Branched chain amino acids (BCAA) are involved in *Heterodera schachtii* development on *Arabidopsis thaliana*

Shahbaz Anwar[1], Erich Inselsbacher[2] and Julia Hofmann[1]

[1]Department of Crop Sciences, University of Natural Resources and Life Sciences, Konrad Lorenzstrasse 24, 3430, Tulln, Austria
[2]Department of Geography and Regional Research, University of Vienna, Universitätsstrasse 7, A-1010, Vienna, Austria
e-mail: shahbazanwarch@hotmail.com

Accepted for publication 16 May 2016

Summary. The plant-parasitic cyst nematode *Heterodera schachtii* is able to infect *Arabidopsis* plants and transform root cells in specialised feeding structures called syncytia. These syncytia have strong metabolic sink character and serve as sole nutrient source for nematodes throughout their life. Unlike plants, animals cannot synthesise branched chain amino acids (BCAA) and depend on their diet for them. Thus, being essential amino acids, this study aimed at determining their role in nematode development on *Arabidopsis*. We found that valine, leucine and isoleucine, among several other amino acids, were enriched in syncytia. Genes coding for threonine synthase and dihydroxyacid dehydratase, common enzymes in BCAA synthesis, were found strongly expressed in syncytia. We use two independent T-DNA insertion mutant alleles of gene coding for dihydroxyacid dehydratase to test its influence on nematode development. Our results showed significant decrease in number of females on mutant plants. Further, the size of cysts and hatching rate of females on mutant plants were also decreased significantly. Mutation did not affect the size of syncytia, revealing that BCAA are important for nematode development but not for syncytium establishment.

Key words: cyst nematode, dihydroxyacid dehydratase, isoleucine, interaction, leucine, syncytia, threonine synthase, valine.

Plant-parasitic nematodes are devastating pathogens that infect most cultivated plant species. Sedentary plant-parasitic nematodes are obligate biotrophs (Sobczak & Golinowski, 2011) and are capable of causing big loss in food and fibre crops in terms of yield and quality (Moens & Perry, 2009). *Heterodera schachtii* induce the development of specialised feeding structures in the roots of host plants (Jones & Northcote, 1972; Golinowski *et al.*, 1996). Such nematodes meet their nutritional requirements solely from modified, but living, root cells of their host plants (Betka *et al.*, 1991; Grundler *et al.*, 1991). These obligate parasites start their life cycle as non-feeding, mobile, infective, second-stage juveniles (J2) in soil, which are able to locate and then penetrate into host roots. J2 migrate inside the root to reach the vascular cylinder, where they initiate the formation of specialised feeding structures called syncytia (Jones & Northcote, 1972; Sijmons *et al.*, 1994; Golinowski *et al.*, 1996; Hussey & Grundler, 1998). To support their sedentary life cycle, nematodes inject parasitism protein secretions into initial feeding cells. The parasitism proteins appear to provide many of the stimuli that result in a dramatically altered host gene expression as observed in nematode-infected plant roots (Gheysen & Fenoll, 2002; Puthoff *et al.*, 2003; Jammes *et al.*, 2005; Ithal *et al.*, 2007a, b). Secreted nematode parasitism proteins, directly or indirectly, modulate the host signalling and metabolic pathways (Doyle & Lambert, 2003; Favery *et al.*, 2004; Hofmann *et al.*, 2010; Anwar *et al.*, 2015). In detail, these secretions provoke several morphological and physiological changes including nuclei enlargements, proliferation of mitochondria and plastids, cytoplasm condensation and disintegration of the central vacuole into several small vacuoles (Golinowski *et al.*, 1996). During the development of syncytia, outer walls thicken to withstand increasing turgor pressure (Golinowski *et al.*, 1996; Grundler *et al.*, 1998). As obligate parasites, the nematodes rely on plant nutrient supply during their entire life span. After 2 weeks, nematodes have developed to the adult stage and males become vermiform and mobile, leaving the roots to search for female mating partners. After

fertilisation, females produce up to several hundred eggs and change into cysts as a survival stage (Wyss, 1992).

Valine, leucine and isoleucine form the small group of branched chain amino acids (BCAA). They are classified by their small branched hydrocarbon residues responsible for the aliphatic character of these molecules. BCAA are *de novo* synthesised by plants from pyruvate, 2-oxobutanoate and acetyl-CoA (Binder, 2010), but cannot be synthesised by animals and are thus classified as essential amino acids (Binder *et al.*, 2007; Binder, 2010). Therefore, animals have to take up these amino acids with their diets (Akman Gunduz & Douglas, 2009). Metabolic pathways involving BCAA in plants are well investigated because they provide precursors for a number of plant secondary metabolites (Lea & Ireland, 1999). Supply of BCAA from plant is important in many plant microbe interactions. For instance, development of bacteroides and symbiotic nitrogen fixation in many legume species (Prell *et al.*, 2009). Lactic acid bacteria are nutritionally demanding bacteria that need BCAA along with other substances for optimal growth (Garault *et al.*, 2000). Previously it has been shown that leucine, isoleucine and valine were required for population growth of the free-living nematode *Caenorhabditis briggsae* (Vanfleteren, 1973). The nematode populations did not grow when any of the three BCAA was deficient in the medium (Balasubramanian & Myers, 1971; Jackson, 1973; Perelman & Lu, 2000). In another study, analyses of extracts from the crushed juveniles of *Heterodera glycines* revealed the presence of most BCAA (Aist & Riggs, 1969). Isoleucine, leucine and valine were found to increase in feeding cells during egg production of *H. schachtii* (Krauthausen & Wyss, 1982).

However, the role of BCAA in the development of plant parasitic nematodes is still not well investigated. There is a significant lack of functional data on BCAA family members during plant nematode interactions. Therefore, in this study we aimed: *i*) to determine the regulation of genes involved in the metabolism of BCAA in developing syncytia; and *ii*) to investigate how these amino acids influence cyst nematode growth and development.

MATERIALS AND METHODS

Plant and nematode cultivation. *Arabidopsis thaliana* mutant seeds were grown in pots in at 16 h light/8 h dark and 25°C for DNA extraction. Plant-nematode interactions were studied in sterile culture on a modified Knops medium (Sijmons *et al.*, 1991) at 16 h light/8 h dark and 25°C. Twelve-day-old seedlings were inoculated with 50-60 freshly hatched mobile second-stage juveniles (J2) each, obtained from a sterile stock culture (Sijmons *et al.*, 1991).

Identification of T-DNA insertion mutants. Independent T-DNA insertion mutants of *Arabidopsis thaliana* (Col-0) for the gene At3g23940 (SALK_075098 and SALK_130404) were obtained from NASC (http://www.arabidopsis.info/) (Table 1). Leaf tissue was harvested from 30-day old plants. DNA was extracted from each sample using a rapid extraction protocol (Edwards *et al.*, 1991). Each mutant was tested for homozygousity by PCR using T-DNA specific and border primers (Table 2).

Table 1. Detail of *Arabidopsis thaliana* T-DNA mutants used for infection essays with *Heterodera schachtii*.

Gene ID	Enzyme	Mutant ID
AT3G23940	dihydroxyacid dehydratase	SALK_130404
AT3G23940	dihydroxyacid dehydratase	SALK_075098

Sample collection, RNA isolation and qRT-PCR. Syncytia from *H. schachtii* infected *Arabidopsis thaliana* (Col-0) and root samples omitting root tips from uninfected control plants were dissected and shock-frozen in liquid nitrogen. Sampling was performed at 5, 10 and 15 days post inoculation (dpi). Samples were collected for three independent biological replicates. Harvesting was always performed at the middle of the day to rule out diurnal effects. Total RNA was extracted using the RNase Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, including a DNase I digest (Qiagen, Hilden, Germany). Quality and quantity of the RNA was analysed by Agilent 2100 bioanalyser (Agilent Technologies, Palo Alto, CA, USA). Reverse transcription of RNA into cDNA was done using random primers [oligo(dN)6] and the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative realtime PCR was done by using an ABI PRISM 7300 Sequence Detector (Applied BioSystems). 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 1min. Primer sequences are listed in Table 2. Internal references were used according to Hofmann & Grundler (2007). Data analysis was carried out using the Sequence Detection Software SDS V2.0 (Applied BioSystems).

Table 2. Primer sequences used in this study to confirm the T-DNA insertion in the respective mutants and for qRT-PCR of respective genes.

Primer name	Primer sequence	
SALK_130404	Forward	CTGTTAAGCCTTCCCATAGC
	Reverse	ATATCATGTGGTTCGGTTTGG
SALK_075098	Forward	CTGTTAAGCCTTCCCATAGCC
	Reverse	ATATCATGTGGTTCGGTTTGG
AT4G29840	Forward	GAGATGAGGCTCGTCGTAATCG
	Reverse	GCCTCCATATCGTGTTCGACATC
AT3G23940	Forward	GTGGAACCATTAAGCCTGGCC
	Reverse	CATTGTATTCGCTGTGTACATGC

Amino acid contents. Syncytia induced by *H. schachtii* infected (10 dpi) *Arabidopsis thaliana* (Col-0) plants and shoots from the same specimen were harvested and shock frozen in liquid nitrogen. Roots and shoots from non-infected plants were sampled at the same time as control material. Samples were collected from five independent biological replicates of each plant line. 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 ground by mixer mill with a stainless steel ball in each tube at maximum frequency for 1 min. One ml of solvent mix was added to each tube and the process was repeated for 3 min. After removing the steel ball, samples were centrifuged for 10 min at 16,000 *g*. The supernatant (aqueous phase) was transferred into microvials and evaporated to dryness with a Speed Vac (Savant Speedvac SPD 111; Thermo Fisher Scientific Inc., Waltham, MA, USA). Amino acid analysis was done by reversed-phase liquid chromatography using a Waters Ultra High Performance (UPLC™) system with a Waters Tunable UV (TUV) detector (Inselsbacher *et al.*, 2011).

Cyst and syncytia size measurement. Size of syncytia was measured at 15 dpi. In detail, six *H. schachtii* induced syncytia each associated with a single female were selected randomly per culture plate and three plates were used from each line resulting in 18 biologically independent replicates. Cysts were collected from 3-month old plates used for infection test assays. Ten cysts were collected from each plate and three plates were used resulting in 30 biologically independent replicates. Photographs of syncytia and cysts were taken with Axiovert 200M Inverted Microscope (Carl Zeiss Light Microscopy) using a Zeiss Axiocam digital camera. The area of longitudinal view was measured using AxioVision LE Rel. 4.3 software.

Hatching rate calculation. Ten *Heterodera schachtii* cysts were collected randomly from each *Arabidopsis thaliana* wild type and mutant culture plate. Cysts were placed in 3mM $ZnCL_2$ solution. Hatched juveniles were counted in 0.7% gelrite solution. Three replicates were taken from each batch and the experiment was repeated three times resulting in three technical and three biological replicates.

Statistical analysis. Statistical variations of the amino acid results were tested using ANOVA and LSD ($P = 0.5$) using the statistical software package STATGRAPHICS Plus Version 5.0. Pearson's correlation analyses were performed using SPSS 15.0 (http://www.spss.com/). Infection test data and syncytium size were analysed by t-Test.

RESULTS

Transcriptional profiling of genes involved in BCAA metabolism. Expression of important genes coding for the involved enzymes was tested by qRT-PCR during nematode development (Table 3). Studied genes were selected on the basis of a previous transcriptome analysis (Szakasits *et al.*, 2009). Syncytia and non-infected control root sections were sampled at 5, 10 and 15 days post inoculation (dpi). Threonine is an important precursor in the valine super pathway (Fig. 1), and one gene coding for the threonine synthase (EC 4.2.3.1)

Table 3. Relative transcript levels (Fold Change – FC) of genes coding for enzymes involved in the BCAA metabolic pathway were studied by qRT-PCR at 5, 10 and 15 dpi. Values are means ± SE, n = 3 (biological replicates), significant differences are indicated by asterisks (t-test, $P < 0.05$).

Gene	EC	FC qRT-PCR			Enzyme
		5dpi	10dpi	15dpi	
AT4G29840	4.2.3.1	1.38±0.29	1.78*±0.14	1.77*±0.08	Threonine synthase
AT3G48560	2.2.1.6	2.25*±0.31	2.22*±0.32	2.64*±0.16	Acetolactate synthase
AT3G58610	1.1.1.86	2.35*±0.10	2.81*±0.16	3.60*±0.08	Ketolacid reductoisomerase
AT3G23940	4.2.1.9	4.36*±0.12	8.27*±1.94	8.02*±1.05	Dihydroxyacid dehydratase
AT3G49680	2.6.1.42	1.81*±0.20	1.87*±0.10	2.33*±0.25	Branched-chain amino acid aminotransferase -3
AT5G65780	2.6.1.42	1.34±0.14	1.36±0.10	1.58*±0.13	Branched-chain amino acid aminotransferase -5

was significantly up-regulated at all three time points tested. Valine, leucine and isoleucine are synthesised in two parallel pathways (Fig. 1), each with a set of four identical enzymes. Most of the genes were significantly up-regulated as determined by qRT-PCR. One gene (At3g23940) coding for the dihydroxyacid dehydratase (EC 4.2.1.9) was highly up-regulated in all three time points tested, with highest expression at 15 dpi (Table 3).

Levels of BCAA are increased in *H. schachtii* induced syncytia. Following on from the results obtained from the transcript analyses, amino acid levels of shoots, roots and syncytia of infected and non-infected Col-0 plants at 5 and 10 dpi were measured by UPLC™. Comparing all analysed tissues, threonine levels were significantly increased in roots at 5 dpi and in shoots at 10 dpi (Fig. 2A). Isoleucine is produced from threonine by a number of intermediate steps (Fig. 1). The levels of isoleucine at 5 dpi were significantly increased in the infected tissues compared to the control. In particular, syncytia showed four times higher isoleucine levels than non-infected control roots (Fig. 2B). At 10 dpi there was no significant difference in isoleucine levels between shoots. Isoleucine levels in syncytia were still highly enriched compared to control roots at 10 dpi. The pathways of valine and leucine are tightly connected. Both amino acids were significantly enriched in syncytia at both analysed time points (Fig. 2C & D). Accordingly, valine levels were significantly enriched at 5dpi in all infected tissue, as observed for isoleucine. At 10 dpi there was no difference between shoots again, but syncytia show three times higher valine levels compared to control roots (Fig. 2C). Finally, leucine levels, with its biosynthesis closely related to that of valine (Fig. 1), were not significantly different between the analysed shoot samples at 5 dpi and 10 dpi. However, syncytia show significantly increased levels of leucine compared to non-infected roots at both studied time points (Fig. 2D).

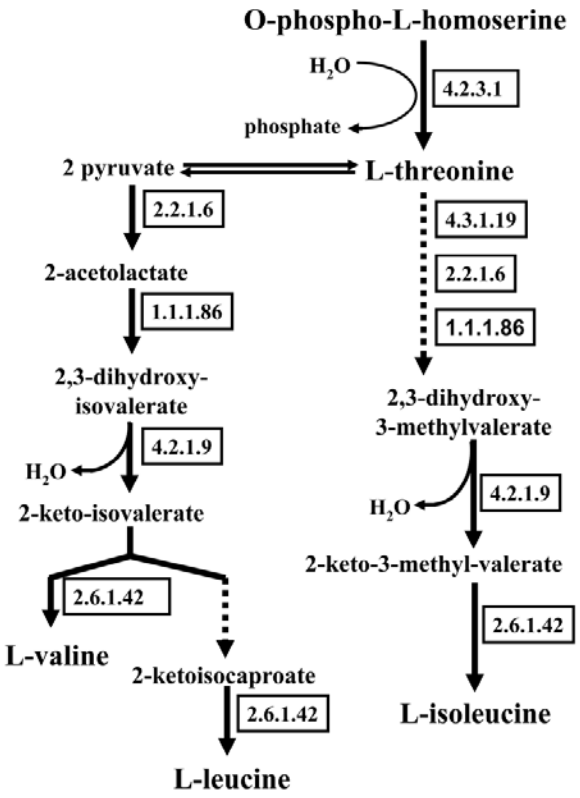

Fig. 1. Branched chain amino acids (BCAA) metabolism in *Arabidopsis thaliana*. Boxes show the respective enzyme. Dotted arrow represents more than one reaction.

Pearson correlation analysis of individual BCAA levels. We hypothesised that individual amino acids would behave in a similar way in different tissues of infected and control plants, which would be reflected in correlating patterns. Pearson correlation analysis was used to analyse how different amino acids correlate in different tissues. In control shoots, significant correlations were found among isoleucine, leucine, threonine and valine (Table 4A). Infected shoots show almost the same trend where leucine, threonine and valine show significant correlation (Table 4B). In roots significant correlations were found between leucine and valine and in syncytia threonine and valine (Table 4C & D).

Nematode development is affected by At3g23940 mutation. We studied the importance of BCAA for nematode development by subjecting two independent T-DNA insertion lines (SALK_075098 and SALK_130404) for the gene At3g23940 (EC 4.2.1.9) to infection essays. This gene codes for a dihydroxyacid dehydratase, which is common in the valine, leucine and isoleucine pathway. Mutation in this gene was supposed to affect the synthesis of all the BCAA described above. Mutant lines showed a significant difference in the number of females per cm of root length. The female-to-male ratio was also decreased significantly compared to the wild type plants (Fig. 3A).

Size of syncytia and cysts induced by female nematode was measured in the above analysed mutant lines. Results showed that interfering with the BCAA pathway of host plant affects the normal growth and development of female nematode. Cysts were significantly smaller in both mutant lines (Fig. 3B). Smaller cysts however, do not necessarily mean fewer offspring. Thus, the hatching rates of the cysts obtained in the infection test experiment were calculated. Three-month-old cysts from control and both mutant plant lines were collected. Hatching of the juveniles from the cysts was calculated as described in the material and methods section. We found a significant difference between the control and mutant lines tested (Fig. 3C). Size of syncytia induced by *H. schachtii* in the roots of wild type and T-DNA insertion mutants described above was calculated at 15 dpi. The data however, revealed no significant difference in size comparing syncytia induced in the mutant lines to those in the wild type (Fig. 3D).

Table 4. Pearson's correlation analysis of the relative changes in amino acid contents of infected and control tissue in *Arabidopsis thaliana* (Col-0). Control shoots (A), infected shoot (B), root (C) and syncytia (D). Significant correlations are indicated by asterisks: * (P = 0.05); ** (P = 0.01); ile – isoleucine, leu – leucine, thr – threonine, val – valine.

A	ile	leu	thr	val
ile	1	0.913**	0.757*	0.722*
leu	0.913**	1	0.781**	0.843**
thr	0.757*	0.781**	1	0.855**
val	0.722*	0.843**	0.855**	1

B	ile	leu	thr	val
ile	1	0.911**	0.839**	0.752*
leu	0.911**	1	0.854**	0.692*
thr	0.839**	0.854**	1	0.601
val	0.752*	0.692*	0.601	1

C	ile	leu	thr	val
ile	1	–0.034	–0.271	–0.248
leu	–0.034	1	–0.168	0.842**
thr	–0.271	–0.168	1	–0.193
val	–0.248	0.842**	–0.193	1

D	ile	leu	thr	val
ile	1	0.563	0.101	–0.148
leu	0.563	1	0.092	–0.365
thr	0.101	0.092	1	0.694*
val	–0.148	–0.365	0.694*	1

DISCUSSION

Previous studies on plant-nematode interactions have found an increase in BCAA in nematode induced syncytia (Betka *et al.*, 1991; Hedin & Creech, 1998; Hofmann *et al.*, 2010). However, the role of BCAA for nematode development has not been studied in detail so far. To this end, we studied the role of valine, leucine, isoleucine and threonine in syncytia induced by *H. schachtii* in the roots of *A. thaliana*. Transcript analyses of several genes coding for enzymes of these pathways revealed an up-regulation of most of the genes. Up-regulation of

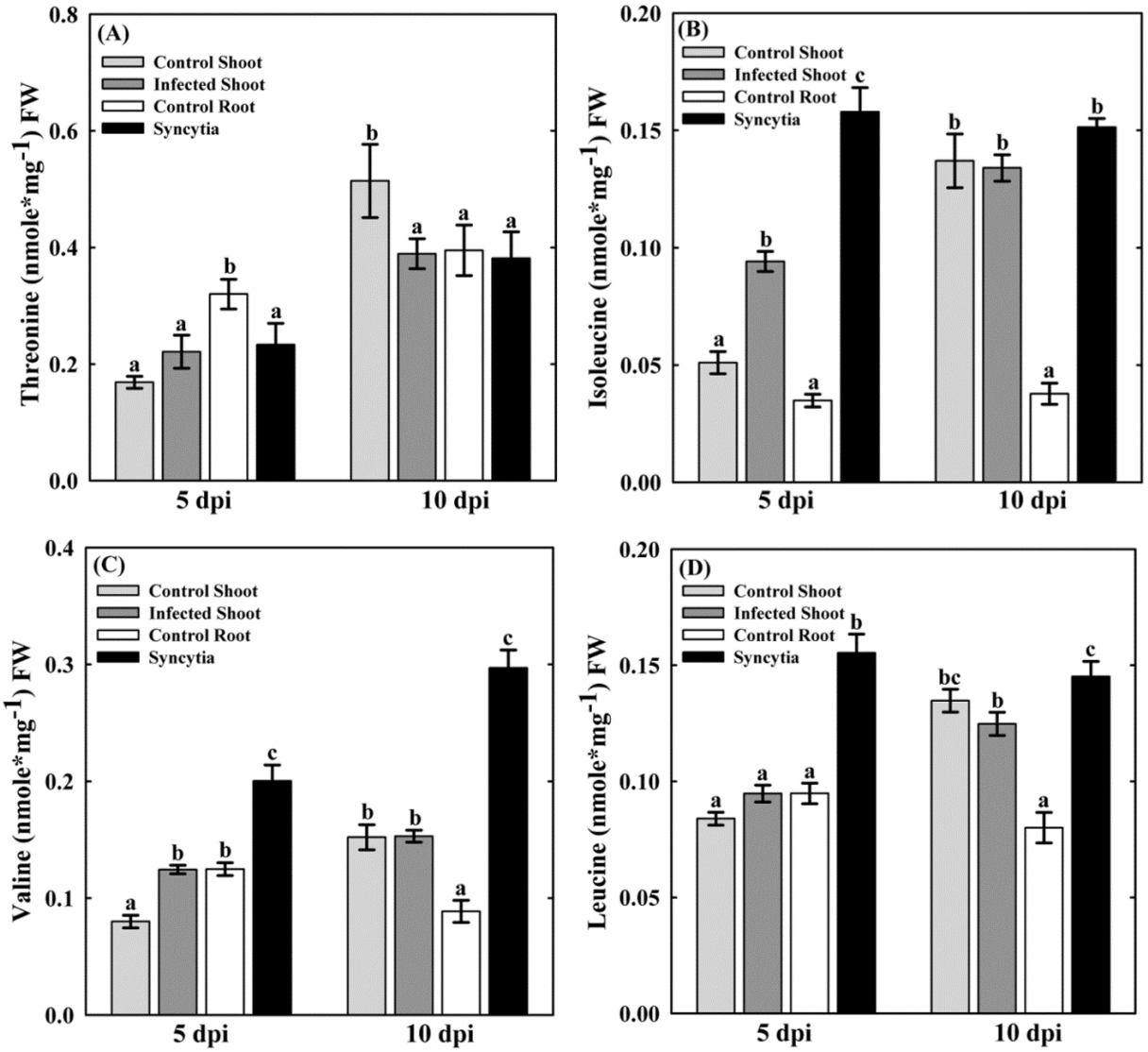

Fig. 2. Levels of BCAA in shoots of control and infected plants, in roots of control plants and in *Heterodera schachtii* induced syncytia of *Arabidopsis thaliana* (Col-0): A: Threonine, B: Isoleucine, C: Valine, D: Leucine at 5 and 10 dpi. Amino acid levels are given in nanomoles per milligram of FW. Values are means ± SE, n = 5 (Biological replicates); different letters indicate significant difference within a time point ($P < 0.05$, one-way ANOVA, LSD).

genes for threonine synthase and dihydroxyacid dehydratase in syncytia indicates a possible shift in metabolism of BCAA towards increased production of valine, leucine and isoleucine. This hypothesis is supported by our finding of higher accumulation of these amino acids in syncytia. Threonine synthase is an important enzyme in threonine biosynthesis. The gene coding for threonine synthase was significantly up-regulated in syncytia in the current studies. This gene competes with cystathionine gamma-synthase for the common substrate O-phosphohomoserine. Cystathionine is involved in the aspartate family pathway, and methionine biosynthesis and catabolism (Curien *et al.*, 1996; Laber *et al.*, 1999; Amir *et al.*, 2002). Isoleucine and valine regulate the activity of homoserine kinase, which catalyses the formation of O-phosphohomoserine from homoserine, and leads to the formation of either threonine or methionine (Baum *et al.*, 1983). In *Arabidopsis* reduced threonine synthase activity results in an increased production of methionine (Bartlem *et al.*, 2000; Zeh *et al.*, 2001). Dihydroxyacid dehydratase is another important enzyme in the BCAA metabolic pathway. All three threonine derived amino acids need this enzyme for their synthesis. This enzyme catalyses the dehydration of 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate to the 2-oxo acids 3-methyl-

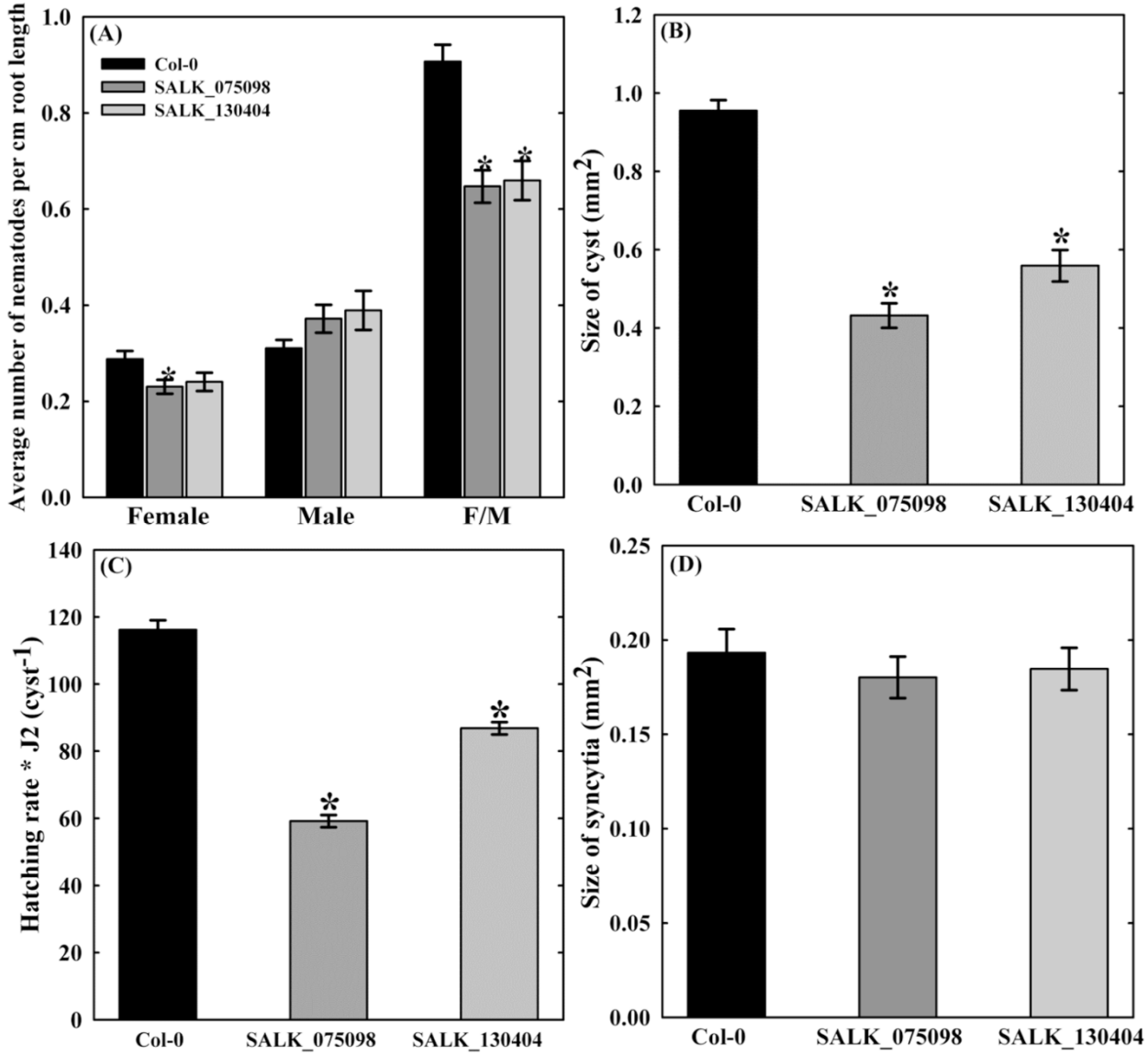

Fig. 3. Nematode infection assays using the *Arabidopsis thaliana* T-DNA insertion lines for gene At3g23940 (SALK_075098 and SALK_130404) compared to the wild type (Col-0). A: average number of nematodes per cm of root length, values are means ± SE, n = 15 (biological replicates), * indicate significant differences from the control (t-test, $P < 0.05$); B: size of cyst was measured 3 months after inoculation, values are means ± SE, n = 30 (biological replicates), * indicate significant differences from the control (t-test, $P < 0.05$); C: hatching rate of second-stage juveniles, values are means ± SE, n = 90 (biological replicates), * indicate significant differences from the control (t-test, $P < 0.05$); D: size of syncytia associated with female nematode juveniles in wild type (Col-0), salk_075098 and salk_130404 T-DNA insertion mutants, values are means ± SE, n = 18 (Biological replicates), (t-test, $P < 0.05$).

2-oxobutanoate or 3-methyl-2-oxopentanoate (Fig. 1). These 2-oxo-acids can now be converted into the corresponding amino acids (Singh & Shaner, 1995; Binder *et al.*, 2007; Binder, 2010). The gene for this enzyme showed a significant up-regulation at all three time points tested, with a gradual increase with the age of syncytia. Since valine, leucine and isoleucine are considered as essential amino acids (Binder *et al.*, 2007; Binder, 2010), nematodes acquire these amino acids from the induced syncytia (Jackson, 1973).

Besides transcript analyses, the levels of BCAA were measured in syncytia induced by *H. schachtii*. Valine, leucine and isoleucine levels were significantly increased in syncytia compared to the roots of control plants. A relative increase in leucine and isoleucine was found under drought stress in *Arabidopsis* foliage (Nambara *et al.*, 1998). Osmotic

stress due to drought, salinity and flooding, is considered the most serious problem for agricultural crop productivity (Ceccarelli & Grando, 1996). As a general response to such stress, all plants accumulate amino acids and other osmolytes in the cytoplasm; in most cases, proline has been found to be the most common amino acid induced by drought stress (Delauney & Verma, 1993; Parida & Das, 2005; Lehmann *et al.*, 2010), but the relative increase of isoleucine (90-fold) and leucine (150-fold) in drought-stressed *Arabidopsis* foliage is greater than the 80-fold foliar proline increase (Nambara *et al.*, 1998). However, in our study shoot BCAA composition did not differ between control and infected plants, indicating that accumulation of these amino acids in syncytia is not a stress response.

The nutritional value of BCAA is well studied in higher animals. Alterations in the composition of free amino acids in the body can result in reduced growth intensity (Ryadchikov *et al.*, 2005). Imbalance of essential amino acids in insects was reported to result in the decrease of life span and fecundity (Grandison *et al.*, 2009). Valine, leucine and isoleucine were considered to impart an important part of diet in animals. In studies on fingerling *Cirrhinus* dietary BCAA valine, leucine and isoleucine were found directly related to growth and weight gain (Ahmed & Khan, 2006). The role of valine was studied extensively in animal metabolism. It was demonstrated that dietary deficiency of valine and isoleucine results in weight loss in animals (Corzo *et al.*, 2009, 2010). In addition, metabolism of BCAA is also important in plant-microbe interactions. Nitrogen fixation in *Rhizobium leguminosarum* by *viciae* bacteroides depends upon the supply of BCAA leucine isoleucine and valine; their transport is essential from plant to bacteria for N_2 fixation (Prell *et al.*, 2009).

In order to find out if potential changes in the biosynthesis of BCAA affect nematode development, an infection assay was performed. In this assay, nematode development in a T-DNA insertion mutant line lacking the expression of the coding for the dihydroxyacid dehydratase was compared to the development in the wild type. The assay revealed that the number of females was significantly decreased in one mutant. Further, a change in the female-to-male ratio in both tested mutant lines was observed. Sex determination was shown to be influenced by environmental conditions such as the availability and quality of available nutrients (Betka *et al.*, 1991; Grundler *et al.*, 1991; Berthou *et al.*, 2003). Thereby, more second-stage juveniles in good nutrient conditions developed females, whilst a higher percentage of those under poor conditions developed males (Trudgill, 1967; Wyss & Grundler, 1992). Studies on *Brassica rapa* showed an influence of amino acids on the development of *H. schachtii*. Many of the studied amino acids play a beneficial role in female development (Krauthausen & Wyss, 1982; Betka *et al.*, 1991; Grundler *et al.*, 1991).

In conclusion, the results of the present work demonstrate the importance of BCAA in nematode development. Mutation in the gene coding for dihydroxyacid dehydratase interfere with the normal growth and development of *H. schachtii* as demonstrated by the decreased number of developing females and thus a decreased female-to-male ratio, reduced cyst size and fewer offspring in the mutant plant lines. Results of the present work give significant information on the role of BCAA for the development of *H. schachtii* in the roots of *Arabidopsis*. However, better understanding of this process can provide the opportunity to regulate plant BCAA pathways in a targeted manner.

ACKNOWLEDGEMENTS

We acknowledge the Austrian Science Fund (project no. P21717-B16) and the Higher Education Commission (HEC) of Pakistan for funding the work. Financial support to Erich Inselsbacher from FORMAS and VR is acknowledged. Help and support from Florian Grundler (Uni. Bonn) is also acknowledged.

REFERENCES

AHMED, I. & KHAN, M.A. 2006. Dietary branched chain amino acid valine, isoleucine and leucine requirements of fingerling Indian major carp, *Cirrhinus mrigala* (Hamilton). *British Journal of Nutrition* 96: 450-460.

AIST, S. & RIGGS, R.D. 1969. Amino acids from *Heterodera glycines*. *Journal of Nematology* 1: 254-259.

AKMAN GÜNDÜZ, E. & DOUGLAS, A.E. 2009. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proceedings of the Royal Society B: Biological Sciences* 276: 987-991.

AMIR, R., HACHAM, Y. & GALILI, G. 2002. Cystathionine gamma synthase and threonine synthase operate in concert to regulate carbon flow towards methionine in plants. *Trends in Plant Science* 7: 153-156.

ANWAR, S., INSELSBACHER, E., GRUNDLER, F.M.W. & HOFMANN, J. 2015. Arginine metabolism of *Arabidopsis thaliana* is modulated by *Heterodera schachtii* infection. *Nematology* 17: 1027-1043.

BALASUBRAMANIAN, M. & MYERS, R.F. 1971. Nutrient media for plant parasitic nematodes: II. Amino acid requirements of *Aphelenchoides* sp. *Experimental Parasitology* 29: 330-336.

BARTLEM, D., LAMBEIN, I., OKAMOTO, T., ITAYA, A., UDA, Y., KIJIMA, F., TAMAKI, Y., NAMBARA, E. & NAITO, S. 2000. Mutation in the threonine synthase gene results in an over-accumulation of soluble methionine in *Arabidopsis*. *Plant Physiology* 123: 101-110.

BAUM, H.J., MADISON, J.T. & THOMPSON, J.F. 1983. Feedback inhibition of homoserine kinase from radish leaves. *Phytochemistry* 22: 2409-2412.

BERTHOU, F., KOUASSI, A., BOSSIS, M., DANTEC, J.P., EDDAOUDI, M., FERJI, Z., PELLE, R., TAGHZOUTI, M., ELLISSECHE, D. & MUGNIERY, D. 2003. Enhancing the resistance of the potato to Southern root-knot nematodes by using *Solanum sparsipilum* germplasm. *Euphytica* 132: 57-65.

BETKA, M., GRUNDLER, F.M.W. & WYSS, U. 1991. Influence of changes in the nurse cell system (syncytium) on sex determination and development of the cyst nematode *Heterodera schachtii*: single amino acids. *Phytopathology* 81: 75-79.

BINDER, S. 2010. Branched-chain amino acid metabolism in *Arabidopsis thaliana*. *Arabidopsis Book* 8: e0137.

BINDER, S., KNILL, T. & SCHUSTER, J. 2007. Branched chain amino acid metabolism in higher plants. *Physiologia Plantarum* 129: 68-78.

CECCARELLI, S. & GRANDO, S. 1996. Drought as a challenge for the plant breeder. *Plant Growth Regulation* 20: 149-155.

CORZO, A., LOAR, R.E. & KIDD, M.T. 2009. Limitations of dietary isoleucine and valine in broiler chick diets. *Poultry Science* 88: 1934-1938.

CORZO, A., DOZIER, W.A., LOAR, R.E., KIDD, M.T. & TILLMAN, P.B. 2010. Dietary limitation of isoleucine and valine in diets based on maize, soybean meal, and meat and bone meal for broiler chickens. *British Poultry Science* 51: 558-563.

CURIEN, G., DUMAS, R., RAVANEL, S. & DOUCE, R. 1996. Characterization of an *Arabidopsis thaliana* cDNA encoding an S-adenosylmethionine-sensitive threonine synthase from higher plants. *FEBS Letters* 390: 85-90.

DELAUNEY, A.J. & VERMA, D.P.S. 1993. Proline biosynthesis and osmoregulation in plants. *Plant Journal* 4: 215-223.

DOYLE, E.A. & LAMBERT, K.N. 2003. *Meloidogyne javanica* chorismate mutase 1 alters plant cell development. *Molecular Plant Microbe Interactions* 16: 123-131.

EDWARDS, K., JOHNSTONE, C. & THOMPSON, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* 19: 1349.

FAVERY, B., CHELYSHEVA, L.A., LEBRIS, M., JAMMES, F., MARMAGNE, A., DE ALMEIDA-ENGLER, J., LECOMTE, P., VAURY, C., ARKOWITZ, R.A. & ABAD, P. 2004. *Arabidopsis* formin AtFH6 is a plasma membrane-associated protein upregulated in giant cells induced by parasitic nematodes. *Plant Cell* 16: 2529-2540.

GARAULT, P., LETORT, C., JUILLARD, V. & MONNET, V. 2000. Branched chain amino acid biosynthesis is essential for optimal growth of *Streptococcus thermophilus* in milk. *Applied and Environmental Microbiology* 66: 5128-5133.

GHEYSEN, G. & FENOLL, C. 2002. Gene expression in nematode feeding sites. *Annual Review of Phytopathology* 40: 191-219.

GOLINOWSKI, W., GRUNDLER, F.M.W. & SOBCZAK, M. 1996. Changes in the structure of *Arabidopsis thaliana* during female development of the plant parasitic nematode *Heterodera schachtii*. *Protoplasma* 194: 103-116.

GRANDISON, R.C., PIPER, M.D.W. & PARTRIDGE, L. 2009. Amino acid imbalance explains extension of lifespan by dietary restriction in *Drosophila*. *Nature* 462: 1061-1064.

GRUNDLER, F.M.W., BETKA, M. & WYSS, U. 1991. Influence of changes in the nurse cell system (syncytium) on sex determination and development of the cyst nematode *Heterodera schachtii*: total amounts of proteins and amino acids. *Phytopathology* 81: 70-74.

GRUNDLER, F.M.W., SOBCZAK, M. & GOLINOWSKI, W. 1998. Formation of wall openings in root cells of *Arabidopsis thaliana* following infection by the plant parasitic nematode *Heterodera schachtii*. *European Journal of Plant Pathology* 104: 545-551.

HEDIN, P.A. & CREECH, R.G. 1998. Altered amino acid metabolism in root-knot nematode inoculated cotton plants. *Journal of Agricultural and Food Chemistry* 46: 4413-4415.

HOFMANN, J. & GRUNDLER, F.M.W. 2007. Identification of reference genes for qRT-PCR studies of gene expression in giant cells and syncytia induced in *Arabidopsis thaliana* by *Meloidogyne incognita* and *Heterodera schachtii*. *Nematology* 9: 317-323.

HOFMANN, J., EL ASHRY, A.E.N., ANWAR, S., ERBAN, A., KOPKA, J. & GRUNDLER, F.M.W. 2010. Metabolic profiling reveals local and systemic responses of host plants to nematode parasitism. *Plant Journal* 62: 1058-1071.

HUSSEY, R.S. & GRUNDLER, F.M.W. 1998. Nematode parasitism of plants. In: *Physiology and Biochemistry of Free-Living and Plant Parasitic Nematodes* (R.N. Perry & D.J. Wright Eds). pp. 213-243. Oxford, UK, CAB International.

INSELSBACHER, E., ÖHLUND, J., JÄMTGÅRD, S., HUSS-DANELL, K. & NÄSHOLM, T. 2011. The potential of

microdialysis to monitor organic and inorganic nitrogen compounds in soil. *Soil Biology and Biochemistry* 43: 1321-1332.

ITHAL, N., RECKNOR, J., NETTLETON, D., HEARNE, L., MAIER, T., BAUM, T.J. & MITCHUM, M.G. 2007a. Parallel genome-wide expression profiling of host and pathogen during soybean cyst nematode infection of soybean. *Molecular Plant Microbe Interactions* 20: 293-305.

ITHAL, N., RECKNOR, J., NETTLETON, D., MAIER, T., BAUM, T.J. & MITCHUM, M.G. 2007b. Developmental transcript profiling of cyst nematode feeding cells in soybean roots. *Molecular Plant Microbe Interactions* 20: 510-525.

JACKSON, G.J. 1973. *Neoaplectana glaseri* essential amino acids. *Experimental Parasitology* 34: 111-114.

JAMMES, F., LECOMTE, P., ALMEIDA-ENGLER, J., BITTON, F., MARTIN-MAGNIETTE, M.L., RENOU, J.P., ABAD, P. & FAVERY, B. 2005. Genome wide expression profiling of the host response to root knot-nematode infection in *Arabidopsis*. *Plant Journal* 44: 447-458.

JONES, M.G.K. & NORTHCOTE, D.H. 1972. Nematode-induced syncytium – a multinucleated transfer cell. *Journal of Cell Science* 10: 789-809.

KRAUTHAUSEN, H.J. & WYSS, U. 1982. Influence of the cyst nematode *Heterodera schachtii* on relative changes in the pattern of free amino acids at feeding sites. *Physiological Plant Pathology* 21: 425-436.

LABER, B., MAURER, W., HANKE, C., GRAFE, S., EHLERT, S., MESSERSCHMIDT, A. & CLAUSEN, T. 1999. Characterization of recombinant *Arabidopsis thaliana* threonine synthase. *European Journal of Biochemistry* 263: 212-221.

LEA, P.J. & IRELAND, R.J. 1999. Nitrogen metabolism in higher plants. In: *Plant Amino Acids: Biochemistry and Biotechnology* (B.K. Singh Ed.). pp. 1-47. New York, USA, Marcel Dekker.

LEHMANN, S., FUNCK, D., SZABADOS, L. & RENTSCH, D. 2010. Proline metabolism and transport in plant development. *Amino Acids* 39: 949-962.

MOENS, M. & PERRY, R.N. 2009. Migratory plant endoparasitic nematodes: a group rich in contrasts and divergence. *Annual Review of Phytopathology* 47: 313-332.

NAMBARA, E., KAWAIDE, H., KAMIYA, Y. & NAITO, S. 1998. Characterization of an *Arabidopsis thaliana* mutant that has a defect in ABA accumulation: ABA dependent and ABA independent accumulation of free amino acids during dehydration. *Plant Cell Physiology* 39: 853-858.

PARIDA, A.K. & DAS, A.B. 2005. Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety* 60: 324-349.

PERELMAN, D. & LU, N.C. 2000. Requirements for branched chain amino acids and their interactions in the nematode *Caenorhabditis elegans*. *Nematology* 2: 501-506.

PRELL, J., WHITE, J.P., BOURDES, A., BUNNEWELL, S., BONGAERTS, R.J. & POOLE, P.S. 2009. Legumes regulate *Rhizobium* bacteroid development and persistence by the supply of branched-chain amino acids. *Proceedings of the National Academy of Sciences of the United States of America* 106: 12477-12482.

PUTHOFF, D.P., NETTLETON, D., RODERMEL, S.R. & BAUM, T.J. 2003. *Arabidopsis* gene expression changes during cyst nematode parasitism revealed by statistical analyses of microarray expression profiles. *Plant Journal* 33: 911-921.

RYADCHIKOV, V.G., TARABRIN, I.V., RADUL, N.P. & ZIGANSHIN, R.K. 2005. [Nutritional behavior of animals at different forms of balance of essential amino acids]. *Sel'skokhozyaistvennaya Biologiya* 5: 3-13 (in Russian).

SIJMONS, P.C., GRUNDLER, F.M.W., VON MENDE, S., BURROWS, P.R. & WYSS, U. 1991. *Arabidopsis thaliana* as a new model host for plant parasitic nematodes. *Plant Journal* 1: 245-254.

SIJMONS, P.C., ATKINSON, H.J. & WYSS, U. 1994. Parasitic strategies of root nematodes and associated host cell responses. *Annual Review of Phytopathology* 32: 235-259.

SINGH, B.K. & SHANER, D.L. 1995. Biosynthesis of branched chain amino acids: from test tube to field. *Plant Cell* 7: 935-944.

SOBCZAK, M. & GOLINOWSKI, W. 2011. Cyst nematodes and syncytia. In: *Genomics and Molecular Genetics of Plant-Nematode Interactions* (J. Jones, G. Gheysen & C. Fenoll Eds). pp. 61-82. Dordrecht, the Netherlands, Springer.

SZAKASITS, D., HEINEN, P., WIECZOREK, K., HOFMANN, J., WAGNER, F., KREIL, D.P., SYKACEK, P., GRUNDLER, F.M.W. & BOHLMANN, H. 2009. The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in *Arabidopsis* roots. *Plant Journal* 57: 771-784.

TRUDGILL, D.L. 1967. The effect of environment on sex determination in *Heterodera rostochiensis*. *Nematologica* 13: 263-272.

VANFLETEREN, J.R. 1973. Amino acid requirements of the free-living nematode *Caenorhabditis briggsae*. *Nematologica* 19: 93-99.

WYSS, U. 1992. Observations of the feeding behavior of *Heterodera schachtii* throughout development, including events during moulting. *Fundamental and Applied Nematology* 15: 75-89.

WYSS, U. & GRUNDLER, F.M.W. 1992. *Heterodera schachtii* and *Arabidopsis thaliana*, a model host parasite interaction. *Nematologica* 38: 488-493.

ZEH, M., CASAZZA, A.P., KREFT, O., ROESSNER, U., BIEBERICH, K., WILLMITZER, L., HOEFGEN, R. & HESSE, H. 2001. Antisense inhibition of threonine synthase leads to high methionine content in transgenic potato plants. *Plant Physiology* 127: 792-802.

URL: http://www.arabidopsis.info/ (accessed: June 14, 2015).

URL: http://www.spss.com/ (accessed: July 5, 2015).

S. Anwar, E. Inselsbacher and J. Hofmann. Аминокислоты с разветвленными цепями вовлечены в процесс развития *Heterodera schachtii* на *Arabidopsis thaliana*.

Резюме. Паразитирующие на растениях цистообразующие нематоды *Heterodera schachtii* способны поражать растения *A. thaliana* и трансформировать клетки корней в особые питающие структуры, называемые синцитиями. Эти синцитии активно потребляют соединения, производимые в процессе фотосинтеза, и служат единственным источником питательных веществ для нематод на протяжении всей их жизни. В отличие от растений, животные не способны синтезировать аминокислоты с разветвленной цепью, и полностью зависят от потребления таких аминокислот из внешних источников. Данное исследование направлено на изучение роли этих необходимых аминокислот в процессе развития нематод в *Arabidopsis*. Показано, что валин, лейцин и изолейцин, среди прочих аминокислот, накапливаются в синцитии. Выявлена мощная экспрессия в синцитии генов, кодирующих треонин-синтазу и дегидратазу диоксикислот (ферменты, играющие важную роль в синтезе аминокислот с разветвленной цепью). Также для оценки воздействия на развитие нематод были использованы два мутанта по гену дегидратазы диоксикислот. Показано, что формирование самок у таких мутантов достоверно снижено. Мутации не влияют на размер синцития, показывая, что аминокислоты с разветвленной цепью важны для развития нематод в растении, но не для формирования самого синцития.