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Full Length Article

Microarray Analysis of Early Gene Expression in Wheat Roots Susceptible to Cereal Cyst Nematode *Heterodera filipjevi*

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Abstract

Wheat root cells undergo dramatic biochemical changes during the infestation of wheat root with cereal cyst nematodes (CCNs). In order to identify the differentially expressed genes prior to the formation of syncytium within the wheat (*Tritium aestivum* L.) roots susceptible to *Heterodera filipjevi*, a gene expression analysis was conducted at 24 h post-inoculation, using Agilent wheat whole-genome cDNA microarray containing 43,803 probe sets. The result of the analysis revealed that after the *H. filipjevi* infection, 820 transcripts were expressed differentially, among which approximately 60% of the transcripts were up-regulated, and the remaining were down-regulated. The expressions of the genes associated with defense, cell structure, and signal transduction, including those for peroxidases, hydroxyproline-rich glycoprotein, and ras-related proteins, were dramatically altered. The present study, therefore, provided novel insights into the CCN–wheat interaction. © 2020 Friends Science Publishers

Keywords: Cereal cyst nematode; Gene expression; *Heterodera filipjevi*; Microarray; Plant-nematode interaction; Wheat

Introduction

Cereal cyst nematodes (CCNs) are the most widely distributed parasites of cereals and grasses, which have caused substantial economic losses to wheat production (Smiley and Nicol 2009). In China, CCNs are known to be distributed across 16 provinces (Chen *et al.* 1991; Peng *et al*. 2012). Among the CCNs, *Heterodera avenae* and *H. filipjevi* negatively impact the wheat production, resulting in significant reductions of 15–80% in the yield annually (Li *et al.* 2010).

Most CCNs are able to complete only one generation of their life cycle during each crop season. The J2s (infective second-stage juveniles) penetrate the epidermal and cortical cells using their robust stylet, following which they migrate intracellularly toward the vascular stele (Jung and Wyss 1999). After the "migration period", the nematodes select an initial syncytium cell (ISC) and stay motionless for approximately eight hours. When this stage, referred to as the "feeding-preparation period", is completed, it is followed by the "feeding site period" which begins at approximately 18 h post-inoculation (hpi). When the nematodes enter into the ISC, they release cell-wall modifying proteins from their esophageal glands, inducing the formation of enlarged feeding cells. Thereafter, the nematodes feed on the water- and nutrient-conductive tissues in the host root, marking the completion of their life cycle (Sobczak and Golinowski 2009).

The infection of wheat roots with CCN leads to impairments in the physiological aspects and growth of the infected host plants. Several histological changes such as the formation of the syncytium, dissolution of surrounding cell walls, enlargement of nucleoli, accumulation of endoplasmic reticulum, deposition of callose, increase in the number of vacuoles, and appearance of nematode secretions near the stylet during the period between 18 hpi and 4 dpi have been reported (Mahalingam and Skorupska 1996). The CCN infection elicits the response of the host defense system, along with tremendous alterations in the gene expression in wheat cells. Signaling within both the nematode and the plant is necessary for the formation and maintenance of the syncytium (Davis *et al.* 2008; Gheysen and Mitchum 2009). Identification of active, differentially-expressed genes might provide insight into the molecular mechanisms of interaction between nematode and plant, and propose technologies for crop protection (Williamson and Kumar 2006; Klink and Matthews 2009). GeneChip microarray technology serves as a useful platform for studying alterations in gene expression during the process of nematode infection. A study was conducted on soybean–SCN (soybean cyst nematode) interaction using soybean array, focusing on the sensitive response of soybean to the SCN infection (Puthoff *et al.* 2003; Ithal *et al.* 2007a). Other related studies focused on SCN infection in susceptible and

resistant varieties (Klink *et al.* 2007b; Mazarei *et al.* 2011). In the present study, customized Agilent 4 × 44 K wheat whole-genome oligo microarray, containing 43,803 probe sets and spanning over 42,000 transcripts, was utilized for investigating early gene expression in the wheat roots infected with *H. filipjevi*. The present research would assist in understanding the wheat–CCN interaction during the onset of the syncytium establishment.

Materials and Methods

Experimental materials

An *H. filipjevi*-susceptible wheat cultivar Chinese Spring was used as a host in the present study (Zhang *et al.* 2012). The seeds from the host plant were surface-disinfected by soaking in 95% ethanol for 3 min, followed by further treatment with 10% sodium hypochlorite for 10 min in a laminar flow hood using sterile culture techniques. Seedlings of wheat were grown in sterile sand in flats (20 × 20 × 10 cm^3) in a growth chamber for one week, following which they were pulled out softly from the sterile sand, rinsed with sterile water, and dried using bibulous paper.

The cysts of *H. filipjevi* (pathotype Hfc-1) were obtained from Xuchang, Henan province, China (34.04°N, 113.74°E) (Li *et al.* 2010).

Treatments

Hatching of the cysts was achieved by following the method described ahead. The full cysts with bright color were separated, sterilized using 0.5% NaClO for 10 min, and washed several times with sterile water. The selected cysts were added into tubes containing 50 mL sterile water and stored at 4°C in a refrigerator for two months to retain moisture. Subsequently, the cysts were incubated at 15°C in an artificial climate incubator.

In order to prepare the pi-J2s (pre-infective second-stage juvenile nematodes), the hatched cysts inside the tubes were poured on a 250-μm mesh cloth and washed with sterile water. The washed pi-J2s were carefully harvested and placed into a clean flask, followed by the addition of sterile water until the volume of 100 mL was reached. After proper mixing, 100 μL of the sterile water containing pi-J2s were pipetted into a hemacytometer to evaluate the exact number of pi-J2s using a microscope. Ten repeats were averaged for the calculation of the concentration of J2s in the suspension. Finally, the J2s were diluted to a final concentration of 4000 individuals mL^{-1}.

Seedlings were placed on moistened filter paper placed inside plastic trays. The inoculum (nematode suspension) was added directly to the roots, at a final concentration of 1000 J2s/root. The blank control (mock-inoculated) was inoculated with the same amount of sterile water. The roots were then covered with a moistened sheet of germinating paper and placed in a plastic tray (size: 45 × 50 × 20 cm^3) with 1 cm water level at the bottom for humidity. The tray was then covered with a translucent plastic film and was maintained under a 16-h light/8-h dark photoperiod. Using the knowledge from previous experiments, the inoculated wheat roots were grown for 24 hpi only. The roots of the inoculated and mock-inoculated were washed; one inoculated seedling root–as a sample, and four samples were prepared as technical replicas using the same method. Four control samples were prepared using the same method. Finally, the root tissues were frozen using liquid nitrogen and stored at −80°C.

Root penetration assay

The inoculated roots were harvested at 12 h, 24 h, and 48 h post-inoculation (hpi), followed by soaking in 1.5% sodium hypochlorite solution for 15 min, and then washing with tap water to remove the excess sodium hypochlorite. The roots were stained using 1 mL of 3.5% acid fuchsin (Bybd *et al.* 1983). The solution was heated until boiling, followed by cooling to room temperature and then rinsing with tap water to remove the excess stain. The stained roots were used to prepare slide specimens, which were then observed under a microscope. Ten seedlings were selected at each sampling time point, and for each of these seedlings, 3 root tips were selected randomly, followed by counting the number of J2s in the root tissue. In order to analyze the number of J2s in the root tissue, one-way ANOVA, and Bonferroni multiple comparison test were performed using S.P.S.S. 20.0 software.

RNA extraction and agilent GeneChip hybridization

The time points selected for the collection of the materials were all within the period between the infusion of nematode in wheat and the formation of syncytium cells. Therefore, rather than the syncytium cells, the inoculated roots and the mock-inoculated roots were collected as the experimental materials. Total RNA was extracted from the experimental group and the control group using the Trizol reagent in accordance with the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). The concentration and purity of the extracted RNA were measured using a spectrophotometer, and the RNA Integrity Number (RIN) was verified using Agilent Bioanalyzer 2100 (Agilent).

Probes were prepared using Low Input Quick Amp Labeling Kit-plus for One-color (Agilent). An aliquot of two micrograms of total RNA from each sample was converted into complementary RNA labeled with fluorophore Cyanine 3-CTP (CY3c). The labeled cRNAs were further purified using RNeasy Mini Kit (QIAGEN) and RNase-Free DNase Set (QIAGEN, GmBH, Germany). The probes were evaluated for yield, concentration, amplification efficiency, and abundance of CY3c, using Nanodrop ND–1000 spectrophotometer (Thermo Scientific, USA) at A260 and A550.

The Agilent 4 × 44 K wheat whole-genome oligo

microarray, with 43,803 probe sets and spanning over 42,000 transcripts (design ID: 022297), was constructed on the basis of the wheat transcriptome information available in WormBase, RefSeq, Unigene, and TIGR databases. A total of eight microarrays of single dye were considered for each sample following the manufacturer's instructions (Agilent Technologies One-Color Microarray-Based Gene Expression Analysis). In brief, each slide was hybridized with 1.65 μg Cy3-labeled cRNA, in a hybridizing oven at 65°C and 10 rpm, using a gene expression hybridization Kit (Agilent Technologies, Santa Clara, C.A., U.S.). After 17 h of hybridization, the slides were washed with gene expression Buffer Kit (Agilent) in a staining dish (Thermo Shandon, Waltham, MA, USA).

Data analysis and gene annotation

Agilent chip scanner G2565CA was used with default settings for hybridized slides scanning at 550 nm. The image processing program of the feature extraction software 10.7 (Agilent) was utilized to process the scanned .tiff files and generate the standard data for statistical analysis. The original data were normalized using the quantile algorithm, followed by the processing of all the samples from the baseline to median using Gene Spring Software 11.0 (Agilent). Detected, undetected, and leaked data were marked as P, A, and M, respectively, and the standardized data were transformed into log2 values (Quackenbush 2002). Coefficients of variations (CVs) were calculated using the signal readings obtained from the ten replicates of the probe spots and were used to determine the stability in the Agilent array system. The detection percentages were also calculated from the number of detected spots (with flags, except A) and the number of total spots. All the CVs were below 10% (range: 4.13−6.19%), while the detection percentages ranged from 70.10 to 76.21%.

The Diffgene programs, including *t*-test and SAM (significant analysis of microarray) (Tusher *et al.* 2001) from Shanghai Biochip Co., Ltd. (SBC) analysis system (http://sas.ebioservice.com/, SAS), were used to screen the differentially expressed genes. The program was connected to the R-software (The R Project for Statistical Computing, http://www.r-project.org), the Gene Ontology website (http://www.geneotology.org), NCBI Entrez Gene, KEGG, Biocarta, MINT, among others. The average expression values for different groups were used to calculate the ratio of expression changes between the treated samples and the control samples. The criteria for the identification of the differentially expressed genes induced by the CCN-infection were as follows: (i) differential expression of genes between nematode-infected plants and mock-infected plants was statistically significant ($P \leq 0.05$); (ii) the fold change (FC) in the expression of inoculated sample and control sample was ≥ 2 (up-regulated) or ≤ 0.5 (down-regulated); and (iii) the flag/call value for each probe site "A" did not occur in either group.

The genes exhibiting a change of greater than 2-fold or less than 0.5-fold in response to the CCN infection were classified according to their logarithmic transformation rate using the hierarchical clustering method (Anderberg 1973). The annotation information was obtained from GenBank. BLASTx search was applied on three databases, which included these genes assigned to the known genes of rice or Arabidopsis–the TIGR rice pseudomolecules database, the KOME peptide sequence database, and the TIGR Arabidopsis annotation database (ATH1). Accession numbers of the wheat mRNA (with certain exceptions such as those for rice) were identified through BLAST search in NCBI. Gene classification based on gene ontology (GO) was performed using the hierarchical clustering program in R software. In the present analysis, the GO terms with an FDR (false discovery rate)-adjusted *P*-value of ≤ 0.05 were retained.

Real-time qRT-PCR verification

A total of six differential genes from various functional categories were selected randomly for the validation of the microarray data using real-time quantitative reverse transcription PCR (qRT-PCR). Gene-specific primers based on the sequences of probe sets in the microarray were designed using Primer Premier 6.0, and the specificity of the primer pairs was verified using the blast program in NCBI (Table 1). The wheat actin gene was used for data normalization. Total RNA samples were treated with DNase to remove residual any traces of genomic DNA before the synthesis of the first strand of cDNA from the Oligo dT primer labeled with SuperScript III RT (Invitrogen). PCR was performed in a 96-well plate inside Bio-Rad iCycler real-time PCR system (Bio-Rad, Hercules, CA) using SYBR Green I PCR Master Mix (Bio-Rad). The PCR reaction conditions were as follows: 95°C for 10 s, followed by 40 cycles at 94°C for 5 s, 60°C for 25 s, and 72°C for 31 s. In order to evaluate the overall specificity, the non-cDNA template was used as a negative control. Each experiment was performed in triplicate. Gene expression was quantified using the relative quantitative method ($2^{-\Delta\Delta CT}$) and was compared with internal control. Data from the real-time PCR populations were analyzed through an independent t-test in S.P.S.S. 20.0 using an alpha of 0.05 (Canales *et al.* 2006).

Results

Time course of *H. filipjevi* infection in wheat roots

The time course of J2s infection was monitored, and the results are presented in Figs. 1 and 2. At 12 hpi and 24 hpi, J2s penetrated the wheat roots and migrated toward the vascular tissue (Fig. 1A and 1B). Subsequently, the nematodes established infection by selecting a cell as the feeding site. The number of J2s in the root tissue at 48 hpi

Table 1: Primer sets used in the qRT-PCR analysis

Gene symbol	Probe set	Primer sequence		Product size (bp)
		Forward	Reverse	
EF368363	A_99_P124345	GTCGTCGGGAGGAAGAAAGG	AGCCGTCGTCGAGGATGT	91
JX679079	A_99_P489907	AGTCGGAGCTACAGCGTGTG	GAGGACGGCTGGTTGTTGTAG	163
DQ013358	A_99_P072835	CCGAGAACAGAGTCCCGAGTT	CCATCCAGCAAGACCAACGA	175
EU082065	A_99_P158407	CGATGATATTCAGGGCACAGC	CCAGCACCAAGGAAGAGGTAAG	108
DQ334410	A_99_P195090	CGGAGTTTATGTAGCTGATATGACTG	CCCTGCCGTTGTTGTGC	139
EF368361	A_99_P148642	CGACTACTCGCTGCTTCCG	CCGCTCGTACATGTTCATCG	64
AB181991	Wheat actin	TCCAATCTATGAGGGATACACGC	GCCAGCAAGGTCCAAACGA	58

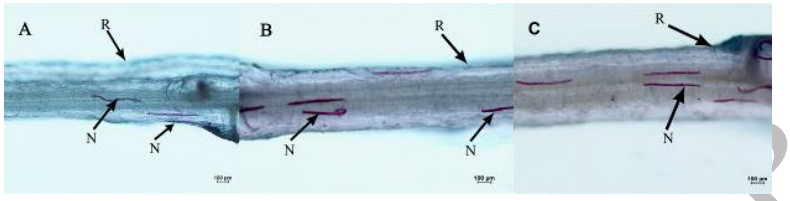

Fig. 1: Micrographs showing progression of *Heterodera filipjevi* infection in Wheat cultivar Chinese Spring roots that were infected with *Heterodera filipjevi* (pathotype Hfc-1) and grown for different times. Nematodes in wheat roots were stained with acid fuchsin stain. Wheat roots sections were bleached with sodium hypochlorite. **A**, at 12 hpi, a small amount of J2s already penetrated the wheat root; **B**, at 24 hpi, more J2s penetrated wheat root and reached the vascular tissue; **C**, 48 hpi, almost entirely invaded J2s reached the vascular tissue. N=nematode and R=wheat root

was significantly higher than that at 12 hpi (Fig. 1C). The number of J2s in the root tissue at 24 hpi exhibited no significant differences from that at 12 hpi and 48 hpi (Fig. 2). Therefore, the 24 hpi time point was selected for subsequent gene profiling analyses, which is also consistent with the time at which the CCN began forming the syncytium.

Gene expression profiling

The microarray analysis identified 820 transcripts that exhibited differential accumulation in the susceptible cultivar with a fold change ratio of ≥ 2 or ≤ 0.5 with a *P*-value ≤ 0.05; in total, 496 transcripts were up-regulated, while 324 transcripts were down-regulated. In all the transcripts exhibiting differential expression, 336 up-regulated transcripts and 292 down-regulated transcripts exhibited an FC ratio between two and four, 109 up-regulated transcripts, and 32 down-regulated transcripts exhibited an FC ratio between four and eight, and 51 up-regulated transcripts exhibited an FC ratio above eight. When a *P*-value of ≤ 0.01 was used, a total of 595 differentially-expressed transcripts were identified, among which 235 were up-regulated and 205 were down-regulated with an FC ratio between two to four, 84 were up-regulated and 23 down-regulated with an FC ratio between four to eight, and 48 were up-regulated at an FC ratio greater than eight (Fig. 3).

Among a total of 820 differentially-expressed genes that were identified, 317 genes had been annotated, and approximately 86% of these annotated genes were identified as having known or speculative functions. Analysis of the annotated data revealed that greater than 17% of these genes were associated with amino acid/protein metabolism, energy metabolism, carbohydrate metabolism, and fatty acid, and lipid metabolism. Further, 24% of these were involved in transcription regulation, 12% were involved in translation, 15% were involved in signal transduction, and the rest were related to cell structure, stress/defense, and transport (Fig. 4). In order to explore the potential pathogenic mechanisms and resistance resources, the focus of the present study was maintained on the functional classifications related to cell structure, stress/defense, transcription factors, signal transduction, and metabolism.

Major functional categories of response genes in wheat roots

Cell wall, defense, and stress response-related genes: Cell wall strengthening and development are generally viewed as a part of early defense response. Genes related to the cell wall, such as the cellulose synthetase (CESA) gene, play a key role in the defense response to nematode infection. In the microarray analysis conducted in the present study, nine cell wall-related genes exhibited significant changes. Among these genes, glycosyl hydrolase family 5 (JV987084), and glucuronosyltransferase (JV945131), which are involved in the synthesis of glucuronoxylan hemicellulose in secondary cell walls, as well as pectin lyase (AK335586) and hydroxyproline-rich glycoprotein (DR737925) that are involved in cell walls maintenance and synthesis, were observed to be up-regulated. In contrast, the cellulose synthesis-related gene cellulose synthase-like H1 (AK332242), pectin lyase (AK335102), and hydroxyproline-rich glycoprotein (AK072978*, rice GenBank accession), which are involved in cell wall synthesis and maintenance, were observed to be down-regulated (Table 2).

Several genes in wheat were also differentially expressed under the cyst pathogen stresses. Among these

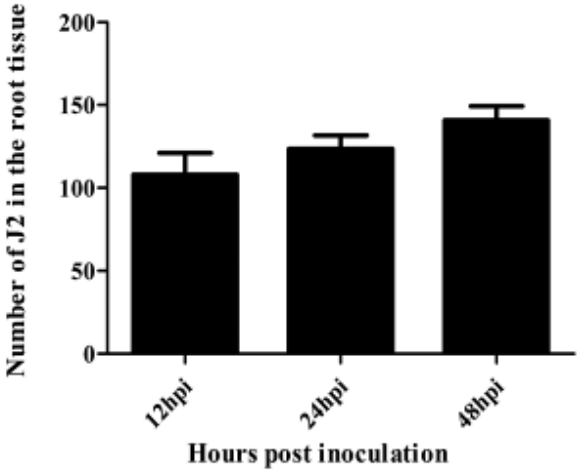

Fig. 2: Number of J2 penetrating into roots of *H. filipjevi* -infected assayed at 12, 24 and 48 hpi. Values are means ±SE. Data were analyzed using one-way analysis of variance and the Bonferroni multiple comparison test in S.P.S.S. 20.0

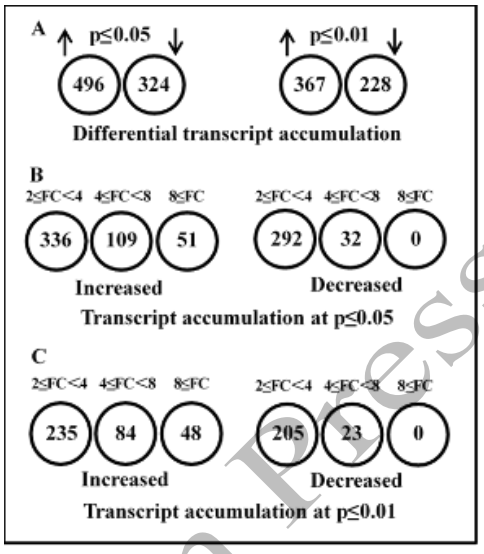

Fig. 3: Venn diagrams depicting the numbers of differential transcripts in the Chinese Spring (susceptible) after *H. filipjevi* infection. A, General numbers of transcripts that displayed differential accumulation at different false discovery rate (FDR) of *p* values and FC $\geq$ 2 (up-regulated) or FC $\leq$ 0.5 (down-regulated). ↑ = up-regulated and ↓ = down-regulated. B, Transcripts that displayed up-regulated or down-regulated accumulation with different FC ratio cutoff at an FDR with a *P* value of $\leq$ 0.05. C, Transcripts that displayed up-regulated or down-regulated accumulation with different FC ratio cutoffs at an FDR with a *P* value of $\leq$ 0.01

alterations, the gene (JV948284) similar to the Arabidopsis gene, which encodes proline-rich extensin, peroxidase (EF028783, JW017812), and monocopper oxidase (JP837828) and performs a [hypothetical] role in the general defense response of plants, was up-regulated. In contrast, two genes, including the pathogenesis-related protein gene (JP233598) and thylakoid-bound ascorbate peroxidase (AF532972), were down-regulated (Table 2). However, a few other defense-response genes were observed, usually 2–8 days after the SCN invasion, and were absent during the 24 h after challenge with *H. filipjevi.*

Three genes encoding the NBS-LRR disease-resistance proteins (GAJL01182435, CJ848889, and CV767657), the rust-resistance protein gene (JP940665), and the mildew-resistance gene (MLO5, GAJL01189557) were up-regulated. Notably, the gene encoding a protein similar to the resistance protein candidate (CV767657) was up-regulated, exhibiting an increase of greater than 10.0 folds and an FC ratio of 15.3837, while the other two genes corresponding to the NBS-LRR disease-resistance proteins (DP000010*, rice GenBank accession; AK336044) were down-regulated (Table 2).

Transcription factors and signal transduction-related genes: The wheat genome is known to contain at least 1,127 predicted transcription factors (TFs), which have been classified into 57 families (Wheat Transcription Factor database-PlantTFDB; http://planttfdb_v1.cbi.pku.edu.cn:9010/web/index.php?sp=ta). In the present study, 43 differentially expressed TFs were identified, which belonged to approximately 18 families, namely, ABI3-VP1, AP2-EREBP, ARF, ARID, bZIP, C2H2, C3HC4, CCAAT, FHA, HB, HLH, HMG, MYB, NAC, TCP, TLP, trihelix, and WRKY. Among these differentially expressed TFs, most of the members of the WRKY, MYB, and AP2-EREBP families were up-regulated, while the members of C2H2, C3HC4, HB, and TLP families were either up-regulated or down-regulated.

Forty-eight signaling-related genes were identified as infection responsive genes, 32 of which were up-regulated. Twenty-one of the signaling-related genes were identified as protein kinase genes based on the query against the database of rice kinase (http://phylomics.ucdavis.edu/kinase/). All the protein kinases in the database are divided into the following seven groups: AGC kinases, CaM kinases, CK1, CMGC, STE, TKL, and the non-classified. Among the differentially-expressed protein kinase genes, ten genes belonging to the TKL family were up-regulated, while one gene from the uridine kinase family was up-regulated remarkably with an FC ratio of 11.8762. Three genes from the CMGC family and one gene from the CK1 family were down-regulated.

The expressions of several other genes relevant to cell signaling and communication were altered as well. Two genes associated with synaptic signaling (GAEF01091554 and GAJL01128233), two ras-related protein genes (CD877975 and GAJL01223381), two signal-transducing G protein genes (JW026210 and BT008975), three calcium-related genes (JV954788, HE996494, and JV998694), the protein phosphatase 2C gene (GAJL01007051), and one cyclin-like F-box domain-containing protein gene (AK332287) were up-regulated. The GTPase-activating

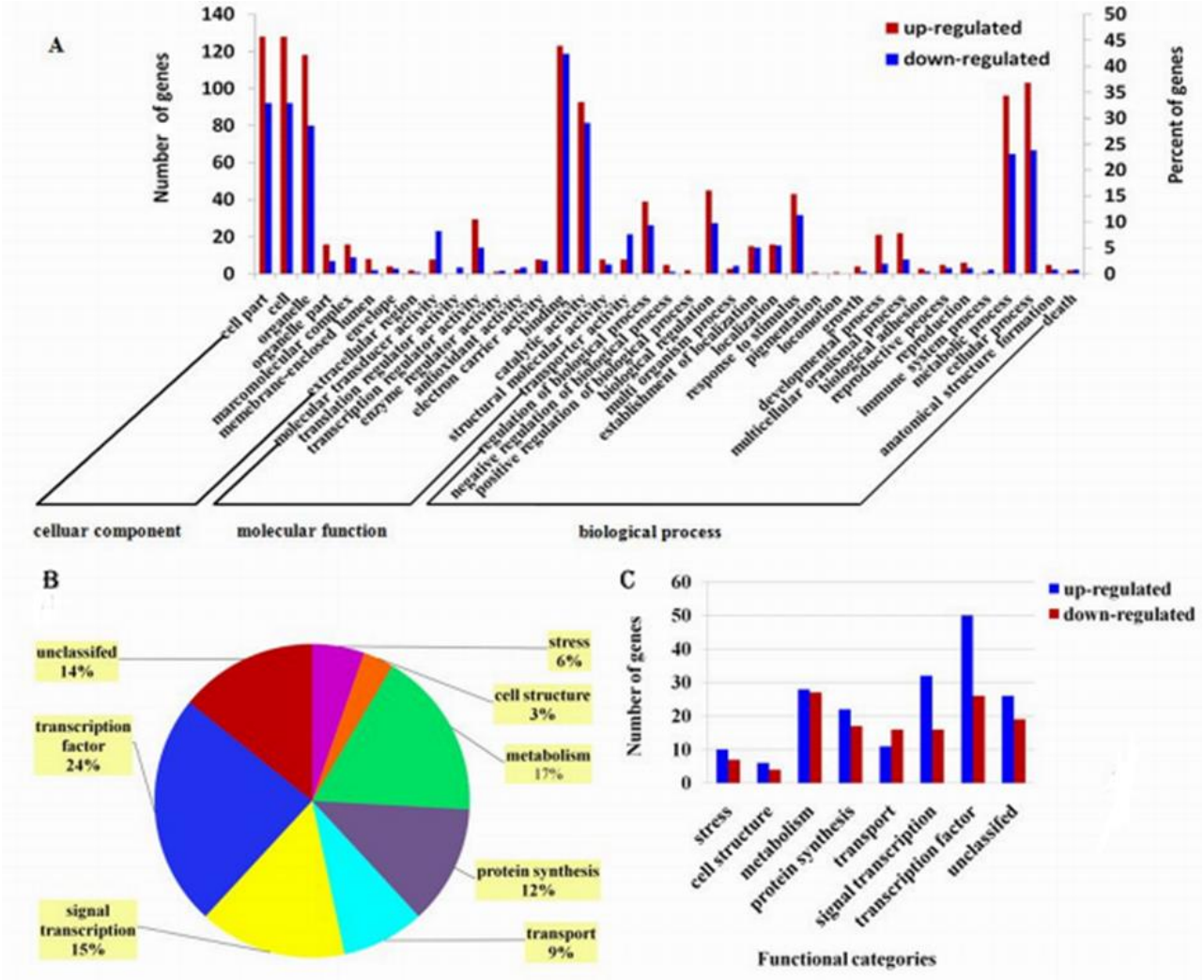

Fig. 4: Functional classification of the 317 annotated genes out of 820 significantly regulated genes in *H. filipjevi* -infected wheat roots.

A, The bar chart representation of Gene Ontology (GO) classification of the 317 annotated genes. It includes three main categories: biological processes, cellular components, and molecular functions. The *y*-axis on the left indicates the number of up-regulated genes and down-regulated genes in a category, respectively. The *y*-axis on the right indicates the percent of a specific category of up-regulated genes and down-regulated genes in that main category, respectively. B, Functional distribution of the 317 annotated genes in different tentative functional categories. Of the 317 annotated genes, 14% are unclassified for function unknown. C, The bar chart representation of the 317 annotated genes in different tentative functional categories. The *y*-axis indicates the number of up-regulated and down-regulated annotated genes.

protein gene (AK332208) and the remorin protein gene (AK330902) were down-regulated (Table 3).

The genes encoding nine different secondary transporters (CJ628086; C99253*, rice GenBank accession; JW033866; AP008217*, rice GenBank accession; AB539586; JP210959; JW014975; CA486682; AK331727), as well as the five ATP-dependent transporter genes (JV979550; BT008921; AK332850; JV980384; AK066618*, rice GenBank accession) were down-regulated, while three genes associated with multidrug resistance (AK332498; GAJL01218900; AP008212*, rice GenBank accession) were up-regulated (Table 3).

Genes involved in metabolism: Metabolic enzymes are classified into six groups: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. A total of 55 genes encoding metabolic enzymes were identified as being differentially expressed, among which 30 genes were up-regulated, including the 14 genes associated with protein and amino acid metabolism and the 14 hydrolase encoding genes. Genes encoding Glucan endo–1,3-β-glucosidase (JP861804), NADP-dependent malic enzyme (JP845417), galactose oxidase (AK100645*, rice GenBank accession;

Table 2: The selected genes from the 317 annotated genes that involved are involved in cell structure, defense, and transcription factor in wheat roots at 24 hpi after *H. filipjevi* inoculation. GenBank accession with "*"is rice gene accession

Functional category	GenBank accession	Fold change	Description
Cell structure			
Up-regulated	DR737925	7.237	Similar to Hydroxyproline-rich glycoprotein DZ-HRGP precursor
	AK335586	2.7477	Pectin lyase fold family protein
	JV945131	2.0119	Probable glucuronosyltransferase involved in the synthesis of glucuronoxylan hemicellulose in secondary cell walls
	JV987084	2.0098	Cellulase, glycosyl hydrolase family 5
Down-regulated	AK072978*	0.4707	Hydroxyproline-rich glycoprotein family protein
	AK335102	0.3277	Pectin lyase fold family protein
	AK332242	0.2467	Cellulose synthase-like H1
Stress/defense related			
Up-regulated	CV767657	15.3837	Similar to NBS-LRR Resistance protein candidate
	GAJL01189557	7.4513	Mildew resistance gene, MLO5
	JP940665	4.319	Rust resistance protein
	JW017812	3.4493	Similar to Peroxidase (EC 1.11.1.7),
	JP837828	2.5819	Monocopper oxidase
	CJ848889	2.2945	NBS-LRR type resistance protein
	JV948284	2.1583	Proline-rich extensin-like family protein
	EF028783	2.0491	Similar to Peroxidase precursor (EC 1.11.1.7)
	GAJL01182435	2.0401	NBS-LRR disease resistance protein
Down-regulated	JP852719	0.4913	Similar to Thylakoid-bound ascorbate peroxidase (EC 1.11.1.11)
	JP233598	0.4531	Similar to Steroid membrane binding protein-like, OsFBT7-F-box and tubby domain-containing protein
	AK336044	0.4076	Similar to NBS-LRR disease resistance protein homolog
	DP000010*	0.328	Similar to NBS-LRR type resistance protein
Transcription factor			
Up-regulated	JF951950	84.2977	MYB-like DNA-binding domain containing protein
	GAJL01041979	42.8642	Similar to AP2 domain containing protein RAP2
	EU665453	5.2611	Similar to WRKY transcription factor 59
	GAEF01013195	4.8624	WRKY transcription factor 74
	JV951032	4.8347	MYB DNA-binding domain containing protein
	JP923124	3.2649	Ethylene-responsive factor,AP2
Down-regulated	GAEF01037412	0.4614	Homeobox protein knotted-1–1, HB
	JP848030	0.4278	OsFBT7 – F-box and tubby domain containing protein, TLP
	HP633405	0.3291	Zinc finger, C3HC4 type, domain containing protein
	AK335450	0.2873	Zinc knuckle domain containing protein, C2H2

GAEF01030908), and 2-oxo acid dehydrogenase acyltransferase domain-containing protein (BJ270556) were up-regulated, while seven sugar-related genes were down-regulated (Table 3). Six of the identified genes were involved in lipase metabolism, among which four genes encoding fatty acid desaturase (JW031840), thioesterase (AP003572*, rice GenBank accession), hydrolase (JP844213), and lipase family protein (CA635498) were down-regulated. In contrast, those encoding lipase-related protein (BJ222289) and esterase/lipase/thioesterase domain-containing protein (JP901515) were upregulated (Table 3). With the only exception of cytochrome P450 (CK206961), most of the genes associated with drug metabolism were up-regulated at 24 hpi. Most of the genes encoding chloroplast-related enzymes were up-regulated, while those encoding photosystem I subunit L (JP832184) and porphobilinogen deaminase (AK333284) were down-regulated (Table 3). Taken together, these results indicated that when comparing the genes associated with catabolism and those associated with anabolism, the number of up-regulated genes was higher [by a small number] in the former group. This possibly reflected the situation around the establishment of the feeding site.

Real-time qRT-PCR to verify microarray data: In order to verify the microarray data and study the dynamic changes occurring in the gene expression, the expression profiles of the six genes that were altered dramatically (five up-regulated and one down-regulated) were analyzed using real-time qRT-PCR (Fig. 5). The results demonstrated that cell wall-associated kinase 4 (WAK4), NADP-dependent malic enzyme (NADP-ME), ethylene-responsive factor-like transcription factor ERFL 2b, WRKY 80, and WRKY 10 were significantly up-regulated after the *H. filipjevi* infection, which was consistent with the microarray data. Nevertheless, certain exceptions were observed. The expression of the WRKY 72-b gene in the real-time qRT-PCR analysis was less than that in the microarray data. However, in general, the data from real-time qRT-PCR confirmed the expression trend of the corresponding genes observed from the microarray analysis.

Discussion

Infection in host plants with the cyst nematode is a complicated process, which could be generally divided into several distinct stages. Although two dpi or three dpi are

Table 3: The selected genes from the 317 annotated genes that are involved in signal transduction, transport, and metabolism in wheat roots at 24 hpi after *H. filipjevi* inoculation. GenBank accession with "*"is rice gene accession

Functional category	GenBank accession	Fold change	Description
Signal transduction			
Up-regulated	CD877975	134.1545	Ras-related protein
	AK332287	7.3067	Cyclin-like F-box domain containing protein
	JV998694	4.9537	calcium ion binding
	BT008975	4.2439	signal-transducing G protein
	JV954788	2.7883	Calreticulin family protein
	HE996494	2.5925	IQ calmodulin-binding region domain containing protein
	GAJL01128233	2.5116	synaptic transmission
	JW026210	2.5073	signal-transducing G protein
	GAJL01007051	2.4449	Similar to Protein phosphatase 2C-like
	GAJL01223381	2.2173	Ras-related protein
	GAEF01091554	2.1911	synaptotagmin
Down-regulated	AK332208	0.4977	GTPase-activating protein
	AK330902	0.3848	Remorin protein
Transport			
Up-regulated	AK332498	75.3024	Multidrug resistance protein
	AP008212*	3.696	Similar to multidrug resistance associated protein 1
	GAJL01218900	3.2219	Multidrug resistance protein
Down-regulated	AK066618*	0.4859	F-ATPase
	CA486682	0.446	Secondary transporter
	JW014975	0.4427	Secondary transporter
	AK331727	0.4408	Secondary transporter
	JP210959	0.4036	Secondary transporter
	AB539586	0.3931	Secondary Transporter
	AP008217*	0.3845	Secondary transporter
	JV980384	0.3718	P-ATPase
	AK332850	0.3347	ATP-Dependent ABCB, ABC, ABCB
	BT008921	0.3235	ATP-Dependent ABCB, ABC, ABCC
	JV979550	0.2769	ATP-Dependent ABCB, ABC, ABCB
	JW033866	0.2622	Secondary transporter
	C99253*	0.2473	Secondary transporter
	CJ628086	0.2135	Secondary transporter
Metabolism			
Up-regulated	AK100645*	7.325	Galactose oxidase
	BJ270556	6.8057	2-oxo acid dehydrogenases acyltransferase domain containing protein
	BJ222289	4.1343	Lipase-related
	GAEF01030908	2.8625	Galactose oxidase
	JP845417	2.4679	NADP-dependent malic enzyme
	JP901515	2.4033	Esterase/lipase/thioesterase domain containing protein
	JP861804	2.3658	Glucan endo–1,3-beta-glucosidase precursor
Down-regulated	CK206961	0.4998	Similar to Cytochrome P450 76C2 (EC 1.14.-.-)
	CA635498	0.4956	Lipase class 3 family protein
	JP844213	0.4392	hydrolysis
	JP832184	0.4075	PSAL (photosystem I subunit L)
	AP003572*	0.3598	Thioesterase family protein
	JW031840	0.3454	Fatty acid acyl-CoA desaturase family protein
	AK333284	0.2012	Similar to Porphobilinogen deaminase

often selected for observing the development of syncytium, the previously proposed time point for the initial establishment of the feeding cell was 18 hpi (Sobczak and Golinowski 2009). In the present study, 24 hpi was selected as an important time point for the screening of early responses of wheat to CCN infections. Traditional techniques for evaluating gene expression could detect a limited number of genes. With the employment of a recently-developed microarray to analyze the alterations in gene transcription in susceptible wheat roots at 24 hpi, the present study became a pioneer in demonstrating the differentially expression of genes in wheat after the CCN infection using a whole-genome microarray. This analysis generated huge data that provided indications regarding the expressions of a large number of wheat genes during the critical period of infection when the CCN began to establish feeding sites. This data also revealed the commonality and uniqueness in the gene expression profiles between the compatible wheat–CCN interaction and soybean–SCN interaction at similar time-points (Puthoff *et al.* 2003; Khan *et al.* 2004).

A few studies investigated the alterations in gene expression after infection with SCN in both susceptible and resistant soybean cultivars, during an extended period of six hpi to eight dpi (Alkharouf *et al.* 2006). These studies demonstrated that the gene expression patterns of soybean

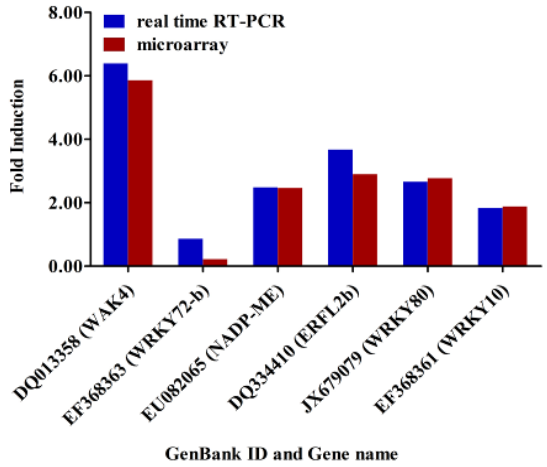

Fig. 5: Quantitative Real-Time PCR verification of the six differential genes expression level. Transcript levels were presented as relative values that were normalized with respect to the level of wheat actin gene

varied remarkably with progress in nematode infection. Remarkably down-regulated gene expression was observed at 24 hpi in the susceptible soybean infected with SCN. Similar to the above-stated finding, the present study also revealed that the number of up-regulated and down-regulated genes in susceptible wheat at 24 hpi accounted for 60 and 40% of the total differentially-expressed genes, respectively, thereby exhibiting a unique gene expression profile. The present study also suggested that the down-regulation of genes upon CCN infection might be as important as the up-regulation of genes for the successful establishment of the feeding site and the subsequent successful parasitism.

The present study revealed an up-regulation of two peroxidase precursors (EF028783 and JW017812) and one monocopper oxidase (JP837828), and the down-regulation of thylakoid-bound ascorbate peroxidase (JP852719) and a protein similar to peroxidase I (JP233598). It is known that among the numerous defense-related proteins, class III peroxidase (Prx, EC 1.11.1.7) is involved in auxin metabolism, cell wall elongation, and stiffening, and the protection of plants against abiotic and biotic stresses (Kawano 2003; Cosio and Dunand 2009). Several studies have demonstrated that peroxidase is involved in the defense response of *H. avenae* (Al-Doss *et al.* 2010; Simonetti *et al.* 2012). Seven groups of peroxides have been identified in wheat, three of which were induced in both susceptible and resistant lines at four dpi and seven dpi, respectively, in a previous study (Simonetti *et al.* 2012). In addition, ascorbate peroxidases (APX, EC 1.11.1.11), which are class I peroxidase members, were demonstrated to be induced in similar and higher magnitudes in the anti-infection genotypes and the susceptible genotypes, respectively (Simonetti *et al.* 2010). Monocopper oxidases are also supposed to participate in cell wall expansion. Plants defend against nematodes by using various mechanisms, such as the pathogenesis-related (PR) proteins, production of phyto-alexins and hypersensitive response (H), lignification, oxidative burst, and reinforcement of cell wall (Almagro *et al.* 2009). When plants are under biotic or abiotic stress, the expression of defense response-related genes is altered significantly after signal burst (Whitham *et al.* 2006; Użarowska *et al.* 2009). The findings of the present study confirmed that peroxidases and monocopper oxidases were important pathogenesis-related proteins (Almagro *et al.* 2009).

Besides Prxs and cellulose (AK332242), one wheat gene (JP233598), which is similar to the pathogenesis-related protein gene in *Arabidopsis*, was down-regulated. The expression of genes encoding SAM22, Kunitz trypsin inhibitor, germin-like protein, chitinases, and lipoxygenase, which have been previously demonstrated to often respond dramatically in susceptible soybean (Alkharouf *et al.* 2006), was not identified in susceptible wheat. Surprisingly, several genes related to defense proteins were observed to be dramatically regulated by the CCN infection, such as those related to the NBS-LRR proteins (DP000010*, rice GenBank accession; AK336044; GAJL01182435; CJ848889; CV767657) and the genes encoding proteins involved in plant resistance (Alkharouf *et al.* 2006; Klink and Matthews 2009). The contrary finding in the present study indicates the importance of down-regulation of resistance genes in susceptible hosts during the formation of the functional syncytium.

The present study revealed that wheat genes encoding proteins similar to *Arabidopsis thaliana* proline-rich extension (JV948284), hydroxyproline-rich glycoprotein (DR737925), glycosyl hydrolase family 5 (JV987084), and pectinesterase (AK335586) were up-regulated, which was consistent with the previous findings in susceptible soybean at a similar time point, although the gene encoding expansin was not altered at 24 hpi. The results demonstrated that most cell wall-related genes were influenced by the different infection patterns of nematodes, which was not completely consistent with the previous studies on the induction patterns of cell wall structural proteins, mainly extensins, in tobacco (Niebel *et al.* 1993) and soybean (Khan *et al.* 2004) in an interaction compatible with cyst nematode. Moreover, several cell wall-related genes such as cellulose synthase-like H1 (AK332242), pectin lyase fold family protein (AK335102), and hydroxyproline-rich glycoprotein (AK072978*, rice GenBank accession) were down-regulated, indicating that the down-regulation of these genes probably plays a major role in the establishment of feeding site of nematodes. The migration and establishment of feeding site often induce degradation of cell walls, triggering a series of alterations in cell wall-related genes. Extensive changes in cell wall structure are the markers for syncytial development (Ithal *et al.* 2007b). Recently, several molecular studies have demonstrated that the genes encoding cell wall modifying proteins, including reversible glycosylated polypeptides, different kinds of

glycosyltransferases, xylose glucan endotransferases, β-1,4-endoglucanases, α-expansion, and repeated proline-rich proteins, are differentially expressed in response to soybean cyst nematode (Ithal *et al.* 2007a; Mazarei *et al.* 2011). In particular, xyloglucan endotransglycosylases were observed to be expressed specifically within the cyst nematode-induced feeding cells (Ithal *et al.* 2007b). In soybean–SCN compatible interaction, the genes encoding repetitive proline-rich protein extensin, cellulose, and expansin, were observed to be up-regulated upon SCN infection (Ithal *et al.* 2007a; Klink *et al.* 2007b). These structural proteins might have a role in strengthening the syncytium walls to protect the nutrient contents during a compatible cyst nematode interaction (Khan *et al.* 2004; Alkharouf *et al.* 2006).

In the present study, most of the signal transduction-related genes (32/48) were regulated after CCN infection. TFs belonging to WRKY, MYB, and AP2-EREBP families were up-regulated, which was consistent with the finding of a previous study on soybean that TFs from the WRKY family were persistently up-regulated in susceptible soybean from six hpi to eight dpi (Alkharouf *et al.* 2006).

In the present study, genes for fifty-five metabolic enzymes were identified as differentially expressed genes, among which 30 genes were up-regulated. Seven sugar-related genes were down-regulated in susceptible wheat at 24 hpi; this was inconsistent with the previously reported observations in susceptible soybean at six dpi and eight dpi, according to which, several sugar-related genes were up-regulated (Alkharouf *et al.* 2006). The findings indicating the involvement of certain identified genes in lipase metabolism was contrary to the previous findings in soybean (Alkharouf *et al.* 2006; Klink *et al.* 2007a, b). A few up-regulated genes were identified to be associated with catabolism, which was contrary to previous findings in soybean (Klink *et al.* 2007a, b); this might be related to the establishment of the feeding site.

Furthermore, transporter-related genes were identified in the present study, with the genes encoding ATP-dependent transporters and secondary transporters being down-regulated and the genes encoding multidrug resistance protein being strongly up-regulated. These findings could reflect the dramatic pathological changes associated with the formation of syncytium.

In the present study, 1.1% of the identified genes were up-regulated genes, and 0.7% were down-regulated genes, similar to the previously reported ratios for the whole root sample of soybean (1.0 and 0.6%, respectively) (Alkharouf *et al.* 2006). However, the complexity of the wheat–CCN interactions suggested more gene expressions are involved in the process of compatible infection.

Conclusion

In conclusion, the results of the present study, first of all, unraveled the gene expression profiles in the susceptible wheat after the CCN infection. A total of 820 transcripts were functionally annotated. With the inclusion of samples from CCN-infected wheat roots, the results obtained in the present study revealed important information regarding the gene expression associated with the wheat response to the infection, in terms of defense, cell structure, and signal transduction. Accordingly, a number of transcripts and their functional annotations provided information that would allow exploring the molecular mechanism underlying CCN resistance in *Tritium aestivum* L. Furthermore, certain genes were identified to be dramatically altered in the CCN-infected wheat, which indicated that further investigation is required to understand the molecular mechanism occurring in CCN-infected wheat.

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Author Contributions

X.X. conceived and designed the research; X.X., H.Y. and Y.G. performed the experiments. The manuscript was written by X.X. with input and corrections from all coauthors.

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