PHYSIOLOGICAL ASPECTS OF NON-PROTEINOGENIC AMINO ACIDS IN PLANTS

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PHYSIOLOGICAL ASPECTS OF NON-PROTEINOGENIC AMINO ACIDS IN PLANTS

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Editorial: Physiological Aspects of Non-proteinogenic Amino Acids in Plants

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Editorial on the Research Topic

Physiological Aspects of Non-proteinogenic Amino Acids in Plants

In addition to the canonical 20 amino acids that constitute the essential building blocks of proteins, plants produce a wide variety of non-proteinogenic amino acids (NPAAs; Fowden, 1981, Rosenthal, 1982, Barrett, 1985, Bell, 2003). Some of these plant metabolites are components of central metabolism, serving as intermediates in biosynthetic pathways or as signaling molecules during plant stress responses. NPAAs such as ornithine, citrulline, arginosuccinate, homoserine, homocysteine, and cystathionine, are well-studied metabolic intermediates and are likely to be present in all plant species. Other commonly encountered plant NPAAs, for instance pipecolic acid with its derivatives, can function as signaling molecules that influence plant development, physiology, and defense responses (Huang et al., 2020).

A particularly noteworthy NPAA, γ-aminobutyric acid (GABA), is essential for many physiological and developmental processes in plants, including energy dissipation, maintenance of carbon/nitrogen balance, pollen tube growth, and fruit development (Kinnersley and Turano, 2000, Palanivelu et al., 2003, Fait et al., 2008, Snowden et al., 2015, Amir et al., 2018). Functioning as both signaling molecule and a regulator of plant metabolism, GABA can modulate plant immune responses (Kim et al., 2013, Wang et al., 2019, Deng et al., 2020, Tarkowski et al., 2020). Numerous studies have shown a role for GABA accumulation in protecting plants against abiotic stresses such as drought and salinity (Bor et al., 2009, Akcay et al., 2012, Vijayakumari and Puthur, 2015, Mekonnen, 2017, Carillo, 2018, Rezaei-Chiyaneh et al., 2018, Jin et al., 2019, Podlesakova et al., 2019).

NPAAs that are not part of primary metabolism are often defense-related, providing protection against pests and pathogens, and typically have a more sporadic distribution in the plant kingdom (Bell, 1976). For instance, many legumes accumulate large amounts of canavanine or other NPAAs that not only function as defensive metabolites but also serve for nitrogen storage in the seeds (Huang et al., 2011). Canavanine is a structural analog of arginine and exerts its toxicity in animals by interfering with arginine-related metabolism, including nitric oxide synthase and incorporation of arginine into proteins (Bence and Crooks, 2003). In new research on the toxicity of canavanine in plants, Staszek et al. show that the canavanine-mediated inhibition of nitric oxide biosynthesis leads to formation of differentially nitrated proteins and a disruption of the antioxidant system in tomato roots.

Another NPAA, 1-aminocyclopropane carboxylate (ACC), is the direct precursor of ethylene, a gaseous hormone regulating a wide ranges of developmental and stress-related processes in plants (e.g., Lee et al., 2019, Seo and Yoon, 2019). However, as discussed by Polko and Kieber, ACC itself also functions as a plant signaling molecule. Physiological processes in plants that are

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influenced directly by ACC include stomatal development, cell wall biosynthesis, stress responses, and pathogen interactions (Xu et al., 2008, Tsuchisaka et al., 2009, Tsang et al., 2011, Yin et al., 2019). The levels of ACC in plants are critical for ethylene production and seem to be influenced by another group of NPAAs, the *D*-Amino acids. *D*-Amino acid isomers of the proteinogenic *L*-amino acids are produced by soil microbes and are taken up by plant roots, but can also be produced by plants themselves (Genchi, 2017). Although some *D*-amino acids are toxic to *Arabidopsis thaliana* (Arabidopsis) at low concentrations (Erikson et al., 2004), the metabolism of *D*-amino acids strongly varies between different Arabidopsis ecotypes (Gordes et al., 2013). Suarez et al. used natural accessions and transgenic mutant lines to identify and investigate AtDAT1, a major *D*-amino acid transaminase in Arabidopsis. Decreased activity of this enzyme leads to enhanced susceptibility to *D*-methionine and increased *D*-amino acid abundance stimulated accumulation of ethylene. In this study it was demonstrated, that the regulation of *D*-methionine and ACC derivatives in plants are interlinked. However, the detailed mechanisms by which *D*-amino acids induce ethylene production remain to be investigated.

β-Amino acids, which have the amino group attached to the β-carbon rather than the adjacent α-carbon, have been reported in many plant species (Kudo et al., 2014). Whereas, some β-amino acids, for instance β-tyrosine, have likely defensive functions in plants (Yan et al., 2015), others are essential components of primary metabolism. Parthasarathy et al. review the biosynthesis and function of β-alanine, which is not only a component of vitamin B_5 and thereby is essential for Coenzyme A function, but also contributes to plant responses to both biotic and abiotic stresses. Although the β-alanine biosynthetic pathways are not yet completely elucidated in plants, spermine, spermidine, propionate, and uracil are known metabolic precursors.

The biosynthetic pathways of proteinogenic amino acids, and by extension the biosynthesis of NPAAs that serve as intermediates in these pathways, have been elucidated in Arabidopsis and other plant species (Jander and Joshi, 2010). However, the biosynthetic pathways and/or metabolic functions have been unraveled for only a few of the hundreds of other plant NPAAs, including *D*-amino acids, β-amino acids, other isomers, and structural mimics. Thus, there are many opportunities for novel discoveries in this research area. In particular, with the development of new research methods for studying non-model plant species at the molecular level, it will be possible to study the biosynthesis pathways, as well as structural, defensive, and signaling functions, of NPAAs that are not present in Arabidopsis.

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GJ wrote the first draft. All authors contributed revisions and approved the published version of the manuscript.

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The Synthesis and Role of β-Alanine in Plants

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Most studies on amino acids are focused on the proteinogenic amino acids given their essential roles in protein synthesis among other pathways. In addition to 20 ubiquitous amino acids used in protein synthesis, plants synthesize over 250 non-proteinogenic amino acids that are involved in the synthesis of compounds that are anti-herbivory, anti-microbial, response to abiotic stresses, nitrogen storage, toxins against both vertebrates/invertebrates, and plant hormones among others. One such non-proteinogenic acid is β-alanine, which is known mainly for studies on humans. β-Alanine forms a part of pantothenate (vitamin B5), which is incorporated into the universal carbon shuttling compounds Coenzyme A and acyl carrier protein, in all organisms including plants. The focus of this review, however, is on the biosynthesis, metabolism, and the role of β-alanine in plants. There are several functions of β-alanine unique to plants. It is accumulated as a generic stress response molecule involved in protecting plants from temperature extremes, hypoxia, drought, heavy metal shock, and some biotic stresses. There is evidence of its participation in lignin biosynthesis and ethylene production in some species. It is further converted to the osmoprotective compound β-alanine betaine in some species and converted to the antioxidant homoglutathione in others. The polyamines spermine/spermidine, propionate and uracil have been shown to be precursors of β-alanine in plants. However, plants vary in terms of their biosynthetic pathways, and the primary metabolism of β-alanine is far from settled.

Keywords: β-alanine, L-alanine, non-proteinogenic, amino acid, secondary metabolites

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INTRODUCTION

β-Alanine is a non-proteinogenic amino acid, where the amino group is at the β-position from the carboxylate group (IUPAC name = 3-aminopropanoic acid). In contrast to L-alanine, which is a proteinogenic amino acid, β-alanine has no stereo center (**Figure 1**). β-Alanine is incorporated into pantothenate (Vitamin B5), and therefore, is a precursor of Coenzyme A (CoA) and acyl-carrier protein, which shuttle carbon within the cell (Voet et al., 2006). β-Alanine is a component of carnosine, a dipeptide concentrated in muscle and brain tissue, which underlies the wide use of β-alanine in humans as a strength enhancing supplement. The vast majority of scientific articles about β-alanine deal with the exercise supplement aspect, please see for example Blancquaert et al. (2017). Much less is known about the role of β-alanine in plants.

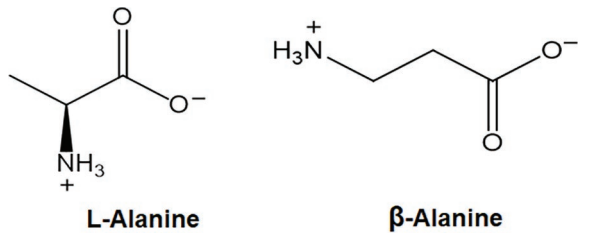

FIGURE 1 | The chemical structures of L-alanine and β-alanine.

The focus of this manuscript is the current state of knowledge on the metabolism and role of β-alanine in plants.

Apart from the universal importance of β-alanine (as the precursor of CoA) in the synthesis of phospholipids, synthesis and degradation of fatty acids, and the operation of the tricarboxylic acid cycle, plants also employ β-alanine in secondary metabolism, including lignin biosynthesis (Broeckling et al., 2005). Moreover, β-alanine is involved in multiple stress responses in plants. LeSvels of β-alanine have been shown to be elevated significantly following heat shock in *Vigna unguiculata* cell cultures (Mayer et al., 1990). Various biotic and abiotic stresses on *Medicago truncatula* cell cultures resulted in elevated β-alanine levels, suggesting changes in the metabolism of CoA and its thioesters, which are essential in secondary metabolism (Broeckling et al., 2005). Both drought and heat stress were found to induce increased β-alanine levels in the model plant *Arabidopsis thaliana* (Kaplan et al., 2004; Rizhsky et al., 2004). Metabolomics studies reveal that in response to cadmium ions (heavy metal stress); *A. thaliana* accumulates multiple compounds, including L-alanine, β-alanine, and the polyamine putrescine (Sun et al., 2010).

β-Alanine aminotransferases from plants have been known since at least the late 1960s (Stinson and Spencer, 1969a,b). Work on enzymes from *Phaseolus vulgaris* (wax bean) cotyledons showed that β-alanine could be converted into the well-known plant signaling molecule ethylene (Stinson and Spencer, 1969a,b). In some plants, β-alanine is additionally converted to β-alanine betaine, an important quaternary ammonium osmoprotective compound that is involved in tolerance to both high salt concentration and low oxygen (Hanson et al., 1991, 1994; van Dongen et al., 2009; Rocha et al., 2010a,b). A role for β-alanine in recovery from waterlogging was suggested in an earlier study employing a hydroponic system (Drakeford et al., 1985). *In vitro* protection against high temperature stress for the enzyme lactate dehydrogenase (LDH) is afforded by β-alanine (Mehta and Seidler, 2005).

In some leguminous plants, β-alanine forms a part of the thiol tripeptide homoglutathione, which is involved in protection against heavy metal toxicity and free radicals (reactive oxygen species) (Klapheck et al., 1988; Moran et al., 2000). For this reason, pathways involving β-alanine are considered attractive targets for the metabolic engineering of plants, potentially including crop plants in a world facing a growing human population and increasing environmental stresses due to climate change. Work that is more recent suggests that β-alanine metabolism in *A. thaliana* involved in a variety of pathways and roles such as nitrogen utilization efficiency, response to hypoxia, osmoprotection, vitamin B5 and CoA metabolism (Parthasarathy et al., 2019).

THE SYNTHESIS OF β-ALANINE IN PLANTS

The biosynthesis of β-alanine in plants may be initiated from at least four different precursors, namely (1) the polyamines spermine/spermidine, (2) the carboxylic acid propionate, (3) the nucleotide base uracil, and (4) the proteinogenic amino acid L-aspartate. Among these, the first three are thoroughly characterized, while the fourth has long been postulated without conclusive experimental evidence. The physiological implications of each pathway are discussed below.

The Synthesis of β-Alanine in Plants: The Polyamine Pathway

The polyamines spermine and spermidine are converted *via* 1,3-diaminopropane to β-alanine (**Figure 2**) in many plants and demonstrated in maize shoots (Terano and Suzuki, 1978; Galston and Sawhney, 1990). Pericarp discs of tomato fruits were also shown to degrade spermidine to putrescine and β-alanine (Rastogi and Davies, 1989). Spermidine is cleaved by the FAD- and heme-containing enzyme spermidine dehydrogenase [EC 1.5.99.6] into 1,3-diaminopropane and 4-aminobutyraldehyde. Spermine degradation is facilitated by the incorporation of water and molecular oxygen by the enzyme polyamine oxidase [EC 1.5.3.14], yielding 1,3-diaminopropane, 4-aminobutyraldehyde, and hydrogen peroxide. The common intermediate 1,3-diaminopropane is then deaminated with the addition of water and molecular oxygen into 3-aminopropionaldehyde, hydrogen peroxide, and ammonia by the enzyme diamine oxidase [EC 1.4.3.22]. Finally, oxidation of 3-aminopropionaldehyde with the addition of water by the NAD(P)-dependent aldehyde dehydrogenase [EC 1.2.1.3] yields β-alanine. Spermidine oxidation *via* these enzymes is induced by abiotic stresses (Kamada-Nobusada et al., 2008; Moschou et al., 2008a,b).

*At*ALDH10A8, an NAD^+-dependent aminoaldehyde dehydrogenase [EC 1.2.1.19] in *A. thaliana* was shown to convert 3-aminopropionaldehyde into β-alanine *in vitro*, and similar enzymes were studied in apple fruit (Zarei et al., 2015, 2016). However, unlike gamma-aminobutyric acid (GABA), which was specifically accumulated in response to salinity, β-alanine was not, suggesting that β-alanine is accumulated as part of a non-specific rather than a specific stress response (Zarei et al., 2016). *A. thaliana* has been shown to survive without the ability to make spermine, but spermidine synthesis is essential to survival. This suggests that spermidine (but not spermine) could be linked to pantothenate biosynthesis *via* β-alanine (Imai et al., 2004). The condensation of β-alanine with pantoate in plants (**Figure 2**) yields pantothenate (vitamin B5), which is essential for all organisms as a precursor to the 4′-phosphopantetheine

moiety of coenzyme A (CoA) and acyl carrier protein (Webb et al., 2004; Webb and Smith, 2011).

As seen in **Figure 2**, there is an intriguing link between the metabolism of the branched chain amino acids (BCAA) and β-alanine. It appears that there is a feedback loop whereby a change in the levels of β-alanine may be effected *via* BCAA degradation. Since valine and isoleucine may serve as precursors for propionyl-CoA, recent studies in this regard are discussed in the next section.

The Synthesis of β-Alanine in Plants: The Propionate Pathway

Propionate may act as the precursor for β-alanine in the pathway involving a β-alanine aminotransferase (**Figure 3**). Propionate is activated with the hydrolysis of ATP to propionyl-CoA A by propionate CoA ligase [EC 6.2.1.17]. Propionyl-CoA is then oxidized to acrylyl-CoA by the FAD-dependent medium chain acyl-CoA dehydrogenase [EC 1.3.8.7]. 3-hydroxypropionyl-CoA dehydratase [EC 4.2.1.116], then hydrates acrylyl-CoA yielding

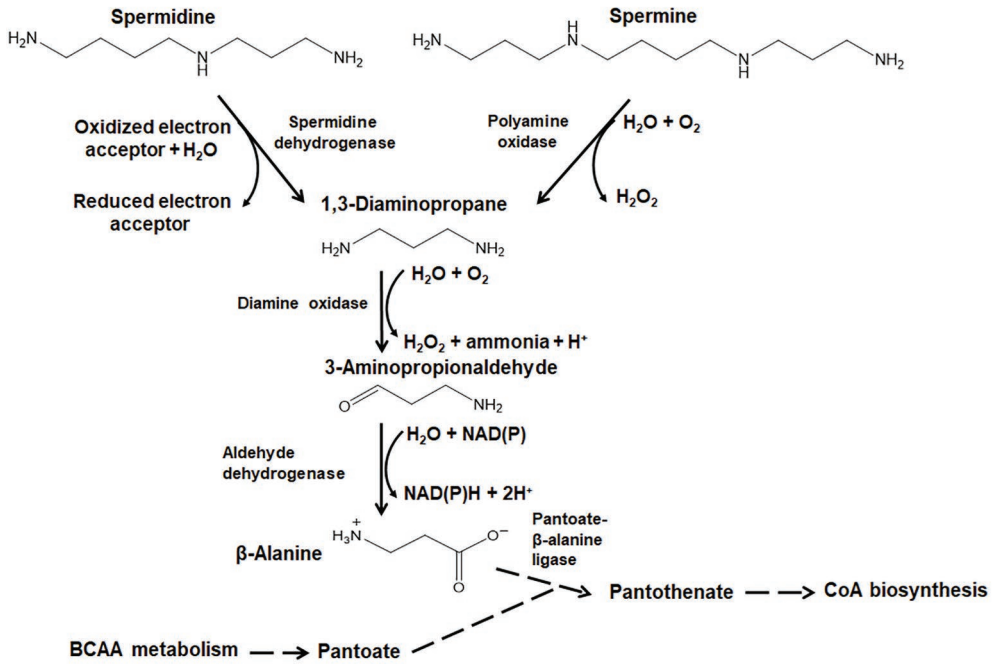

FIGURE 2 | Anabolism of β-Alanine via the spermine and spermidine pathways. The enzyme class (EC) numbers shown in figure correspond to the following enzymes: spermidine dehydrogenase [EC 1.5.99.6], polyamine oxidase [EC 1.5.3.14], diamine oxidase [EC 1.4.3.22], aldehyde dehydrogenase [EC 1.2.1.3], and pantoate-β-alanine ligase (AMP-forming) [EC 6.3.2.1]. The dotted lines show the pantothenate and CoA biosynthesis pathway into which β-alanine can be fed. BCAA, branched chain amino acid.

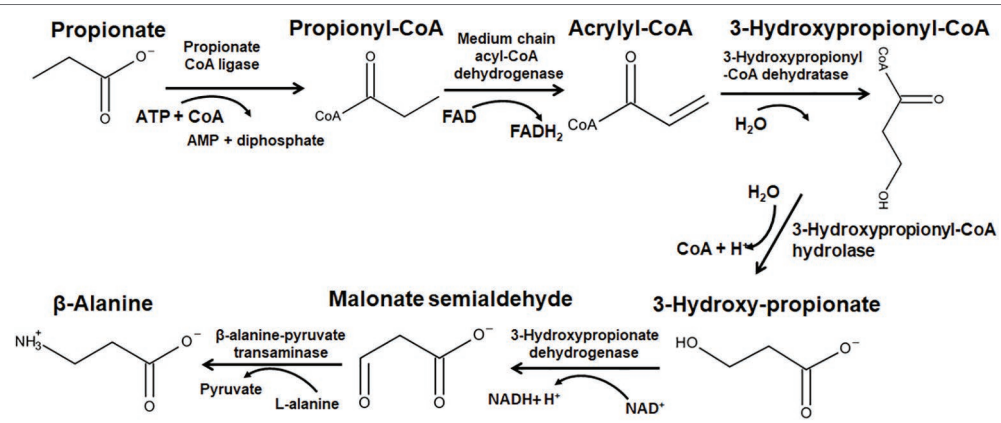

FIGURE 3 | The anabolism of β-alanine *via* the propionate pathway. The enzymes shown in the figure correspond to the following EC numbers: propionate CoA ligase = [EC 6.2.1.17]; medium chain acyl-CoA dehydrogenase = [EC 1.3.8.7]; 3-hydroxypropionyl-CoA dehydratase = [EC 4.2.1.116]; 3-hydroxypropionyl-CoA hydrolase = [EC 3.1.2.4]; 3-hydroxypropionate dehydrogenase = [EC 1.1.1.59], and β-alanine-pyruvate transaminase = [EC 2.6.1.18].

3-hydroxypropionyl-CoA. Hydrolysis of the CoA ester by 3-hydroxypropionyl-CoA hydrolase [EC 3.1.2.4] generates 3-hydroxyproiponate, which is further oxidized to malonate semialdehyde by the NAD-dependent 3-hydroxypropionate dehydrogenase [EC 1.1.1.59]. The final step in this pathway is the transamination catalyzed by the pydridoxal-5′-phosphate (PLP)-dependent β-alanine-pyruvate transaminase [EC 2.6.1.18], whereby an amino group is transferred from L-alanine to malonate semialdehyde, yielding β-alanine and pyruvate (Hayaishi et al., 1961; Stinson and Spencer, 1969a,b). After 2001 study identified a β-alanine aminotransferase in *A. thaliana* (Liepman and Olsen, 2001), it was hypothesized that this pathway may be involved in pantothenate production. In *A. thaliana*, there is a gene encoding for the pantothenate synthase (PtS) enzyme, which condenses pantoate with β-alanine to yield pantothenate (Genschel et al., 1999; Ottenhof et al., 2004). It should be noted that homologous gene/s encoding aspartate decarboxylase (ADC), the enzyme that generates β-alanine in the bacterial pantothenate pathway were not identified in the plant (Ottenhof et al., 2004).

The propionate pathway connects the metabolism of L-alanine and β-alanine in plants, since the final step requires the former amino acid as the amino donor (**Figure 3**). Therefore, a change in the levels of one or the other of the two amino acids may have wide-ranging physiological effects. Gene regulation studies of alanine aminotransferase has been studied in many plants in response to low-oxygen stress, light, and nitrogen, and it was demonstrated that hypoxia induced the expression of two distinct alanine aminotransferase genes in *A. thaliana* (Miyashita et al., 2007). The function of the gene product from the locus tag At3g08860 was unknown for a long time, but was recently shown to encode a β-alanine/L-alanine aminotransferase using an *in vivo* functional complementation approach (Parthasarathy et al., 2019). The same study reported that a definitive preference of the corresponding enzymatic activity toward the synthesis of β-alanine. The gene corresponding to the β-alanine/L-alanine aminotransferase was shown to be most highly expressed in *A. thaliana* roots (Schmid et al., 2005). The enrichment of the β-alanine aminotransferase enzyme in the roots suggests that β-alanine possibly has a specific protective function in the root tissue. Earlier, hydroponics studies in *Helianthus annuus L.* revealed that roots which were excised and flooded for 24 h had reduced uptake of radioactive β-alanine than non-flooded roots and also that they exuded more radioactive β-alanine into distilled water (Drakeford et al., 1985). This suggests, first, that there is a regulation of β-alanine uptake (if available in the medium), and second, that flooded roots may exude β-alanine for protective purposes. Therefore, it is likely that plants require enhanced expression of the β-alanine aminotransferase in the roots to facilitate a β-alanine based defense response when faced with hypoxia or flooding.

Additional studies have addressed the links between branched-chain amino acids and β-alanine, since valine and isoleucine degradation can produce propionyl-CoA. Using radiolabeled precursors of both isoleucine and valine, it was shown in *A. thaliana* that only isoleucine directly generated β-alanine. However, if seedlings were treated with valine, an increase in the levels of β-alanine resulted, suggesting an indirect effect *via* amino acid homeostasis (Perrett et al., 2017). Seedlings harboring a mutated methylmalonate semialdehyde dehydrogenase (MMSD, At2g14170) gene cannot convert valine into propionyl-CoA (since the Mmsd-1 protein catalyzes the final step leading to propionyl-CoA); but the equilibrium is shifted toward β-alanine, presumably by the amino acid homeostatic mechanism (Perrett et al., 2017). More recently, feeding of 13C-labeled isoleucine and propionate were shown to result in the production of C^{13}-labeled 3-hydroxypropionate and β-alanine in *A. thaliana* seedlings, offering direct evidence for the metabolic link between isoleucine and β-alanine *via* propionyl-CoA (Goldfarb and Rouhier, 2019). Further studies in transgenic *A. thaliana* and wheat seedlings confirmed that isoleucine degradation could initiate β-alanine synthesis (Rouhier et al., 2019). This suggests that isoleucine may serve as an additional precursor for β-alanine in plants (apart from those known already) and since valine is a precursor of pantoate, pantothenate production could depend on BCAA catabolism.

The Synthesis of β-Alanine in Plants: The Uracil Pathway

The nucleotide base uracil is reduced by NADPH-dependent dihydrouracil dehydrogenase [EC 1.3.1.2] into dihydrouracil (**Figure 4A**); in *A. thaliana,* this gene is identified with the At3g17810 locus. The hydrolysis of dihydrouracil into 3-ureidopropionate is catalyzed by dihydropyrimidinase [EC 3.5.2.2] (**Figure 4A**); the accession IDs in *A. thaliana* are At5g12200 and At5g12200.1. Further hydrolysis by β-ureidopropionase [EC 3.5.1.6] eliminates ammonia and carbon dioxide, yielding β-alanine as shown in **Figure 4A** (Campbell, 1960; Traut and Loechel, 1984); the *A. thaliana* accession IDs are At5g64370 and At5g64370.1. The degradation of uracil and thymine to produce carbon dioxide, ammonia, β-alanine, and γ-aminoisobutyrate has been shown to occur in seedlings of *Brassica napus* (rapeseed), while the β-ureidopropionase enzyme of maize was characterized by its overexpression in *Escherichia coli* (Tsai and Axelrod, 1965; Walsh et al., 2001). The existence of this pathway in pine trees was inferred already in the 1960s (Barnes and Naylor, 1962). In the halotolerant members of the leadwort or plumbago family (*Plumbaginaceae*) of perennial plants, uracil is only one of many sources for β-alanine (Duhazé et al., 2003). Thus, the distribution of this pathway in higher plants varies widely.

The Synthesis of β-Alanine in Plants: The Aspartate Pathway

L-aspartate may be decarboxylated by aspartate 1-decarboxylase [EC 4.1.1.11] also yielding β-alanine, which may enter Coenzyme A biosynthesis (**Figure 4B**). This enzyme abbreviated ADC and encoded by the *panD* gene has been well characterized in prokaryotes (Williamson and Brown, 1979; Merkel and Nichols, 1996). However, its presence in plants has never been identified and or confirmed. Bioinformatics analysis based on sequence homology using the FUGUE tool failed to find homologs in either *A. thaliana* or the yeast *Saccharomyces cerevisiae* genomes, casting doubt on whether this pathway

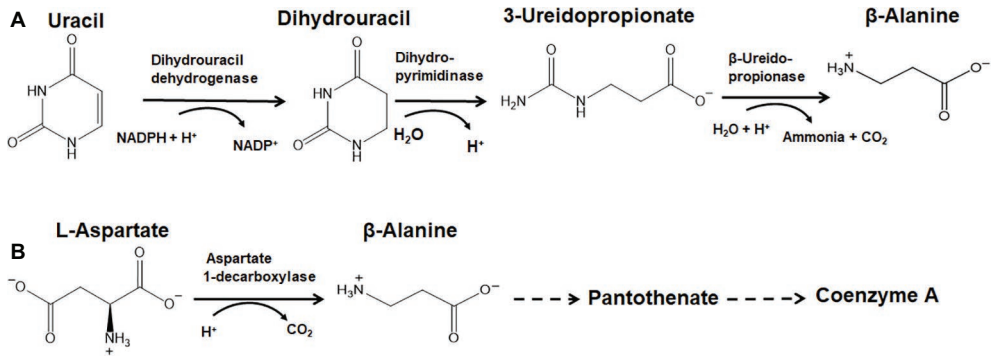

FIGURE 4 | The anabolism of β-Alanine biosynthesis *via* uracil and L-aspartate. **(A)** The nucleotide base uracil can be degraded to β-alanine. The enzymes in this pathway are dihydrouracil dehydrogenase [EC 1.3.1.2], dihydropyrimidinase [EC 3.5.2.2] and β-ureidopropionase [EC 3.5.1.6]. **(B)** Aspartate 1-decarboxylase [EC 4.1.1.11] can directly decarboxylate L-aspartate into β-alanine, which may feed into Coenzyme A biosynthesis *via* pantothenate.

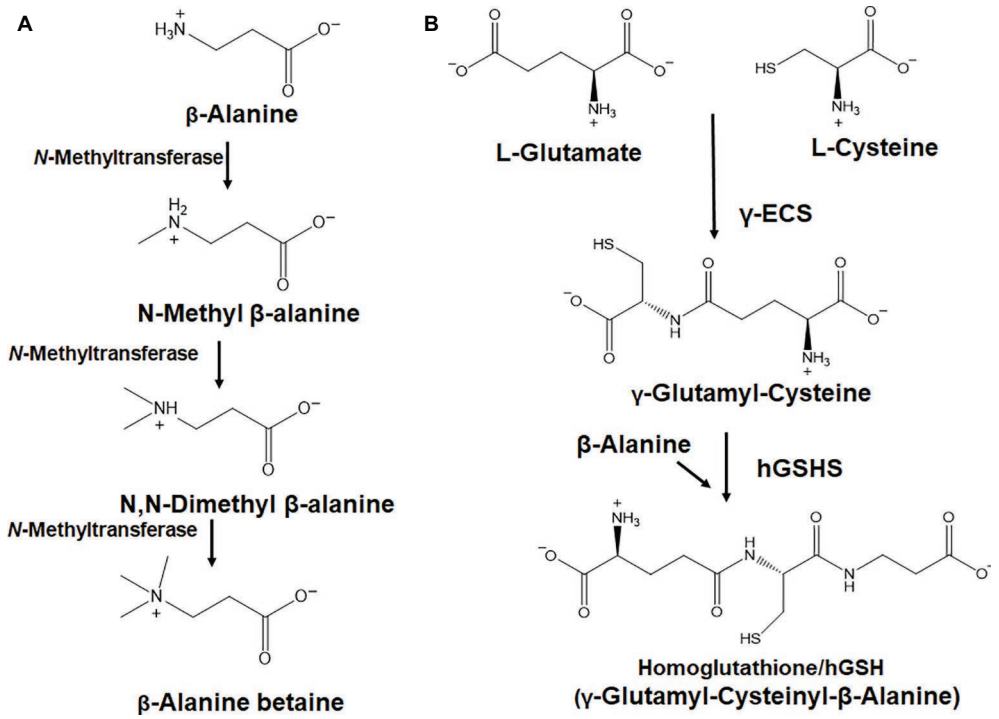

FIGURE 5 | The conversion of β-alanine to important secondary metabolites. **(A)** A trifunctional, *S*-adenosyl-L-methionine (SAM)-dependent *N*-methyltransferase [EC 2.1.1.49], performs iterative *N*-methylation of β-alanine to β-alanine betaine. **(B)** Homoglutathione biosynthesis involves the enzymes, glutamate cysteine ligase (GCL) or γ-ECS (γ-glutamyl-cysteine synthetase) [EC 6.3.2.2] and a β-alanine specific homoglutathione synthase (hGSHS) [EC 6.3.2.23], both of which perform specific amino acid condensations at the expense of ATP.

crossed the divide between prokaryotes and eukaryotes (Shi et al., 2001; Ottenhof et al., 2004). With the availability of numerous genome sequences of plants and fungi available since 2004, further inquiry of *panD* orthologs using bioinformatics tools is warranted.

Since only plants and microbes synthesize pantothenate *de novo*, there is significant interest in engineering this pathway in plants in order to fortify food crops with pantothenate, which is essential for animals since they lack the enzymes required to biosynthesize the compound (Coxon et al., 2005; Chakauya et al., 2008). Also, since only plants and microbes contain the pathway for pantothenate, it is also considered an attractive target for herbicides, fungicides and antibiotic development (Coxon et al., 2005). Increased heat tolerance *via* overproduction of β-alanine and pantothenate content could be achieved in transgenic tobacco plants harboring the gene for the *E. coli* ADC enzyme (Fouad and Rathinasabapathi, 2006). Later, the same system was also shown to enhance photosynthesis and to augment biomass production in response to higher temperatures (Fouad and Altpeter, 2009). These are important

practical advances that could pave the way for heat resistant transgenic food crops that are better suited in warmer climate zones and in changing climates, where loss in agricultural productivity with increasing temperatures might be a concern.

THE UTILIZATION OF β-ALANINE IN OTHER PLANT PATHWAYS

β-Alanine Betaine

β-Alanine betaine is an osmoprotective compound accumulated by most members of the highly stress-tolerant leadwort or plumbago family (*Plumbaginaceae*). β-Alanine betaine is synthesized by S-adenosyl-*L*-methionine (SAM)-dependent enzymatic *N*-methylation of β-alanine (**Figure 5A**) *via* *N*-methyl β-alanine and *N,N*-dimethyl β-alanine (Raman and Rathinasabapathi, 2003). Most members of the highly stress-tolerant *Plumbaginaceae* accumulate β-alanine betaine instead of glycine betaine (Hanson et al., 1991, 1994). It was proposed that β-alanine betaine is a more suitable osmoprotectant than glycine betaine under saline hypoxic conditions because the first step in glycine betaine synthesis catalyzed by choline monooxygenase [EC 1.14.15.7] requires molecular oxygen (Hanson et al., 1991, 1994). Further, β-alanine betaine accumulation was proposed to be an evolutionary strategy to avoid the metabolic limitations existing for choline oxidation under hypoxic conditions, because β-alanine betaine is synthesized from the ubiquitous primary metabolite β-alanine (Hanson et al., 1994).

Homoglutathione

In many legumes, the thiol tripeptide homoglutathione (hGSH; γGlu-Cys-βAla) (**Figure 5B**) can partially or fully replace the better-known thiol, glutathione. γ-Glu-Cys is formed from L-glutamate and L-cysteine by glutamate cysteine ligase (γ-Glutamylcysteine synthetase or γ-ECS) at the expense of ATP, and γ-Glu-Cys and β-alanine are condensed at the expense of ATP by a specific hGSH synthetase (hGSHS), an enzyme which has affinity for β-alanine and low affinity for glycine (Macnicol, 1987; Klapheck, 1988; Klapheck et al., 1988). Multiple cellular compartments are involved in the biosynthesis of hGSH and fractionation of the root nodules demonstrated that the bacteroids contain high hGSH concentrations and the highest specific activities of GSHS, suggesting a critical role in nitrogen fixation processes in the root nodules of legume plants (Moran et al., 2000). More recent work in *M. truncatula* also showed that hGSH is essential for the growth of parasitic nematode worms, which infect plant roots and force the differentiation of root cells into giant cells. In addition, it was also shown that hGSH-depleted roots did not suffer similar damage by nematodes (Baldacci-Cresp et al., 2012). Thus, hGSH may play key roles in plant defense and could be the target of future interventions into improving plant resistance to nematodes.

CONCLUSIONS

Although underappreciated, the non-proteinogenic amino acid β-alanine has important roles in plant physiology and metabolism, directly as a defense compound that enables plants to withstand various stresses such as hypoxia, waterlogging and drought, and indirectly as a precursor to the compounds pantothenate and CoA, which are involved in a variety of functions. Furthermore, the amino acid is converted into β-alanine betaine, which has additional protective functions such as salt tolerance, and homoglutathione, which may be critical for nitrogen fixation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Canavanine-Induced Decrease in Nitric Oxide Synthesis Alters Activity of Antioxidant System but Does Not Impact S-Nitrosoglutathione Catabolism in Tomato Roots

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Canavanine (CAN) is a nonproteinogenic amino acid synthesized in legumes. In mammalians, as arginine analogue, it is an inhibitor of nitric oxide synthase (NOS) activity. The aim of this study was to investigate the impact of CAN-induced nitric oxide level limitation on the antioxidant system and *S*-nitrosoglutathione (GSNO) metabolism in roots of tomato seedlings. Treatment with CAN (10 or 50 μM) for 24–72 h led to restriction in root growth. Arginine-dependent NOS-like activity was almost completely inhibited, demonstrating direct effect of CAN action. CAN increased total antioxidant capacity and the level of sulphydryl groups. Catalase (CAT) and superoxide dismutase (SOD) activity decreased in CAN exposed roots. CAN supplementation resulted in the decrease of transcript levels of genes coding CAT (with the exception of *CAT1*). Genes coding SOD (except *MnSOD* and *CuSOD*) were upregulated by CAN short treatment; prolonged exposition to 50-μM CAN resulted in downregulation of *FeSOD, CuSOD*, and *SODP-2*. Activity of glutathione reductase dropped down after short-term (10-μM CAN) supplementation, while glutathione peroxidase activity was not affected. Transcript levels of glutathione reductase genes declined in response to CAN. Genes coding glutathione peroxidase were upregulated by 50-μM CAN, while 10-μM CAN downregulated *GSHPx1*. Inhibition of NOS-like activity by CAN resulted in lower GSNO accumulation in root tips. Activity of GSNO reductase was decreased by short-term supplementation with CAN. In contrast, GSNO reductase protein abundance was higher, while transcript levels were slightly altered in roots exposed to CAN. This is the first report on identification of differentially nitrated proteins in response to supplementation with nonproteinogenic amino acid. Among nitrated proteins differentially modified by CAN, seed storage proteins (after short-term CAN treatment) and components of the cellular redox system (after prolonged CAN supplementation) were identified. The findings demonstrate that due to inhibition of NOS-like activity, CAN leads to modification in antioxidant system. Limitation in GSNO level is due to lower nitric oxide formation, while GSNO catabolism is less affected. We demonstrated that monodehydroascorbate reductase, activity of

which is inhibited in roots of CAN-treated plants, is the protein preferentially modified by tyrosine nitration.

Keywords: canavanine, cellular antioxidant system, GSNOR—GSNO reductase, nitrated proteins, nitric oxide—NO, nonproteinogenic amino acid, NOS-like activity, reactive nitrogen species (RNS)

INTRODUCTION

Canavanine (CAN) is a nonproteinogenic amino acid (NPAA) of a legume plant origin (Rosenthal, 1982). It is a structural analogue of L-arginine (Arg), thus its primary mode of action is linked to disturbances in Arg-dependent reactions or incorporation into proteins instead of Arg [review by Staszek et al. (2017)]. Incorporation of CAN disturbs the correct structure of target proteins and leads to protein dysfunction or prevention of repair of DNA damage. Some studies have revealed that CAN may reduce proliferation of tumor cells (Nurcahyanti and Wink, 2016). CAN is also a selective inhibitor of an inducible isoform of nitric oxide (NO) synthase (NOS), an enzyme involved in the generation of NO from Arg in mammalians (Abd el-Gawad and Khalifa, 2001; Li et al., 2001). CAN-dependent restriction of NOS activity was accompanied by a limitation in NO emission (Luzzi and Marletta, 2005), and induction of an oxidative burst (Demiryürek et al., 1997; Riganti et al., 2003).

Based on experiments conducted in our laboratory, we have suggested that also in plant research, CAN may be used as a convenient biochemical tool for the modification of NO metabolism (Krasuska et al., 2016c; Staszek et al., 2017). Arg-dependent formation of NO in plants is still under investigation and discussion, although there are many reports confirming contribution of this oxidative pathway of NO synthesis in various plant tissues (review by Jeandroz et al., 2016 and Corpas and Barroso, 2017).

Over the past two decades, due to publication of numerous reports, NO has been accepted as a signaling molecule in plants development and responses to stresses (review by Baudouin and Hancock, 2014 and Domingos et al., 2015). It is widely accepted that the activity of reactive nitrogen species (RNS), including NO, should be considered on the basis of modifications in reactive oxygen species (ROS) metabolism (Corpas and Barroso, 2013; Groß et al., 2013; Begara-Morales et al., 2016; Farnese et al., 2016).

In our previous experiments, tomato (*Solanum lycopersicum* L.) seedlings were cultured for 24 or 72 h in 10-μM or 50-μM CAN resulting in inhibition of root growth in 50 or 100%, respectively (**Figure 1**). Malformations in root morphology (shorter and thicker roots, limited number of lateral roots) were accompanied by modification in NO and peroxynitrite ($ONOO^-$) production and localization (Krasuska et al., 2016a; Krasuska et al., 2016b). CAN restricted NO emission and transiently (after 24 h) enhanced $ONOO^-$ production suggesting direct impact of this NPAA on NO biosynthesis (Krasuska et al., 2016a; Krasuska et al., 2016b). In the current work, we have measured Arg-dependent NOS-like activity in tomato roots to prove that CAN is an inhibitor of Arg-dependent NO formation in plants.

Supplementation of tomato seedlings with CAN led to increased superoxide radical ($O_2^{\bullet-}$) generation and elevated hydrogen peroxide (H_2O_2) concentration in the roots, accompanied by stimulation of protein carbonyl groups formation (Krasuska et al., 2016a). Based on these data, we have proposed that CAN-induced oxidative burst could be potentially due to the disruption of the cellular antioxidant system, as NO regulates many enzymes involved in modulation of ROS level (Begara-Morales et al., 2016). The aims of this work were to a) analyze the impact of CAN-induced NO limitation on activity of the enzymatic antioxidant system: superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC1.11.1.6), glutathione (GSH) reductase (GR; EC 1.8.1.7), and GSH peroxidase (GPx; EC 1.11.1.9) and to b) examine how CAN influences cellular antioxidants: total content of thiols and total antioxidant capacity.

The intracellular level of NO depends on its biosynthesis and detoxification (Astier et al., 2018; Lindermayr, 2018). NO and GSH form nitrosoglutathione (GSNO). The GSNO pool is regulated by the activity of GSNO reductase (GSNOR; EC 1.2.1.1), which converts GSNO into the oxidized form of GSH and ammonia (Petřivalský et al., 2015). GSNOR is considered as a key regulator of plant development and is induced in response to stresses (Leterrier et al., 2011; Kubienová et al., 2014). Increased activity of GSNOR was observed as a plant reaction to various abiotic stresses, e.g., cold or high temperature (Kubienová et al., 2014; Petřivalský et al., 2015; Tichá et al., 2016). However, it was difficult to find a general tendency in modulation of GSNOR activity in plants exposed to a distinct type of abiotic stressors, e.g., cadmium or arsenate decreased gene expression and GSNOR activity (Petřivalský et al., 2015). Lindermayr (2018) proposed GSNOR as a main component of the cross-talk between ROS and NO. According to his concept, during oxidative burst GSNOR loses its activity due to a modification of cysteine, leading to an accumulation of GSNO, which initiates NO signaling and induces the cellular antioxidant machinery. Therefore, the additional aim of our study was to investigate GSNOR activity, protein abundance, and gene transcription in roots of tomato plants characterized by CAN-induced secondary oxidative stress accompanied by lower NO production.

The chemical nature of NO and NO-derived molecules suggests that the signal transduction involves post-translational modifications of proteins (PTM), with *S*-nitrosylation and

Abbreviations: CAT, catalase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione reduced form; MDAR, monodehydroascorbate reductase; PTM, post-translational modification of protein; NPAA, Nonproteinogenic Amino Acid; $O_2^{\bullet-}$, superoxide radical; ROS, Reactive Oxygen Species; SOD, superoxide dismutase.

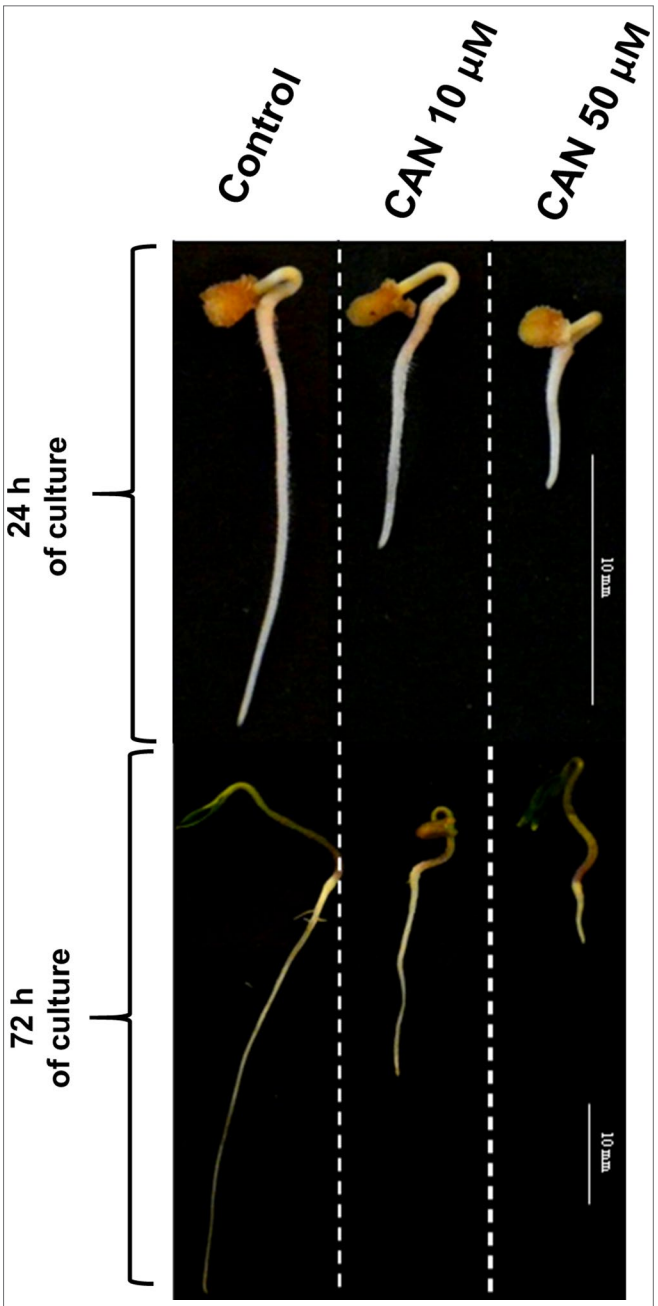


FIGURE 1 | Tomato seedlings grown for 24 or 72 h in water (control) or CAN aqueous solutions (10 or 50 μM). Images of representative seedlings are presented.

nitration being the most widely studied (Mata-Pérez et al., 2016). Therefore, protein modifications are expected to be the key mechanisms when considering the downstream effects of the NO molecule. Protein nitration is the reaction of nitrating agent with a tyrosine residue of the target proteins and results in formation of stable 3-nitrotyrosine (3-NT) (Kolbert et al., 2017). Formation of 3-NT modifies protein structure leading to changes in protein activity. This PTM, mediated by $ONOO^-$, could be considered as a marker of nitro-oxidative stress. As demonstrated earlier, short term (24 h) CAN supplementation of tomato seedlings resulted in an increase in 3-NT concentration in root proteins as compared with plants growing in water and corresponds well to $ONOO^-$ formation (Krasuska et al., 2016a). For that reason, a next step of this work was to identify 3-NT-immunopositive proteins that were abundant in roots of CAN-treated plants. We suspected that some of them could be stress-related proteins, elements of the cellular antioxidant system, as they were previously identified in other plant material to be targets of tyrosine nitration (Mata-Pérez et al., 2016).

MATERIALS AND METHODS

Plant Material

Tomato seeds (*Solanum lycopersicum* L. cv. Malinowy Ożarowski) (obtained commercially from PNOS Sp. z o.o.) were germinated in water at 20°C in darkness for 3 days. After this period, seedlings of equal roots' length (5 mm) were selected and transferred to Petri dishes (ϕ 15 cm) filled with filter paper wetted with water (control) or CAN (L-stereoisomer, Sigma-Aldrich) dissolved in distilled water. CAN at concentrations of 10 and 50 μM (inhibiting root growth in 50 and 100%, respectively) was used according to Krasuska et al. (2016b). Control seedlings and seedlings treated with CAN were cultured in a growth chamber at 23/20°C, 12/12 h day/night regime, and light intensity 150-μmol PAR m^{-2} s^{-1} for 24 or 72 h as described by Krasuska et al. (2016b).

Measurement of Arginine-Dependent Nitric Oxide Synthase-Like Activity

Arg-dependent NOS-like activity was measured according to a method described by Dawson and Knowles (1999) with modification by Krasuska et al. (2016c). Freshly collected roots were washed in distilled water and homogenized in an ice bath in 50-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–potassium hydroxide (HEPES) pH 7.0 containing: 5-mM dithiothreitol (DTT), 300-mM sucrose, 10% (w/v) glycerol, 0.1% (w/v) Triton X-100, 1% (v/v) cocktail of protease inhibitors (Sigma-Aldrich), and 2% (w/v) polyvinylpolypyrrolidone (PVPP). After centrifugation at 13,000*g* for 15 min at 4°C, the supernatant was desalted using protein concentrator PES, 3K MWCO (Thermo Scientific™).

After desalting, supernatants were collected for further analyses. The reaction mixture contained: 50-mM HEPES pH 7.5 with 1-mM nicotinamide adenine dinucleotide phosphate (NADPH), 10-μM DTT, 100-nM calmodulin, 10-mM magnesium chloride ($MgCl_2$), 10-Mm calcium chloride ($CaCl_2$), 20-μM oxyhemoglobin, 25-μM flavin adenine dinucleotide (FAD), 10-μM flavin mononucleotide (FMN), 10-μM tetrahydrobiopterin, 1-mM ascorbic acid, and 1-mM Arg in 0.1-M hydrochloric acid (HCl). Oxyhemoglobin was freshly prepared from 1-mM hemoglobin (Sigma-Aldrich, H2625) dissolved in 20-mM potassium phosphate buffer pH 7.0 treated with 5-mM sodium dithionite. Oxyhemoglobin

concentration, after desalting with Zeba Spin Desalting Columns, 7K MWCO (Thermo Scientific™) was measured at 415 nm (ε = 131 mM^{-1} cm^{-1}).

Extracts were preincubated for 10 min at 37°C. One portion of the extract was preincubated with equal volume of 12.5-mM N^{ω}-nitro-L-arginine methyl ester (L-NAME) in 20% dimethyl sulfoxide (DMSO) and the second one only with 20% DMSO. The reaction was started by adding a reaction mixture. An Arg-dependent NOS-like activity was measured using microplate reader (Sunrise, Tecan) at 401 and 421 nm at 37°C as NO-dependent conversion of oxyhemoglobin to methemoglobin. The activity was calculated as a difference between results obtained for both reaction mixtures and using the results obtained from calmodulin-dependent endothelial nitric oxide synthase (eNOS; Sigma-Aldrich, N1533) reaction carried out as described for the plant enzymatic extracts. Arg-dependent NOS-like activity was presented as nanomole NO per minute per gram protein, based on the activity of eNOS (Sigma-Aldrich, N1533). The activity of eNOS was 0.23 nmol NO min^{-1} mg^{-1} protein.

Measurement of Total Antioxidant Capacity

Total antioxidant capacity in roots was determined by reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Molyneux, 2004). Roots (0.1 g) were homogenized in 0.5 ml of 80% (v/v) methanol and incubated in an ultrasonic bath for 5 min at 4°C. Homogenates were centrifuged at 7,000*g* for 10 min at 4°C. After homogenization, 2 µl of the extract was added to 298 µl of 60-µM DPPH dissolved in methanol. The reaction mixture was incubated for 15 min in darkness at room temperature. Concentration of reduced DPPH was measured at 517 nm using microplate reader (Sunrise, Tecan). Antioxidant capacity was expressed as reduction of DPPH defined as $[(A_0-A_s)/A_0]$ x 100%, where A_0 is absorbance of a blank, and A_s is absorbance of the sample.

Measurement of Total Thiol Content

The total thiol content in roots was measured using Ellman's reagent (Chan and Wasserman, 1993). Roots (0.2 g) were washed with distilled water and homogenized in an ice bath in 0.5 ml of 0.1-M potassium phosphate buffer (pH 7.5) with 2-mM DTT, 10% (w/v) glycerol, 0.1% (w/v) triton X-100, 1% (v/v) cocktail of protease inhibitors (Sigma-Aldrich), and 2% (w/v) PVPP. Homogenates were centrifuged at 13,000*g* for 15 min at 4°C. Thiol content assay was performed in the mixture: 50 µl of the supernatant, 50 µl of 5,5'-dithiobis (2-nitrobenzoic acid) (DNTB), and 200 µl of 0.1-M potassium phosphate buffer (pH 7.5). The mixture was incubated for 10 min in the darkness at room temperature. The total thiol content was measured at 412 nm using microplate reader (Sunrise, Tecan). The concentration of 2-nitro-5-thiobenzoate (TNB) was calculated using the extinction coefficient ε = 14.15 mM^{-1} cm^{-1}. The concentration was expressed as micromole TNB per gram fresh weight (FW).

Determination of the Enzymatic Activity of the Cellular Antioxidant System

Preparation of the Protein Extract for Catalase and Superoxide Dismutase Activity Determination

Roots (0.2 g) were homogenized in an ice bath in 0.1-M potassium phosphate buffer (pH 7.2) with 1-mM ethylenediaminetetraacetic acid (EDTA), 5% (w/v) glycerol, 5-mM DTT, 1% (v/v) cocktail of protease inhibitors (Sigma-Aldrich), and 2% (w/v) PVPP. After centrifugation at 13,000*g* for 15 min at 4°C, supernatant was collected and used for further analysis.

Measurement of Catalase Activity in the Polyacrylamide Gel Under Non-Denaturing Conditions

CAT activity was analyzed in a gel according to Woodbury et al. (1971). Soluble protein (10 µg) were separated using 8% polyacrylamide gel electrophoresis under non-denaturing and non-reducing conditions at 4°C. Visualization of CAT activity was performed with 0.003% (v/v) H_2O_2 (20 min in darkness); next, the gels were washed in distilled water and stained with the mixture of (1:1) 2% (w/v) iron(III) chloride and 2% (w/v) potassium ferricyanide.

Measurement of Superoxide Dismutase Activity in the Polyacrylamide Gel Under Non-Denaturing Conditions

The activity of SOD was analyzed in the gel according to Kuo et al. (2013). Soluble protein (20 µg) samples were subjected to 10% polyacrylamide gel and electrophoretically separated under non-denaturing and non-reducing conditions at 4°C. After an electrophoresis, the gel was incubated in 0.1% (w/v) Nitro blue tetrazolium chloride (NBT) and then in riboflavin solution (28-µM riboflavin and 28-mM N,N,N',N-tetramethylethylenediamine) in 0.1-M potassium phosphate buffer, (pH 7.4). The visualization of the bands was done by exposure of the gel to UV light for 10 min.

To distinguish SOD isoforms, we used potassium cyanide, which inhibits CuZnSOD activity and H_2O_2, which inhibits both CuZnSOD and FeSOD activities.

Measurement of Superoxide Dismutase Activity in Extracts of Tomato Roots

SOD activity in extracts of roots was determined with epinephrine assay (Misra and Fridovich, 1972). Roots (0.1 g) were washed with distilled water and homogenized in an ice bath in 0.5 ml of 0.1-M Tris-HCl buffer (pH 7.0) with 5-mM DTT, 0.1% (w/v) sodium deoxycholate, 1% (v/v) cocktail of protease inhibitors (Sigma-Aldrich), and 2% (w/v) PVPP. Homogenates were centrifuged at 13,000*g* for 15 min at 4°C. SOD activity was measured in a mixture: 10, 15, or 20 µl of the supernatant, 10 µl of freshly prepared 10-mM epinephrine in 10-mM HCl, and an appropriate volume of 0.1-M glycine buffer (pH 10) containing 0.1-M sodium chloride (NaCl). Total volume of the assay mixture was 300 µl. Adrenochrome content

was measured at 480 nm using microplate reader (Sunrise, Tecan). The amount of extract required for 50% inhibition of the oxidation of the epinephrine was taken for the calculation of SOD activity. Results were compared with a standard curve for SOD activity prepared using a commercial SOD (Sigma-Aldrich, S7571-30KU). The activity was expressed as units per milligram protein.

Preparation of Enzymatic Extracts for Glutathione Reductase and Glutathione Peroxidase Activity Determination

Roots (0.15 g) were homogenized in an ice bath in 0.5 ml of 0.1-M potassium phosphate buffer (pH 7.0) with 5-mM DTT, 1% (v/v) cocktail of protease inhibitors (Sigma-Aldrich), and 2% (w/v) PVPP. After centrifugation at 13,000*g* for 15 min at 4°C, supernatant was desalted using protein concentrator PES, 3K MWCO (Termo Scientific™).

Glutathione Reductase

A measurement of GR activity was performed according to Esterbauer and Grill (1978). An enzymatic extract (25 µl) was incubated in a 200-µl reaction mixture [0.05-M potassium phosphate buffer (pH 7.0) with 0.625-mM oxidized form of glutathione (GSSG) (Sigma-Aldrich)] in the dark for 10 min at room temperature. The measurement of GR activity was started by 25 µl of 2-mM NADPH. The GR activity was measured as a decrease of absorbance at 340 nm, using microplate reader (Sunrise, Tecan). The activity was expressed as units per milligram protein. Unit (U) was defined as nanomoles of substrate utilized by the enzyme in 1 min.

Glutathione Peroxidase

A measurement of GPx activity was performed according to Flohé and Günzler (1984) with some modifications (Fontaine et al., 1994) as described by Krasuska and Gniazdowska (2012). An enzymatic extract (25 µl) was incubated with a 0.2-ml reaction mixture: 0.05-M potassium phosphate buffer (pH 7.0) with 0.1-M aminotriazole, 2.5-mM EDTA, 1.25-mM GSH, and 1.5 U of GR (Sigma-Aldrich, G3664) at 25°C for 10 min. After incubation, 50 µl of 2-mM H_2O_2 was added. The reaction was started by adding 25 µl of 2.5-mM NADPH. The GPx activity was determined as an absorbance decrease monitored at 340 nm using a microplate reader (Sunrise, Tecan). The activity was expressed as nanomoles NADPH per minute per microgram protein.

Catalase, Superoxide Dismutase, Glutathione Reductase, Glutathione Peroxidase, and Nitrosoglutathione Reductase Gene Expression Analysis

The expression of genes was assessed in roots using quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted and purified using an RNAzol RT (Sigma-Aldrich) according to manufacturer's instructions. RNA samples were DNase treated with DNase I (Thermo Scientific™). Total RNA (200 ng) was used to generate first-strand complementary DNA (cDNA) by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™) with oligo(dT)18 Primer in a total volume of 35 µl, as is described in the manufacturer's guideline. qRT-PCR was performed in a CFX Connect™ Real-Time PCR System. iTaq™ Universal SYBR® Green Supermix (Bio-Rad) was used as the basis for the reaction in a total volume of 12 µl (6-µl PCR Supermix, 1-µl primer, 4-µl H_2O, and 1-µl cDNA).

Table S1 shows the primer pairs used to amplify the genes.

For the normalization of the expression levels, housekeeping genes *EF1*α and *PP2Acs* were used as a reference genes; cDNA from untreated material was used as a reference sample.

Immunofluorescence Nitrosoglutathione Localization in Root Axis of Tomato Seedlings

Fragments of root tips (0.3 cm) were immediately fixed in 4% (w/v) paraformaldehyde in 0.1-M microtubule stabilizing buffer (pH 6.9) with 0.1% (w/v) Triton X-100 for 2 h at room temperature as described by Gubler (1989). Samples were dehydrated in ethanol with 10-mM DTT and infiltrated in a mixture of butyl–methyl–methacrylate (BMM) resin with ethanol in dilutions: 1:3, 1:1, and 3:1, and finally in a pure BMM. Polymerization was done for 20 h at -20°C. Acetone was used to remove the BMM from 2.0-µm sections collected on silane-coated slides (Thermo Scientific™). An immunofluorescence analysis was carried out after preincubation in 3% (w/v) bovine serum albumin in phosphate-buffered saline (PBS) (3.2-mM sodium hydrogen phosphate, 0.5-mM monopotassium phosphate, 135-mM NaCl, 1.3-mM potassium chloride, pH 7.2) for 1 h at room temperature. Sections were incubated with primary rat anti-GSNO antibodies (Agrisera, AS08 361) in PBS buffer (pH 7.2) (dilution 1:500) for 2 h at room temperature in a humid chamber. Controls for background staining were performed by replacing the primary antibody with the incubation buffer (**Figure S1**). Slides washed with PBS with Tween 20 buffer were treated at room temperature in the dark for 2 h with secondary goat anti-rat immunoglobulin G conjugated to TexasRed-X (Thermo Scientific™, T-6392) in PBS buffer (dilution 1:500). An Olympus AX70 Provis (Olympus Poland) with a UM61002 filter set and equipped with an Olympus SC35 camera was used for fluorescence imaging.

Nitrosoglutathione Reductase Activity Assay in Extracts of Tomato Roots

Activity of GSNOR was measured according to Sakamoto et al. (2002) with some modifications by Krasuska et al. (2017). Root were homogenized in an ice bath with 50-mM Tris-HCl pH 8.0, 1-mM EDTA, 5% (w/v) glycerol, 0.1-mM phenylmethylsulfonyl fluoride (PMSF), 5-mM DTT, 5-mM $MgCl_2$, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), and 2% (w/v) PVPP centrifuged at 10,000*g* 10 min. A supernatant was concentrated and desalted using concentrator PES, 3K MWCO (Thermo Scientific™) at 10,000*g* for 30 min. A reaction mixture (300 µl) contained: 8 µg of protein extract, 0.5-mM EDTA, 0.2-mM nicotinamide adenine dinucleotide (reduced form) (NADH) in 50-mM Tris-HCl pH 8.0. The reaction was initiated by addition of GSNO to the reaction

mixture at a final concentration of 0.6 mM. A GSNOR activity was measured as absorbance decrease at 340 nm for 6 min, using a microplate reader (Sunrise, Tecan). The activity was calculated using the extinction coefficient $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$ and expressed as micromole NADH per gram FW.

Nitrosoglutathione Reductase Activity Detection by Staining Following Native Gel Electrophoresis

Detection of the GSNOR activity in the gel was done according to Kubienová et al. (2014) with some modifications as described by Krasuska et al. (2017). Roots (0.2 g) were homogenized in 0.5-ml extraction buffer 50-mM Tris-HCl pH 7.5, 0.2% (w/v) Triton X-100, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), 5% (w/v) glycerol, and 2% (w/v) PVPP. Extracts were centrifuged for 10 min at 10,000*g* and desalted using protein concentrators PES, 3K MWCO (Termo Scientific™). Samples of 30 µl containing 50-µg proteins were mixed with 10 µl of 60% glycerol and loaded for electrophoretic separation using 10% native polyacrylamide gels. Then, gels were rinsed in deionized water, placed in the mixture: 0.1-M sodium phosphate buffer, pH 7.4, 2-mM NADH for 15 min. Next, two filter papers soaked with 4-mM GSNO in 0.1-M sodium phosphate buffer pH 7.4 were placed on the gel and incubated in darkness for 15 min. Detection of GSNOR activity in the gel was done after UV excitation.

Detection of Nitrosoglutathione Reductase Protein Level

Detection of GSNOR protein was done by immunoblotting as was described by Kubienová et al. (2016). Roots were homogenized in 0.1-M Tris-HCl, pH 7.5 with 1-mM EDTA, 2% (w/v) PVPP, 1-mM DTT, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), 0.1% (w/v) Triton X-100, 1-mM $MgCl_2$, and 10% (w/v) glycerol in an ice bath. After centrifugation at 10,000*g* for 15 min at 4°C, the supernatant was collected for further analysis. Protein samples were suspended in the sample buffer: 63-mM Tris-HCl, pH 6.8, 1% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) glycerol, 0.01% (w/v) bromophenol blue, and 20 mM DTT. After incubation at 95°C for 5 min, 7.5-µg proteins were loaded per lane and separated on 10% polyacrylamide gels with SDS (Laemmli, 1970) and then electrotransferred to nitrocellulose membranes (Pure Nitrocellulose Membrane, Sigma-Aldrich) using a Bio-Rad wet blotting apparatus (Towbin et al., 1979). The membranes were blocked overnight at 4°C with nonfat dry milk in Tris-buffered saline and Tween 20 (TBST). After blocking, membranes were washed three times in TBST and immunolabeled with anti-GSNOR polyclonal rabbit antibodies diluted 1:1,000 (Kubienová et al., 2013). As secondary antibodies, anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma-Aldrich, A3687) at a dilution of 1:100,000 at room temperature was used. Visualization of GSNOR band was done after addition of 0.1-M Tris-HCl pH 9.5, 0.1-M NaCl, 5-mM $MgCl_2$, 0.2-mM NBT, and 0.21-mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Mass Spectrometry Analyses of Proteins

Protein Extraction and Purification

Roots (2 g) were homogenized in liquid nitrogen, and then, proteins were extracted by adding 5 ml of 0.1-M Tris-HCl buffer (pH 7.0) with 1% (w/v) Triton X-100, 2% (w/v) glycerol, 2-mM DTT, 0.15-M NaCl, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), and 5% (w/v) PVPP. After centrifugation, supernatant was concentrated with Pierce™ Protein Concentrator PES, 3K MWCO (Thermo Scientific™) to obtain 2 ml of protein extract. Protein concentration was measured and equalized in all samples. Monoclonal anti-nitrotyrosine (Agrisera, AS10 706-100) antibodies were added to protein samples (2 µl of the antibodies for each 1 mg of proteins) and mixed gently at 4°C overnight. Then, 5-µl protein G agarose (Thermo Scientific™) was added and incubated for 8 h at 4°C with gentle mixing. After centrifugation for 2 min at 2,500*g* at 4°C, supernatant was discarded, and the pellet was dissolved in TBS (0.1 M Tris-HCl with 0.15-M NaCl, pH 8.0) and kept overnight in 4°C. Mixture was centrifuged for 3 min at 2,500 *g* at 4°C, supernatant was discarded, and pellet was suspended in sample buffer [63-mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (w/v) glycerol, 0.01% (w/v) bromophenol blue, and 20 mM DTT] and denatured for 10 min in 95°C. Then, proteins were separated on two 10% polyacrylamide gels with SDS according to Laemmli (1970). One gel was stained with Coomassie, and the second was electrotransferred to nitrocellulose membranes (Pure Nitrocellulose Membrane, Sigma-Aldrich) according to Towbin et al. (1979) using a Bio-Rad wet blotting apparatus. The membrane was blocked overnight at 4°C with nonfat dry milk in TBST. After blocking, membranes were washed three times in TBST, and immunolabeling of 3-NT was carried out by incubating the membranes with monoclonal anti-3-NT antibody (Agrisera, AS10 706-100), conjugated with alkaline phosphatase, at a dilution of 1:100,000 at room temperature. Visualization of proteins containing 3-NT was done after addition of 0.1-M Tris-HCl pH 9.5, 0.1-M NaCl, 5-mM $MgCl_2$, 0.2-mM NBT, and 0.21 mM BCIP

In-Gel Digestion

Protein bands of nitrated proteins that differentiate between control and CAN treated were cut out (**Figure S2**). Destaining, trypsin digestion, and peptide extraction were done as was described by Eckermann et al. (2002). Gel pieces were washed in a mixture of 40% (v/v) acetonitrile and 60% (v/v) 50-mM ammonium bicarbonate for 1–2 h. Destained gel bands were dried by vacuum centrifugation, and modified trypsin (a sequencing grade, Roche) (30 ng μl^{-1}), dissolved in 50-mM ammonium bicarbonate was added. Trypsin digestion was performed at 37°C (overnight). The gel was incubated in sequence: 1) water, 2) acetonitrile, 3) 5% (v/v) formic acid, and again 4) acetonitrile. After each step, supernatants were collected. The combined supernatant was lyophilized and resolved in a mixture of 10% (v/v) acetonitrile and 90% of 0.1% trifluoroacetic acid.

Matrix-Assisted Laser Desorption/ Ionization Analyses

Tandem mass spectrometry (MS/MS) analysis of extracted proteins was performed with a Thermo LTQ XL, as a matrix served α-cyano-4-hydroxy cinnamic acid [5 mg ml^{-1}, dissolved in 84% (v/v) acetonitrile]. For an identified protein, at least 2 peptides were confirmed by MS/MS analysis (**Table S2**). A database search was performed using the MASCOT search engine (Matrix-Science). Peptide tolerance was ±1.2 Da and MS/MS tolerance 0.5 Da.

Protein Concentration Measurement

Protein content determination was performed using Bradford reagent (Bradford, 1976). As a standard, fatty acid-free bovine serum albumin was used.

Densitometry Analysis

Densitometry analysis was done using Image J.

Statistics

All data were obtained in at least three independent experiments with at least two repetitions each. Data were analyzed using Statistica Software. Mean differences were calculated using t-test; standard deviation (SD) was also provided to indicate the variations associated with the particular mean values.

RESULTS

Canavanine Inhibited Arginine-Dependent Nitric Oxide Synthase-Like Activity in Roots of Tomato Seedlings

An Arg-dependent NOS-like activity in extracts of roots of control tomato seedlings did not differ during the culture period and was about 0.33–0.38-nmol NO min^{-1} g^{-1} protein (**Table 1**). After a short-term supplementation, an Arg-dependent NOS-like activity was inhibited by 60% in 10-μM CAN and by 70% in 50-μM CAN. After extending the experiment for an additional 48 h, 10-μM CAN inhibited Arg-dependent NOS-like activity in root extracts by around 50%, while 50-μM CAN by more than 70% (**Table 1**).

TABLE 1 | Arg-dependent NOS-like activity in extracts from roots of the control plants grown in water or from roots of tomato seedlings treated with CAN (10 or 50 μM) for 24 and 72 h.

Plant treatment	Arg-dependent NOS-like activity (nmol NO min^{-1} g^{-1} protein)	
	24 h	72 h
Control (water)	0.33 ± 0.05	0.38 ± 0.01
CAN 10 μM	0.12 ± 0.02*	0.20 ± 0.03*
CAN 50 μM	0.09 ± 0.01*	0.10 ± 0.01*

Values are average ± SD of three to four repetitions. Asterisks (*) indicate significance between treatments and the control at the same time of culture period at $P \leq 0.05$, based on Student's test.

Canavanine Increased Total Antioxidant Capacity and Level of Total Thiols in Extracts of Tomato Roots

The total antioxidant capacity in root extracts of control plants after 24 h of culture was about 41% reduction of DPPH and increased to 57% as the experiment was prolonged (**Table 2**). Short-term treatment of seedlings with 50-μM CAN enhanced total antioxidant capacity to 64%, while CAN at lower concentration had no effect on this parameter. As the culture period was extended, antioxidant capacity of root extracts of CAN-exposed plants increased as compared with that of the control.

The content of total thiols in roots of the control plants did not differ during the culture period and was 0.15-μmol TNB g^{-1} FW (**Table 2**). A supplementation with CAN for 24 h resulted in 40% increase (after 50-μM CAN) and almost doubled the amount of total thiols after exposition to 10 μM CAN. Prolonged treatment with CAN led to further accumulation of total thiols; their level was more than twice higher in the roots of treated plants compared with that in the control (**Table 2**).

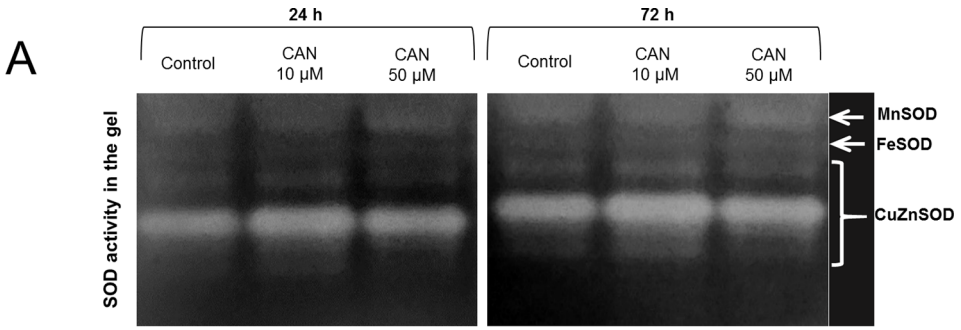
Activity of Enzymatic Antioxidant System Was Inhibited by Canavanine

CAN declined **SOD activity** both measured in-gel (**Figure 2A**) and by spectrophotometric method (**Figure S3**). In control and CAN-supplemented roots, the strongest was CuZnSOD isoform, while MnSOD and FeSOD were weaker (**Figure 2A**). Activity of CuZnSOD in roots treated with 50-μM CAN was 15 and 23% lower than that in the control after 24 and 72 h, respectively (**Figure 2A**). MnSOD was inhibited in CAN-exposed roots in about 25% after 24 h, whereas after additional 48 h, bands corresponding to MnSOD activity were slightly brighter, showing less dissimilarity in all combinations (**Figure 2A**). Inhibition by CAN of total activity of SOD was more spectacular in the spectrophotometric assay (**Figure S3**). SOD activity in roots exposed to 10-μM CAN was reduced by 24 and 39% after 24 and 72 h, respectively. CAN at higher concentration inhibited SOD activity in tomato roots by 39% after 24 h and 60% after an additional 48 h (**Figure S3**).

TABLE 2 | Total antioxidant capacity and concentration of total thiols in extracts from roots of the control plants growing in water or from roots of tomato seedlings treated with CAN (10 or 50 μM) for 24 and 72 h.

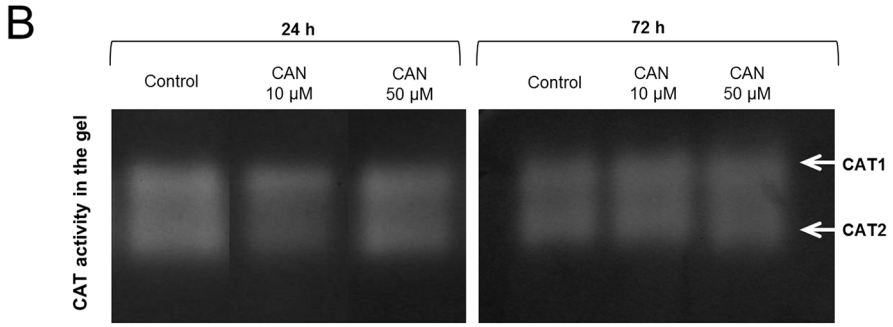
Plant treatment	Total antioxidant capacity (% of DPPH reduction)		Total thiol content (μmol TNB g^{-1} FW)	
	24 h	72 h	24 h	72 h
Control (water)	41.2 ± 8.0	57.1 ± 9.5	0.15 ± 0.02	0.16 ± 0.03
CAN 10 μM	36.6 ± 5.5	65.9 ± 11.2	0.29 ± 0.04*	0.33 ± 0.05*
CAN 50 μM	64.2 ± 4.3*	87.4 ± 5.1*	0.21 ± 0.02*	0.38 ± 0.03*

Values are average ± SD of three to four repetitions. Asterisks (*) indicate significance between treatments and the control at the same time of culture period at $P \leq 0.05$, based on Student's test.



densitometry analysis

Treatment	Izoform	24 h	72 h
Control	MnSOD	88.2±4.2	116.5±7.3
	FeSOD	95.7±6.1	96.0±6.5
	CuZnSOD	92.6±5.8	113.8±6.9
CAN 10 μM	MnSOD	67.6±3.1*	121.3±7.1
	FeSOD	88.9±5.2	97.3±4.8
	CuZnSOD	82.9±4.7	106.8±5.2
CAN 50 μM	MnSOD	65.2±2.8*	104.9±6.7
	FeSOD	78.2±3.9*	83.9±5.1*
	CuZnSOD	77.0±3.5*	88.7±3.8*



densitometry analysis

Treatment	Izoform	24 h	72 h
Control	CAT 1	86.5±4.3	65.4±3.5
	CAT 2	85.3±4.9	72.4±4.7
CAN 10 μM	CAT 1	67.3±3.7*	74.7±3.8
	CAT 2	57.0±3.1*	76.2±4.1
CAN 50 μM	CAT 1	70.1±3.8	72.8±4.2
	CAT 2	67.1±2.9*	72.6±3.1

FIGURE 2 | In-gel detection of SOD **(A)** and CAT **(B)** activities in extracts from roots of the control seedlings growing in water and roots of seedlings treated with CAN (10 or 50 μM) for 24 and 72 h. Total soluble proteins (20 μg per lane for SOD activity measurement and 10 μg per lane for CAT activity measurement) were electrophoretically separated using 10% (for SOD) and 8% (for CAT) gels under non-denaturing and non-reducing conditions. For visualization of SOD activity gels were incubated in 0.1% (w/v) NBT and then in riboflavin solution, for visualization of CAT activity gels were stained with the mixture of (1:1) 2% iron(III) chloride and 2% potassium ferricyanide. For SOD activity, visualization was done by gel exposure to UV light for 10 min. SOD and CAT isoforms are marked by arrows. Experiments were performed three times, and representative data are shown.

In-gel **CAT activity** analysis indicated two CAT isoforms present in tomato roots extracts (**Figure 2**B). The highest CAT activity was noticed for the control plants after 24 h of the culture. Less visible two bands of CAT activity have been seen after 24 h of 10-μM CAN supplementation. The culture of tomato seedlings for 24 h in 50-μM CAN decreased CAT2 activity. No statistically significant differences in CAT activity were noticed after 72 h of CAN treatment (**Figure 2B**).

Activity of GR in control tomato roots was stable during the culture period (**Figure 3A**). Short-term exposure of the seedlings to CAN in low concentration resulted in a drastic reduction of GR activity, while after additional 48 h, GR activity in roots of these plants increased twice and was at the level of the control. CAN at higher (50 μM) concentration had no effect on GR activity after 24 h supplementation. After 72-h treatment with 50-μM CAN, activity of GR was similar as after 24 h and insignificantly higher than that in the control (**Figure 3A**).

Activity of GPx in roots of control plants increased less than 20% during the experiment (**Figure 3B**). Short-term treatment with CAN at both concentration slightly stimulated GPx activity. Prolonged supplementation of tomato seedlings with 10-μM CAN resulted in drastic drop in GPx activity in roots, while CAN at higher concentration (50 μM) led to further stimulation of GPx activity (**Figure 3B**).

Canavanine Modified Expression of Genes Coding Antioxidant Enzymes

SOD transcript levels in roots was determined for five genes coding three Mn-FeSOD family enzymes (*MNSOD*-ID:101256386, *SOD3*-ID:101256231, and *FESOD*-ID: 544259) and two isomers of CuZnSOD family enzymes (*SODCP.2*-ID: 543981 and *CUSOD2*-ID:101264296), NCBI database (**Figure 4A**). After 24 h of CAN supplementation, *FeSOD*, *SODCP.2*, and *SOD3* were upregulated. In contrast, prolongation of the experiment led to downregulation of *FeSOD* and *SODCP.2* (by CAN at both tested concentration) and downregulation of *CUSOD* by 50-μM CAN (**Figure 4A**). *SOD3* and *MnSOD* were upregulated in roots of plants growing in CAN for 72 h (**Figure 4A**).

Three genes encoding different isoforms of CAT—*CAT1* (ID: 543990), *CAT2* (ID:543585), and *CAT3* (ID: 101259333)—are present in tomato roots. Irrespective of the concentration and duration of the culture, two of the genes, *CAT2* and *CAT3*, were downregulated by CAN (**Figure 4B**). In contrast, *CAT1* was upregulated by 50-μM CAN (**Figure 4B**).

Two genes (*GR* and *GR1*) coding different isoforms of GR are present in tomato roots (**Figure 4C**). Irrespective of the concentration and duration of the culture, both of the genes were downregulated by CAN, with the exception of *GR1*, expression of which was at the level of the control after 24 h of CAN at both concentration and GR for 10-μM CAN after 24 h and 50-μM CAN after 72 h (**Figure 4C**).

In tomato roots, there are five genes coding various isoforms of GPx (**Figure 4D**). After 24 h of the experiment in CAN-stressed plants, *GSHPx1* was downregulated. Twenty-four-hour exposition to CAN (both 10 and 50 μM) resulted in upregulation of *GPx* (**Figure 4D**). Higher transcript level was characteristic also for *GSHPx1* and *pGPx8*, as the 50-μM CAN treatment was prolonged. The exception was *GPx*, expression that was not detected in roots grown in 50-μM CAN for 72 h; for other genes, no significant changes were noted (**Figure 4D**).

Canavanine Restricted the Accumulation of Nitrosoglutathione in Root Tips

Localization of GSNO on longitudinal sections of the tomato seedlings root apex was investigated by a single immunofluorescence labeling technique. Control roots showed GSNO red fluorescence signal in root caps, predominantly in separated root border cells, but also in promeristem division zone (**Figures 5B**, **D**). After CAN treatment, the fluorescent signal was reduced as compared with that in control plants (**Figures 5B**, **D**). Moreover, GSNO was localized mainly in the rhizodermis and root caps (depending on the analyzed root zone). The longer the CAN was supplemented, the weaker the GSNO signal was observed (**Figure 5D**). After exposition of tomato seedlings to 50 μM, CAN for 72-h fluorescence signal in roots was observed almost only in the external part of root cap cells (**Figure 5D**). Additionally, in root apex sections where primary antibodies were omitted, there was no red fluorescence signal noticed (**Figure S1**).

Canavanine Slightly Modified Nitrosoglutathione Reductase Activity, Enlarged Nitrosoglutathione Reductase Protein Content, and Had No Effect on Nitrosoglutathione Reductase Gene Expression in Tomato Roots

Short-term exposition of seedlings to CAN lowered GSNOR activity determined in extracts; it was around 21–23 nmol NADH min^{-1} g^{-1} FW. In extracts of roots of control plants, GSNOR activity declined twice during the culture period (**Figure 6A**). After 72 h of the treatment, GSNOR activity in extracts of tomato roots was at the level observed after 24 h of culture but 25 and 43% higher than in control for 10- and 50-μM CAN, respectively (**Figure 6A**).

The tests on GSNOR activity following native electrophoresis showed no CAN dependence (**Figure 6B**). Bands corresponding to activity of GSNOR were visibly thicker for younger seedlings irrespective of the treatment. Only after 72 h that GSNOR activity staining in the gel showed its enhancement by 50-μM CAN (**Figure 6B**).

Content of GSNOR protein in roots was investigated by Western blot analysis. Single bands corresponding to a protein of molecular weight of about 43 kDa were clearly visible in protein roots' extracts of the control and CAN-treated seedlings (**Figure 6C**). In extracts from treated plants, bands corresponding to a protein of molecular weight of 43 kDa were thicker than in control irrespective of the duration of CAN application and its concentration though, in general, were more visible in extracts from roots of younger seedlings (after 24 h of culture).

GSNOR gene expression in tomato roots was not affected by CAN supplementation. *GSNOR* expression in CAN treated plants was found to be higher compared to roots of control plants after 24 h of culture in 10 μM CAN (**Figure 6D**).

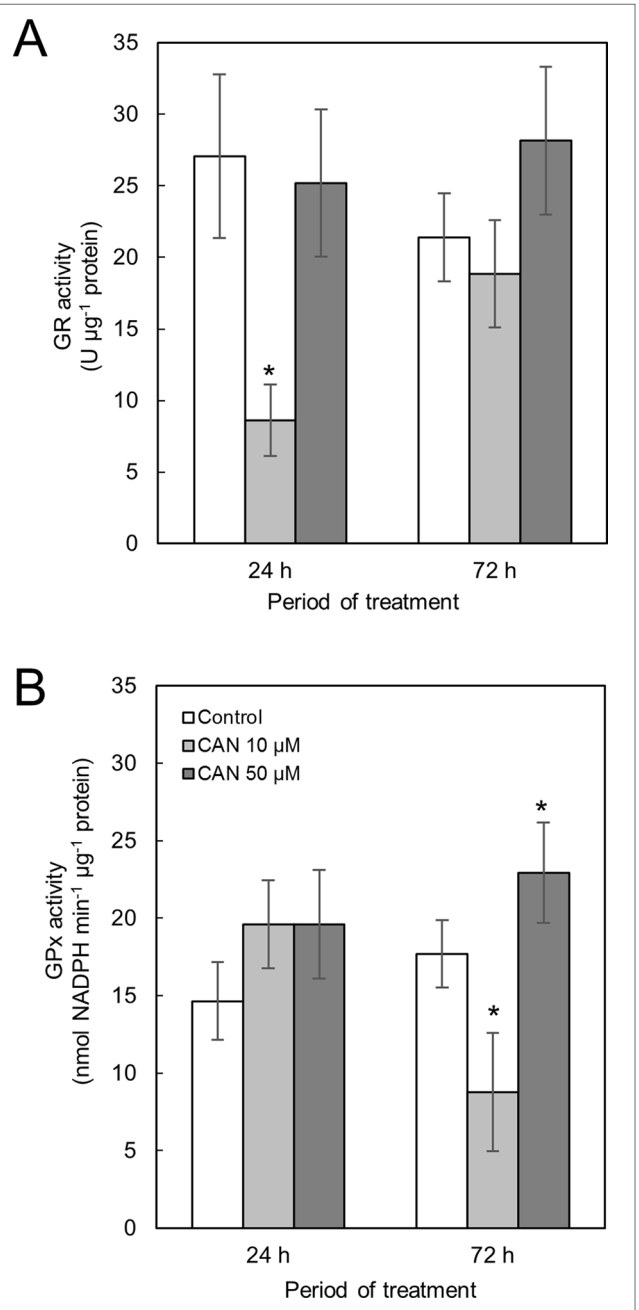


FIGURE 3 | Activity of GR **(A)** and GPx **(B)** in extracts from roots of the control seedlings growing in water and roots of seedlings treated with CAN (10 or 50 µM) for 24 and 72 h. Values are average ± SD of at least three independent experiments and three biological repetitions each. Asterisks (*) indicate significance between treatments and the control at the same time of culture period at $P \leq 0.05$, based on Student's test.

Nitrated Proteins in Roots of Canavanine-Stressed Plants Belong to Seed Storage Proteins or Components of the Cellular Redox System

In protein extracts from roots of CAN-supplemented seedlings, we have found several bands differently nitrated in comparison with the control (**Table 3**). In roots of CAN-treated plants irrespective of the duration of the experiment and the concentration of the tested NPAA, phosphoglycerate kinase was identified as nitrated protein. For plants after 24 h of culture, seed storage proteins (11s globulin seed storage of protein 2-like, 12s seed storage of protein CRA1-like, vivilin precursor) and luminal-binding protein 5 were identified as nitration targets. Prolongation of the CAN treatment resulted in nitration of monodehydroascorbate reductase, peroxidase 3-like, and polyphenol oxidase (**Table 3**).

DISCUSSION

Arginine-Dependent Formation of Nitric Oxide Was Inhibited by Canavanine

Over the last few years, there has been an intense debate about the existence of NOS-like enzyme in the plant kingdom. Based on sequence analysis methods, the main "advocates" of the occurrence of NOS-like protein in plant cells (Jeandroz et al., 2016; Astier et al., 2018) demonstrated that no typical mammalian NOS-like sequences can be found, even in species in which Arg-dependent NOS activity and/or effects of mammalian NOS inhibitors (N^{ω}-nitro-L-arginine methyl ester or N^{ω}-methyl-L-arginine) have been reported. Although the possibility that plants have NOS protein or adequate protein complex of a structure unrelated to a mammalian type is under consideration, functionality of the reaction was demonstrated in various plant tissue (Corpas and Barroso, 2017). In our previous research, it was shown that CAN, a commonly used inhibitor of mammalian isoform of NO synthase, led to restriction in NO formation in tomato roots (Krasuska et al., 2016a). This observation was the impulse to check CAN impact on one of the putative pathways of NO synthesis in plants. Thus, in the current work, we have clearly confirmed that, in plants, CAN may be used as an inhibitor of Arg-dependent NO formation (**Figure 7**). Prolonged (72 h) supplementation of tomato seedlings with CAN resulted in dose-dependent inhibition of Arg-dependent NOS-like activity. No differences (in comparison with the control) in NO_2^- concentration observed in CAN-supplemented plants (Krasuska et al., 2016b) indicated that CAN does not play an important role in the regulation of reductive pathway of NO biosynthesis. Therefore, low NO emission resulted from CAN application could be explained by demonstrated inhibition of Arg-dependent NO synthesis. Similarly, a negative effect of CAN on NO emission was noticed in apple (*Malus domestica* Borkh.) embryos and was also accompanied by reduced Arg-dependent NOS-like activity (Krasuska et al., 2016c).

Canavanine Decreased Nitrosoglutathione Level in Root Tips and Had No Effect on Nitrosoglutathione Reductase

A plant reaction to biotic and abiotic stresses involves activation of RNS- and ROS-dependent signaling pathways linked to, e.g., PTMs of proteins. *S*-nitrosylation, which consists of the reversible achievement of NO into cysteine residue, belongs to one of NO-dependent PTMs and led to formation of nitrosothiols (SNOs) (Mur et al., 2013). GSNO is a low molecular SNO and is considered to be one of the main signaling molecules among

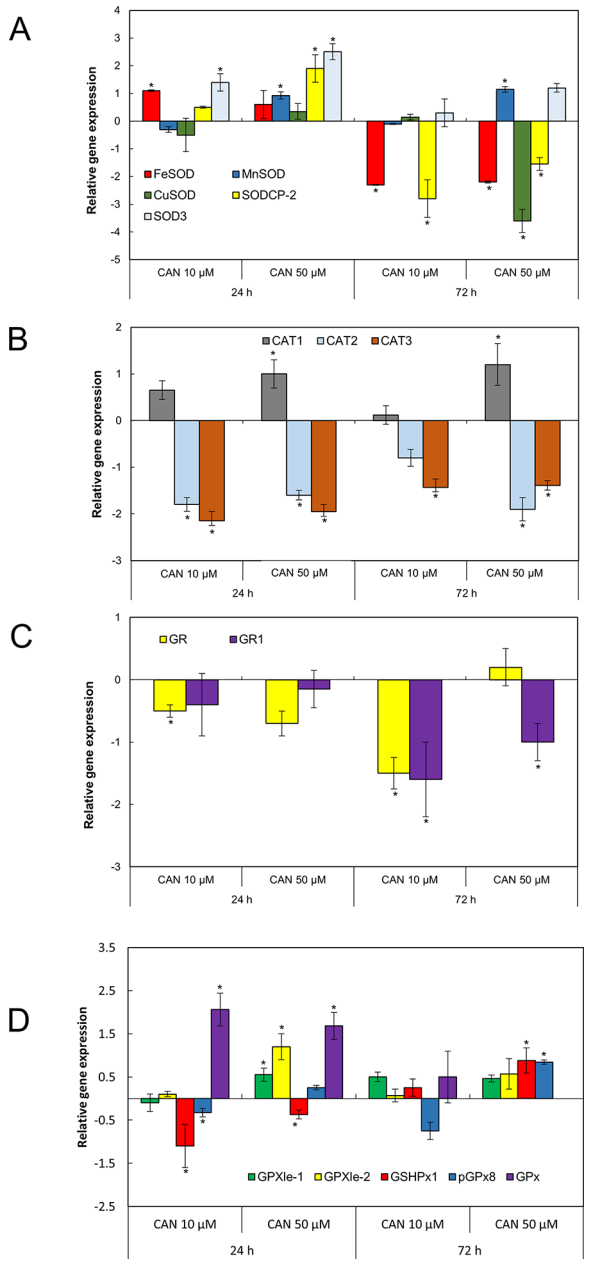


FIGURE 4 | The expression level of genes encoding different isoforms of SOD: *MNSOD*, *CUSOD2*, *FESOD*, *SODCP.2*, *SOD3* **(A)**; CAT: *CAT1*, *CAT2*, *CAT3* **(B)**; GR: *GR*, *GR1* **(C)**; and GPx: *GPXle-1*, *GPXle-2*, *GSHPx1*, *pGPx8*, *GPx* **(D)** analyzed by qRT-PCR as described in the section of *Material and Methods*. Roots of tomato seedlings were collected after 24 or 72 h of CAN (10, 50 µM) treatment; as a control, roots of plants cultured in water were used. Asterisks (*) indicate significance between treatments and the control at the same time of culture period at $P \leq 0.05$, based on Student's test.

SNOs and a reservoir of NO. Restriction in NO formation, due to inhibition of Arg-dependent NOS-like activity, in CAN-treated tomato seedlings resulted in decline of the content of GSNO in root tips (**Figure 7**). After prolonged exposition of seedlings to CAN (particularly at higher concentration), GSNO localization was limited only to external part of root tips, which fits well to NO localization (Krasuska et al., 2016b). Similarly, after a high-temperature treatment (Chaki et al., 2011). GSNO was localized in cortex and epidermal cells instead of vascular tissues and suggested that such redistribution of GSNO could be a consequence of the protective mechanisms against stress factors. GSNO moves in vascular tissue and can propagate message about environmental signals into other organs or tissues (see review by Begara-Morales, 2018). Therefore, its absence or limitation may disturb reactions to stresses particularly those linked to protein *S*-nitrosylation. The intracellular level of GSNO is controlled by GSNO formation and its catabolism by GSNOR, which in turn is regulated by PTMs, e.g., *S*-nitrosylation (Guerra et al., 2016) or oxidation (Kovacs et al., 2016). Kubienová et al. (2014) demonstrated stimulation of GSNOR activity in plants exposed to various stressors (low, high temperature, mechanical injury, or pathogens), while Chaki et al. (2011) noticed downregulation of GSNOR after mechanical wounding. Exposition of tomato to *meta*-tyrosine (NPAA released into the environment as root exudates of fescues) led to an increased GSNOR activity and higher abundance of the protein (Krasuska et al., 2017). Although the morphological consequences of CAN supplementation in tomato seedlings were similar to that observed after *meta*-tyrosine (Andrzejczak et al., 2018), its impact on GSNOR was opposite. We noticed slightly higher content of the protein, only small inhibition of the enzyme after short-term treatment with CAN and constant activity as the experiment was prolonged. In addition, expression of *GSNOR* was not affected by CAN. Therefore, we suspect that GSNO level in CAN-treated roots depends rather on its formation than catabolism (**Figure 7**). These results are in agreement with the model proposed by Guerra et al. (2016), suggesting that at low NO concentration in the cells, GSNOR activity is maintained at constitutive level.

Canavanine Induced Alterations in the Cellular Antioxidant System

The key role of ROS and RNS in plant response to stresses was demonstrated in many experiments and reviewed in details (Molassiotis and Fotopoulos, 2011; Farnese et al., 2016). Their cross-talk has become a more and more fascinating topic, as development in research methodology allows for identification of proteins that are post-translationally modified by ROS or/and RNS. Stress induced by application of allelochemicals is commonly associated with induction of oxidative stress (Gniazdowska et al., 2015). CAN (naturally synthesized in legumes and stored mostly in seeds) is a toxic molecule of well-established negative effect against herbivores (Rosenthal, 2001; Staszek et al., 2017), but its action in plants is only fragmentarily investigated. CAN supplementation resulted in overproduction of H_2O_2 and $O_2^{\bullet-}$ in tomato roots (Krasuska et al., 2016a; Krasuska et al., 2016b), and it was accompanied by elevated activity of ROS-producing enzymes (polyamine oxidase, NADPH oxidase) (Krasuska et al., 2016a). In the current work, we proved that CAN modified cellular antioxidant activity and enzymatic antioxidant system. Relatively small oxidative damages [electrolyte leakage, malondialdehyde (MDA) content, and DNA fragmentation] (Krasuska et al., 2016b) observed in CAN-stressed roots could be a result of the

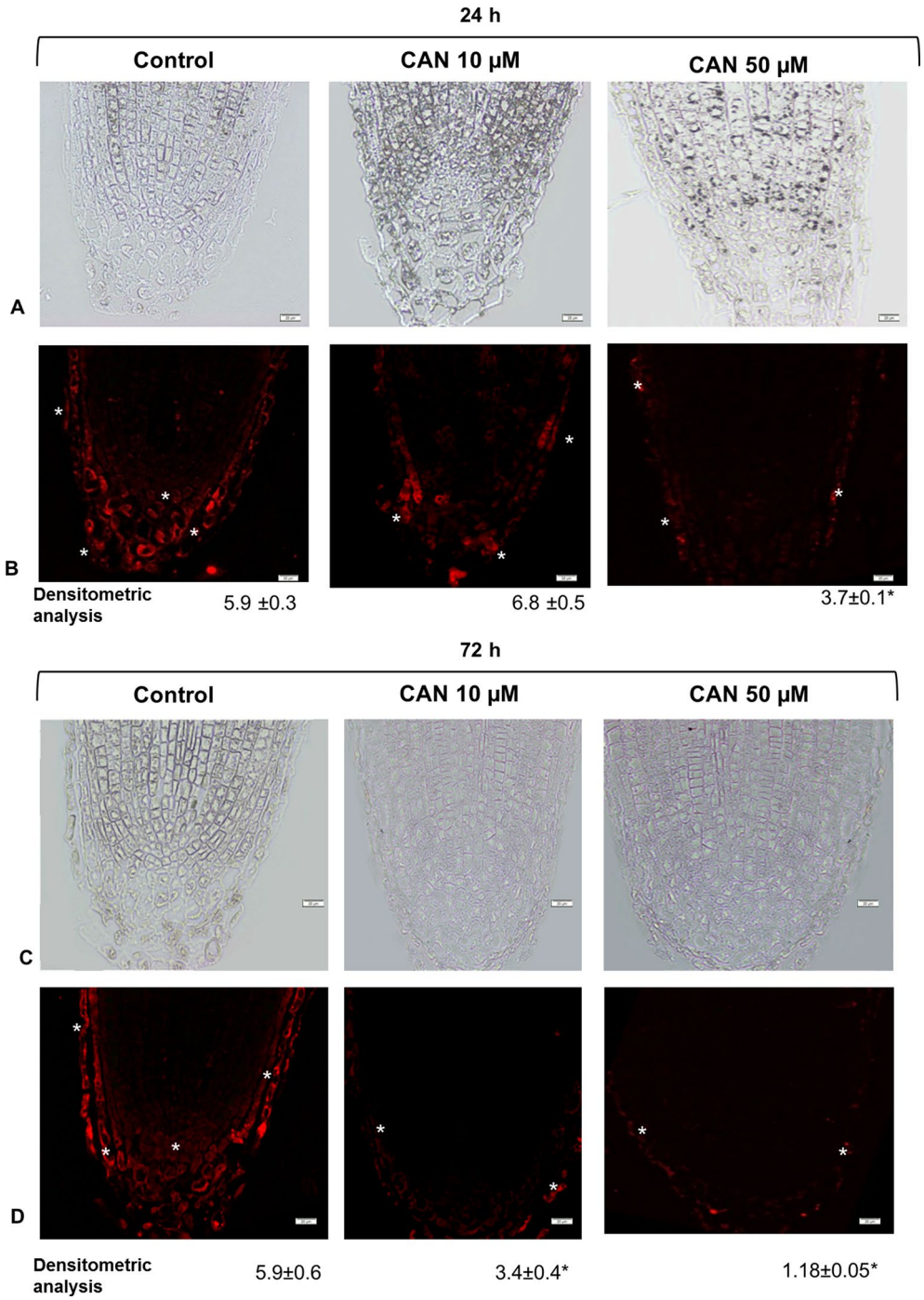


FIGURE 5 | GSNO-related red fluorescence signal (*) in root apex of tomato seedlings growing in CAN (10 or 50 µM) for 24 h (panels **A** and **B**) and 72 h (panels **C** and **D**). Panels **(A)** and **(C)** present the bright field, panel **(B)** and **(D)** corresponding to them fluorescence images. Scale bars 20 µm. Experiments were performed three times, and representative data are shown.

high total antioxidant capacity of the tissue. The elevated total antioxidant capacity in CAN-supplemented seedlings was related to the total thiols concentration (**Figure 7**). Increase of the total thiols was a rapid reaction to CAN, and its gradual increase was characteristic for prolongation of an NPAA treatment. We suspect that CAN leads to increased GSH content. A similar situation was detected in stress induced by some allelochemicals, e.g., a strong influence of *Achillea santolina* L. shoot extract on wheat (*Triticum aestivum* L.) plants was accompanied by accumulation of GSH (Hatata and El-Darier, 2009). It cannot be

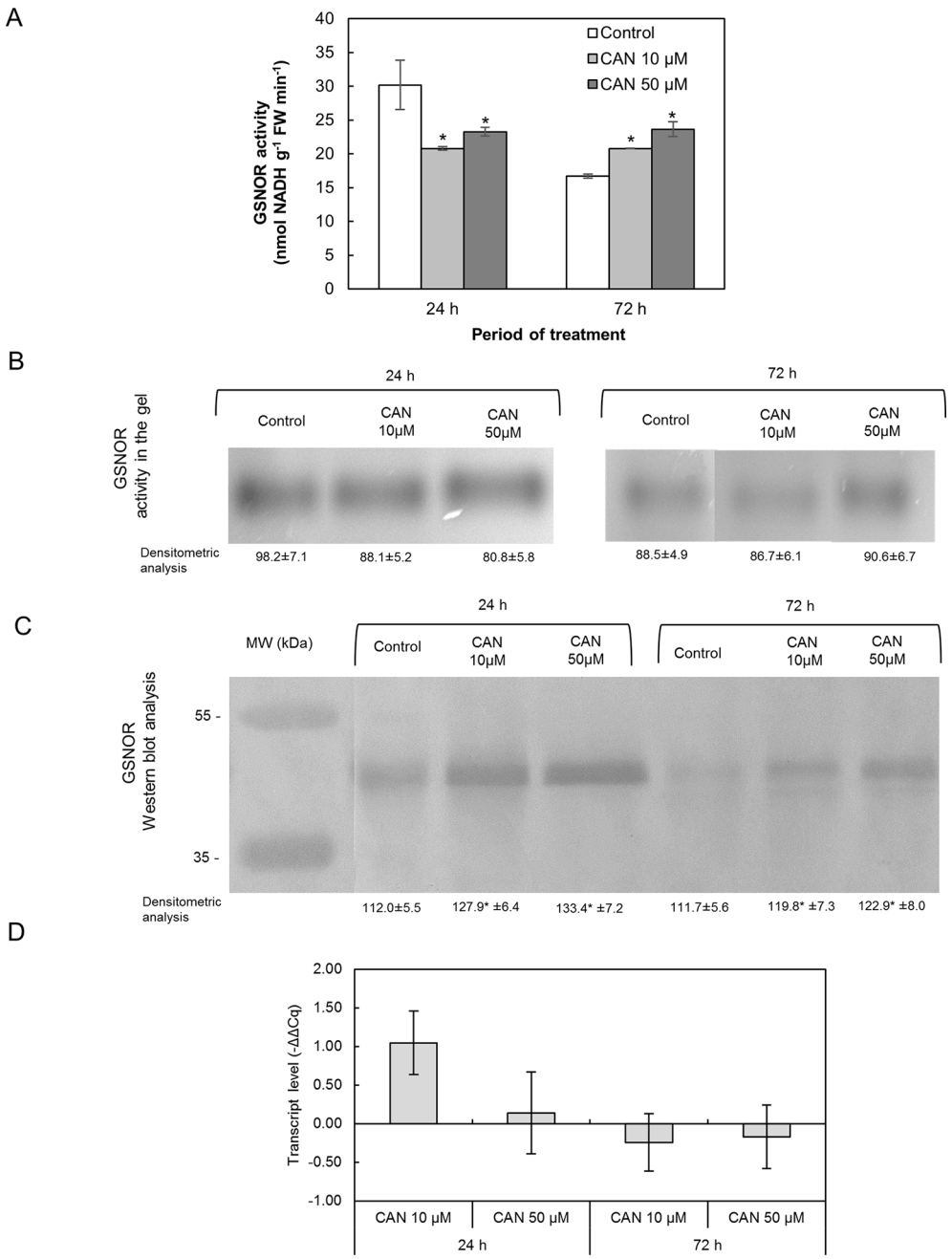


FIGURE 6 | Activity of GSNOR determined in extracts using spectrophotometric assay **(A)**, visualization of GSNOR activity in the gel **(B)**, GSNOR protein **(C)** and transcript level of *GSNOR* **(D)** in roots of tomato seedlings growing in water (control) or treated with CAN (10 or 50 µM) after 24 or 72 h of culture period. Asterisk (*) indicates significance from control at the same time of culture period at $P \leq 0.05$, based on Student's test. Values are average ± SD of at least three independent experiments and three biological repetitions each.

excluded that relatively high total antioxidant capacity in CAN-exposed roots may be due to a stimulation of biosynthesis of other nonenzymatic antioxidants, particularly phenolic compounds. Phenols accumulation was observed in tomato roots treated with *meta*-tyrosine, and we proposed that it could be regarded as a protective mechanism against stress induced by this NPAA (Andrzejczak et al., 2018).

Plants contain several types of enzymes that are able to modulate ROS level. Among them, SOD is responsible for $O_2^{\bullet-}$ disproportionation into H_2O_2. An activity of this enzyme was lower after CAN treatment. A decline in activity of all tested isoforms, MnSOD, FeSOD, and CuZnSOD, was noticed particularly after 24 h of exposition to CAN. This finding corresponds to the high level of $O_2^{\bullet-}$ in CAN-supplemented

TABLE 3 | Matrix-assisted laser desorption/ionization MS/MS identification after trypsin in-gel digestion of the nitrated proteins of tomato roots treated with CAN (10 or 50 μM) for 24 or 72 h.

	Culture period (h)	Description	NBCI ID	Identified peptides (no)
CAN 10 μM	24	Luminal-binding protein 5	XP_004234985.1	3
		Phosphoglycerate kinase, chloroplastic	XP_004243968.1	2
		11s globulin seed storage protein 2-like (fragment)	XP_004247523.1	3
		12s seed storage protein CRA1-like (fragment)	XP_004246943.1	2
	72	Polyphenol oxidase D, chloroplastic	NP_001334885.1	4
		Prohibitin-3 mitochondrial	XP_004250114.1	3
		Monodehydroascorbate reductase	NP_001318117.1	4
		Phospoglycerate kinase, chloroplastic	XP_004243968.1	2
CAN 50 μM	24	Aconitate hydratase, cytoplasmic	XP_004251517.2	3
		Luminal-binding protein 5	XP_004234985.1	2
		Phosphoglycerate kinase. chloroplastic	XP_004243968.1	2
		Vivilin precursor (fragment)	NP_001308118.1	2
		Prohibitin-1, mitochondrial-like	XP_004251498.1	4
		11s globulin seed storage protein 2-like (fragment)	XP_004247523.1	3
		12s seed storage protein CRA1-like (fragment)	XP_004246943.1	2
	72	Peroxidase 3-like	XP_006367274.1	2
		Polyphenol oxidase D, chloroplastic	NP_001334885.18	3
		Monodehydroascorbate reductase	NP_001318117.1	4
		Phospoglycerate kinase, chloroplastic	XP_004243968.1	3

tomato roots (Krasuska et al., 2016a). What more, prolongation of the culture period resulted in a downregulation of most genes encoding SOD, particularly *FeSOD*, *CuSOD*, and *SODCP-2*. *MnSOD* was upregulated by 50-μM CAN both after short and prolonged treatment. As *MnSOD* is highly induced by $O_2^{\bullet-}$ (Wang et al., 2016), elevated level could be one of the explanations of modification of the MnSOD activity. FeSOD and CuZnSOD are inhibited by H_2O_2 (Wang et al., 2016); therefore, accumulation of H_2O_2 after CAN application may result in lowering activity of these SOD isoforms. Different isoforms of SOD undergo inactivation by nitration (Holzmeister et al., 2015). Therefore, as CAN induced a transient increase in $ONOO^-$ (Krasuska et al., 2016a), the decrease in total SOD activity could be explained by nitration of the enzyme, although we did not identify SOD as a CAN-induced target of this PTMs. A more interesting suggestion for an explanation of lowering total SOD activity by CAN may be the impact of this NPAA on NO synthesis. It was shown that exogenous spermidine (Spd) increased activity of SOD in bluegrass (*Poa pratensis* L.) (Puyang et al., 2015). Spd enhanced NO emission in germinating apple embryos (Krasuska et al., 2014); therefore, we cannot exclude that limitation of NO production by CAN may result in a restriction of SOD activity.

CAT is regarded as the first cellular weapon against H_2O_2. Its activity can be decreased by NO donors, e.g., SIN-1 (producing $ONOO^-$) (Chaki et al., 2015). In tomato plants, an activity of CAT decreased only after the short period of CAN supplementation, while downregulated expression of *CAT2* and *CAT3* was noticed after both 24- and 72-h CAN application. *CAT1* expression level in control roots was lower than two other genes encoding CATs (data not shown). Thus, we can suspect that although we have observed upregulation of *CAT1* by CAN, it could not influence CAT total activity (sum of activity of CAT1 and CAT2 isoforms). It needs to be mentioned that in roots of Arabidopsis, expression of only *CAT2* (*CAT3* in tomato) and *CAT3* (*CAT2* in tomato) is detected (Mhamdi et al., 2012). Therefore, it is possible that products of these genes (*CAT2* and *CAT3*) are of more importance for regulation of H_2O_2 concentration. As a result, downregulation of these genes after CAN application could explain a decrease in CAT activity in tomato roots and H_2O_2 accumulation (Krasuska et al., 2016a). In addition, lower than in the control activity

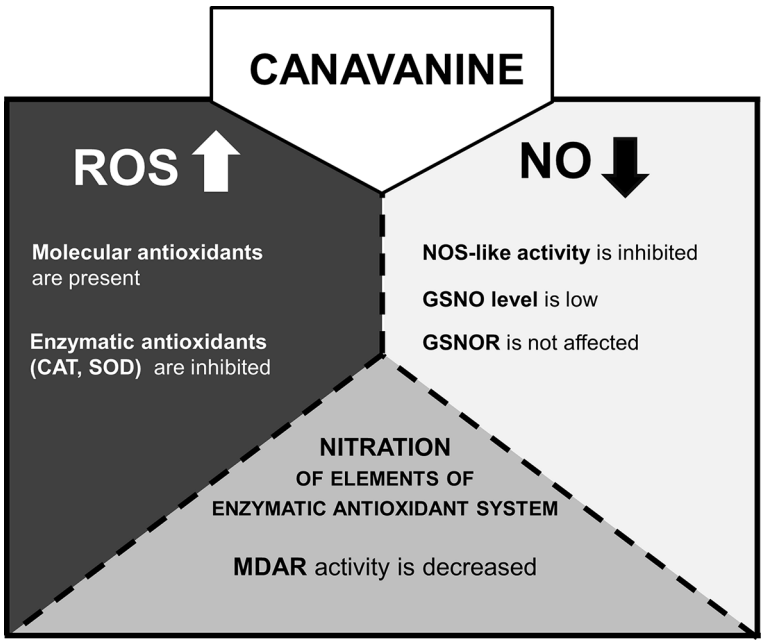


FIGURE 7 | The model of CAN action in tomato roots after prolonged (72 h) supplementation of seedlings with the NPAA. CAN increases level of ROS (Krasuska et al., 2016a) and decreases NO emission (Krasuska et al., 2016a). ROS are accumulated (CAN secondary mode of action) and could inhibit GSNOR activity by oxidative PTMs (Lindermayr, 2018). However, the gene expression and activity of GSNOR are not affected. GSNO level is lowered probably due to limitation of NO resulting from restriction of NOS-like activity (direct mode of action of CAN). ROS over-accumulation is accompanied by stimulation of molecular antioxidant system. CAT or SOD gene expression is downregulated, CAT and SOD enzymatic activities are inhibited. MDAR is an enzymatic antioxidant and the target of differential nitration in CAN-supplemented plants.

of CAT after 24 h of CAN application may be due to putative protein nitration, although as for SOD, we did not find CAT in the group of proteins preferentially nitrated in response to CAN.

GR is one of the enzymes of the enzymatic antioxidant system that sustains the reduced status of GSH and plays a crucial role in maintenance of sulfhydryl (–SH) group. GR has been identified as both nitration and *S*-nitrosylation target (Begara-Morales et al., 2015), although in pea plants in contrast to humans, no effect of these PTMs on activity of the enzyme was shown (Begara-Morales et al., 2016). CAN, in general, had no influence on activity of GR in tomato roots except the drastic drop observed after 24 h of supplementation with CAN at a lower concentration. This phenomenon is hard to explain since downregulation of the genes coding GR was more pronounced after longer CAN treatment. The studies of GR in various plant species have shown an increased GR activity under stresses (Yousuf et al., 2012; Gill et al., 2013). Based on experiments performed using transgenic plants, it is suggested that GR plays an important role in plant resistance to oxidative stress induced by abiotic stress. In addition, it was proved that activity of GR2 is necessary for root growth and maintenance of root apical meristem (Yu et al., 2013). Arabidopsis mutants *miao* (displaying reduction of GR) were characterized by an inhibition of root growth and severe defects in root apical meristem similar to those that were observed in tomato seedlings exposed to CAN for a longer period.

In plants, GPxs are regarded not only as ROS scavenging agents but also as redox sensors taking part in redox transduction signaling pathways as regulators of other regulatory proteins, e.g., transcription factors (Bela et al., 2015; Passaia and Margis-Pinheiro, 2015). GPxs in plants are suggested to be more efficient in reducing peroxides different from H_2O_2, e.g., organic hydroperoxides and lipid peroxides. In tomato, activity of GPx was not affected by CAN even after longer period of treatment with NPAA at a high concentration. It may be due to a relatively low abundance of putative substrates, e.g., lipid peroxides, since oxidative damages of membranes were not observed in CAN-supplemented seedlings (Krasuska et al., 2016b). Generally, GPx gene expression levels were higher after 24-h CAN application (with the exception of *GSHPx1*) and declined as the experiment was prolonged. This is in agreement with some observation indicating that GPx mRNA levels usually increase under various biotic and abiotic stresses (as cited by Bela et al., 2015), but it is not the only possible pattern. Arabidopsis mutants *Atgpx1*, A*tgpx4*, *Atgpx6*, *Atgpx7*, and *Atgpx8* had a significantly greater lateral root density than the wild type, similarly as observed in CAN-stressed tomato roots (Krasuska et al., 2016b), suggesting the importance of GPx activity for root architecture (Passaia et al., 2014). What more, transgenic tomato with GPx genes overexpression exhibited a high tolerance to abiotic stress but lower to biotic stresses (pathogens and parasites), probably because of GPx interference with elements of H_2O_2-mediated signal transduction under pathogen infection (Herbette et al., 2011). We suspect that the decrease in NO and transient increase in

$ONOO^-$ formation, as the reaction to CAN application, may impact GPx genes expression in tomato in an atypical way.

Elements of the Cellular Antioxidant System and Seed Storage Proteins Were Identified as Targets of Tyrosine Nitration in Seedlings Supplemented With Canavanine

In tomato roots, growing in CAN content of 3-NT increased during the culture period. A pattern of nitrated proteins was similar in both CAN-stressed and control plants (Krasuska et al., 2016a). Additional performing immunoprecipitation and Western blot analysis allowed us to distinguish several bands, characterized by stronger reaction with antibodies against 3-NT. Similarly, as in other plant material (Begara-Morales et al., 2016; Kolbert et al., 2017), monodehydroascorbate reductase (MDAR) was identified as nitrated protein in extracts from roots of plant exposed to CAN (10 and 50 μM) for 72 h (**Figure 7**). In tomato roots, MDAR activity was significantly reduced by CAN after 24 and 72 h in concentration-dependent manner (**Table S3**). This observation corresponds well to data on MDAR activity regulation by NO-PTMs. In pea (*Pisum sativum* L.) plants, peroxisomal MDAR was shown to be deactivated by both *S*-nitrolysation and nitration (Begara-Morales et al., 2015). Reduced MDAR activity in CAN-supplemented tomato roots could limit regeneration of ascorbate and lead to disturbance in the glutathione-ascorbate cycle. Among nitrated proteins identified in extracts from roots of CAN-treated plants after 72 h of culture peroxidase 3-like, polyphenol oxidase D were found, which are involved in plant reaction to biotic stresses (herbivores) or have been implicated in the biosynthesis of pigments and other secondary metabolites (Araji et al., 2014). A predicted inhibition of these enzymes being the result of 3-NT formation may induce alterations in secondary metabolites in tomato, mainly in phenylpropanoid pathways. In younger plants after 24 h of CAN supplementation, predominantly seed storage proteins were identified as nitration targets. They are probably preferentially degraded, as is suggested also for carbonylated storage proteins (Job, 2005). Similarly, in apple embryos, legumin A-like protein was nitrated during dormancy alleviation (Krasuska et al., 2016a). A nitration of binding proteins (BiP) localized in the lumen of endoplasmatic reticulum (ER) detected in roots stressed with CAN for short-term may suggest a disruption in secretion of proteins from ER. It is possible that nitrated BiP cannot stabilize proteins in ER or do not prevent aggregation of malformed proteins. The proper function of BiP proteins is necessary for plant immunity or osmotic stress (Carvalho et al., 2014). In addition, it requires ATP, while nitration of phosphoglycerate kinase identified as differentially nitrated protein in CAN-exposed roots could lead to ATP limitation. An interesting observation is CAN induced nitration of mitochondrial prohibitin-1 or prohibitin-3. Although loss of the prohibitin complex in yeast did not affect the mitochondrial membrane potential and respiration, overexpression of prohibitin-1 in endothelial cells decreased the accumulation of ROS, suggesting that prohibitins protect against oxidative stress (Merkwirth and Langer, 2009). Therefore, prohibitin loss of function due to formation of 3-NT may accelerate ROS accumulation in CAN-stressed tomato roots.

Conclusions

In the current work (**Figure 7**), the inhibition of Arg-dependent NOS-like activity was demonstrated as a direct mode of action of CAN in tomato roots. Limitation in NO synthesis resulted in alteration in cellular antioxidant system; CAN increased antioxidant capacity and the level of sulphydryl groups. Enzymatic antioxidants were suppressed particularly after prolonged culture of seedlings with tested NPAA. Plant supplementation with CAN lowered GSNO accumulation in root tips and does not influenced GSNOR. In seedlings exposed to CAN, we have also shown a new data regarding differentially nitrated proteins. Among them, seed storage proteins (after short-term CAN treatment) and components of the cellular redox system (after prolonged CAN supplementation) were identified. We identified MDAR as a protein nitration target and demonstrated that the activity of the enzyme in roots was lowered after CAN application into the culture medium (**Figure 7**).

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

Conceptualization, AG and PS; Methodology, PS and UK; Investigation, PS; Data Curation, PS and UK; Writing—Original Draft Preparation, AG and PS; Writing—Review and Editing, AG and PS; Supervision of the experimental work, UK, JF, and KO-K; Project Administration, AG and PS; Funding Acquisition, AG and PS. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01077/full#supplementary-material

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REVIEW
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1-Aminocyclopropane 1-Carboxylic Acid and Its Emerging Role as an Ethylene-Independent Growth Regulator

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1-Aminocyclopropane 1-carboxylic acid (ACC) is the direct precursor of the plant hormone ethylene. ACC is synthesized from *S*-adenosyl-L-methionine (SAM) by ACC synthases (ACSs) and subsequently oxidized to ethylene by ACC oxidases (ACOs). Exogenous ACC application has been used as a proxy for ethylene in numerous studies as it is readily converted by nearly all plant tissues to ethylene. However, in recent years, a growing body of evidence suggests that ACC plays a signaling role independent of the biosynthesis. In this review, we briefly summarize our current knowledge of ACC as an ethylene precursor, and present new findings with regards to the post-translational modifications of ACS proteins and to ACC transport. We also summarize the role of ACC in regulating plant development, and its involvement in cell wall signaling, guard mother cell division, and pathogen virulence.

Keywords: 1-aminocyclopropane 1-carboxylic acid, plant physiology, plant hormones, plant signaling, ethylene

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1-AMINOCYCLOPROPANE 1-CARBOXYLIC ACID AS A PRECURSOR OF ETHYLENE

Four decades ago, 1-aminocyclopropane 1-carboxylic acid (ACC), a non-proteinogenic amino acid, was discovered to be an intermediate in the biosynthesis of the plant hormone ethylene (Adams and Yang, 1979). Ethylene regulates a wide range of developmental processes and responses to biotic and abiotic stresses, in part by complex interactions with other phytohormones (Muday et al., 2012; Vandenbussche et al., 2012; Merchante et al., 2013; Dubois et al., 2018). Its biosynthesis starts with the conversion of the amino acid methionine to *S*-adenosyl L-methionine (SAM) by SAM synthetase and the subsequent conversion of SAM to ACC, which is catalyzed by ACC synthase (ACS) (**Figure 1**) (Adams and Yang, 1977; Adams and Yang, 1979). The by-product of this reaction, 5'-methylthioadenosine (MTA), is recycled back into the Yang cycle while ACC is oxidized to ethylene by ACC oxidase (ACO) (Murr and Yang, 1975). In *Arabidopsis*, ACO proteins are encoded by five genes (*ACO1–5),* which belong to a superfamily of oxygenases/oxidases (Dong et al., 1992; Zhang et al., 2004). In general, ACS is the rate-limiting step in ethylene biosynthesis, though in some instances, ACO activity is limiting (Vriezen et al., 1999; Van de Poel et al., 2012). This topic, along with current knowledge on ACO phylogeny and their regulation and importance in agriculture, has been comprehensively discussed in a recent review (Houben and Van de Poel, 2019).

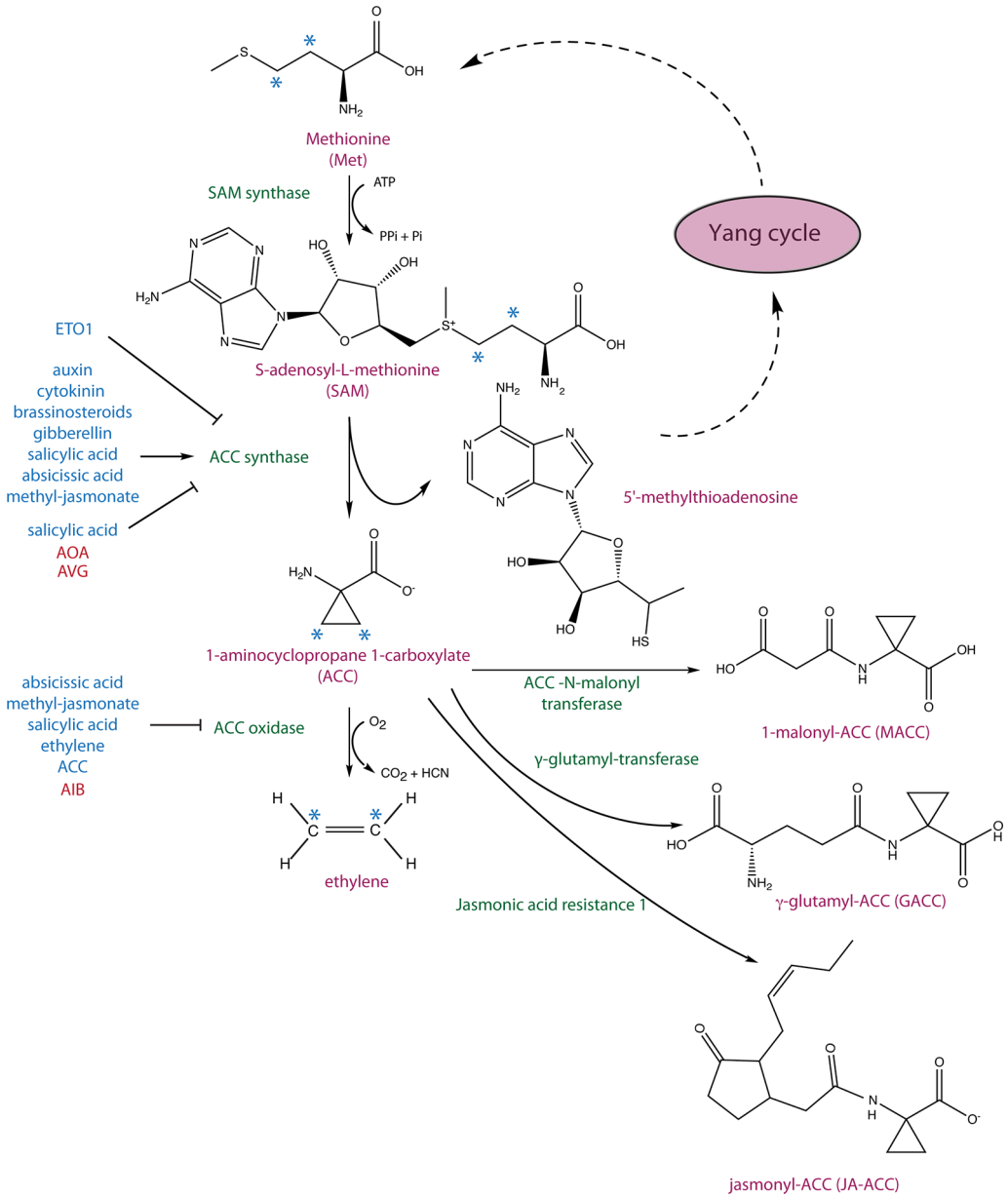

FIGURE 1 | Ethylene biosynthetic pathway and 1-aminocyclopropane 1-carboxylic acid (ACC) conjugation. *S*-adenosyl-L-methionine (SAM) synthase converts methionine to SAM, which is subsequently converted to ACC and 5'-methylthioadenosine (MTA) by ACC synthase (ACS). MTA is recycled back to the Yang cycle to recover methionine, and ACC is oxidized to ethylene by ACC oxidase (ACO). The hormonal inputs that regulate *ACS* and *ACO* expression as well as ACS stability are depicted in blue. ACC has been shown to be converted to three derivates: 1-malonyl-ACC (MACC) by the ACC-N-malonyl transferase, γ-glutamyl-ACC by a glutamyl-transferase, and jasmonyl-ACC (JA-ACC) by jasmonic acid resistance1 (JAR1). The asterisks mark carbons that give rise to ethylene.

Conjugation of ACC has long been hypothesized to play a role in regulating the level of ethylene biosynthesis but may also generate novel signaling molecules. ACC can be conjugated to 1-malonyl-ACC (MACC), γ-glutamyl-ACC (GACC), jasmonyl-ACC (JA-ACC) (Amrhein et al., 1981; Martin et al., 1995; Staswick and Tiryaki, 2004) (**Figure 1**), and perhaps other yet-to-be discovered conjugates. ACC conjugation regulates the availability of ACC to be converted to ethylene and, therefore, can be utilized by plants to control the level of ethylene biosynthesis. The ACC-to-MACC conversion is catalyzed by the enzyme ACC *N*-malonyl transferase (Martin et al., 1995; Peiser and Yang, 1998). MACC is the most abundant ACC conjugate in ripening *Lycopersicon esculentum* (tomato) fruit, and its formation can be induced by ethylene (Liu et al., 1985; Martin et al., 1995; Peiser and Yang, 1998). ACC was shown to be hydrolyzed back to ACC in *Nasturtium officinale* (watercress) stems, *Nicotiana tabacum* (tobacco) leaf discs, and senescing *Dianthus caryophyllus* (carnation) petals (Jiao et al., 1986;

Hanley et al., 1989;). The formation of GACC is catalyzed by the enzyme γ-glutamyl-transferase (GGT) (Martin et al., 1995; Martin and Slovin, 2000) which, in *Arabidopsis*, is encoded by four widely expressed *GGT* (*1–4*) genes, two of which (*GGT3* and *GGT4)* encode catalytically inactive or minimally active enzymes. Interestingly, GGT1 and GGT2 appear to be localized extracellularly (Martin et al., 2007).

JA-ACC is the second most abundant JA conjugate detected in *Arabidopsis* leaves and is formed by JAR1, a JA-amino synthetase. Similar to MACC and GACC, JA-ACC might regulate levels of ACC available for the biosynthesis of ethylene, and may also regulate JA levels in the plant (Staswick and Tiryaki, 2004). The availability of ACC can also be controlled by plant and bacterial encoded ACC deaminases that irreversibly convert ACC to ammonia and α-ketobutyric acid (Glick et al., 1998). Multiple species of plant growth–promoting bacteria from various phyla, including *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes,* contain ACC deaminases that can decrease host plant ACC levels. A decrease of ACC often facilitates stress-coping mechanisms under various unfavorable conditions (reviewed in Glick, 2014; Nascimento et al., 2014; Van de Poel and Van Der Straeten, 2014). For example, tomato plants grown in the presence of ACC deaminase–producing *Enterobacter* or *Pseudomonas* strains exhibit an increased tolerance to flooding stress, likely as a result of decreased ethylene (Grichko and Glick, 2001a). Moreover, *Arabidopsis*, *Populus* (aspen), and tomato plants have been shown to contain ACC deaminases, but their role in plant growth and development has not as yet been elucidated (McDonnell et al., 2009; Plett et al., 2009).

ACS is generally encoded by a multigene family in most plant species. For example, in *Arabidopsis*, ACS proteins are encoded by a family of 12 genes, though only *ACS2–ACS9* and *ACS11* encode functional ACS enzymes; *ACS10* and *ACS12* encode aminotransferases (Liang et al., 1995; Yamagami et al., 2003), *ACS3* is a pseudogene, and ACS1 is catalytically inactive due to deletion of a highly conserved tripeptide Thr–Asn–Pro (TNP) (Liang et al., 1995). The remaining *Arabidopsis* ACS proteins can potentially form up to 45 different functional homo- and heterodimers, comprising a family of ACS enzymes with diverse biochemical properties (Tsuchisaka and Theologis, 2004; Tsuchisaka et al., 2009). ACS enzymes share an N-terminal catalytic domain and can be divided into three classes based on the presence of regulatory residues within their C-termini (Harpaz-Saad et al., 2012). The C-terminal domain of type-1 ACSs (ACS2 and ACS6 in *Arabidopsis*) have target residues for both calcium-dependent and mitogen-activated protein kinases (CDPKs and MAPKs, respectively) (Liu and Zhang, 2004; Sebastià et al., 2004). Joo et al. (2008) showed that phosphorylation of the Ser residues within the C-terminus of ACS6 by MPK6 increases its stability and is associated with increased rates of ethylene production. Type-2 ACS proteins have a target site for CDPKs and an overlapping Target of ETO1 (TOE) motif. Type-2 ACSs are targeted for degradation by the 26S proteasome pathway upon binding of ETHYLENE OVERPRODUCER1 (ETO1) or one of its paralogs, ETO-like1 or 2 (EOL1/2) (Chae et al., 2003; Wang et al., 2004; Christians et al., 2009;). Phosphorylation of the C-terminus of type-2 ACS proteins reduces their targeting for degradation. Type-3 ACS proteins (ACS7 in *Arabidopsis*) have a short C-terminus that lacks an apparent regulatory domain, though ACS7 levels can be mediated through the activity of the E3 ligase XBAT32 (Prasad et al., 2010; Lyzenga et al., 2012). The 14-3-3 phospho-specific binding proteins bind various ACS isoforms *in planta* to regulate their stability. 14-3-3s also bind to ETO1 and EOL2, but in these cases, it results in their destabilization. Therefore, 14-3-3s control the level of ACS proteins through a bipartite mechanism—on one hand stabilizing them through direct binding, but also destabilizing the ubiquitin ligases involved in their degradation (Yoon and Kieber, 2013).

There is a complex crosstalk between ethylene and other plant hormones at the level of signaling and/or biosynthesis, the latter of which includes both transcriptional and post-transcriptional regulation of ACS (reviewed in Kazan and Manners, 2012; Muday et al., 2012; Van de Poel et al., 2015; Shigenaga and Argueso, 2016; Hu et al., 2017; Liu et al., 2017; Zemlyanskaya et al., 2018; Bürger and Chory, 2019; Qin et al., 2019). For example, cytokinin and brassinosteroid additively increase the stability of type-2 ACS proteins independently of their TOE domains (Hansen et al., 2009). The effect of various phytohormones on the rates of degradation of type-1, -2, and -3 ACS proteins was comprehensively investigated using etiolated *Arabidopsis* seedlings as a model (Lee et al., 2017). Consistent with previous results, auxin was found to increase *ACS2* and *ACS5* transcript levels (type-1 and type-2, respectively) as well as to stabilize their encoded proteins. Gibberellin, brassinosteroid, and cytokinin were also found to decrease the turnover of ACS2 and ACS5 proteins. Salicylic acid (SA) stabilized ACS5 but decreased the stability of ACS2 protein, the latter of which is different from the effect in light-grown seedlings in which SA stabilizes ACS2 (Liu and Zhang, 2004; Lee et al., 2017). Abscisic acid (ABA) and methyl-jasmonate (MeJA) did not affect ACS2 protein levels but increased the stability of ACS5, which is distinct from the negative effect of these hormones on levels of ethylene production (Lee et al., 2017). The reduced ethylene biosynthesis in response to ABA and MeJA is likely explained by the downregulation of *ACO* genes in response to these hormones (Lee et al., 2017). Interestingly, the turnover of ACS7 (a type-3 ACS) was not regulated by any of the hormones examined in the study, and the analysis of its half-life showed that ACS7 is the most stable protein, confirming previous suggestions (Chae and Kieber, 2005). Moreover, the heterodimerization with ACS7 increased the stability of both ACS2 and ACS5 as compared to the respective homodimers, which suggests that dimerization among various ACS isoforms may regulate their turnover rate and, as a result, ethylene biosynthesis (Lee et al., 2017). ACS5 proteins are also stabilized when etiolated *Arabidopsis* seedlings are moved to the light, promoting ethylene biosynthesis and hypocotyl elongation during this transition (Seo and Yoon, 2019).

ACC TRANSPORT AND LYSINE HISTIDINE TRANSPORTERS

Ethylene is involved in various stress-related responses such as wounding, pathogen infection, neighbor proximity, elevated temperatures, drought, soil waterlogging, and submergence (Vandenbussche et al., 2005; Sasidharan and Voesenek, 2015; Huang et al., 2016; Loreti et al., 2016; Valluru et al., 2016; Dubois et al., 2018). Following the demonstration that ethylene leads to epinasty of petioles in waterlogged tomato plants (Jackson and Campbell, 1975), Bradford and Yang showed that waterlogging and root anoxia correlated with the shootward transport of ACC, its subsequent conversion to ethylene, and leaf epinasty (Bradford and Yang, 1980). This spatial separation between the biosynthesis of ACC and the its conversion to ethylene is the result of the oxygen dependence of the ACO enzyme (Murr and Yang, 1975). Multiple studies confirmed the phenomenon of ACC transport between roots and shoots in several plant species (e.g. Else and Jackson, 1998). During the de-submergence of flood-tolerant *Rumex palustris* (marsh dock) plants, ACC delivered from the root contributes to the pool of ACC that accumulated in the shoot during submergence to stimulate petiole elongation (Voesenek et al., 2003). In contrast, flood-intolerant *Rumex acetosa* (common sorrel) does not accumulate ACC in roots or shoots and consequently fails to recover from the detrimental effects of flooding. The root-to-shoot transport of ACC is thought to occur primarily in the xylem, though there is evidence for phloem-translocated ACC as well (Amrhein et al., 1981; Hume and Lovell, 1983). Radio-labeled ACC application to the abaxial side of *Gossypium hirsutum* (cotton) leaves resulted in both basipetal and acropetal transport of ACC throughout the plant as well as rapid conversion to [^{14}C]MACC, which was not translocated from the source leaf. ACC is compartmentalized within the tonoplast of *Zea mays* (maize) leaf mesophyll cells *via* a mechanism dependent on an electrochemical gradient (Saftner and Martin, 1993). Translocation of ACC conjugates into the vacuole likely plays a role in regulating ACC availability and/or ethylene levels. In *Acer pseudoplatanus* (sycamore maple) protoplasts treated with [^{14}C]ACC, there was a steady transport of the [^{14}C]MACC conjugate into the vacuole (Bouzayen et al., 1988). Furthermore, Tophof et al. (1989) showed that MACC accumulated to higher levels than ACC in vacuoles in both wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) plants.

The ability of plants to transport ACC both within the cell and throughout the plant suggests the existence of ACC transporters. ACC and its structural analog α-aminoisobutyric acid (AIB) are taken up by tomato pericarp cells; this uptake is inhibited by neutral but not by acidic or basic amino acids (Saftner and Baker, 1987). Tophof et al. (1989) speculated that ACC might be translocated to the tonoplast by a neutral amino acid transporter and as MACC competitively inhibited the transport of malate to the vacuole, they suggested that these molecules likely shared a common translocator. Recently, the identification of an ACC-resistant (*are2*) *Arabidopsis* mutant that displayed a reduced uptake of exogenous ACC led to the identification of the LYSINE HISTIDINE TRANSPORTER1 (LHT1) as a potential ACC transporter (Shin et al., 2014). LHT1 localized to the plasma membrane of leaf mesophyll and epidermal root cell and is not detected in the vasculature (Chen and Bush, 1997; Hirner et al., 2006). The *lht1* mutant displays severe growth defects on media with aspartate and glutamate as the sole nitrogen source and is impaired in the uptake of [^{14}C]-labeled amino acids. The *are2* mutant, allelic to *lht1-5*, was resistant to ACC but displayed a normal triple response when exposed to ethylene. Isolated *are2/lht1-5* protoplasts display reduced accumulation of [^{14}C]ACC. Additionally, competition experiments showed that the presence of alanine and glycine can reduce the triple response morphology elicited in response to ACC, consistent with Tophof's (1989) speculation that ACC is translocated through the tonoplast by a neutral amino acid transporter. However, Hirner et al. (2006) showed that lysine and histidine are the best substrates for LHT1, suggesting that multiple distinct transporters may act in the movement of ACC. It is possible that the ACC uptake and transport are mechanistically different and require distinct transport proteins. The subject of alternative ACC transporters and strategies to identify them has been recently discussed in a comprehensive review (Vanderstraeten and Van Der Straeten, 2017). Further studies are needed to identify and distinguish transporters involved in the short- and long-distance ACC translocation, its uptake into cells, as well as its intracellular trafficking.

ACC IN PLANT DEVELOPMENT AND BEYOND

A growing body of evidence indicates a role for ACC as a signaling molecule distinct from its role in ethylene biosynthesis. One of the first findings consistent with this was the discovery of the involvement of ACC in the regulation of cell wall function in the FEI pathway (Xu et al., 2008). FEI1 and FEI2 are leucine-rich repeat receptor-like kinases (LRR-RLKs) that have been linked to cellulose biosynthesis. *fei1 fei2* loss-of-function mutants display root swelling under high concentrations of salt and sucrose, decreased biosynthesis of cellulose, hypersensitivity to the cellulose inhibitor isoxaben, thickening of etiolated hypocotyls, and a decrease in the formation of cellulose rays in seed coat mucilage (Xu et al., 2008; Harpaz-Saad et al., 2011), which together indicate a role of the FEI proteins in regulating cellulose biosynthesis. Intriguingly, inhibition of ethylene biosynthesis [via aminooxy-acetic acid (AOA) or AIB; **Figure 1**] reverted the swollen root phenotype of *fei1 fei2* mutants, but blocking ethylene perception, using either the inhibitors 1-methylcyclopropane (1-MCP) or silver thiosulfate, or by introducing ethylene-insensitive *ein2* and *etr1* mutations into the *fei1 fei2* background, had no effect. Furthermore, the FEI kinase domain was shown to directly interact with type-2 ACS proteins, suggesting a direct link to ACC synthesis (Xu et al., 2008).

An analysis of cell elongation in roots treated with the cellulose biosynthesis inhibitor isoxaben provided further support that ACC acts as a signal. Tsang et al. (2011) analyzed root trichoblast length upon treatment with isoxaben in presence or absence of various ethylene inhibitors. Interestingly, inhibitors of ethylene biosynthesis [amino-ethoxyvinylglycine (AVG), AOA, or 2-anilino-7-(4-methoxy-phenyl)-7,8-dihydro-5(6*H*)-quinazolinone] reversed the isoxaben-induced elongation defects, but inhibitors of ethylene perception did not. Moreover, short-term ACC treatment induced shortening of trichoblasts in ethylene-insensitive *ein3 eil1* mutants, further supporting the hypothesis that ACC acts independently of ethylene signaling. Additionally, the authors found that both cell wall damage–induced and ACC-mediated growth inhibition is dependent on auxin signaling since the growth inhibition was absent when combined with α-(phenylethyl-2-one)- indole-3-acetic acid (PEO-IAA), a transport inhibitor response 1 (TIR1) receptor antagonist This is consistent with the suppression of the *fei1 fei2* root swelling phenotype by auxin biosynthesis mutants (Steinwand et al, 2014). Together, these studies suggest that ACC plays a role in the response to cell wall perturbations, triggered by either chemical or genetic disruption of cellulose synthesis, and that auxin is involved in this pathway (Xu et al., 2008; Tsang et al., 2011).

Genetic analysis of disruption of *ACS* genes in *Arabidopsis* also supports a function for ACC in addition to its role as an ethylene biosynthetic precursor. A comprehensive genetic study of all members of the ACS gene family in *Arabidopsis*, including the generation and analysis of single, double, triple, and high-order *acs* mutants, suggested novel roles for ACS beyond ethylene biosynthesis (Tsuchisaka et al., 2009). Analysis of the mutants revealed both synergistic and antagonistic relationships among various *ACS* genes in ethylene biosynthesis and in regulation of hypocotyl and rosette growth, and flowering time. Disruption of multiple *ACS* genes led to a progressive increase in plant size, concomitant with a decreased level of ethylene biosynthesis. Remarkably, an octuple *acs 2, 4, 5, 6, 7, 9, amiR acs8 acs11* mutant, which had a ~90% decrease in the level of ethylene production, displayed embryonic/gametophytic lethality and/or unfertilized ovules. The octuple *acs* mutant inflorescences are significantly taller than wild-type or lower-order *acs* mutants, despite their initial reduced growth rate. An independent octuple mutant line analyzed in the study could only be propagated when the amiR transgene was heterozygous, consistent with embryo/gametophytic lethality or infertility. The striking phenotypes of the octuple *acs* mutant is distinct from the full reproductive viability of even very strong ethylene signaling mutants, suggesting that *ACS* genes play a role beyond acting as

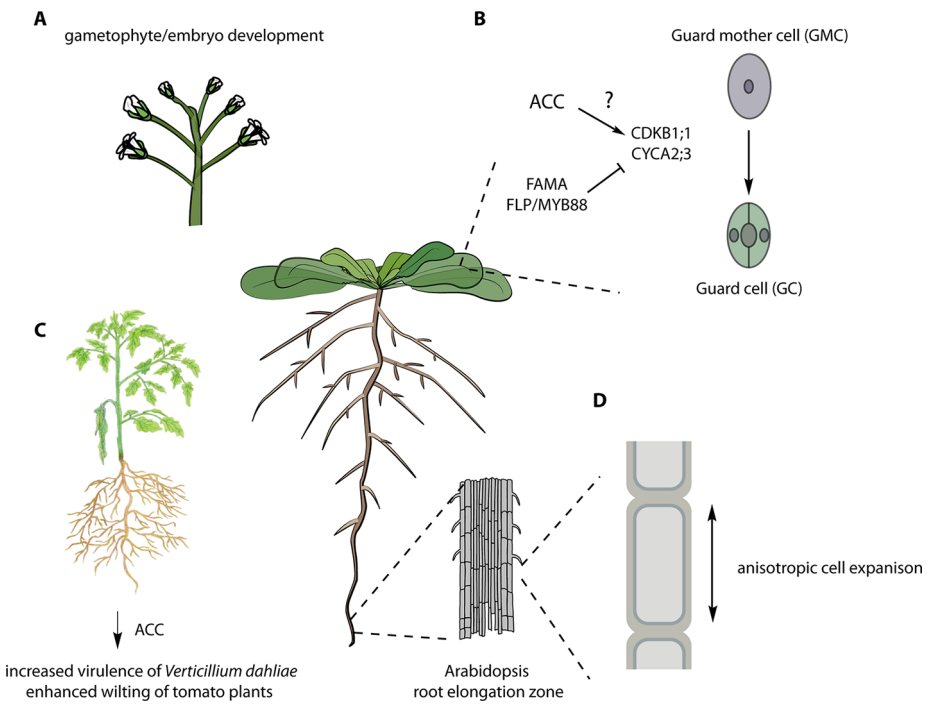

FIGURE 2 | Various ACC-dependent processes. **(A)** The infertility of the octuple *acs 2, 4, 5, 6, 7, 9, amiR acs8 acs11* mutants suggests an essential role in gametophytic and/or embryonic function (Tsuchisaka et al., 2009). **(B)** Role of ACC in stomatal development. ACC is required for the division of the guard mother cell (GMC) likely *via* the regulation of the indicated cell cycle regulators (Shin et al., 2014). **(C)** Overexpression of the ACC deaminase from the fungal pathogen of tomato, *Verticillium dahliae,* enhances its virulence, and pre-treatment of tomato plants with ACC reduced the symptoms of *V. dahliae* infection even in ethylene-insensitive mutants (Tsolakidou et al., 2019). **(D)** ACC is involved in cell wall signaling regulating anisotropic elongation of root cells (Xu et al., 2008; Tsang et al., 2011). See text for more details. Figure adapted, with permission, from Figshare [**A** (Bouché, 2018a); **B** (Bouché, 2018b); **C** (Davis and Mitra, 2019); **D** (Bouché, 2017)].

precursors in ethylene biosynthesis. Alternatively, ACS proteins may have a moonlighting function, and the reported lethality of the octuple mutants may result from disruption of an unrelated process. The precise nature of the embryo/gametophyte lethality of the octuple *acs* mutant needs further characterization.

ACC was recently shown to play a role in stomatal development. The terminal division of the guard mother cell (GMC) produces the two guard cells (GCs) that comprise the mature stomata (Yin et al., 2018). Application of the ACS inhibitor AVG induced the formation of pore-less, single guard cell (SGCs). Similarly, the octuple *acs 2, 4, 5, 6, 7, 9, amiR acs8 acs11* mutant (Tsuchisaka et al., 2009) developed SGCs. This SGC phenotype was not observed in the presence of ACO inhibitors (AIB or Co^{2+}) or the ethylene binding inhibitor 1-MCP, nor was it present in various ethylene-signaling mutants (*etr1*, *ein2, ctr1, ein3 eil2*). ACC did not increase cell division in GMCs in wild-type plants but did so (as did ethylene) in *fama* and *four lips* (*flp*)/*myb88* mutants, which developed clusters of thin cells on the epidermis. FAMA and FOUR LIPS (FLP)/MYB88 are central regulators of the last cell division of GMC, acting upstream of the core cell cycle genes (*CYCA2;3*, *CDKB1;1*, and *CDKA;1*) (Xie et al., 2010; Vanneste et al., 2011; Yang et al., 2014). Because ACC induced extra divisions in these mutant backgrounds, it was concluded that FAMA and FLP/MYB88 might antagonize the effect of ACC on the GMC division. Moreover, ACC, but not ethylene, stimulated the expression of *CYCA2;3* and *CDKB1;1* in *fama* and *flp/my88* mutants, and conversely, AVG downregulated the expression of these genes. ACC partially rescued the SGC formation in the *acs 2, 4, 5, 6, 7, 9, amiR acs8 acs11* line, but not in *cyca2;3* and *cdkb1;1* mutants, suggesting that it acts upstream of the cell cycle–dependent control of the GMC division.

Recent studies suggest that the signaling role of ACC could extend beyond the plant kingdom. Since many plant growth–promoting rhizobacteria (PGPR) possess ACC deaminase genes and utilize ACC as a source of nitrogen, Li et al. (2019) examined whether ACC could act as a chemoattractant. Indeed, *Pseudomonas putida* displayed a chemotactic response to ACC, but not to ethylene, and the ability to respond to ACC was correlated to the ability of *P. putida* to colonize wheat roots.

The fungal pathogen, *Verticillium dahliae* is a soil-borne pathogen of many plant species, causing vascular wilt disease. Tsolakidou et al. (2019) found that genetic modulation of ACC levels in *V. dahliae* affected its microsclerotia development and hyphae growth. Overexpression of ACC deaminase in *V. dahliae* led to increased virulence on tomato and eggplant, including enhanced wilting and greater fungal growth. On the contrary, the disruption of ACC deaminase in *V. dahliae* resulted in reduced virulence and less fungal biomass. To test if ACC was acting as a signal controlling plant defense, wild-type and the ethylene-insensitive mutant *Never-ripe* (*Nr*) tomato plants were treated with ACC and then infected with *V. dahliae*. ACC increased the resistance of both wild-type and *Nr* plants, suggesting that ACC and not ethylene promotes disease resistance against *V. dahliae*. It will be interesting to investigate if similar responses occur in other plant–pathogen interactions.

CONCLUSIONS AND OUTSTANDING QUESTIONS

An increasing number of studies have established that ACC acts as a signaling molecule beyond its function in ethylene biosynthesis (**Figure 2**). ACC appears to be involved in regulating multiple processes, including stress responses, cell expansion, cell wall function, stomatal development, pathogen interactions, and fertilization-related events. Additional studies are needed to elucidate the mode of action of ACC and to further define its role in plant growth and development. For example, the embryo/gametophyte lethality of the octuple *acs* mutants (Tsuchisaka et al., 2009) raises questions about the precise basis for ACC action during gametophyte development, fertilization, or embryogenesis. The recent link between LHT and ACC uptake (Shin et al., 2014) provides tantalizing clues to the mechanisms of ACC uptake/transport, but as LHT is part of a large gene family, other amino acid transporters may also participate in ACC uptake and translocation (reviewed in Vanderstraeten and Van Der Straeten, 2017). The biological roles of ACC derivatives, including the JA-ACC, GACC, and MACC, also need to be investigated. Finally, in light of the findings presented in this review, studies that use ACC as a proxy for ethylene need to be interpreted with caution.

AUTHOR CONTRIBUTIONS

Both authors wrote the manuscript.

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AtDAT1 Is a Key Enzyme of D-Amino Acid Stimulated Ethylene Production in *Arabidopsis thaliana*

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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

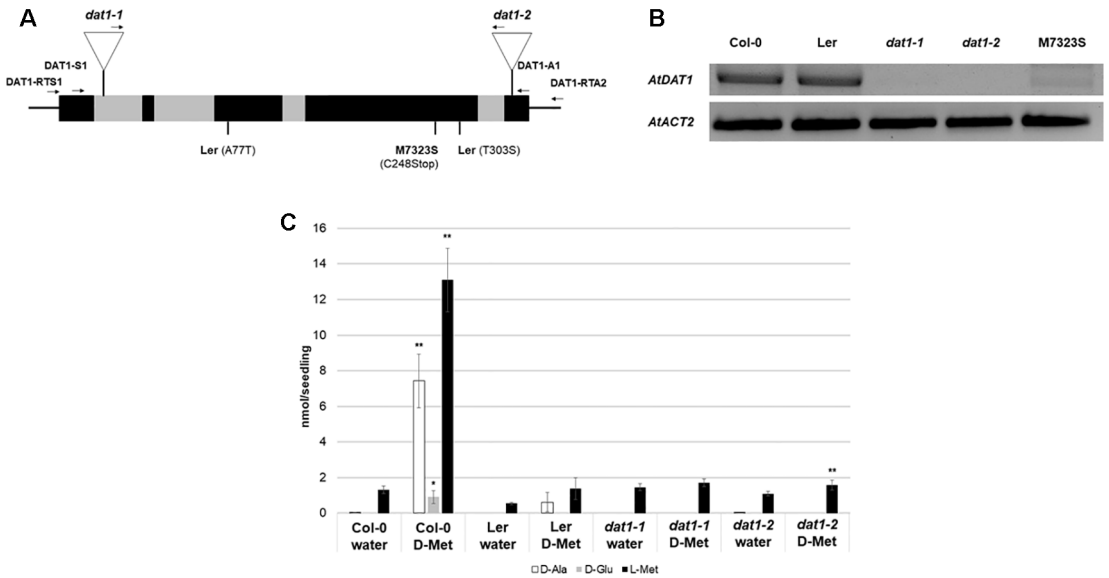

FIGURE 1 | AtDAT1 as a candidate protein for the metabolism of D-AAs in *Arabidopsis.* **(A)** Scheme of the genomic structure of *AtDAT1* (exons and introns in black and grey, respectively) with the positions of T-DNA insertions in *dat1-1* and *dat1-2* as well as the mutations found in L*er* and M7323S. 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).

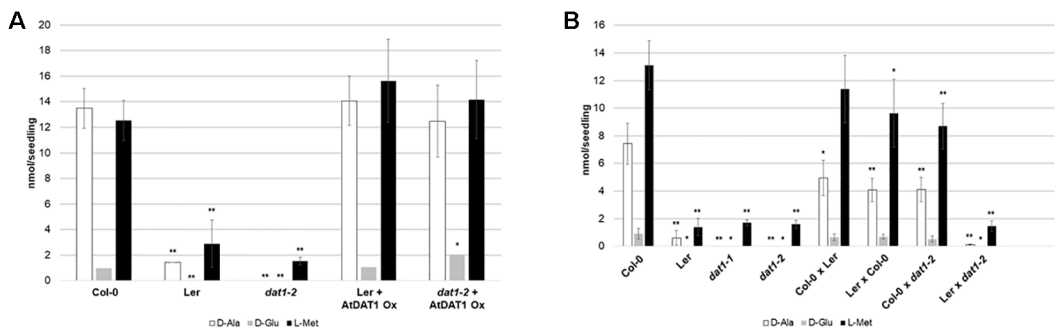

FIGURE 2 | D-Met metabolism in lines overexpressing *AtDAT1* and in F1 seedlings from crosses of Col-0, L*er* and *dat1-2*. Contents of D-Ala, D-Glu, and L-Met after overnight exposure to D-Met **(A)** in L*er* and *dat1-2* seedlings overexpressing *AtDAT1* (AtDAT1 Ox) and their corresponding background 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

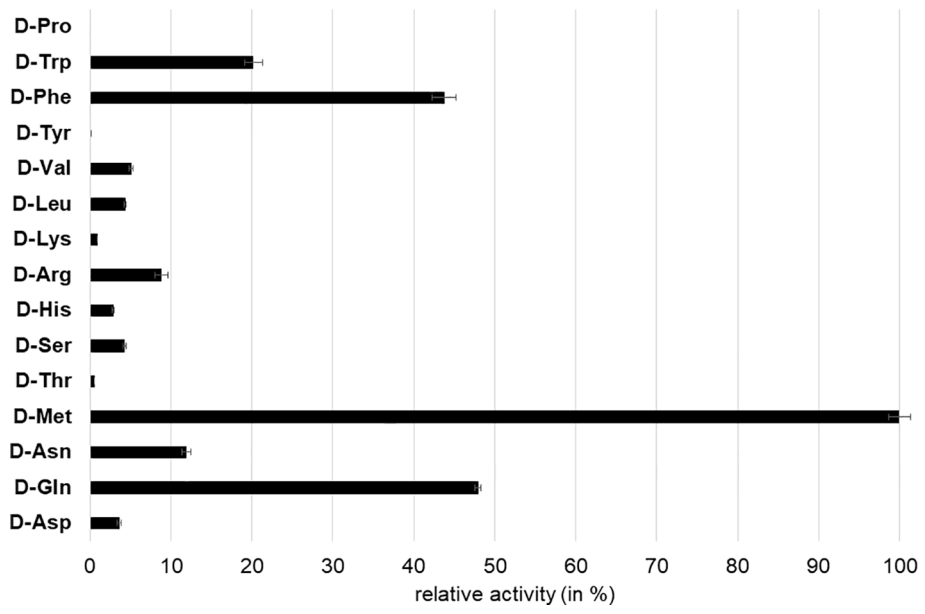

FIGURE 3 | Relative D-Ala producing activity of AtDAT1 with different D-AAs as amino group donor and pyruvate as acceptor. Activity of reaction with D-Met was set to 100% and all other reactions were calculated in relation to it. Each bar represents the mean of measurement of three independent assays.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

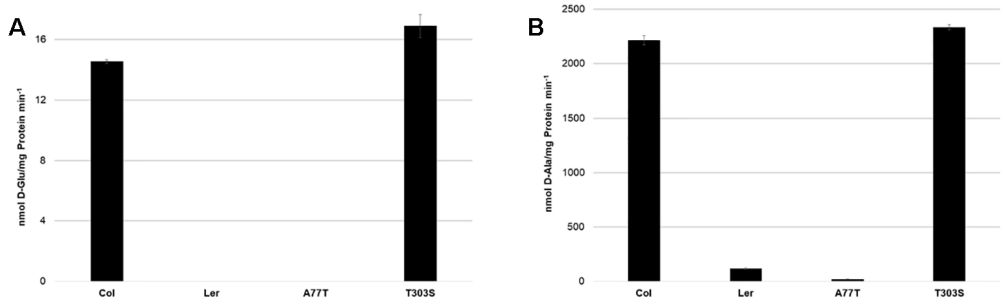

FIGURE 4 | Activities of AtDAT1 variants. Transaminase activities of AtDAT1$_{(Col\text{-}0)}$, AtDAT1$_{(Ler)}$, AtDAT1$_{(A77T)}$, and AtDAT1$_{(T303S)}$ with D-Met as amino group donor and **(A)** 2-oxoglutarate or **(B)** pyruvate as acceptor molecule are displayed; for further information, see **Figure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

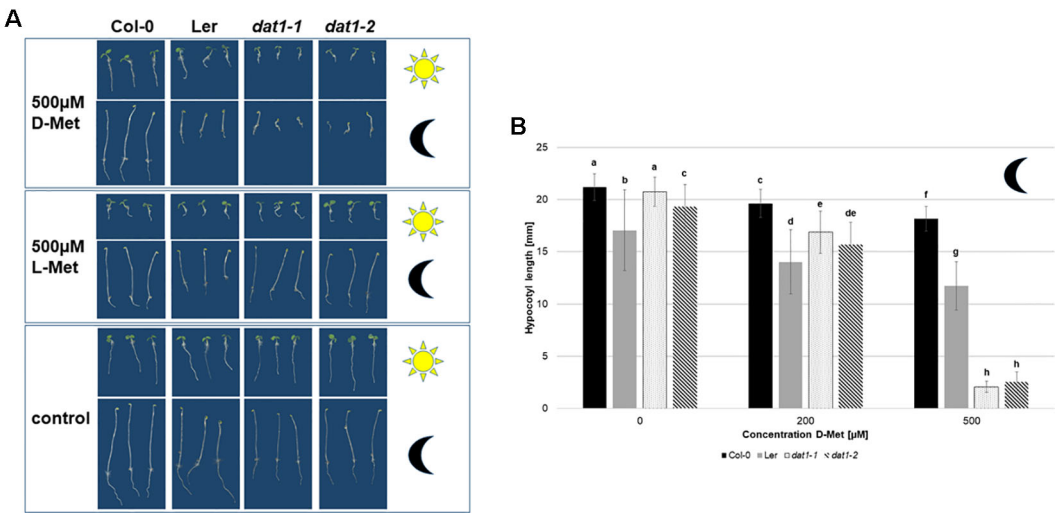

FIGURE 5 | Seedling growth is differentially suppressed by D-Met in *AtDAT1* knock out-lines. **(A)** Seeds of Col-0, *dat1-1*, and *dat1-2*, and L*er* were germinated either in continuous light (sun) or darkness (moon) on different solid growth media (with 500 µM D-Met, with 500 µM L-Met supplemented 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).

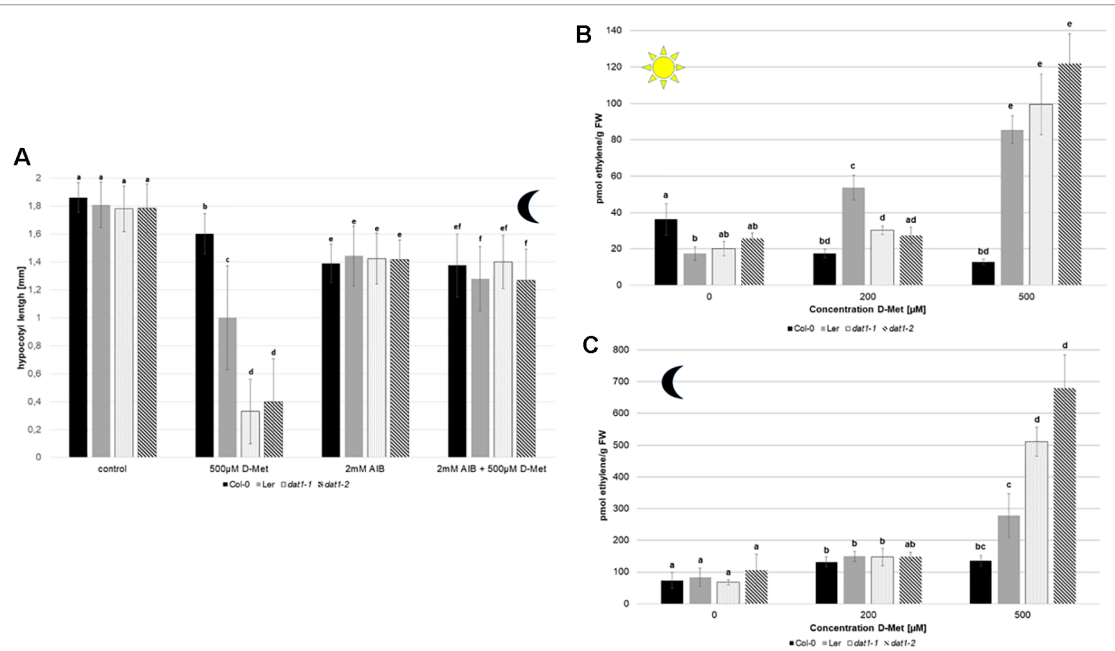

FIGURE 6 | D-Met leads to an increase of ethylene in *AtDAT1* knock out-lines. **(A)** Seeds of Col-0, *dat1-1*, and *dat1-2*, and L*er* were germinated in continuous darkness on solid growth media without any supplementation (control), supplemented just with 500 µM D-Met, supplemented just with 2 mM 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

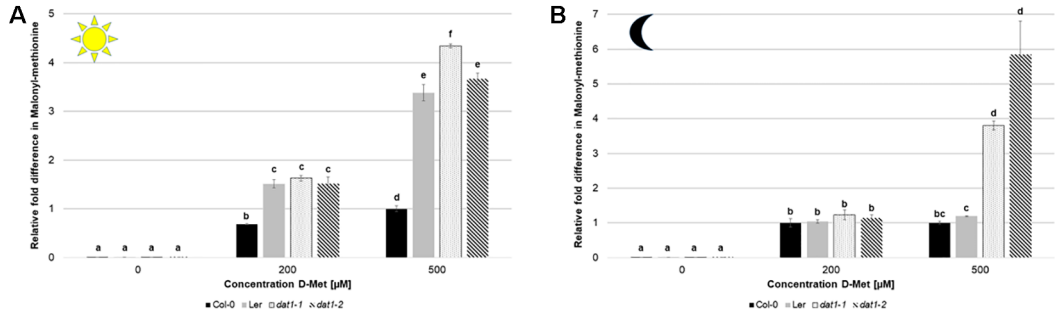

FIGURE 7 | D-Met affects formation of malonyl-methionine differently in Col-0, L*er*, and *dat1* mutants. Malonyl-methionine contents in seedlings of Col-0, L*er*, *dat1-1*, and *dat1-2* were measured after growth **(A)** in continuous light or **(B)** in darkness on agar plates supplemented with 200 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).

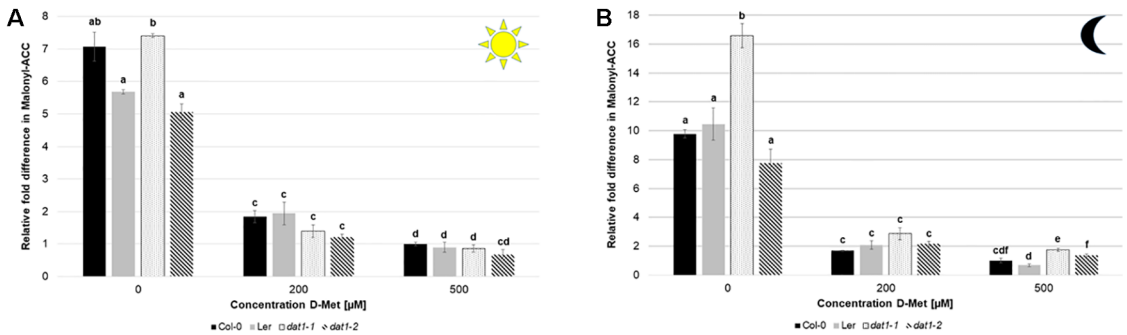

FIGURE 8 | D-Met leads to a decrease of malonyl-ACC levels in all tested lines. Malonyl-ACC contents in seedlings of Col-0, L*er*, *dat1-1*, and *dat1-2* were measured after growth **(A)** in continuous light or **(B)** in darkness on agar plates supplemented with 200 and 500 µM D-Met, and without supplementation. 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-

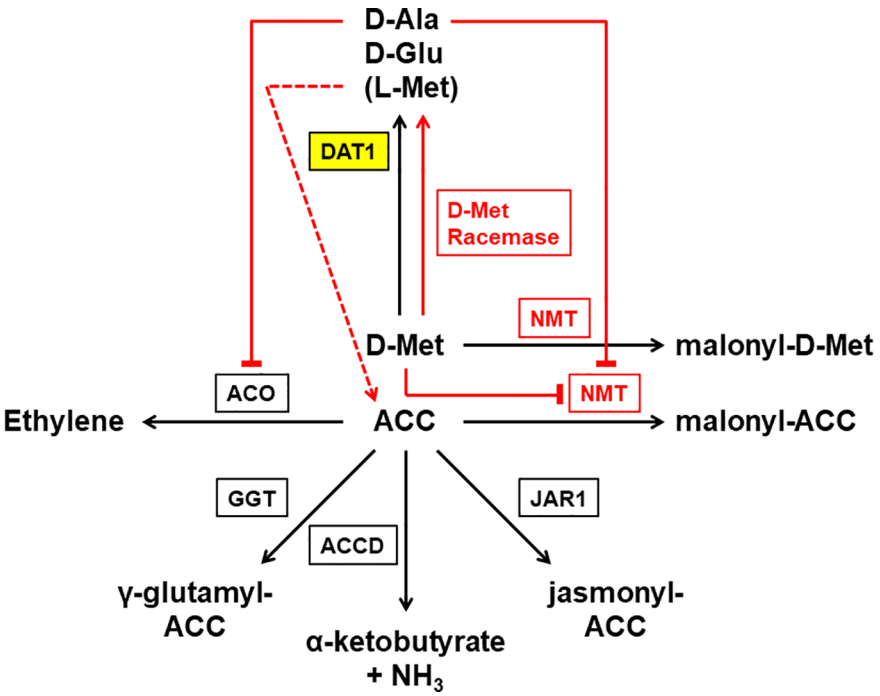

FIGURE 9 | Working model of the different reactions leading to D-Met stimulated ethylene production in plants. This reaction scheme summarizes proven (black) and postulated (red) enzymes and reactions involved in the process of D-Met stimulated ethylene production in plants. As the central enzyme of 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.

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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

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