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Analysis of *Arabidopsis* amino acid metabolism in response to *Heterodera schachtii* infection

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Abstract

In the present study, a detailed amino acid analysis based on Ultra Performance Liquid Chromatography (UPLC™) was performed. The results showed significant increase in arginine, proline and glutamine amounts in syncytia. Ammonium ions were also increased as an important precursor in arginine, proline and glutamine biosynthesis. In addition, branched chain amino acids and amino acids of shikimic acid based pathway were also enriched in syncytia. This study opens a door to understand the remodeling of amino acid metabolism in response to *H. schachtii* infection in plants.

Keywords: *Arabidopsis thaliana*, amino acids, cyst nematode, *Heterodera schachtii*, interaction, syncytia, threonine synthase, valine, arginine, threonine, UPLC.

Plant-parasitic nematodes cause significant damage to a vast number of important crops worldwide (Moens & Perry, 2009). Especially cyst nematodes are responsible for big losses in agriculture. They are obligate parasites developing highly specific relationship with host plants (Sobczak & Golinowski, 2011). *Heterodera schachtii*, commonly known as sugar beet cyst nematode is of particular importance. This parasite gets nutrition from modified plant root cells called syncytia (Betka *et al.*, 1991), which significantly weakens the host due to severe nutrient losses caused by nematode feeding (Böckenhoff *et al.*, 1996). Infection process involves the penetration of second-stage juveniles (J_2) into roots and their migration towards the vascular bundles. Then each J_2 larvae pierces one single cell with its stylet and secretes saliva containing specific effectors (Sijmons *et al.*, 1994; Hussey & Grundler, 1998). Nematode secretions stimulate several structural and functional reorganizations of selected cells.

These changes include nucleus enlargement, mitochondria and chloroplast proliferation, condensation of cytoplasm and breakdown of central vacuole into dispersed smaller ones (Golinowski *et al.*, 1996). In addition, cell walls of neighboring cells dissolve at plasmodesmata integrating these cells into a syncytial multinucleated structure (Golinowski *et al.*, 1996; Grundler *et al.*, 1998). Nematode depends upon plant derived nutrients and solutes, which are concentrated in feeding structures. During the course of development, cyst nematode

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continuously withdraws nutrients from syncytium. Syncytia are very much evolved sink tissues in plant roots. Studies on phloem transport via fluorescent dye carboxy fluorescein (CF) and ^{14}C-labeled solutes reveal that Syncytia are symplastically isolated from the surrounding host cells and nematodes have direct access to phloem derived solutes of the host plant (Böckenhoff *et al*., 1996).

Parasitism proteins secreted by nematodes directly or indirectly modify the host signaling and metabolic pathways (Hofmann *et al*., 2010b; Anwar *et al.*, 2015; 2016). In order to get a complete picture of plant nematode interactions, several transcriptomic and metabolic studies have been previously performed (Hammes *et al.*, 2005; Szakasits *et al.*, 2009; Hofmann *et al.*, 2010a).

Highly regulated metabolic networks play central role in plant cell metabolism. Therefore, metabolic networks connected to amino acid biosynthesis gained considerable interest in plant research. Amino acids are not only used in protein synthesis but also serve as precursors for a large array of metabolites. Thus, amino acids play a vital role in multiple plant functions during growth and development and response to various stresses. Metabolites like hormones, cell wall components and a large group of secondary metabolites require amino acids as their precursors (Pichersky *et al.*, 2006; Korkina, 2007).

Arginine plays a central role in plant nematode interactions (Anwar *et al.*, 2015). Proline is involved in many abiotic stress responses in plants (Rontein *et al.*, 2002). Threonine, glycine and isoleucine play vital role during seed development and cellular energy production (Jander *et al.*, 2004; Joshi *et al.*, 2006). Branched chain amino acids play a central role during the growth and development of plant-parasitic nematodes (Anwar *et al.*, 2016). Methionine amino acid is an important part of many metabolic pathways. It serves as precursor for ethylene, polyamine and glucosinolates synthesis. It also plays a role in DNA methylation, chloroplast and cell wall biosynthesis (Amir *et al.*, 2002; Rebeille *et al.*, 2006; Goyer *et al.*, 2007). Studies on plant amino acid metabolism have so far been emphasized on their biosynthesis, which is regulated by end product feedback inhibition. During this control process, specific enzymes for particular amino acid biosynthesis pathway are inhibited by the amino acids they produced (Galili, 1995).

Plants deploy defense responses against microbes by physiochemical processes such as cell wall modifications and biochemical responses like production of reactive oxygen species (Bolwell & Wojtaszek, 1997; Hewezi *et al.*, 2008; Gill & Tuteja, 2010; Sharma *et al.*, 2012). These responses require high level of energy and need modifications of the physiology and metabolism of infected plant locally and systemically (Bolton, 2009). Some pathogens and symbionts induce the formation of specific local structures for feeding.

These structures are characterized by increased production of metabolites like amino acids (Desbrosses *et al.*, 2005; Hofmann *et al*., 2010a; Anwar *et al.*, 2015). Plant pathogens use many strategies to manipulate their hosts into synthesizing metabolites essential for the growth of pathogen. *Agrobacterium tumefaciens* is extensively investigated pathogen on plants. This bacterium induces the synthesis of opines as its food source, which cannot be used up by plants (Beck von Bodman *et al.*, 1992). Necrotrophic fungus *Sclerotinia sclerotiorum* deploys the same strategy in plants and triggers conversion of carbohydrates into fungal polyols (Jobic *et al.*, 2007).

Nematode induced syncytia consist of root cells, which undergo extensive proliferation and reorganization. The ultimate proliferation of host plant cells and high nutrient demand of the pathogen results in extensive changes in the plant primary metabolism. Nematodes are found to trigger many metabolic processes for

biosynthesis of essential nutrients for their diet. This results in the induction of new metabolic links within the host plants.

In the present study, we used an amino acid analysis approach in order to study the shift in metabolite levels of the affected plant tissue upon nematode infection. We compared the increase of specific amino acids in shoots and syncytia at specific time points. We also compared the changes in gene expression of relevant enzymes from available microarray of the model plant *Arabidopsis thaliana*. Finally, we analyzed the potential function of anabolic enzymes in amino acid biosynthesis upon *H. schachtii* infection.

Material and Methods

Plant growth and nematode inoculation: Plants were grown in sterile culture on a modified Knops medium (Sijmons *et al*., 1991) at 16 h light/8 h dark and 25°C. Roots of 12 day-old seedling were inoculated with 50-60 freshly hatched mobile second-stage juveniles (J_2). Briefly ~200-300 *H. schachtii* cysts were harvested from *in vitro* stock cultures on roots of mustard plants (*Sinapis alba* cv. Albatros) growing on 0.2X Knop medium (Sijmons *et al*., 1991) supplemented with 2% sucrose. Cysts were transferred from mustard-plates into a sterile 100µm sieve placed in a hatching funnel filled with 3m M $ZnCl_2$ solution.

The larvae hatch in darkness at 25°C. After 3 days Juveniles (J_2) that sediment at the bottom of the funnel were collected in 15µm sieve for inoculation. Before *in vitro* inoculation the larvae were additionally sterilized with the autoclaved solution of 0.05 % $HgCl_2$ for 3 min, washed several times with sterile distilled water and mixed with 0.7 % gelrite solution (Duchefa;www.duchefa-biochemie.nl/).

Sample collection: Root segments containing syncytia induced by *H. schachtii* in *A. thaliana* (Col-0) plants and shoots from the same specimen were harvested and shock frozen in liquid nitrogen. Roots without root tips and shoots from non-infected plants were sampled at the same time as control material. Sampling was performed at 5 and 10 days post inoculation (dpi).

Samples were collected for five independent biological replicates. Harvesting was always performed in the middle of the day to rule out diurnal effects. Control shoots and roots were harvested from non-infected plants of the same developmental stage. Control plants were cultivated under the same growth conditions parallel to the nematode-inoculated plants.

Amino acid analysis: Samples were collected from five independent biological replicates of *A. thaliana* (Col-0) infected and control plants. Shoots and syncytial material were collected at 5dpi and 10 dpi (days post inoculation). These two time points cover the important stages in nematode development.

At 5 dpi, most of the inoculated nematodes are in the second developmental stage called J_2. However, at 10 dpi sexual differentiation starts and this stage is called J_3.

Amino acid extraction was done with a solvent mix containing 20% (v/v) chloroform, 20% (v/v) distilled water and 60% (v/v) methanol. Briefly, samples were grinded by mixer mill (MM 2000, Retsch technology, Haan, Germany) with a stainless steel ball in each tube at vibrational frequency of 25 Hz for 1 min.

One ml of solvent mix was added to each tube and the grinding process was repeated for 3 min. After removing the steel ball, samples were centrifuged for 10 min at 16000 g. The supernatant (aqueous phase) was transferred into microvials and dried with a Speed Vac (Savant Speedvac SPD 111; Thermo Fisher Scientific Inc., Waltham, MA, USA). Amino acid profiling was performed using Waters Ultra-Performance Liquid Chromatography (UPLC^{TM}) system with a Waters Tunable UV (TUV) detector. Amino acids were quantified as nanomole per milligram

fresh weight of the sample (Inselsbacher *et al.*, 2011).

Statistical analyses and data evaluation: Comparison of the same plant organs in the presence and absence of nematodes at same stage of plant development allowed us to calculate fold changes in the amino acid levels upon nematode infection. The fold change (FC) is calculated as the log transformation of the ratio between the mean of amino acid contents in the infected tissue relative to the control tissue.

Fold change (FC) for genes presented in Tables 1 to 5 was calculated from available microarray data representing *H. schachtii*-induced syncytia (5dpi and 15 dpi) vs control roots (Szakasits *et al*., 2009). Difference between the geometric mean of the normalized ratios within each condition is represented as fold change as presented before in Hofmann *et al*., (2010a).

Amino Acid Pathways and Data source: Pathways for amino acid metabolism were designed using the AraCyc a highly curated species-specific database present at the Plant Metabolic network (PMN: https://plantcyc.org/). Genes encoding biosynthetic enzymes catalyzing the major steps of the amino acids in *A. thaliana* were identified from individual amino acid pathways. Genes and enzymes were further confirmed by literature search and by their annotation in TAIR (http://www.arabidopsis.org) database.

Results

Amino acids were divided into five groups on the basis of their metabolic connections. First group comprises of arginine, glutamate, glutamine and proline. In addition to these amino acids ammonium ions (NH_4^+) were also quantified as precursors in glutamine and glutamate biosynthesis (Fig. 1). Fold changes for all amino acids and NH_4^+ are highly increased in syncytia in all time points tested with the exception of glutamine, which is decreased in syncytia at 5dpi (Fig. 2A). Glutamine serves as precursor for the synthesis of glutamate, which in turn provides raw material for arginine biosynthetic pathway in *A. thaliana* (Fig. 1).

In shoots decreasing trend was found for NH_4^+ at both time points. Significant increase in fold changes of glutamate (10dpi), glutamine (05dpi) and proline (05 & 10 dpi) was found in shoots of all tested plants. However, arginine fold changes remained similar in shoots at both 5 dpi and 10 dpi (Fig. 2A). The fold changes of the genes coding for the relevant enzymes in arginine, glutamate, glutamine and proline metabolism are shown in Table 1. According to Szakasits *et al.*, (2009) most of these genes are up-regulated with argininosuccinate synthase (*AS*), argininosuccinate lyase (*AL*) and gamma-glutamyl-phosphate reductase (*AGPR*) showing the strongest up-regulation (Table 1).

Second group comprises branched chain amino acids (BCAAs): isoleucine, leucine and valine. The biosynthesis pathways of these amino acids are tightly connected. They are produced from threonine by a number of intermediate steps (Fig. 3). In this pathway two genes threonine synthase (*MTO2*) and dihydroxyacid dehydratase (*DHAD*) show strong up-regulation (Table 2).

All amino acids show a significant increase in syncytia at both time points tested. Valine shows significant increase at 10 dpi compared to early time point. In shoots, levels of all three amino acids are increased at 5dpi, whereas at 10 dpi fold changes are significantly decreased for all the three amino acids (Fig. 2B). Third group consists of threonine, glycine, serine, cysteine and alanine. In *A. thaliana* serine serves as precursor for glycine, cysteine and alanine. Threonine is precursor for glycine as well as valine, leucine and isoleucine (Fig. 3 & 4).

Table 1. Important enzymes and relevant genes involved in the metabolism of arginine, glutamate, glutamine and proline. FC-fold change calculated from available microarray data representing *H. schachtii*-induced syncytia at 5 and 15 dpi (Szakasits *et al.*, 2009). *n.d*- not determined.

EC	Locus	Symbol	Enzyme	FC
2.1.3.3	AT1G75330	OTC	Ornithine carbamoyltransferase	2.2
6.3.4.5	At4G24830	AS	Argininosuccinate synthase	4.9
4.3.2.1	AT5G10920	AL	Argininosuccinate Lyase	3.7
3.5.3.1	At4g08900	ARGAH1	Arginase 1	*n.d*
3.5.3.1	At4g08870	ARGAH2	Arginase 2	*n.d*
1.5.1.2	At5g14800	P5CR	Pyrroline-5-carboxylate reductase	2.4
4.1.1.19	At2g16500	ADC1	Arginine decarboxylase 1	0.4
4.1.1.19	At4g34710	ADC2	Arginine decarboxylase 2	1.7
6.3.1.2	At5g37600	GLN1	Glutamine synthetase 1	-0.7
6.3.1.2	At5g35630	GLN2	Glutamine synthetase 2	-0.6
1.4.7.1	AT5G04140	GLU1	Glutamate synthase 1	1.8
2.7.2.11	AT3G55610	P5CS2	Delta 1-pyrroline-5-carboxylate synthetase 2	-0.6
2.7.2.11	AT2G39800	P5CS1	Delta1-pyrroline-5-carboxylate synthetase 1	-0.6
2.6.1.13	AT2G39800	δ -OAT	Ornithine-δ-aminotransferase	*n.d*
1.2.1.88	AT5G62530	P5CDH	1-pyrroline-5-carboxylate dehydrogenase	2.7
2.7.2.8	AT3G57560	NAGK	Acetyl-glutamate kinase	1.6
1.2.1.38	AT2G19940	AGPR	gamma-glutamyl-phosphate reductase	4.6

In this pathway only two genes serine hydroxymethyl transferase (*SHMT*) and cysteine synthase-c (*ATCS-C*) show strong up-regulation (Table 3). Concerning amino acids, threonine shows significant decrease in syncytia at both time points and 10dpi shoot as compared to 5dpi shoot. Levels of glycine are significantly increased in 10dpi syncytia compared to shoot and 5dpi syncytia. Serine also shows the same trend as glycine but its level increases in 10dpi syncytia, however not that much as seen for glycine. Cysteine shows a little increase in shoot at 5dpi followed by the significant drop in shoots and high increase in syncytia. Alanine shows a significant drop in syncytia at 5dpi followed by a big increase at 10dpi (Fig. 2C). Fourth group of amino acids consists of aspartate, asparagine, lysine and methionine (Fig. 5). In this pathway, aspartate kinase homoserine dehydrogenase I (*AK-HSDH 1*), aspartate kinase homoserine dehydrogenase II(*AK-HSDH 2*), aspartate semialdehyde dehydrogenase (*ASDH*), L,L-diaminopimelate aminotransferase (*AGD2)*, dihydrodipicolinate synthase 2 (*DHDPS2*) and threonine synthase (*MTO2*) are the strongly up-regulated genes (Table 4). Among amino acids, aspartate is increased in 5dpi shoots and 10dpi syncytia with a significant decrease in 10dpi shoot and 5dpi syncytia. Asparagine shows decreasing trend at all tested stages. In contrast, 5dpi shoot and syncytia at both time points show significant increase in lysine level. Methionine is decreased in shoots of nematode-infected plants and enriched in syncytia (Fig. 2D). Fifth group is a diverse group comprising of phenylalanine, tyrosine and histidine. Tyrosine and phenylalanine are produced from common substrate chorismate in *A. thaliana*. The expression levels of genes coding for relevant

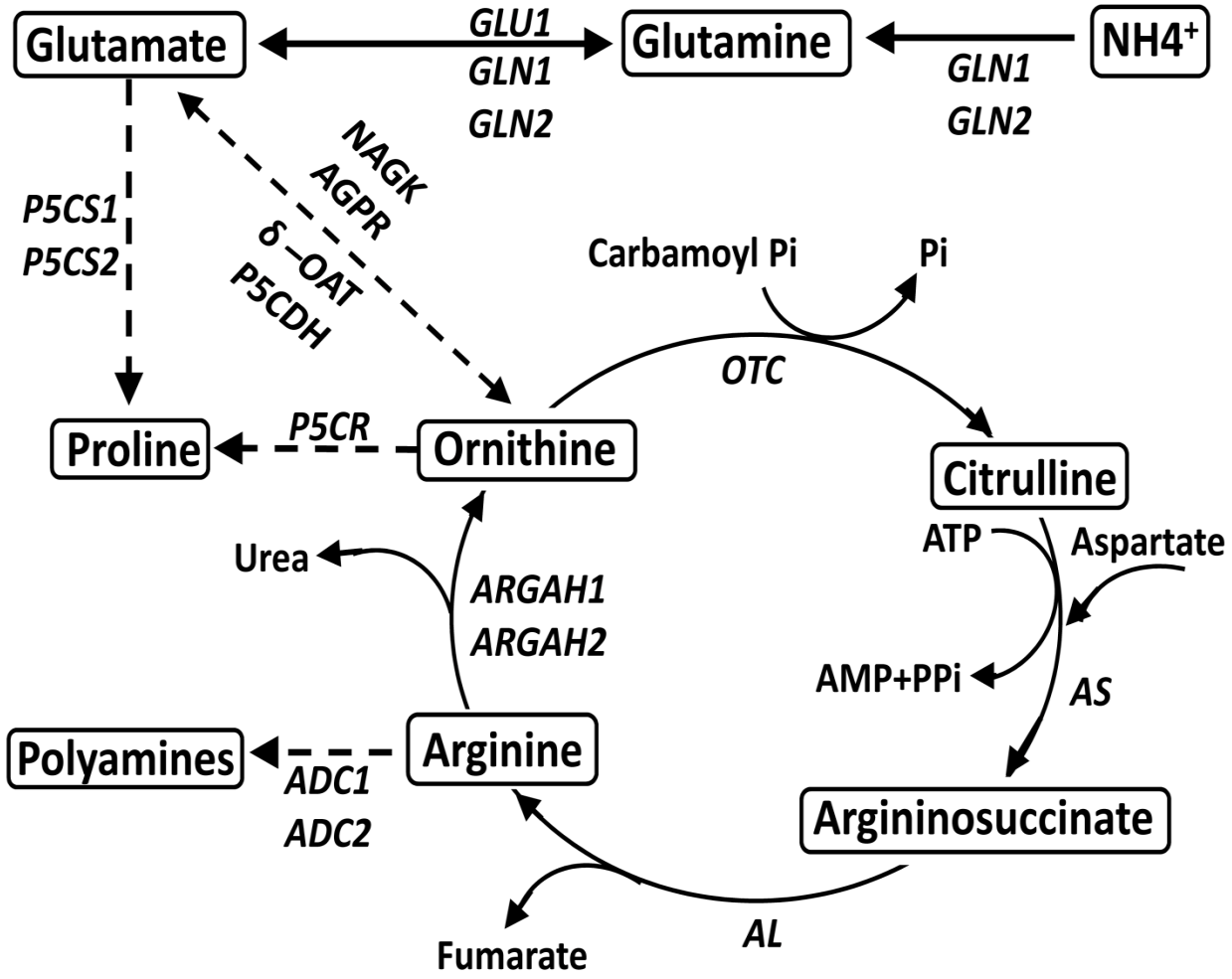

Fig. 1. Arginine, glutamate, glutamine and proline metabolism in *A. thaliana*. Important enzymes are shown for each reaction. Dotted arrows represent more than one reaction (PMN: https://plantcyc.org/).

Table 2. Important enzymes and genes involved in metabolism of isoleucine, leucine and valine. FC-fold change calculated by available microarray data representing *H. schachtii*-induced syncytia at 5 and 15 dpi (Szakasits *et al.*, 2009).

EC	Locus	Symbol	Enzyme	FC
4.2.3.1	At4g29840	MTO2	Threonine synthase	3.1
4.3.1.19	At3g10050	OMR1	L-threonine ammonia lyase	2.9
2.2.1.6	At3g48560	ALS	Acetolactate synthase	2.3
1.1.1.86	At3g58610	KARI	Ketol-acid reductoisomerase	2.2
4.2.1.9	At3g23940	DHAD	Dihydroxyacid dehydratase	3.9
2.6.1.42	At3g49680	BCAT3	Branched-chain amino acid aminotransferase -3	1.6
2.6.1.42	At5g65780	BCAT5	Branched-chain amino acid aminotransferase -5	1.7

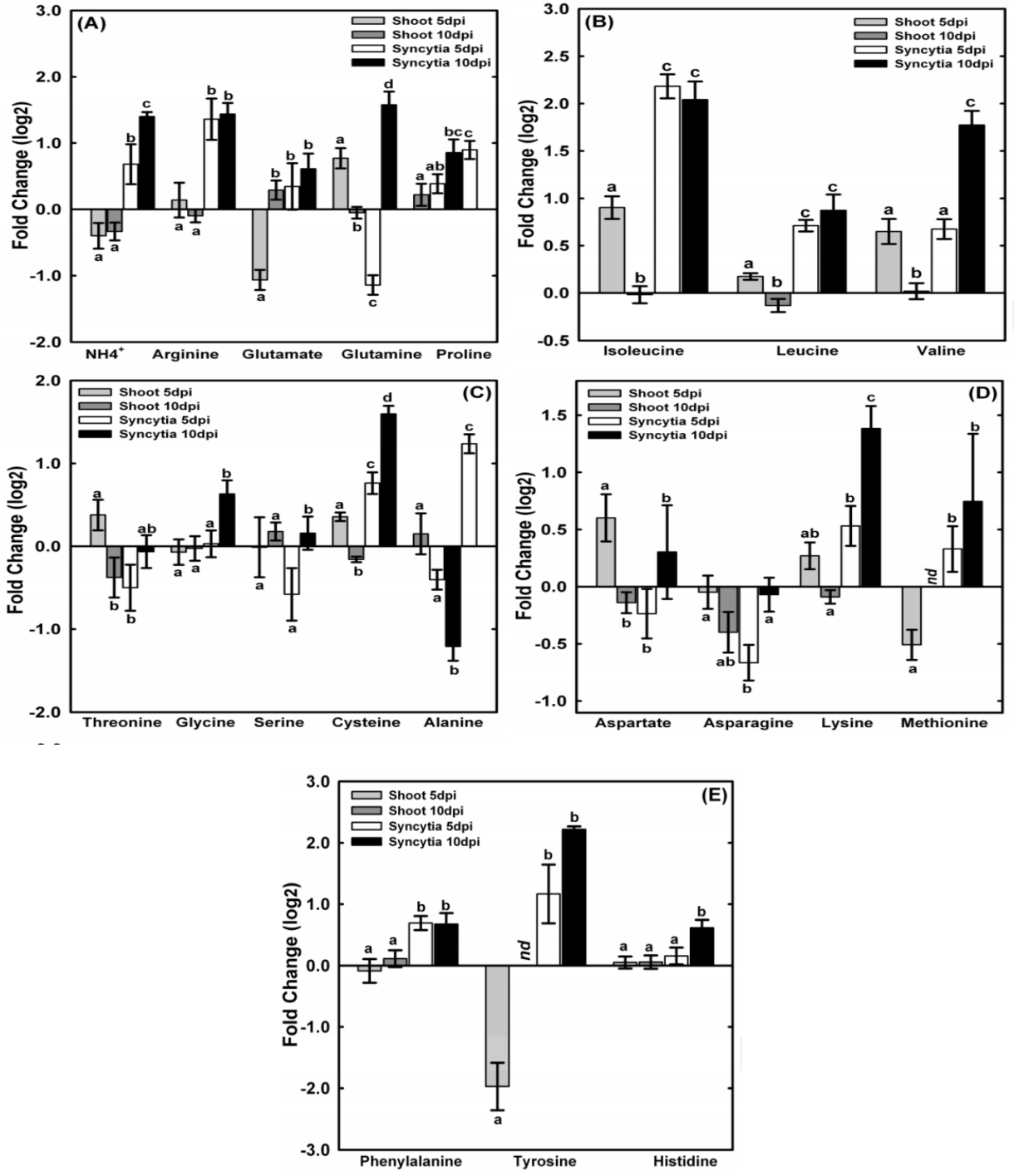

Fig. 2. Fold changes (log2) of studied amino acids and ammonium ions in shoots and syncytia of *A. thaliana* (Col-0) infected with *Heterodera schachtii*. Values are means ± SE, n=5 (biological replicates), different letters indicate significant difference within and between various time points ($p<0.05$, one-way ANOVA, LSD).

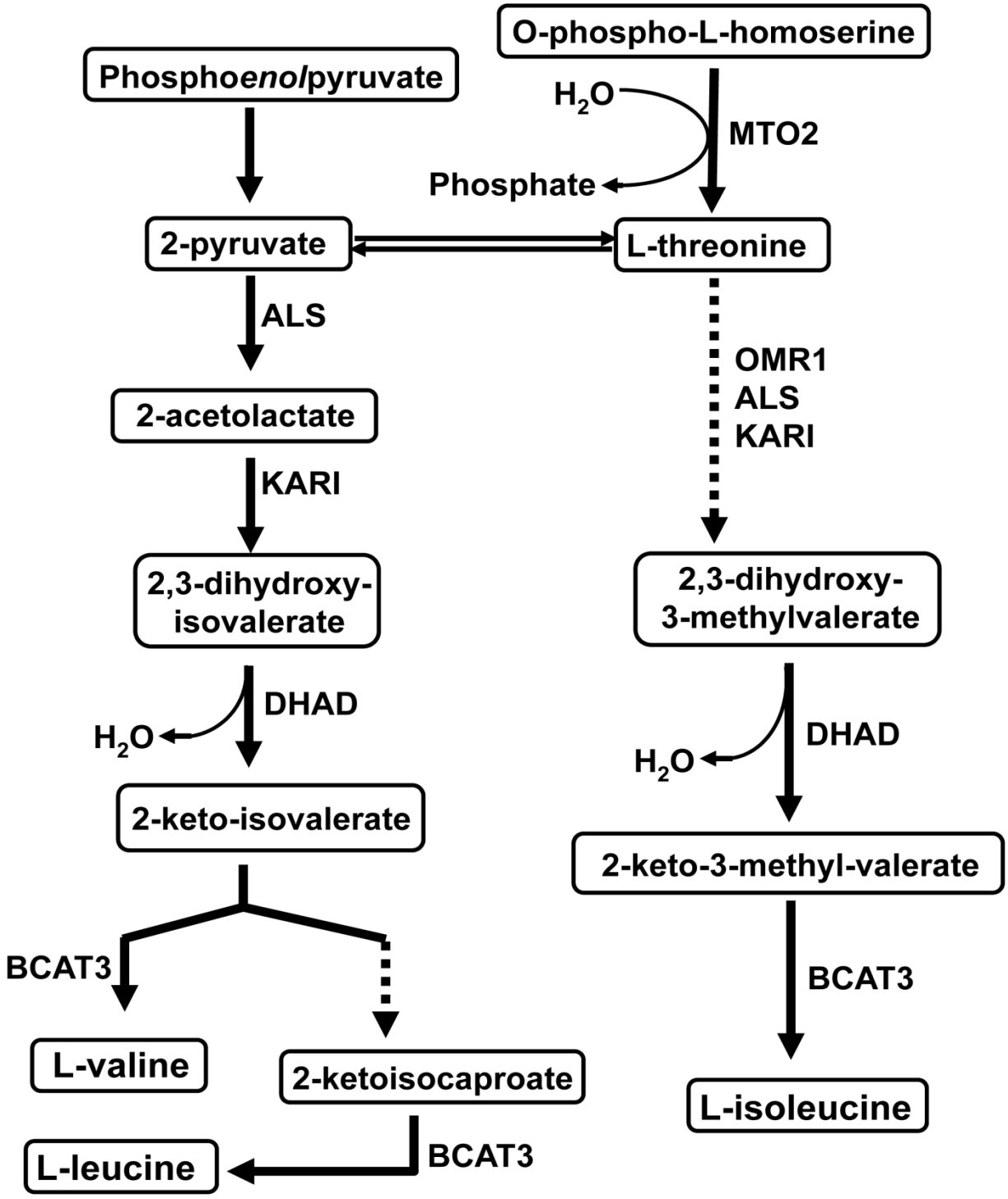

Fig.3. Isoleucine, leucine and valine metabolism in *A. thaliana*. Important enzymes are shown for each reaction. Dotted arrows represent more than one reaction (PMN: https://plantcyc.org/).

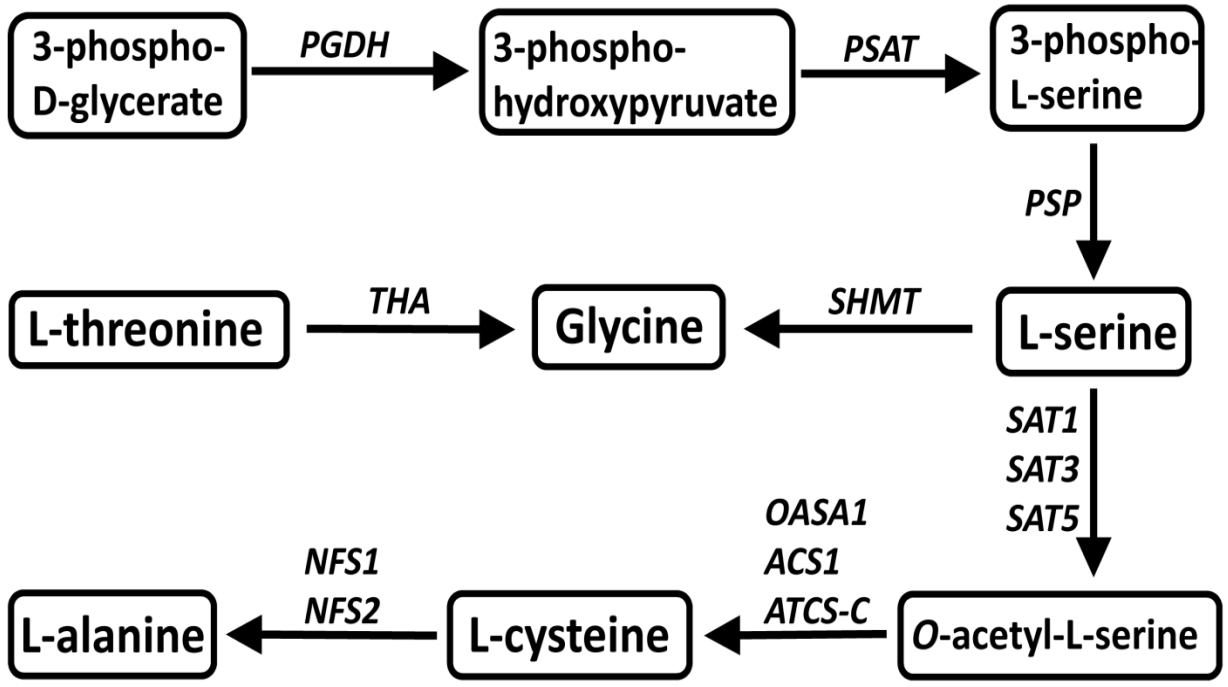

Fig.4. Threonine, glycine, serine, cysteine and alanine metabolism in *A. thaliana*. Important enzymes are shown for each reaction (PMN: https://plantcyc.org/).

Table 3. Important enzymes and relevant genes involved in the metabolism of threonine, glycine, serine, cysteine and alanine. FC- fold change calculated by available microarray data representing *H. schachtii*-induced syncytia at 5 and 15 dpi (Szakasits *et al.*, 2009).

EC	Locus	Symbol	Enzyme	FC
1.1.1.95	AT1G17745	PGDH	Phosphoglycerate dehydrogenase	-0.7
2.6.1.52	AT4G35630	PSAT	Phosphoserine aminotransferase	1.2
3.1.3.3	AT1G18640	PSP	Phosphoserine phosphatase	2.0
2.1.2.1	AT4G37930	SHMT	Serine hydroxymethyltransferase	3.2
4.1.2.48	AT3G04520	THA	Threonine aldolase	-1.7
2.5.1.47	AT4G14880	OASA1	O-acetylserine (thiol)-lyase	0.7
2.5.1.47	AT2G43750	ACS1	Cysteine synthase 1	0.8
2.5.1.47	AT3G59760	ATCS-C	Cysteinsynthase-c	2.9
2.3.1.30	AT1G55920	SAT1	Serine acetyltransferase 1	-3.3
2.3.1.30	AT3G13110	SAT3	Serine acetyltransferase 3	-1.0
2.3.1.30	AT5G56760	SAT5	Serine acetyltransferase 5	0.4
2.8.1.7	AT5G65720	NFS1	Cysteine desulfurase	0.5
4.4.1.16	AT1G08490	NFS2	Selenocysteine lyase	0.4

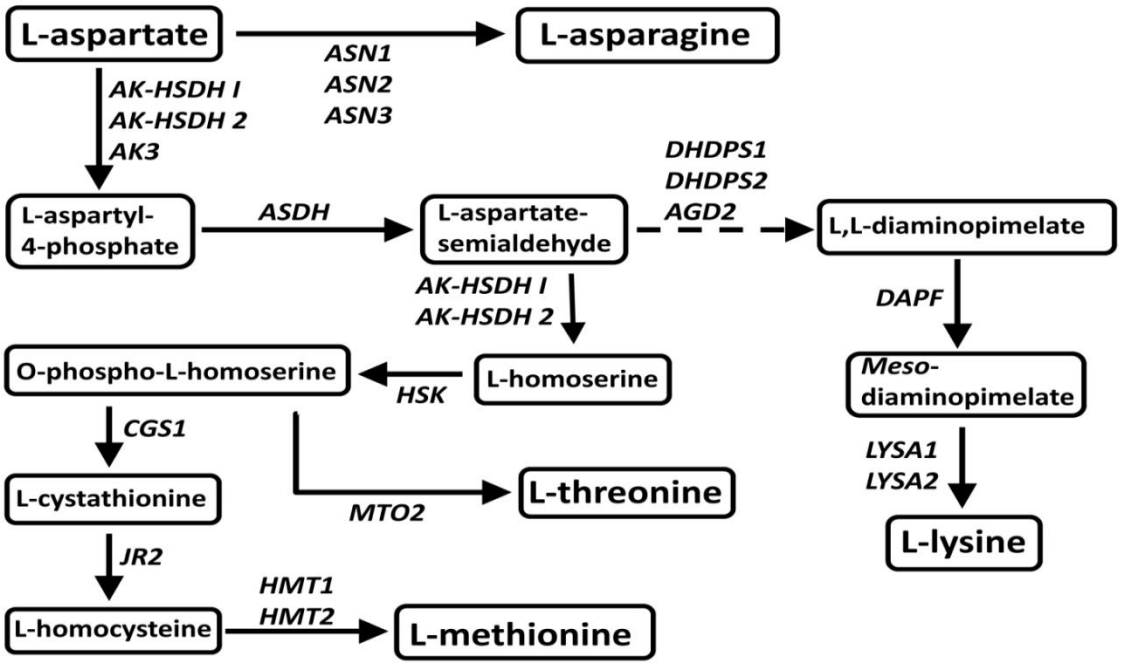

Fig.5. Asparagine, aspartate, threonine, lysine and methionine metabolism in *A. thaliana*. Important enzymes are shown for each reaction. Dotted arrows represent more than one reaction (PMN: https://plantcyc.org/).

Table 4. **Important enzymes and relevant genes involved in the metabolism of aspartate, asparagine, threonine, lysine and methionine. FC- fold change calculated by available microarray data representing *H. schachtii*-induced syncytia at 5 and 15 dpi (Szakasits *et al.*, 2009).*n.d*- not determined.**

EC	Locus	Symbol	Enzyme	FC
6.3.5.4	AT3G47340	ASN1	Asparagine synthetase 1	0.2
6.3.5.4	AT5G65010	ASN2	Asparagine synthetase 2	2.6
6.3.5.4	AT5G10240	ASN3	Asparagine synthetase 3	2.9
2.7.2.4	AT1G31230	AK-HSDH I	Aspartate kinase homoserine dehydrogenase I	3.6
2.7.2.4	AT4G19710	AK-HSDH 2	Aspartate kinase homoserine dehydrogenase II	4.6
2.7.2.4	AT3G02020	AK3	Aspartate kinase 3	*n.d*
1.2.1.11	AT1G14810	ASDH	Aspartate semialdehyde dehydrogenase	3.6
2.6.1.83	AT4G33680	AGD2	L,L-diaminopimelate aminotransferase	3.2
4.3.3.7	AT3G60880	DHDPS1	Dihydrodipicolinate synthase 1	2.3
4.3.3.7	AT2G45440	DHDPS2	Dihydrodipicolinate synthase 2	3.0
5.1.1.7	AT3G53580	DAPF	Diaminopimelate epimerase	1.4
4.1.1.20	AT3G14390	LYSA1	Diaminopimelate decarboxylase 1	*n.d*
4.1.1.20	AT5G11880	LYSA2	Diaminopimelate decarboxylase 2	*n.d*
2.7.1.39	AT2G17265	HSK	Homoserine kinase	1.1
4.2.3.1	AT4G29840	MTO2	Threonine synthase	3.1
2.5.1.48	AT3G01120	CGS1	Cystathionine gamma-synthetase 1	0.6
4.4.1.35	AT4G23600	JR2	Cystathionine beta-lyase	*n.d*
2.1.1.10	AT3G25900	HMT1	Homocysteine S-methyltransferase 1	0.5
2.1.1.10	AT3G63250	HMT2	Homocysteine S-methyltransferase 2	2.2

enzyme in phenylalanine, tyrosine and histidine biosynthesis are shown in Table 5. Histidine is synthesized from the products of phosphoribosyl-α-1-diphosphate (PRPP) biosynthesis (Fig. 6). All three amino acids were increased in syncytia. Shoots did not show any changes in levels of phenylalanine and histidine. Only tyrosine shows a significant decrease in shoots at 5dpi (Fig. 2E).

Discussion

Exploitation of infected plant tissue by the pathogens is very demanding not only due to substantial withdrawal of nutrients but also due to significant amounts of energy and resources deployed for defense responses (Bolton, 2009). Among the others, the amino acid biosynthesis is often significantly affected, which was observed in different plant-microbe interactions. This is not surprising as many plant pathogens fully depend on essential amino acids and organic nitrogen from their hosts (Desbrosses *et al.*, 2005; Depuydt *et al.*, 2009; Hofmann *et al.*, 2010a; Anwar *et al.*, 2015; 2016). Present analysis was performed to unravel potential systemic and local changes in amino acid metabolism in *A. thaliana* during *H. schachtii* infection. The obtained results show that nematode parasitism triggers significant changes in infected tissues in all studied amino acid groups.

First amino acid group that was analyzed is tightly connected to arginine metabolism. In *A. thaliana*, arginine is produced from citrulline, and then further hydrolyzed into urea and ornithine in many reactions (Tischner *et al.*, 2007). It plays central role in biotic and abiotic stress as well as serves as storage and transport molecules in plants (Walters, 2003; Kuznetsov *et al.*, 2006; Micallef *et al.*, 2009). Arginine is a precursor for many important metabolites like glutamate, γ-aminobutyric acid, nitric oxide (NO) and polyamines (Wu & Morris, 1998). Arginine serves as precursor for the synthesis of nitric oxide by NO-synthase (NOS), an important molecule in plant defense and developmental responses (Delledonne *et al.*, 1998; Flores *et al.*, 2008). Nematode-triggered up-regulation of arginine pathway is already well documented. As shown recently, arginine levels are increased in *A. thaliana* roots infected by *H. schachtii* (Anwar *et al.*, 2015). Moreover, arginine has been found to be essential diet component of the free-living nematode *Caenorhabditis briggsae* (Vanfleteren, 1973). Arginine was also found in crushed nematode *Heterodera glycines* (Aist & Riggs, 1969).

Proline is produced in *A. thaliana* from ornithine through several reactions, with the δ-pyrroline-5-carboxylate reductase (P5CR) catalyzing the final reaction (Kenklies *et al.*, 1999). However, proline can also be produced from glutamate (Funck *et al.*, 2008). Proline acts as an important osmolyte in plants accumulating during drought and salt stress (Delauney & Verma, 1993; Verbruggen & Hermans, 2008). Further, proline is associated with the metabolism of free radicals (Smirnoff & Cumbes, 1989; Saradhi *et al.*, 1995; Siripornadulsil *et al.*, 2002), which are produced as a result of incompatible nematode infections as well as during many other plant-pathogen interactions (Stoian *et al.*, 1996; Muckenschnabel *et al.*, 2002).

Proline has a well investigated role in stabilization of membranes and sub-cellular components (Delauney & Verma, 1993), which explain its increase in nematode-induced syncytia. It can also assist the syncytia to cope with high osmotic pressure (Böckenhoff, 1995). The ammonia (NH_3) is a gas molecule which protonates rapidly to form the NH_4^+ ions (Kleiner, 1981). Ammonium ions are major source of nitrogen for plants. Plants assimilate these ions and produce glutamine with the help of enzyme glutamine synthase (GS). Glutamine is a central amino acid for the synthesis of a number of amino acids (Tabuchi *et al.*, 2007). Second group studied is small group of branched chain amino acids (BCAAs). These amino acids are synthesized in plants from pyruvate, 2-oxobutanoate and acetyl-CoA (Binder, 2010).

Table 5. Important enzymes and relevant genes involved in the metabolism of phenylalanine, tyrosine and histidine. FC- fold change calculated by available microarray data representing *H. schachtii*-induced syncytia at 5 and 15 dpi (Szakasits *et al.*, 2009).*n.d*- not determined.

EC	Locus	Symbol	Enzyme	FC
5.4.99.5	AT3G29200	CM1	Chorismate mutase 1	-0.9
5.4.99.5	AT5G10870	CM2	Chorismate mutase 2	-0.2
5.4.99.5	AT1G69370	CM3	Chorismate mutase 3	2.7
2.6.1.1	AT2G22250	AAT	Aspartate aminotransferase	-0.3
4.2.1.51	AT2G27820	ADT3	Arogenate dehydratase 3	-0.5
4.2.1.91	AT3G44720	ADT4	Arogenate dehydratase 4	0.8
4.2.1.91	AT1G08250	ADT6	Arogenate dehydratase 6	-0.1
1.3.1.78	AT5G34930	TYRAAT1	Arogenate dehydrogenase 1	*n.d*
1.3.1.78	AT1G15710	TYRAAT2	Arogenate dehydrogenase 2	2.8
2.4.2.17	AT1G09795	ATP-PRT2	ATP-phosphoribosyl transferase	3.9
3.6.1.31	AT1G31860	HISN2	Phosphoribosyl-ATP pyrophosphohydrolase	1.6
5.3.1.16	AT2G36230	HISN3	Phosphoribosyl-formimino-5-aminoimidazole-4-carboxamide ribonucleotide isomerase	-0.9
2.4.2.- 4.1.3	AT4G26900	HISN4	Imidazoleglycerol phosphate synthase	3.3
4.2.1.19	AT3G22425	HISN5A	Imidazoleglycerol-phosphate dehydratase	1.8
2.6.1.9	AT5G10330	HISN6A	Histidinol-phosphate transaminase	*n.d*
3.1.3.25	AT4G39120	HISN7	Inositol-phosphate phosphatase	0.4
1.1.1.23	AT5G63890	HISN8	Histidinol dehydrogenase	2.0

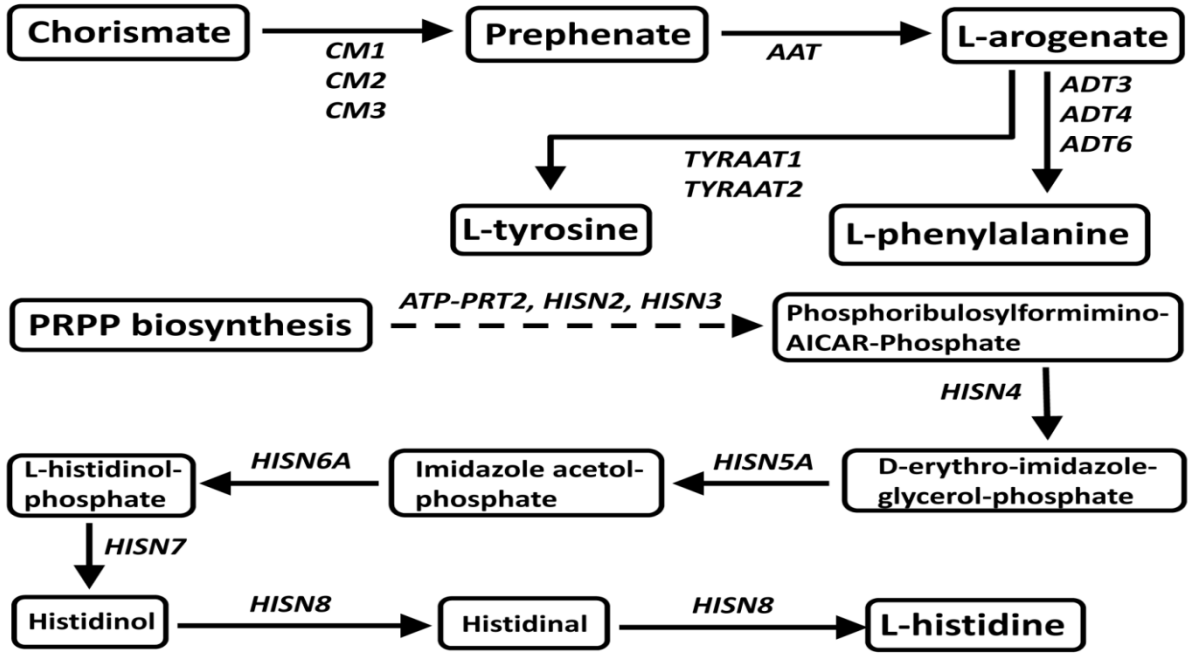

Fig. 6. Phenylalanine, tyrosine and histidine metabolism in *A. thaliana*. Important enzymes are shown for each reaction. Dotted arrows represent more than one reaction (PMN: https://plantcyc.org/).

Animals cannot synthesize these amino acids thus classified as essential amino acids (Binder *et al.*, 2007; Binder, 2010) and they fully rely upon their diet to get them (Akman Gunduz & Douglas, 2009). BCAAs serve as precursors for a number of plant secondary metabolites (Lea & Ireland, 1999). Metabolism of these amino acids has direct linkage to plant-microbe interactions. Development of nitrogen fixing bacteria in many legume species depends on BCAAs supply from plant and is regulated by the plant (Prell *et al.*, 2009). BCAAs are required for the optimal growth of nutritionally demanding lactic acid bacteria (Garault *et al.*, 2000). Population growth of free-living nematode *Caenorhabditis briggsae* depends on the supply of BCAAs (Vanfleteren, 1973). During egg production of *H. schachtii* these amino acids are found to be increased in syncytia (Krauthausen & Wyss, 1982). Increase in syncytial valine, isoleucine and leucine contents is recorded in many studies during plant-nematode interactions (Betka *et al.*, 1991; Hedin & Creech, 1998; Hofmann *et al.*, 2010a). Previous studies showed the accumulation of isoleucine, leucine and valine in *H. schachtii* induced syncytia. Deficiency of BCAAs interferes with the normal growth and development of nematodes. These amino acids are found to be necessary for nematode growth and development but not for syncytium establishment (Anwar *et al.*, 2016).

Third amino acid group studied here consists of threonine, glycine, serine, cysteine and alanine. Threonine serves as precursor for glycine and enzyme responsible for this conversion is threonine aldolase. Glycine is synthesized from serine by the action of serine hydroxymethyl transferase (Kastanos *et al.*, 1997). Cysteine is produced from serine via O-acetyl-L-serine by the enzyme group called O-acetylserine (thiol) lyases (Droux *et al.*, 1998; Jost *et al.*, 2000; Berkowitz *et al.*, 2002). Alanine has many precursors like pyruvate, propanoate and cysteine. Cysteine is converted into alanine in a single step reaction driven by NFS1 and NFS2 (Leon *et al.*, 2002; Pilon-Smits *et al.*, 2002). Threonine serves as precursor for branched chain amino acids so its depletion during plant-nematode interaction is obvious, even though major enzyme threonine synthase is up-regulated at later stages of plant-nematode interaction (Anwar *et al.*, 2016). Serine also serves as metabolic signal during various energy consuming reactions in plants (Timm *et al.*, 2013). Serine hydroxymethyl transferase was found to play an important role during biotic and abiotic stress responses (Moreno *et al.*, 2005). Glycine also serves as signal for many processes in *A. thaliana* like ligand-mediated gating of calcium and regulation of hypocotyl elongation (Dubos *et al.*, 2003). Nematodes are known to have a high glycine-rich protein demand that they withdraw from their feeding sites (Potenza *et al.*, 2001; Karimi *et al.*, 2002).

Forth group comprises aspartate, asparagine, lysine and methionine. Aspartate and asparagine serve as precursors for many amino acids. Conversion of aspartate to asparagine is controlled by a group of three asparagine synthases: ASN1, ASN2 & ASN3 (Lam *et al.*, 1998; Miesak & Coruzzi, 2002; de la Torre *et al.*, 2006). Depletion of asparagine in infected tissue can be a result of its conversion into aspartate by the action of enzyme asparaginase (Bruneau *et al.*, 2006). Aspartate is an important substrate in the synthesis of threonine and methionine. Threonine is used for the production of BCAAs which are essential for nematode growth and development (Anwar *et al.*, 2016). Increase of lysine in syncytia has been studied recently. A lysine and histidine specific amino acid transporter was found to be active in nematode-induced syncytia (Elashry *et al.*, 2013). Enrichment of methionine in *H. schachtii* induced syncytia can be explained by the up-regulation of methionine-related transcripts in the feeding site (Szakasits *et al.*, 2009).

Fifth group of amino acids is a diverse group comprising tyrosine and phenylalanine being part of the same pathway. Histidine is produced from the products of phosphoribosyl-α-1-

diphosphate (PRPP) biosynthesis in a separate pathway. Phenylalanine is produced from chorismate via arogenate by the action of chorismate mutase (Eberhard *et al.*, 1996; Mobley *et al.*, 1999) and arogenate dehydratase (Warpeha *et al.*, 2006; Cho *et al.*, 2007). Tyrosine is produced from arogenate by the enzymes arogenate dehydrogenase1 &2 (Rippert & Matringe, 2002). Phenylalanine and tyrosine are important amino acids of shikimic acid based pathway (Cho *et al*., 2007).

Significant increase has been found in phenylalanine and tyrosine levels in syncytia as compared to the shoots of infected plants. Nematodes are known to induce shikimic acid-dependent pathways. Chorismate mutase which is normally a part of shikimic acid pathway is known to be secreted by potato cyst nematode. Sequence analysis of chorismate mutase related gene reveals its involvement in host-parasite interactions (Lambert *et al.*, 1999; Jones *et al.*, 2003).

In conclusion, we present a detailed amino acid analysis based on Ultra Performance Liquid Chromatography (UPLC^{TM}). This technique has an advantage over GC–MS analysis as far as quantification of charged, polar and basic amino acids is concerned.

Nematodes are known to manipulate the plant metabolic network to induce sophisticated feeding sites that provide high amounts of nutrients for these parasites. This study has been represented the changes in 19 amino acid levels as a result of *H. schachtii* infection.

Study of individual metabolic pathways of amino acids and comparison of expression data of related genes provide insights related to their spatial and temporal accumulation. Comparison of metabolite levels in specific plant tissue helps to understand the role of their metabolic pathways during nematode parasitism. Complete dissection of these pathways may provide new insights into plant–nematode interactions.

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