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Research Article

Serendipita indica modulates expression of arginine metabolism genes during colonization of *Arabidopsis thaliana* roots

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Abstract
Endophytic fungus *Serendipita indica* belongs to the order Sebacinales. It is an extremely versatile root endophyte colonizing more than 150 different plant species including the model plant *Arabidopsis thaliana*. Colonization of *S. indica* triggers enhanced growth, early flowering, increase in seed content, alteration of secondary metabolite pathways and adaption to different biotic and abiotic stresses. This is why this fungus got ample attention by the plant researchers. In this study, we report modulation in expression of argininosuccinate synthase (*AS*), argininosuccinate lyase (*AL*) and arginase 2 (*ARGAH2*) genes in *A. thaliana* as a result of *S. indica* colonization. Similarly, expression of GUS reporter gene driven by *AS* and *ARGAH2* promoters in roots was altered. *AS*, *AL* and *ARGAH2* genes were down-regulated at early stage of root colonization i.e at 3 days post inoculation (dpi), with a subsequent up-regulation at later stages (7 and 14 dpi). Other arginine catabolism genes, Arginine decarboxylase-1 (*ADC1*), Arginine decarboxylase-2 (*ADC2*) and Pyrroline-5-carboxylate reductase (*P5CR*) were strongly up-regulated at later stages of *S. indica* colonization. Our findings show significant changes in expression of several important genes in arginine metabolic pathway upon *S. indica* colonization. These changes are very specific and strongly depend on the colonization phase of the fungus.
Keywords: *AL*; *ARGAH1*; *ARGAH2*; *AS;* Arginine metabolism; *GUS*; Quantitative RT-PCR; *Serendipita indica*

Introduction

Abiotic and biotic stresses are the major causes of crop losses throughout the world. They primarily include unfavorable climate conditions, different plant diseases and plant pests. One commonly used solution to cope with these stresses is the high chemical input to increase the plant production **[1]**. However, these soil and plants treatments cause severe damage to environment. Use of biological agents instead of chemical treatments is therefore gaining more attention and importance. Mycorrhizae are already established as beneficial associations of fungi with plants increasing their growth and production. Moreover, endophytic arbuscular mycorrhizal fungi (AMF) are proven to enhance abiotic stress tolerance. These fungi

have mutualistic relationships with 80% plant species worldwide [2]. In addition, many AMF associations were shown to be effective against soil born plant pathogens [3, 4] and play an important role in nutrient acquisition of host plants [5].

Endophytic fungus *Serendipita indica* (previously known as *Piriformospora indica*) belongs to the order Sebacinales. It colonizes roots, grows inter- and intracellularly, and forms spores in the roots as well as on the root surface. Its colonization on *A. thaliana* roots can be divided into four different phases: (1) extracellular (approx. 1 dpi); (2) biotrophic (less than 3 dpi); (3) cell death associated (approx. 7 dpi) and (4) fungal reproduction (approx. 14 dpi), whereupon external and intracellular sporulation occurs approx. at 7 and 14 dpi, respectively [6]. Sebacinales got much attention due to its role in enhancement of plant growth and development [7]. *S. indica* is also reported to confer stress tolerance against heavy metals, drought, acidity and enhanced resistance against plant pathogens. It has been demonstrated that it is beneficial during the attack of different plant pathogens [8].

Recently the role of arginine metabolism in plant-microbe interactions gained more attention [9, 10]. In general, arginine pathway is involved in different plant defense responses where it antagonizes pathogens by enhancing nitric oxide production [11]. In plants, arginine is produced from argininosuccinate via argininosuccinate lyase (*AL*). Argininosuccinate is synthetized from citrulline by the enzyme argininosuccinate synthase (*AS*). Further, arginine is catabolized by arginase (*ARGAH1 & ARGAH2*) to ornithine, which is a precursor for the production of proline via many steps. The final reaction is catalyzed by pyrroline-5-carboxylate reductase (*P5CR*). Arginine is also catabolized to polyamines in many reactions, where the first step involves the synthesis of agmatine by the action of arginine decarboxylase (*ADC1 & ADC2*).

We are still lacking knowledge on involvement of arginine metabolic pathway during colonization of plants by endophytic fungi. Since *S. indica* induces different modifications in host roots, this study is aimed to elucidate different parts of this pathway during colonization process. Therefore, our work aims at studying the involvement of arginine pathway in *A. thaliana, S. indica* interaction. Our findings showed clear modulation of arginine metabolic pathway upon *S. indica* colonization. *GUS* reporter analysis and gene expression analysis reveals down-regulation of *AS, AL* and *ARGAH2* genes at early stages of colonization with subsequent up-regulation at later stages. Expression of catabolic gene was also found significantly increased at later stages of fungus development.

Materials and methods

Plant growth and inoculation

A. thaliana surface-sterilized seeds were grown on 0.2% Knop medium at 16 h light/8 h dark and 25°C. Potato dextrose agar (Fluka, Germany) was used to culture *S. indica* at 28°C in the dark. Inoculation of 12-day-old *A. thaliana* plants was done by adding potato dextrose agar plugs (5 mm) containing *S. indica* hyphae 1cm away from the roots. Empty plugs were used in control plates.

Plasmid construction and transformation of *A. thaliana*

GUS promoter line (p*AS*::GUS) was constructed as described in [9]. Briefly, a 418 bp fragment upstream to start codon of the argininosuccinate synthase gene (At4g24830) was amplified from genomic DNA of *A. thaliana*. Sgs*I*/Asc*I* and Hind*III* restriction sites were introduced in the fragment by the specific primer sequences. The PCR products were cloned into binary vector pMDC139 [12, 13]. Presence of the promoter was confirmed by sequencing of

the resulted plasmid. Floral dip technique [14] was used for Agrobacterium-mediated plant transformation to insert the binary vectors in *A. thaliana* (Col-0). Screening of transformed seeds was done by antibiotic selection. Homozygous T3 (Third generation) seeds of lines p*AS*::GUS [9] and p*ARGAH2*::GUS [15] were collected after segregation analysis and used for final characterization of expression patterns.

Histochemical GUS assay

Histochemical GUS expression was performed using X-GLUC (5-bromo-4 chloro-3-indolyl β-D-glucuronic acid) (Biomol, Germany). Three, 7 and 14 days post inoculation (dpi) petri dishes containing inoculated plants were opened and X-GLUC solution was added. Dishes were then vacuum-infiltrated for 3 min and incubated at 37°C for eight hours. After incubation, dishes were washed with 70 % ethanol and observed under dissection microscope for GUS staining. Photographs were taken with Axiovert 200M (Zeiss, Austria) using an Axiocam digital camera (Zeiss, Austria).

Sample collection, RNA isolation and quantitative RT-PCR

Root segments were harvested at 3, 7 and 14 dpi from colonized and uncolonized (control) plants of the same age omitting root tips. Samples were immediately shock-frozen in liquid nitrogen. Three plates were used for harvesting at each time point representing one biological replicate. Three biological replicates from independently grown batches were collected for RNA isolation. All experiments were repeated three times. RNA was extracted as described in [9].

Quantitative RT-PCR was performed using an ABI PRISM 7300 Sequence Detector (Applied BioSystems; USA). The PCR was carried out at 50°C for 2 min, 95°C for 5 min followed by 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 1 min. Primer sequences are listed in (Table 1). *18S RNA* primers were used as internal reference. Fold changes were calculated using 2^{-ddCt} method [16]. RNA samples were used from three independent biological replicates. Each biological replicate was run in a set of three technical replicates to minimize the experimental errors.

Table 1. Presents the sequences of the primers used for qRT-PCR. *AS*, Argininosuccinate synthase; *AL*, Argininosuccinate Lyase; *ARGAH1*, Arginase 1; *ARGAH2*, Arginase 2; *ADC1*, Arginine decarboxylase-1; *ADC2*, Arginine decarboxylase-2; *P5CR*, Pyrroline-5-carboxylate reductase

Locus	Gene	Direction	Primer Sequence 5'- 3'
At4G24830	*AS*	Forward	AAAGCACGGGATTGGGCGGA
		Reverse	GCGGATCAAACCATCTTCCTGCG
At2G10920	*AL*	Forward	GCAGCTCGAACGTGATGCTGGT
		Reverse	CCTCGGAAGCCCACAGTACCCA
At4G08900	*ARGAH1*	Forward	AGCTTGGAGGGCCTGTGGAC
		Reverse	GCCTTGTTCCCGTCCTTCCTGG
At4G08870	*ARGAH2*	Forward	GGAAGGTGGCTATGCGCGGC
		Reverse	ACACTCCGTGCGCGAATCCC
At2G16500	*ADC1*	Forward	GACCCGTGCTGTGATGGGCC
		Reverse	CCGCTGCGGTGAGAGCGTTT
At4G34710	*ADC2*	Forward	GCTCGATCAAAGGCCCGGGG
		Reverse	CGCTCTGGGAGACCCGGACA
At5G14800	*P5CR*	Forward	AGCCTTGGCACAGGAGCAACG
		Reverse	GCTCCAAGAACGGTCTGTGAAG
At3G41768	*18S RNA*	Forward	TGACACGGGGAGGTAGTGACA
		Reverse	AGTCTGGTAATTGGAATGAGTACAATCTAA

Statistical analysis

Gene expression was calculated using 2^{-ddCt} method. Statistical variations were tested using ANOVA and LSD tests ($p < 0.05$) using the statistical software package STATGRAPHICS Plus Version 5.0.

Results

Characterization of promoter::*GUS* transgenic lines

Temporal expression patterns of previously published p*AS*::GUS and p*ARGAH2*::GUS fusion lines were analyzed [9, 15]. Here, the promoter activity of *AS* and *ARGAH2* was studied in *S. indica*-colonized roots of *A. thaliana*. p*AS*::GUS lines showed very weak promoter activity at 3 dpi (Figure 1A). In case of *pARGAH2*::GUS line promoter activity showed the same trend at 3 dpi (Figure 1D). In contrast, at 7 dpi p*AS*::GUS activity was observed only in vascular bundles (Figure 1B), and in p*ARGAH2*::GUS line GUS staining could be observed scattered all over the roots (Figure 1E). At 14 dpi in p*AS*::GUS line, strong GUS activity was found in most regions of the analyzed root tissue (Figure 1C). In case of p*ARGAH2*::GUS line strong promoter activity was also found all over the roots (Figure 1F). These findings were confirmed by at least two independent transgenic lines for both of the genes with consistent and reproducible results.

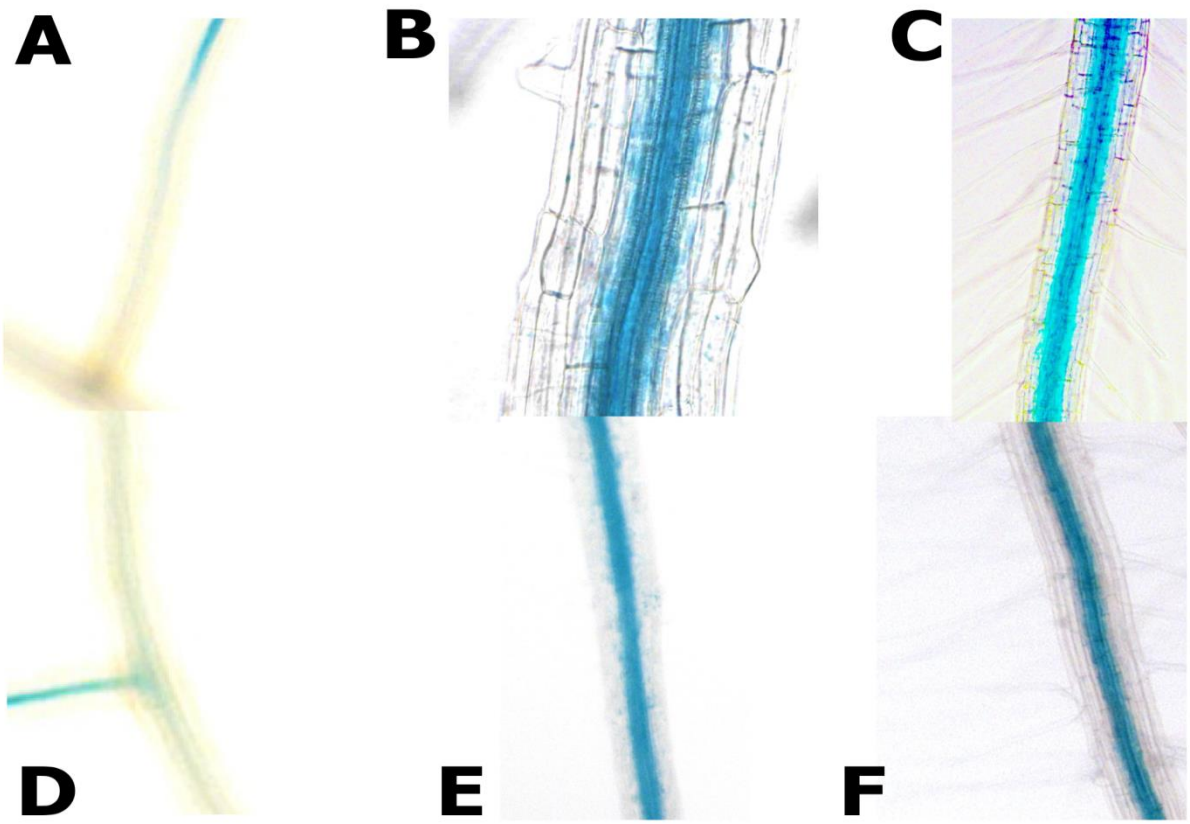

Figure 1. GUS staining upon *S. indica* colonization in roots of p*AS*::GUS line at A) 3 dpi, B) 7 dpi, C) 14 dpi; and *S. indica*-colonized roots of the p*ARGAH2*::GUS line at D) 3 dpi, E) 7 dpi, F) 14 dpi

Expression analysis of arginine cycle genes
Important genes for two anabolic and three catabolic enzymes of arginine metabolic pathway were selected for expression analysis. The entire arginine pathway is presented in (Figure 2). Expression of these genes was analyzed using qRT-PCR at three different time points: early 3 dpi, mid 7 dpi and late 14 dpi. At an early stage of development (3 dpi), argininosuccinate synthase (*AS*) showed down-regulation as compared to un-colonized control roots, with a subsequent up-regulation at later time points. However, gene expression was decreased at 14 dpi as compared to 7 dpi. Similarly, argininosuccinate lyase (*AL*) showed the same trend i.e down-regulation at 3dpi and up-regulation at later time points. But in this case gene expression was not as strong as in case of *AS* (Figure 3A & B). In case of arginase related genes, arginase 1 (*ARGAH1*) gene was found up-regulated at all three time points with a strong up-regulation at 7dpi followed by a decreasing trend. Arginase 2 (*ARGAH2*) showed a significant down-regulation at 3dpi with almost two-fold up-regulation at later time points (Figure 3C & D). Among other catabolic genes, i.e. *ADC1*, *ADC2* and *P5CR*, an increasing trend was found at all-time points tested. Strongest up-regulation was found at 14 dpi in all three genes (Figure 3E, F & G).

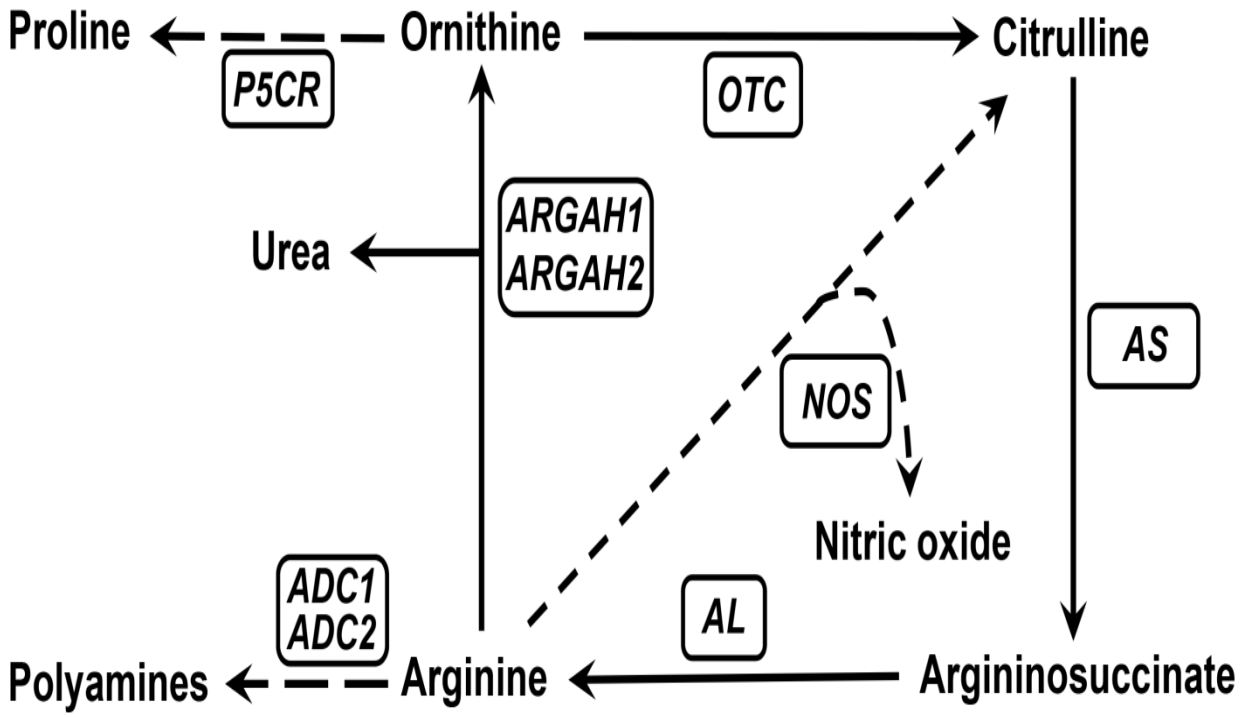

Figure 2. Arginine metabolism in *A. thaliana*. Important enzymes are shown for each reaction. Dotted arrows indicate more than one reaction (modified from PMN: https://plantcyc.org/)

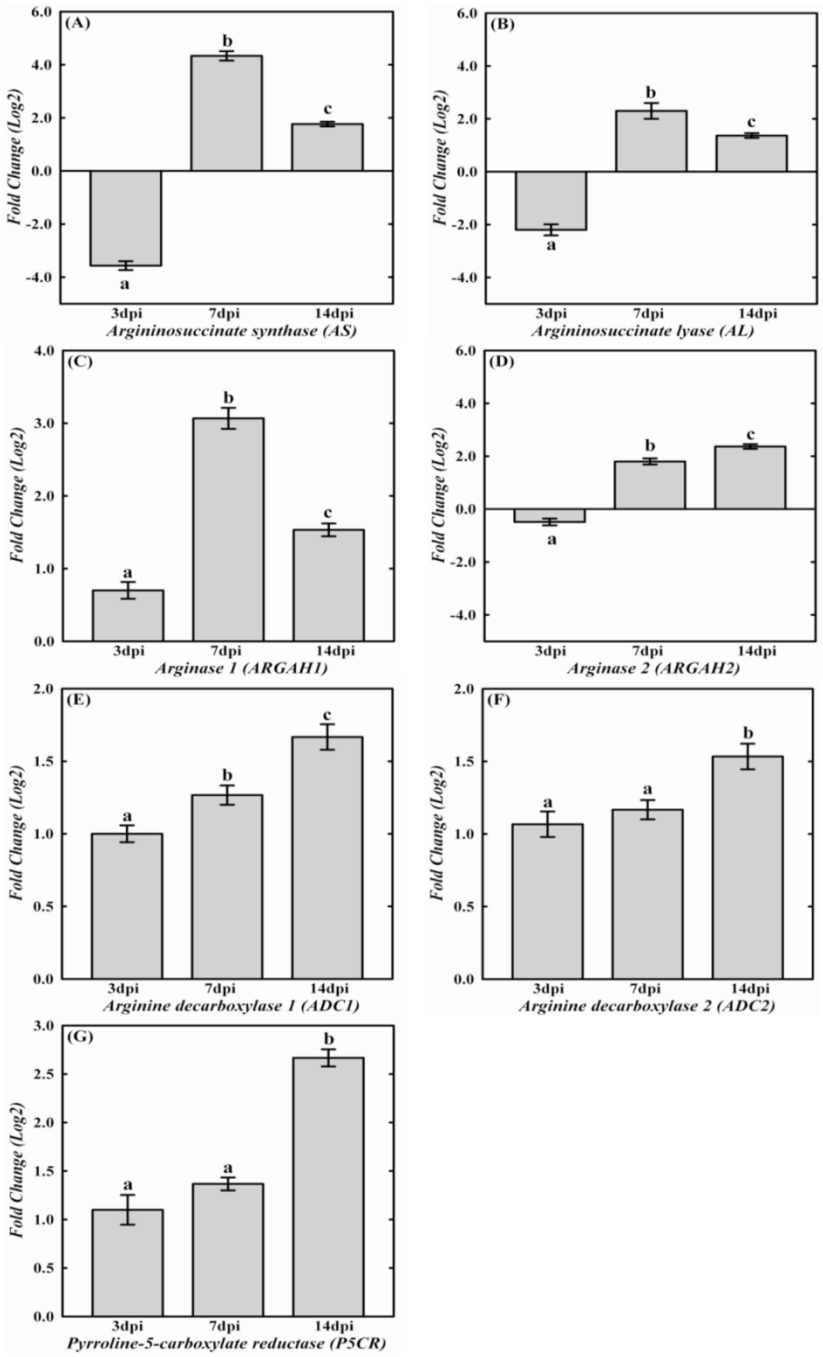

Figure 3. Gene expression patterns of A) argininosuccinate synthase (*AS*), B) argininosuccinate lyase (*AL*), C) arginase 1 (*ARGAH1*), D) arginase 2 (*ARGAH2*), E) arginine decarboxylase 1 (*ADC1*) F) arginine decarboxylase 2 (*ADC2*) and G) pyrroline-5-carboxylate reductase (*P5CR*) in roots of *A. thaliana* colonized by *S. indica* at 3dpi, 7dpi and 14 dpi. Results were obtained using qRT-PCR and fold changes were calculated using 2^{-ddCT} method. Un-colonized roots from the plants of same age were used as control. Values are means ± SE, n = 3 (biological replicates), different letters indicate significant difference within time points ($p<0.05$, one-way ANOVA, LSD)

Discussion

There are only few studies on arginine cycle regulation in plants [17, 18] and little is known about its role in plant-microbe interactions. Arginine metabolism is reported to be modulated e.g. during *A. thaliana*-nematode interaction [9, 19] and to be involved in plant defense responses antagonizing pathogens by supporting the nitric oxide production [20]. However, its involvement in the interaction between plants and fungal endophytes is still not known. Therefore, this work was conducted to study involvement of genes connected to this pathway during *S. indica* colonization of *A. thaliana* roots. Our data showed significant modulation at gene expression level in colonized roots. For instance, at 3 dpi we observed strong down-regulation of AS, which is the rate limiting enzyme and its decreased activity have direct relation to the amount of produced arginine [21]. Argininosuccinate, the product of AS action, is the immediate precursor of arginine in urea cycle and its conversion to arginine is catalyzed by AL [22]. AL was also strongly down-regulated at 3 dpi, which might result in decreased arginine biosynthesis in *S. indica* colonized roots at early colonization phase. Interestingly, reduction of arginine content by silencing the *AS* and *AL* results in better growth of pathogens on *A. thaliana* [9]. The product of AS and AL is arginine, which is an important amino acid not only for plants but also for microbes as an important nitrogen source [23]. For instance, during mycorrhizal symbioses, amino acids are the compounds responsible for the transfer of nitrogen to host plant [24]. Studies on ectomycorrhizal symbioses showed that during the initial stages of development fungi try to assimilate nitrogen by its own nitrate reductase. To accomplish this, the fungus down-regulates the host genes associated with assimilation of nitrogen [25, 26]. Further, arginine is connected with many amino acid pathways and serves as important raw material for the synthesis and modification of many proteins, polyamine and alkaloids. These compounds have a vital role in many plant metabolic processes [27] and are reported to be involved in abiotic stress responses as well as in plant-pathogen interactions [28]. During the interaction with pathogens, arginine production can be enhanced by the plant in order to produce higher amounts of nitric oxide (NO) [29], which play a key role in many plant defense responses against invading organisms [30]. *S. indica* at early colonization phase suppresses plant defense responses [31, 32]. Therefore, the initial down-regulation of *AS* and *AL* gene at 3 dpi, that was observed here, might be the strategy of the endophyte to manipulate plant defenses related to nitric oxide production. At later stages, the host defense responses against *S. indica* are attenuated and the relationship becomes mutualistic [32]. Our results show that at 7 and 14 dpi *AS* and *AL* are strongly up-regulated, which might suggest that the arginine synthesis during these phases is enhanced to cover growing nitrogen demands of *S. indica*.

Arginine is catabolized to ornithine via enzyme arginase, which is coded by a set of duplicated arginase genes called *ARGAH1* and *ARGAH2*. Differential expression of these two genes was found in *A. thaliana* roots colonized by *S. indica* with *ARGAH2* being down-regulated during the initial stage of fungus colonization. In contrast, at later stages both genes were significantly up-regulated. Arginase competes with nitric oxide synthase (NOS) for the common substrate arginine [33]. Hence, it can be speculated that at later stages when arginine is abundant, the fungus tries to attenuate the production of nitric oxide by increasing ornithine production. This phenomenon has been studied in animal parasite systems. For instance, trypanosomes, unicellular parasitic flagellate protozoa, are reported to evade host

NO-mediated defense responses by stimulating the expression of arginase genes [34], which might result in better stress tolerance and regulation of reactive oxygen species [35]. In *A. thaliana*, arginase genes mediate a cross talk between NO and polyamine biosynthesis [36]. Here, we show that two important genes in this pathway, arginine decarboxylase 1 (*ADC1*) and arginine decarboxylase 2 (*ADC2*), are strongly up-regulated upon *S. indica* colonization.

Proline is an important product of arginine catabolism and can be produced from arginine via many reactions. The final reaction catalyzing ornithine to proline is driven by pyrroline-5-carboxylate reductase (P5CR) [37]. As shown here, the gene encoding this enzyme is up-regulated at 7 and 14 dpi. Increased proline production can be beneficial for many plant responses. For instance, it is involved in osmotic stress management and accumulates as important osmolyte during stress conditions [38] and as a result of dehydration [39]. Accumulation of proline can be a result of incompatible plant microbe interactions [40] and can enhance the root growth by increasing the root meristematic zone [41]. Similarly, AMF are known to be triggering increased root growth [42].

Conclusion

This study demonstrates that *S. indica* triggers sophisticated modulations of the arginine pathway in colonized *A. thaliana* roots on the gene expression level. Genes encoding key enzymes in this pathway such as *AS, AL* and *ARGAH2* show significant down-regulation at early time points, whereas during later stages they are strongly and significantly up-regulated. These alterations are tightly interconnected with specific colonization phases of the fungus and are most probably indispensable for successful colonization and further development of the mutualistic relationship. Controlled arginine metabolism might not only help *S. indica* to avoid initial plant defense responses but also might improve conditions to sustain its further growth. Whether *S. indica* does this either to cope with NO-mediated plant defenses or to synthesize important metabolites essential for its growth and development should be in focus of further studies.

Authors' contributions

Conceived and designed the experiments: S Anwar & K Wieczorek, Performed the experiments: S Anwar, Analysed the data: S Anwar, Contributed materials/ analysis/ tools: K Wieczorek, Wrote the paper: S Anwar & K Wieczorek.

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