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Amino acid permease 6 modulates host response to cyst nematodes in wheat and Arabidopsis

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Summary – Cyst nematodes are plant parasites that cause significant crop loss in wheat and other crops. Infective juveniles invade roots and induce syncytial feeding structures as the only source of nutrients throughout their life. A previous genome-wide association study in wheat identified amino acid permease 6 (*TaAAP6*) to be linked to susceptibility to the cereal cyst nematode *Heterodera filipjevi*. To characterise the role of *AAP6* during nematode parasitism, we analysed the expression of *TaAAP6* and the Arabidopsis orthologue *AtAAP6*. *TaAAP6* was found to be highly expressed in nematode-infected roots of susceptible wheat, whereas it was not upregulated in nematode-infected roots of resistant accessions. *AtAAP6* was also found to be highly upregulated in nematode-induced syncytia compared with non-infected roots. Infection assays with an *AtAAP6* knock-out mutant revealed reduction in developing females, female size, and size of female-associated syncytia, thus indicating the importance of AAP6 in cyst nematode parasitism.

Keywords – cereal cyst nematode, gene expression, *Heterodera filipjevi*, *Heterodera schachtii*, susceptible, syncytia.

The cyst nematodes, *Heterodera filipjevi* and *H. schachtii*, are sedentary biotrophic obligate parasites that cause significant yield losses. Nematode second-stage juveniles (J2) invade epidermal and cortical root cells, and migrate intracellularly towards the vascular cylinder, where they become sedentary. The non-sexed J2 induce a highly specific syncytial feeding structure in the host roots. These feeding structures accumulate nutrients that are processed and supplied to the nematodes (Böckenhoff & Grundler, 1994). Syncytia are the only source of nutrients throughout the nematode life cycle. J2 become sedentary and undergo several moults before developing into adults. Functional syncytia lead to the development of females, which continue feeding until egg development is completed. Syncytia providing only a limited food supply trigger formation of males, which require much less food. In resistant plants, syncytium formation and function are strongly limited, leading to a high proportion of males. Since the number of females is low, reproduction of nematodes is strongly reduced under these circumstances. This phenomenon of 'male-based resistance' is commonly observed with cyst-forming nematodes in different crops (Roberts & Stone, 1983; Acedo *et al*., 1984; Rice *et al*., 1985).

During syncytium induction, the affected root cells undergo drastic morphological and physiological changes. The nuclei increase in size, whereas smooth endoplasmic reticulum, ribosomes, mitochondria and other organelles proliferate (Golinowski *et al*., 1996). At the same time, the levels of various amino acids, phosphorylated metabolites, sugars and organic acids in syncytia rise substantially (Hofmann *et al*., 2010). Accordingly, syncytia are strong metabolic sinks in the plant transport system (Böckenhoff *et al*., 1996). Availability of nutrients such as sugars and amino acids depends strongly on translocation processes (Hofmann *et al*., 2007). Since syncytia have been found to be symplasmically isolated during early nematode development, translocation of assimilates is de-

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pendent on the activity of transport proteins (Hammes *et al*., 2005; Hofmann *et al*., 2007). Thus, amino acid transporters (AATs) are very important at this stage. At later stages, secondary plasmodesmata are formed between the phloem and syncytium facilitating symplasmic translocation of assimilates (Böckenhoff & Grundler, 1994).

Amino acids represent the major form of reduced nitrogen that is transported in plants. All proteinogenic amino acids are found in phloem and to a lesser extent in xylem (Lalonde *et al*., 2004). Furthermore, the concentration of amino acid varies upon response to attack by pathogens such as aphids and nematodes (Szakasits *et al*., 2009; Hunt *et al*., 2010; Elashry *et al*., 2013). AATs are integral membrane proteins that transport amino acid from source to sink tissue across cellular membranes in higher plants.

AATs comprise two families: amino acid/auxin permeases (AAAP) and amino acid-polyamine-cholines (APC) (Fischer *et al*., 1998; Wipf *et al*., 2002; Tegeder & Rentsch, 2010). Amino acid permease (AAP) is a member of the AAAP family group (Saier *et al*., 2009; Okumoto *et al*., 2011; Tegeder, 2012). AAP are the most studied amino acid transporters for nematode parasitism (Hammes *et al*., 2005; Barcala *et al*., 2010). Previous studies showed *AtAAP1*, *AtAAP3*, *AtAAP6* and *AtAAP7* to be upregulated upon root-knot nematode infection. *AtAAP6* has also been found to be upregulated upon cyst nematode infection (Puthoff *et al*., 2003). This was further confirmed by genechip analysis, *in situ* rtPCR, and infection assays with promoter::GUS lines (Szakasits *et al*., 2009; Elashry *et al*., 2013). Although AATs in Arabidopsis (*AtAAP1-AtAAP8*) and rice (*OsAAP1-OsAAP19*) are functionally well characterised, much less is known in wheat (Okumoto & Pilot, 2011; Zhao *et al*., 2012).

Bread wheat (*Triticum aestivum* L.) is an allohexaploid composed of three closely related but independently maintained genomes (A, B and D) formed by multiple hybridisation events among the three different progenitor species (Huang *et al*., 2002; Dubcovsky & Dvorak, 2007; Shewry, 2009; Matsuoka, 2011). The wheat genome forms a substantial barrier for genomic analysis, firstly due to its large size (*ca* 17 Gbp) with more than 80% of repetitive DNA in sub-genomes (Gill & Gill, 1994) and, secondly, the lack of genomic and transcriptomic data in relation to host-pathogen interaction limits the annotation for large numbers of genes and proteins in wheat. Therefore, it is very difficult to study complex interactions using reverse genetics at the molecular level. However, current advances in genome sequencing and high density marker genotyping has made it possible to locate QTLs and identify related genes modulating various agronomic traits. Our previous GWAS (Genome-Wide Association Studies) with a set of wheat accessions infected with *H. filipjevi* revealed a QTL linked to nematode development (Pariyar *et al*., 2016a). This study identified a genomic region of 74.9 cM on chromosome 2BL associated with a putative amino acid transporter gene linked to nematode susceptibility.

Here, we investigated the role of *TaAAP6* during nematode parasitism in two susceptible and two resistant wheat accessions. Due to several restrictions in the wheat system, it was not possible to study gene function in this context. We therefore used the model *A. thaliana*/*H. schachtii system* to analyse the role of *AAP*6 in nematode parasitism. For this analysis we used the amino acid permease 6 orthologue gene '*AtAAP6*' in *A. thaliana*. This gene has been described as being upregulated in nematode-induced syncytia (Szakasits *et al*., 2009; Elashry *et al*., 2013). Using a T-DNA mutant for infection assays, and following the expression pattern of the *AtAAP6* in syncytia at different time intervals, we show that *AAP6* plays an important role in nematode parasitism.

Materials and methods

Confirmation of *TaAAP6* at wheat chromosome 2B and comparison with *AtAAP6*

In order to confirm *TaAAP6* at significant marker location 74.9 cM on chromosome 2BL, we downloaded the available wheat transcriptome assemblies from the MAS Wheat dataset. Then, all datasets were imported in the CLC genomic workbench and analysed. Database sequences lower than e-value 0.0e-15 were considered positive (Krasileva *et al*., 2013). To locate the genomic region of significant QTL at chromosome 2B, a Manhattan plot was created with SAS 9.2, the extent of LD was estimated, and pairwise measures of LD (r^2) were illustrated as a heat-map by Haploview4.2 (Barrett *et al*., 2005). To identify the gene linked to the QTL, the flanking sequences of SNP (single nucleotide polymorphism) marker were annotated against gene models of *Brachypodium distachyon*, *Oryza sativa* and *Sorghum bicolor* available at the International Wheat Genome Sequencing Consortium (IWGSC), the Institute for Genomic Research (TIGR), National Center for Biotechnology Information (NCBI) and the *Arabidopsis* Information Resource (TAIR). The computational analysis revealed

amino acid transporter (AAT) transmembrane family protein linked to the ORF and identified it as *TaAAP6* (NCBI accession no. AK334879). DNA coding sequence and protein sequences were downloaded from the available Ensembl Plants database. The genomic sequences were translated into amino acid sequences by ExPASy (Expert Protein Analysis System) translate tool and compared by Clustal Omega multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo). In order to compare the structure of *AAP6* in wheat and Arabidopsis, amino acid sequence of *AAP6* ORF, conserved motifs, and domains were analysed by OrthoDB. In this way the Arabidopsis orthologue *AtAAP6* was identified.

Nematode infection assays

Nematode infection assay and host response to *H. filipjevi* in wheat were performed following methods in a previous study (Pariyar *et al*., 2016b). Cysts of *H. filipjevi* were extracted from soil and roots of glasshouse-cultured wheat 'Bezostaya 1'. Cysts were handpicked and sterilised with 0.5% NaOCl for 10 min and subsequently rinsed several times with sterile distilled water (SDW). The sterile cysts then were transferred into a funnel filled with SDW and stored at 4°C for hatching. Freshly hatched J2 were collected and used as inoculum.

Seven seeds from each spike of four wheat accessions (Table S1 in the Supplementary material) were germinated on moistened tissue placed in Petri dishes for 3 days at 22°C. After germination, five seedlings of similar size and development were selected. RLC4-pine tubes (25 mm diam. × 160 mm height; Ray Leach Cone-tainer™, Stuewe & Sons) were filled with a sterilised potting mixture of sand, field soil and organic matter (70:29:1, v/v). Each plant was inoculated with 250 freshly hatched J2 of *H. filipjevi* 7 days after transplanting and set in a completely randomised design. Plants were grown at 23 ± 2°C, 16 h of artificial light and 65% relative humidity. Plants were harvested at 63 days post-inoculation (dpi) and cysts from both the soil and the roots were extracted. Females and cysts from both roots and soil were collected and counted under a dissecting microscope. The response of the tested wheat accessions was determined and categorised into five groups based on the mean number of females and cysts recorded per plant (Pariyar *et al*., 2016b).

For nematode infection in Arabidopsis, cysts of *H. schachtii* were collected from *in vitro* cultured mustard roots *Sinapis alba* 'Albatros' (Sijmons *et al*., 1991). The cysts were soaked in 3 mM $ZnCl_2$ at 25°C for 4 days to induce hatching of J2. Freshly hatched J2 were collected, washed three times with SDW, and re-suspended in 0.5% (w/v) GelRite (Duchefa) to enhance nematode infection; 10-day-old plants were each inoculated with 60 J2. Two plants per plate with 20 replications were used. The Petri plates were sealed and placed in a growth chamber at 25°C under a 16 h light/8 h dark cycle. The numbers of male and female nematodes were counted at 14 dpi. The mean sizes (mm^2) of female nematodes and associated syncytia were measured at 15 dpi in longitudinal optical sections as described previously (Siddique *et al*., 2009). Around 60 syncytia and associated female nematodes were randomly selected and photographed with a Leica DM2000 dissection microscope. The Diskus Kontour tool was used to outline the female and syncytia, and the area was calculated using LAS software (Leica Microsystems). These experiments were repeated three times.

Plant materials and growth conditions

To analyse the role of *TaAAP6* gene in nematode parasitism, two moderately resistant and two highly susceptible wheat accessions were selected (Table S1). The seed material was obtained from CIMMYT-ICARDA germplasm collections. Seeds of each accession were multiplied in a glasshouse culture and later used for infection assays.

Seeds of Arabidopsis plants (T-DNA insertion mutant and wild-type (WT-Colombia-0)) were obtained from Nottingham Arabidopsis Stock Centre (Alonso *et al*., 2003). Seeds were surface-sterilised by soaking them in 5% (W/V) sodium hypochlorite for 5 min, and subsequently washed three times with SDW. Sterilised seeds were grown in Petri dishes containing 0.2% Knop's medium supplemented with 2% sucrose. To analyse gene function, an *AtAAP6* knock out mutant SALK_013231 (N661540) was cultured. The T-DNA insertions were confirmed by PCR using gene-specific primers in combination with a T-DNA left border primer, followed by gel electrophoresis. Primer sequences were obtained from the SIGnAL website (http://signal.salk.edu/tdnaprimers.2.html).

DNA isolation and polymerase chain reaction

Total genomic DNA from individual leaves from both wild type and mutant was isolated using a modified CTAB method (Murray & Thompson, 1980; Sambrook & Russell, 2001). Quality and quantity of the DNA were anal-

Table 1. Primers used for polymerase chain reaction (PCR) and reverse transcriptase (RT) polymerase chain reaction.

Sequence (5′ →3′)	Gene	Gene locus	Product (bp)	Annealing temperature (°C)	Efficiency (%)	Plant
CACGGTTCAACAACATCCAG	UBQ-FP	At4g05320	180	59	2.1	Arabidopsis
TGAAGACCCTGACTGGGAAG	UBQ-RP					
CAACACTGACAGGAGTTACGGT	AtAAP6-FP	At5g49630	137	55	2.1	Arabidopsis
TTCGCGCTCTGGCTCTCTA	AtAAP6-RP					
TGCATGGAACTTGTGTTCTTG	Salk_013231C-RP	At5g49630	1163	60	PCR	T-DNA insertion
ATTGCATTTGCCTACGCATAC	Salk_013231C-LP					
ATTTTGCCGATTTCGGAAC	LBb1.3		568-868	60		
GTGGAACTGGCTCTGGC	qTubulin-FP		234	55	1.9	Wheat
CGCTCAATGTCAAGGGA	qTubulin-RP					
TCTGGCCCAATACGTCAACT	TaAAP6-FP		214	59	2.0	Wheat
GGAGAGCCACCAGATCTTGT	TaAAP6-RP					
GTCAACTTGGTTGGCGTGAC	TaAAP6-FP		1072	60	PCR	Wheat
AGGAGACCTTGAACGGCTTG	TaAAP6-RP					

LBb1.3: left border primer; UBQ: ubiquitin; FR: forward primer; RP: reverse primer; AtAAP6: *Arabidopsis thaliana* amino acid permeases 6; TaAAP6: *Triticum aestivum* amino acid permeases 6.

ysed by optical density (OD) at $\lambda = 260$ nm measured in Nano Drop 2000C spectrophotometer (Peqlab), and DNA purity was determined by the ratio of absorptions at A260/A280. Gene amplification was performed in 25 μl reactions containing 100 ng μl^{-1} of DNA, following polymerase chain reaction (PCR) scheme of sterile deionised water: 16.9 μl, 5× buffer: 5 μl, 10 mM dNTP mix: 0.5 μl, forward, reverse primer, and left border primer: 0.5 μl, *Taq* DNA Polymerase: 0.2 μl, and DNA: 1 μl. Annealing temperatures for each reaction were optimised using a C1000™ Thermal Cycler (Bio-Rad 1000 Series Thermal Cycling Platform). The annealing temperature with desired PCR product range was selected. PCR was performed as follows: 4 min at 94°C, followed by 35 cycles of 1 min 94°C, 1 min at 59°C, 1min at 72°C and final extension of 5 min at 72°C. The PCR products were stained on 1% agarose gels containing 100 ml of 1 × TBE (Tris base, Boric acid, EDTA) buffer with 5 μl of peqGreen at 80°C for 60 min for visualisation, and photographed under ultraviolet light using Gel Documentation System in Bio-Rad Gel Doc™ XR. Primers sequences for the candidate genes and product size are designed by Primer3Plus software (Table 1).

RNA ISOLATION AND QUANTITATIVE REAL-TIME (RT) PCR

Wheat and Arabidopsis plants were grown and infected as described previously. To determine the abundance of *TaAAP6* in wheat, total RNA was isolated from 20 mg of the non-infected root and infected root at 10 dpi using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Similarly, to determine regulation of *AtAAP6* in parasitism, total RNA was isolated from 20 mg of the non-infected root section and syncytia of Arabidopsis (WT) plants at 1, 10 and 15 dpi using the same kit. RNA from non-infected roots was used as a control. The quantity of RNA concentration was measured by Nanodrop 2000C spectrophotometer (Peqlab). To assess RNA integrity, 6 μl of the total RNA was mixed with 2 μl 6× gel loading dye, loaded on a 1% agarose gel and stained on 1% agarose gels containing 100 ml of 1 × TBE (Tris base, Boric acid, EDTA) buffer with 5 μl peqGreen at 80°C for 60 min then photographed under ultraviolet light using Bio-Rad Gel Documentation System, Gel Doc™ XR. Contaminating DNA was digested with DNase1 using a DNA-*free*™ DNA Removal Kit (Ambion). The RNA was used to synthesise cDNA following the manufacturer's instructions by a High Capacity cDNA Reverse transcription Kit (Applied Biosynthesis). To maintain homogeneity, 1 μg of clean and stable RNA was used to synthesise complementary DNA (cDNA) (Schmittgen & Livak, 2008). Quantitative reverse transcription-PCR (qRT-PCR) was performed with the StepOne Plus Real-Time PCR System (Applied Biosystems). Each sample contained 10 μl Fast SYBR Green qPCR Master Mix (Invitrogen), 2 mM $MgCl_2$, 0.5 μl each of forward and reverse primers (10 μM), 2 μl cDNA, and water in a 20 μl

total reaction volume. The wheat gene *qTubulin* and Arabidopsis gene *ubiquitin* were used as internal control (Simonetti *et al*., 2010; Giménez *et al*., 2011). The specificity of the primers was analysed by standard PCR. The PCR amplification efficiency was determined for each primer combination by the slope of the standard curve obtained by plotting the fluorescence *vs* given concentration of a mixture of all sample cDNA (ranging from 1:1 to 1:5000 dilution of the cDNA mixture sample) using the equation: $E = 10^{(-1/\text{slope})} - 1$ (Pfaffl, 2001). The list of tested and reference genes and their respective primer sequences are given in Table 1. The analysis was performed in a MicroAmp® fast optical 96-well reaction plate (Applied Biosynthesis), with an ABI Step One Plus TM Real Time PCR System (Applied Biosynthesis). The PCR reaction was performed as follows: 95°C for 10 min, followed by 40 cycles, each cycle 95°C for 15 s, and 60°C for 60 s. Changes in transcript abundance were calculated using $\Delta\Delta C_t$ method (Pfaffl, 2001; Schmittgen & Livak, 2008). Three technical replicates were used.

Statistical analysis

Data of the bioassay experiments were analysed by one way analysis of variance (ANOVA). Results are considered significant based on Duncan's multiple comparison tests at $P < 0.05$ using Sigma plot 12.5 Software. Regarding RT-PCR data, significant differential expression between infected plants and control tissue was determined by Student's *t*-test at $P \leqslant 0.05$ (Pfaffl, 2001).

Results

Confirmation of *TaAAP6* at wheat chromosome 2B and comparison with *AtAAP6*

To confirm that *TaAAP6* is linked to QTL located at 74. cM on chromosome 2BL (Fig. S1 in the Supplementary material), we first blasted the flanking sequences of the markers against gene models of *B. distachyon*, *O. sativa* and *S. bicolor* at IWGSC, TIGR, NCBI and TAIR and identified the *AAP6* full ORF (Fig. S1) that explained 12% of the phenotypic variation (Pariyar *et al*., 2016b). Then, we illustrated the position of ten SNP markers that are in a cluster of high linkage disequilibrium (LD) with *TaAAP6* (Fig. S2 in the Supplementary material). The analysis of *AAP6* ORF revealed an amino acid transporter (*AAT*), transmembrane family protein that has a full-length complementary DNA (cDNA) sequence AK334879.1, which codes for a putative transmembrane AAT protein belonging to the APC superfamily and has a PF01490 (Aa_trans) consensus domain (http://pfam.janelia.org). The amino acid sequence of AK334879.1 is identical to predicted *Aegilops tauschii* subsp. *tauschii* amino acid permease 6 (XM_020316794.1, Table S2 in the Supplementary material). *Aegilops tauschii* subsp. *tauschii* is the D genome progenitor of modern bread wheat. Thus, we confirmed the gene in wheat as *T. aestivum* amino acid permease 6 (*TaAAP6*).

The comparison of *AAP6* structure between wheat and Arabidopsis identified *AtAAP6* (NP_199774.1) as an orthologue in Arabidopsis (Fig. S3 in the Supplementary material). The result revealed 69% of sequence similarities between *AtAAP6* and *TaAAP6*. They share a common Aa_trans domains and SLC5-6-like_sbd super family protein, which can be found in many amino acid transporters including UNC-47 and MTR. UNC-47 encodes a vesicular amino butyric acid (GABA) transporter with ten predicted transmembrane domains. MTR is an N system amino acid transporter system protein involved in methyl tryptophan resistance. The other members of this family include proline transporters and amino acid permeases (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi).

AAP6 expression in nematode-infected wheat roots

Nematode resistance against cyst nematodes in plant is characterised by failure or limitation to produce functional feeding sites, resulting in a reduced number of females. To assess the regulation of *TaAAP6* during nematode parasitism in susceptible plants, we analysed the expression of *TaAAP6* at 10 dpi in the susceptible and resistant wheat accessions. The response of these selected wheat accessions was categorised previously and also confirmed in this study (Table S1). Since highest nematode infection was recorded at 10 dpi in wheat, the expression of *TaAAP6* was analysed at 10 dpi (Pariyar *et al*., 2016b). The result showed that *TaAAP6* is significantly upregulated in the infected roots of susceptible wheat accessions 'Bezostaya 1' and 'Trapbow' compared with non-infected roots. By contrast, the expression of *TaAAP6* in infected roots of resistant plants 'Lantian 12' and 'BC380' remained low and insignificant compared with non-infected roots (Fig. 1). The results showed that *APP6* in infected roots of susceptible accessions is highly upregulated compared with the infected roots of resistant plants. These analyses indicate that regulation of *TaAAP6* depends on both nematode infection and host susceptibility.

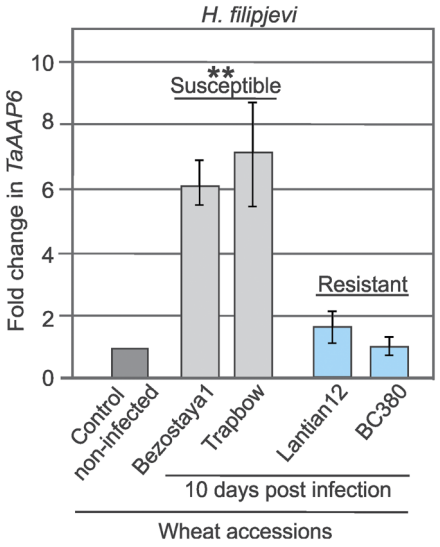

Fig. 1. RT-PCR analysis of *TaAAP6* gene regulation in infected root of susceptible and resistant wheat accessions compared with non-infected roots at 10 days post-infection with *Heterodera filipjevi*. Data were analysed using one-way ANOVA ($P \leqslant 0.05$, $n = 3$) and Holm-Sidak test. Asterisks represent statistically significant difference to corresponding Bezostaya 1 as a control. $^{**}P < 0.01$. Bar indicates the standard error of the mean. The experiment was repeated three times. The gene expression levels were normalised to the endogenous control gene *q-Tubulin* as housekeeping gene and the relative expression was measured as fold change by $\Delta\Delta C_t$ method. *TaAAP6* is highly upregulated in infected roots of susceptible lines compared with infected roots of resistant accessions.

AAP6 expression in Arabidopsis during nematode parasitism

In order to characterise the role of *AAP6* in nematode parasitism, we switched to the model host Arabidopsis. Molecular analyses in wheat are still complex and difficult, whereas working with Arabidopsis is highly efficient, since knock-out mutant plants are available for almost all genes. To assess the role of *AtAAP6* during different stages of nematode parasitism, we evaluated the expression of *AtAAP6*. Results of a RT-PCR analysis of nematode feeding sites confirmed that *AtAAP6* was significantly upregulated in the syncytial samples at 1, 10 and 15 days post *H. schachtii* infection compared with non-infected roots (Fig. 2). The expression of *AtAAP6* gradually increased with nematode infection, significantly progressed with nematode development, and became highest at 10 dpi. These results indicated that nematode infection and development significantly modulates *AtAAP6* expression in the affected root cells.

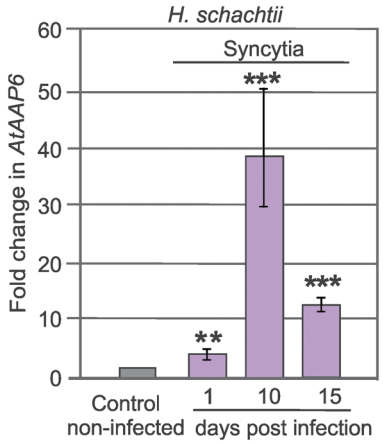

Fig. 2. RT-PCR analysis of *AtAAP6* gene regulation in syncytia at different time points after infection with *Heterodera schachtii* compared with non-infected roots. Data were analysed using one-way ANOVA ($P \leqslant 0.05$, $n = 3$) and Holm-Sidak test. Asterisks represent statistically significant differences to control non-infected roots. $^{**}P < 0.01$ and $^{***}P < 0.001$. Bar indicates the standard error of the mean. The experiment was repeated three times. Gene expression levels were normalised to the endogenous control gene *UBQ* as housekeeping gene and the relative expression was measured as fold change values by $\Delta\Delta C_t$ method.

AAP6 knock-out in Arabidopsis decreases susceptibility to *H. schachtii*

To characterise the role of *AtAAP6* in nematode parasitism, we first screened for *AtAAP6* knock-out mutant in the Arabidopsis information database and selected the mutant line SALK_013231 (Fig. S4A in the Supplementary material). T-DNA insertion in SALK_013231 mutant line was confirmed by PCR with gene-specific primers (Fig. S4B). Further, expression analysis by RT-PCR confirmed that *AtAAP6* is not expressed in the SALK_013231 line (Fig. S4C). We used this line for nematode infection assays and compared it with Col-0. The results revealed that female numbers (Fig. 3A), size of females and associated syncytia were significantly reduced in these plants (Fig. 3B). In the absence of functional *AAP6*, performance of nematode parasitism is strongly limited.

Discussion

AATs play a crucial role in transporting amino acids across cellular membranes. In plants, most of the sink tissues are connected to the source tissue *via* phloem. Nematode-induced syncytia are symplasmically isolated

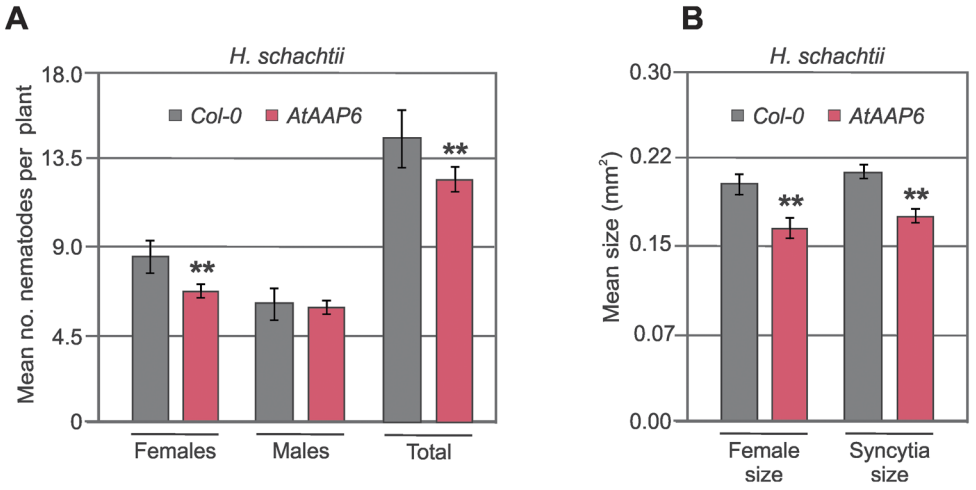

Fig. 3. Nematode development is reduced in *AtAAP6* gene knockout mutant. A: Mean numbers of female *Heterodera schachtii* in *AtAAP6* gene knockout mutant compared to wild-type Arabidopsis Col-0 at 14 days post-infection. Data were analysed using one-way ANOVA ($P \leqslant 0.05$, $n = 30$) and Holm-Sidak test. Asterisks represent statistically significant difference to corresponding Col-0 at ($^{**}P < 0.01$); B: Mean size of females and syncytia produced by *H. schachtii* (mm^2) in *AtAAP6* knockout mutant compared with wild-type Arabidopsis Col-0 at 15 days post-infection. Data were analysed using one way ANOVA at ($P \leqslant 0.05$, $n = 60$) and Holm-Sidak test. Asterisks represent statistically significant difference to corresponding Col-0. $^{**}P < 0.01$. Bar indicates the standard error of the mean.

in the early phase of nematode development. Direct connection between phloem and syncytia through secondary plasmodesmata is established at later stages at 10 dpi (Böckenhoff & Grundler, 1994; Hoth *et al*., 2008). For the early phase, the apoplastic pathway of assimilate translocation plays an important role. Several sugar transporters genes were shown to be upregulated in syncytia and found to have an important influence on nematode development (Hofmann *et al*., 2007). Besides sugars, many amino acids such as glutamic acid, aspartic acid and glutamine are accumulated in syncytia (Betka *et al*., 1991; Tegeder & Rentsch, 1998; Hammes *et al*., 2005; Barcala *et al*., 2010; Hofmann *et al*., 2010). *AtAAP6* is expressed in the xylem parenchyma cells and thought to regulate phloem sap amino acid composition and be involved in xylem-phloem transfer (Okumoto *et al*., 2011). Amino acid concentration in the xylem is lower than in phloem sap, therefore a high affinity transporter like *AtAAP6* is needed to enhance the transfer of amino acids into the sieve elements (SE) (Okumoto *et al*., 2002).

A large number of potential AATs from many different plants were identified *via* functional analysis and sequence similarity in model plants such as Arabidopsis and rice (Wipf *et al*., 2002; Rentsch *et al*., 2007; Hunt *et al*., 2010). Both genomes have been used to find orthologues in wheat (Clarke *et al*., 2003; Tulpan *et al*., 2015). In a recent study, we therefore used GWAS to identify putative genes linked to nematode development in wheat. By using comparative genome analysis, we identified *TaAAP6* in wheat similar to the predicted *A. tauschii* amino acid permeases 6. As hexaploid wheat genome has a high percentage of repetitive DNA in all three sub-genomes, *TaAAP6* might have appeared either spontaneously or imported from *A. tauschii* during evolution. However, we showed that *APP6* is highly conserved in wheat and its wild progenitor species *A. tauschii*.

Many cereal genes have been reported to share low sequence similarities with Arabidopsis genes but show a higher degree of sequence conservation within protein functional domains (Blümel *et al*., 2015). Our *in silico* analysis identified *AtAAP6* as an orthologue of *AAT* transmembrane protein (NP_199774.1) in Arabidopsis by comparing amino acid sequences of *AAP6* ORF and the functional domain (Fig. S3). Thus, domain analysis may play an important role in finding orthologous proteins in wheat and elucidating their function.

As obligate parasites, cyst nematodes take up required amino acids from syncytial feeding sites induced in the host root. During nematode development, cyst nematodes alter the expression of several amino acid transporters in syncytia (Szakasits *et al*., 2009; Elashry *et al*., 2013). The high expression of *AAP6* in roots of infected susceptible

wheat and Arabidopsis indicated its importance in accumulating amino acids in syncytia (Fig. 1). A detailed functional analysis of *AtAAP6* revealed that total amino acid concentration in SE was reduced by 30% in the *AtAAP6* mutant (Hunt *et al*., 2010). Expression in resistant wheat accessions and *AtAAP6* Arabidopsis knock-out line remained low and may also lead to reduce AA accumulation, which may not meet the high demand of nematode females. Thus, increased transcript levels of *AAP6* in susceptible plants support nematode performance, while lower levels limits the function of feeding sites in resistant plants (Fig. 1).

Previously, Elashry *et al*. (2013) reported a noticeable decrease but no significant reduction of *H. schachtii* infection in *AtAAP6* mutant. Compared with our study, they used a different method for infection assay, and a mutant with a different background (WS), which might explain the results. *AtAAP6* is highly expressed in syncytia at 1, 10 and 15 dpi with a peak in 10 dpi (Fig. 2). These results are in line with the results obtained from a gene chip analysis of syncytial transcripts that showed an upregulation of *AtAAP6* in syncytia (Szakasits *et al*., 2009). The high expression at 10 dpi may reflect the important function in AA accumulation during the phase of symplasmic isolation. Later, expression is lower, as AAs can be translocated through secondary plasmodesmata (Böckenhoff & Grundler, 1994; Hammes *et al*., 2005). The localisation of *AtAAP6* gene expression in and around the syncytium at 15 dpi was shown using promoter::GUS lines and *in situ* RT-PCR (Elashry *et al*., 2013). A high level of *AtAAP6* transcripts was clearly detected in syncytia (Szakasits *et al*., 2009).

Our mutant analysis in Arabidopsis revealed a reduced number of female nematodes and a decrease in sizes of females and female-associated syncytia compared to the wildtype (Fig. 2A, B). These data support our hypothesis that loss of *AtAAP6* function reduces host susceptibility. Similar results of reduced *Meloidogyne incognita* infection, development and egg mass production in *AtAAP6* mutant were reported earlier (Marella *et al*., 2013). In fact, this nematode is highly dependent on the function of AA transporters, since its feeding structure is permanently symplasmically isolated (Hammes *et al*., 2005).

Here, we show that *AtAAP6* and *TaAAP6* are important factors for syncytium function and nematode development. These findings increase understanding of nematode parasitism and the involved plant responses, and facilitate future approaches to breed for durable resistance against nematodes.

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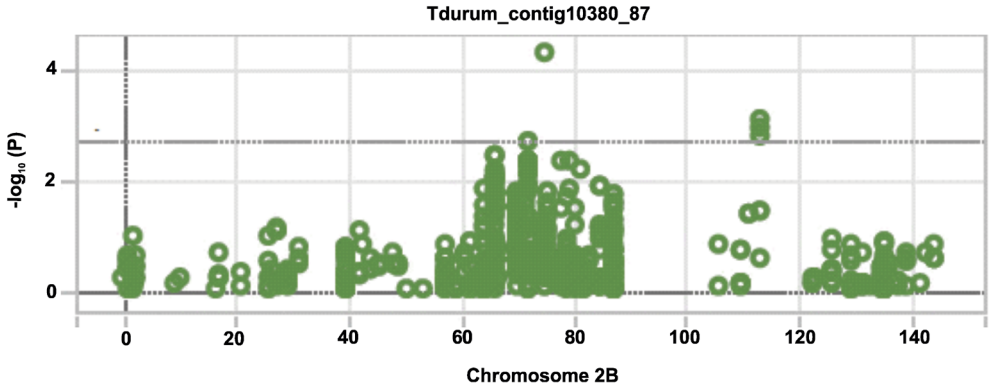

Fig. S1. Manhattan plots shows genomic regions associated with cereal cyst nematode *Heterodera filipjevi* susceptibility to MLM (P+K). The dotted black line represents reference line at false discovery rate of at $P \geqslant 0.05$. The x-axis shows SNP markers along wheat chromosome; the y-axis is the $-\log_{10}$ (P-value).

Table S1. List of selected wheat accessions, origin, response to *Heterodera filipjevi* and host status.

Common name	Accessions status	Pedigree	Origin	Cyst/plant	Host status
Bezostaya 1	Cultivar	LUT17/SRS2	Russia	35[a]	HS
Trapbow	Breeding line	494J6.11//TRAP#1/BOW	Mexico	28[b]	HS
Lantian 12	Cultivar	Qingnong-4/Xiannong-4	China	6[c]	MR
Bezenchukskaya 380			Russia	5[c]	MR

Columns with different letters are significantly different based on one-way ANOVA (Holm-Sidak) analysis ($P \leqslant 0.05$, $n = 15$), and $a > b > c$. HS: highly susceptible; MR: moderately resistant.

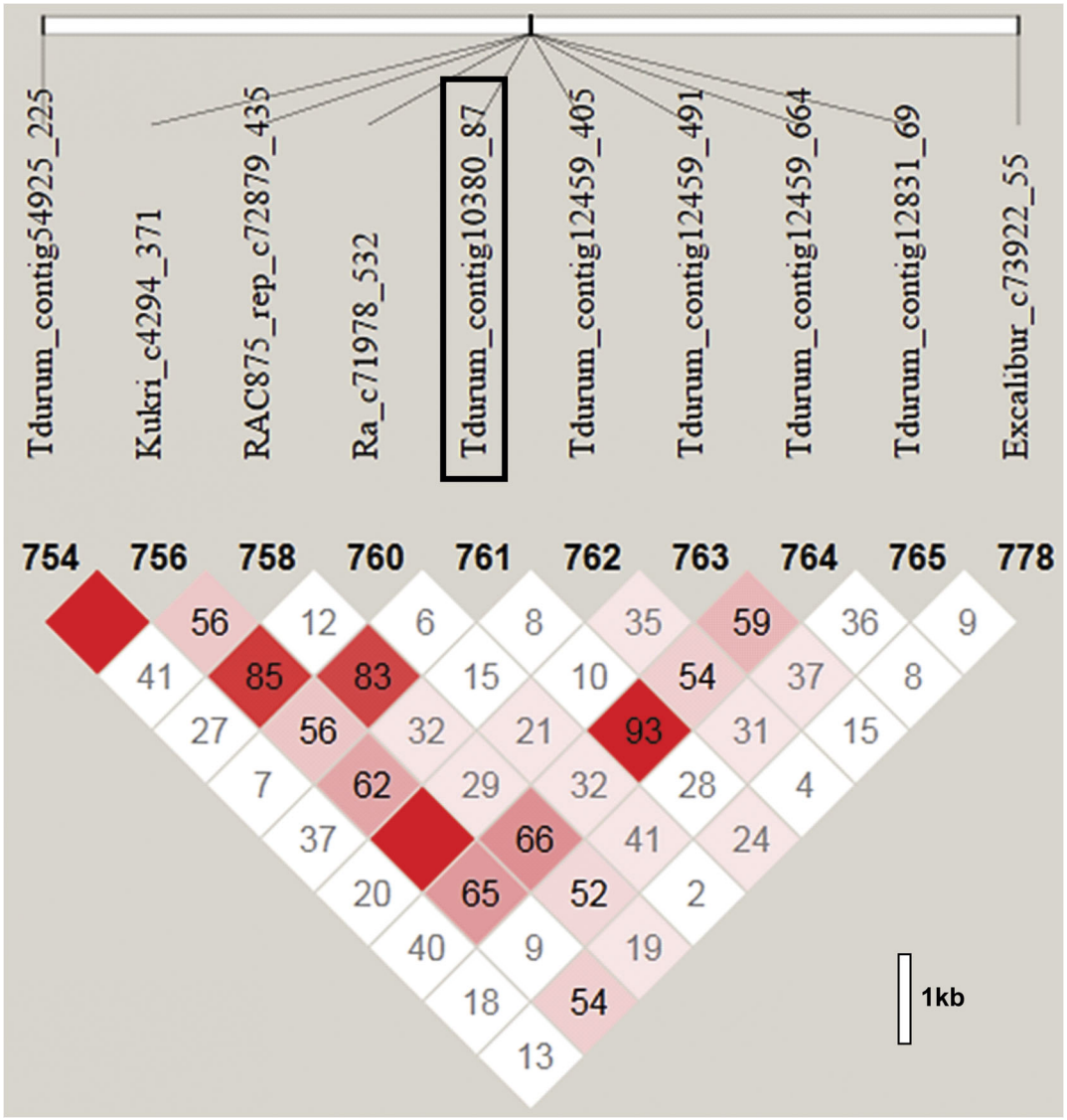

Fig. S2. Linkage disequilibrium (LD) heat-map of ten single nucleotide polymorphic markers close to the QTL linked to Tdurum_contig10380_87 on chromosome 2B. Colour represents a rough measure of significance LD block, bright red colour is better than pink, grey and white. $r^2 = 0$ (white colour), $0 < r^2 < 1$ (shades of grey) and $r^2 = 1$ (bright red). The SNP within the box is significantly associated with susceptibility to *Heterodera filipjevi*.

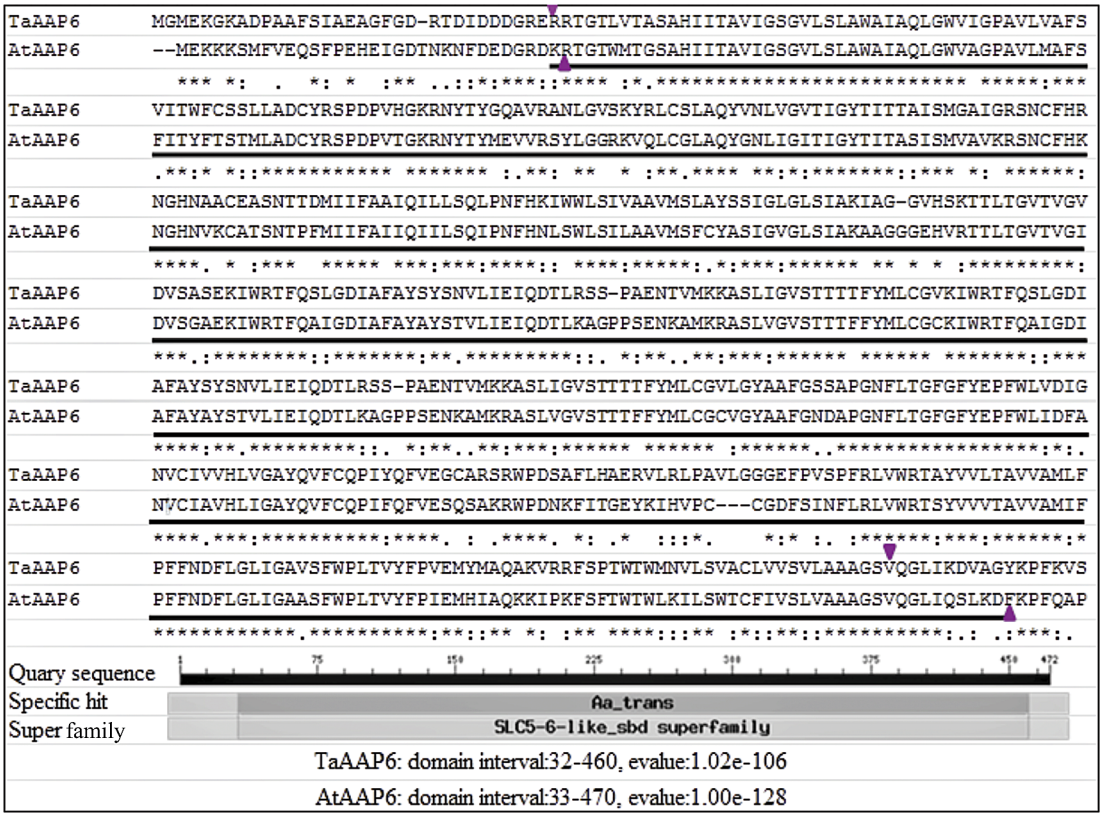

Fig. S3. Sequence alignment of TaAAP6 and AtAAP6 including conserved domain Aa_trans and SLC5-6 like_sbd superfamily protein. The underlined bold black line with (▼/▲) indicate the Aa_trans domain in wheat and Arabidopsis encoded by amino acids. *e-value = expected value, (*) similar amino acid, (. and :) indicates amino acid substitution.

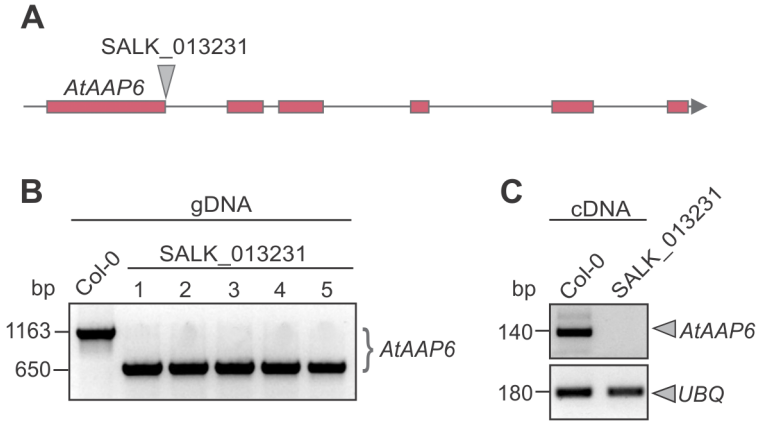

Fig. S4. Characterisation of *AtAAP6* by *Arabidopsis* mutant. A: A graphic gene model for *AtAAP6* with intron/exon structure and location of T-DNA insertions; B: T-DNA insertion was confirmed by PCR using gene-specific primers and the T-DNA primer; and C: Expression analysis of *AtAAP6* confirmed the homozygosity of the mutant SALK_013231. *1-5 represents individual plants used for genotyping from same batch of mutant seed. Specific primers for the Arabidopsis ubiquitin (UBQ) were used as control.

Table S2. Comparison of AAP6 ORF amino acid sequences in wheat (*Triticum aestivum*) and its D genome progenitor species (*Aegilops tauschii*).

Name	Sequence
AK334879	MGMEKGKADPAAFSIAEAGFGDRTDIDDDGRERRTGTLVTASAHIITAVIGSGVLSLAWAIAQLGWVIGPAVL
XM_020316794	MGMEKGKADPAAFSIAEAGFGDRTDIDDDGRERRTGTLVTASAHIITAVIGSGVLSLAWAIAQLGWVIGPAVL
	**********
AK334879	VAFSVITWFCSSLLADCYRSPDPVHGKRNYTYGQAVRANLGVSKYRLCSLAQYVNLVGVTIGYTITTAISMGA
XM_020316794	VAFSVITWFCSSLLADCYRSPDPVHGKRNYTYGQAVRANLGVSKYRLCSLAQYVNLVGVTIGYTITTAISMGA
	**********
AK334879	IGRSNCFHRNGHNAACEASNTTDMIIFAAIQILLSQLPNFHKIWWLSIVAAVMSLAYSSIGLGLSIAKIAGGV
XM_020316794	IGRSNCFHRNGHNAACEASNTTDMIIFAAIQILLSQLPNFHKIWWLSIVAAVMSLAYSSIGLGLSIAKIAGGV
	**********
AK334879	HSKTTLTGVTVGVDVSASEKIWRTFQSLGDIAFAYSYSNVLIEIQDTLRSSPAENTVMKKASLIGVSTTTTFY
XM_020316794	HSKTTLTGVTVGVDVSASEKIWRTFQSLGDIAFAYSYSNVLIEIQDTLRSSPAENTVMKKASLIGVSTTTTFY
	**********
AK334879	MLCGVLGYAAFGSSAPGNFLTGFGFYEPFWLVDIGNVCIVVHLVGAYQVFCQPIYQFVEGCARSRWPDSAFLH
XM_020316794	MLCGVLGYAAFGSSAPGNFLTGFGFYEPFWLVDIGNVCIVVHLVGAYQVFCQPIYQFVEGCARSRWPDSAFLH
	**********
AK334879	AERVLRLPAVLGGGEFPVSPFRLVWRTAYVVLTAVVAMLFPFFNDFLGLIGAVSFWPLTVYFPVEMYMAQAKV
XM_020316794	AERVLRLPAVLGGGEFPVSPFRLVWRTAYVVLTAVVAMLFPFFNDFLGLIGAVSFWPLTVYFPVEMYMAQAKV
	**********
AK334879	RRFSPTWTWMNVLSVACLVVSVLAAAGSVQGLIKDVAGYKPFKVS
XM_020316794	RRFSPTWTWMNVLSVACLVVSVLAAAGSVQGLIKDVAGYKPFKVS
	**********

Results show 100% sequence similarities with 0% gaps as analysed by Clustal Omega multiple sequence alignment. An asterisk (*) indicates similiar amino acid.