.....260.....270.....280.....290.....300.....310.....320

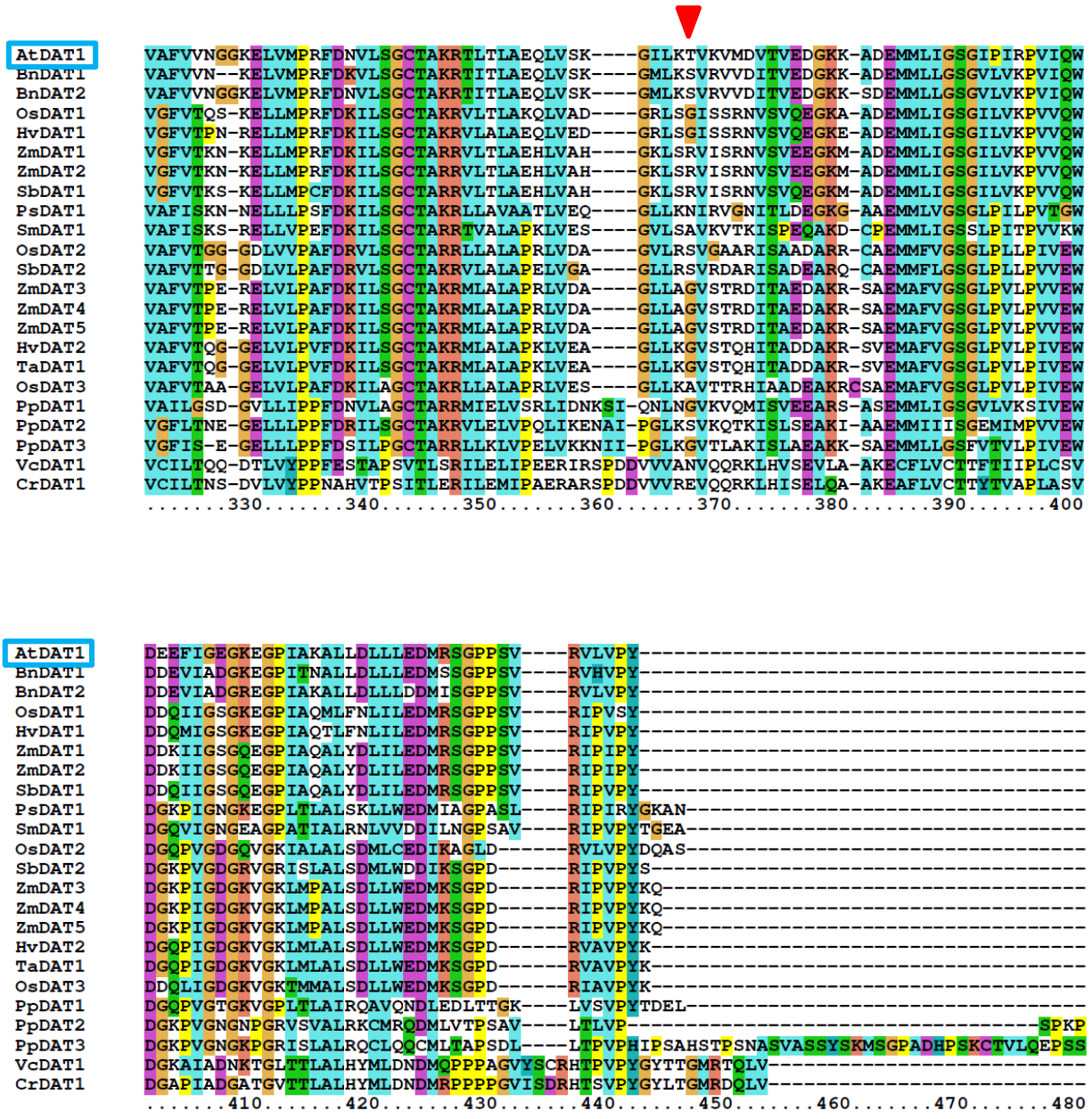


Figure S5: Alignment of DAT1 protein sequences from different plants and algae
Protein sequences were taken from the Phytozome 12 database (https://phytozome.jgi.doe.gov/pz/portal.html#) with AtDAT1 as search sequence. The alignment was constructed with ClustalX 2.1 (Larkin et al. 2007). AtDAT1 in the 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.

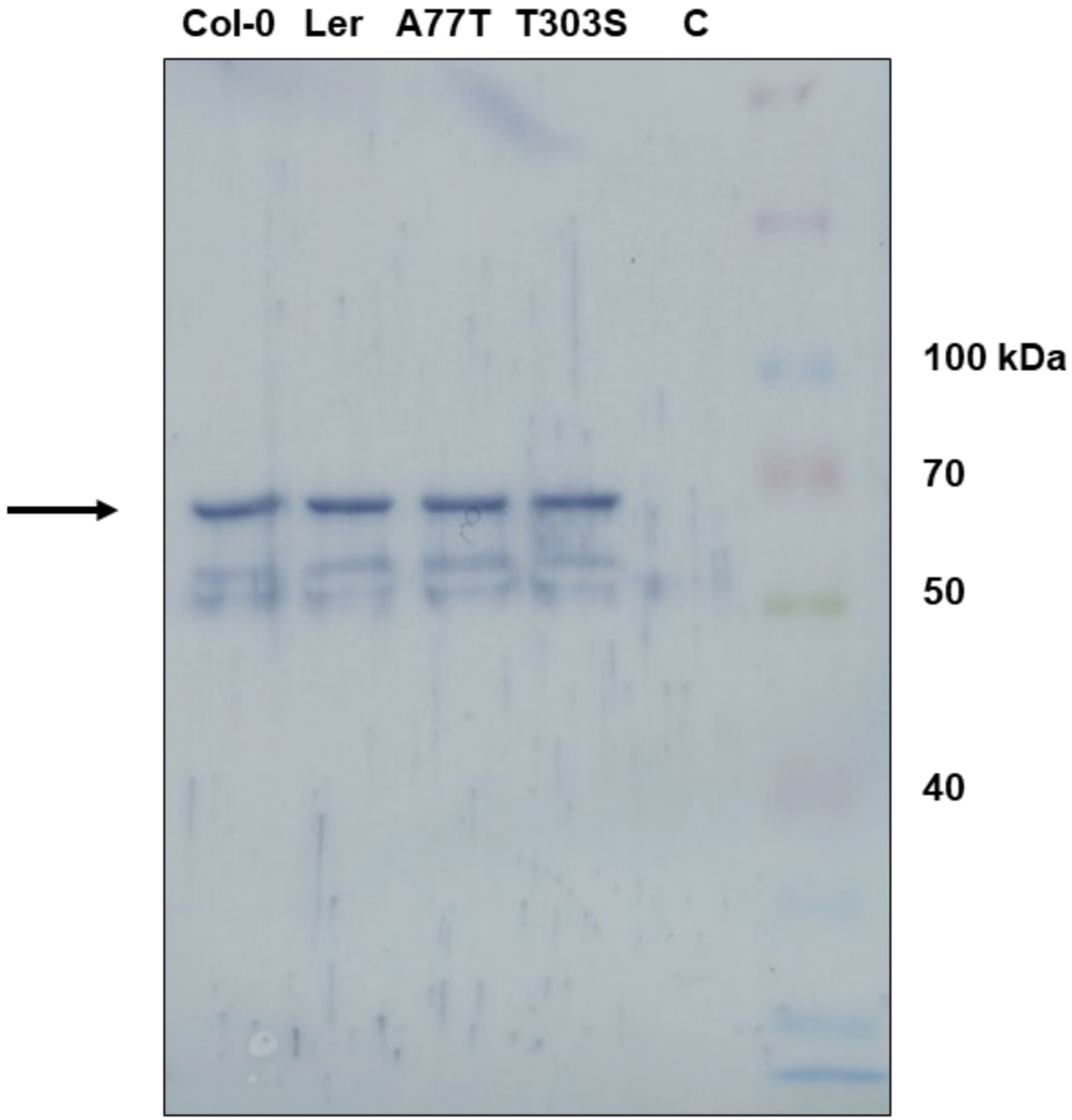


Figure S4: Western blot of the purified AtDAT1 variants
In this figure a western blot is shown of purified His tagged protein from BL21 with pGEX-2TM(Col-0) (lane 1), pGEX-2TM(Ler) (lane 2), pGEX-2TM(A77T) (lane 3), pGEX-2TM(T303S) (lane 4), empty vector (C, lane 5) and the protein size standard (Spectra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