



**Full Length Article**

## Analysis of Early Transcriptomes of Huipizhiheidou and Liaodou 15 Infected by Soybean Cyst Nematode (*Heterodera glycines*)

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### Abstract

We used the Illumina HiSeq™ 2000 system to analyze the transcriptomes of the disease-resistant soybean variety Huipizhiheidou (ZDD2315) and the susceptible soybean variety Liaodou 15 before and after the infection of the soybean cyst nematode (SCN), race 3 (HG type -7). According to the reference genome, we found that 10 days after inoculation with SCN, 389 genes were up-regulated and 344 genes were down-regulated in the disease-resistant soybean variety of Huipizhiheidou, whereas 222 genes were up-regulated and 691 genes were down-regulated in the susceptible soybean variety of Liaodou 15. Gene ontology functional enrichment analysis showed that the differentially expressed genes were mainly involved in energy metabolism, secondary metabolism and the metabolism of macromolecules, such as carbohydrates, lipids and amino acids. KEGG metabolic pathway analysis showed that the differentially expressed genes were associated with Plant-Pathogen Interactions,  $\alpha$ -linoleic acid metabolism and phenylpropane synthesis and participated in the inhibition of the soybean cyst nematode. © 2018 Friends Science Publishers

**Keywords:** Soybean; Soybean cyst nematode; Infection; Transcriptome analysis

### Introduction

Plant parasitic nematodes are obligate parasites causing serious reduction in crop yields (Abad *et al.*, 2008; Ali *et al.*, 2015). Soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, one of the most devastating pathogens of soybean, causes more than \$1 billion in yield losses annually in the US alone (Koenning and Wrather, 2010). Methods to prevent and control SCN mainly include the cultivation of disease-resistant varieties, crop rotation, and the use of chemical nematode killer, of these prevention and treatment methods, the cultivation of disease-resistant varieties is the most cost-effective (Liu *et al.*, 2011). Among the many soybean germplasm resources resistant to SCN, the small black soybean, which has a black seed coat and small black beans, presents good resistance to SCN. In the United States, Peking and PI88788 were used as the source of resistance to cultivate more than 100 resistant varieties, which have been widely used in soybean production in the United States. The code number of Huipizhiheidou in the national germplasm resources is ZDD2315, this soybean has a black seed coat and is native to the Xing County of Shanxi Province, China. Li *et al.* (1991) screened a Huipizhiheidou variety with excellent resistance to the SCN physiological races of 1, 3, 4 and 5, showing strong resistance and stability. Wu (2003) conducted a classification and deduction study on the SCN-resistant genes of Chinese

small black soybean varieties and showed that Huipizhiheidou was the best source for resistant materials, as this variety showed resistance to SCN races 1, 2, 3, 4, 5, 7 and 14. Wang *et al.* (2002) used Huipizhiheidou as the paternal line in a large hybridization study to select nematode-resistant varieties, such as Zhonghuang 12, Zhonghuang 13 and Zhonghuang 17. To understand the differential gene expression of SCN resistance in Huipizhiheidou, we used the Liaodou 15 variety as the disease-susceptible control and applied digital gene expression profile analysis to detect the gene expression levels in Huipizhiheidou infected with SCN for 10 days. Based on differences in the expression of resistance genes, the genes related to SCN resistance in Huipizhiheidou were screened to establish the mechanism of resistance and to lay the foundation for the molecular breeding of nematode resistance.

### Materials and Methods

#### Soybean Varieties

The soybean variety Huipizhiheidou (ZDD2315), which is resistant to SCN race 3, and the soybean variety Liaodou 15, which is susceptible to SCN race 3, were supplied by the Nematology Institute of Northern China at Shenyang Agricultural University. The soybean seeds were soaked in

a 0.5% NaOCl solution for 10 min and then rinsed with sterile water. The seeds were placed in a large culture dish containing wet sterile cotton gauze and germinated in a 25°C incubator with light for 48 h. After the radicle had grown to approximately 1 cm, the seeds were transplanted to a plastic pot (15 cm x 15 cm) containing sterilized sandy soil, with three plants per pot.

### Nematode Inoculation

The nematode for inoculation was SCN race 3, which was bred in the susceptible soybean variety Liaodou 15 in a 25°C greenhouse. The cysts were separated from the collected soil samples using the panning-sieving method. The cysts were first disinfected with 0.5% NaOCl solution for 3 min, washed three times with sterile water and stored at 4°C after drying. According to the method of Liu (1995), full cysts were placed into the tissue breaker, 5-10 mL of distilled water was added using a pipet after gently twisting the tissue breaker core one or two times, and the cyst debris and eggs were washed into a beaker and then poured into 200- and 500-mesh sieves. The eggs on the 200-mesh sieve were gently washed with water, and the eggs on the 500-mesh sieves were collected in a beaker to produce an egg-suspension of approximately 2000 eggs·mL<sup>-1</sup>. In the soybean seedling stage, the plants were inoculated with the SCN race 3 egg suspension at approximately 2000 eggs/plant. Plants that were not inoculated with nematodes were used as controls.

### Sampling and RNA Extraction

Ten days after inoculation, five plants from each variety were randomly selected and the roots of the inoculated and uninoculated Huipizhiheidou and Liaodou 15 were collected. The roots were washed with sterile water, dried, quickly frozen in liquid nitrogen and stored in a -80°C freezer. When the adventitious roots of the germinated soybean reached up to 2 cm, the apical root was inoculated with nematodes and the plants were incubated in the dark at 28°C for two days. The soybean roots were then rinsed with sterile water to wash away the second instar larvae that failed to invade, and the plants were cultivated in sterile soil. After 10 days of inoculation, the apical and elongated areas of the roots were sampled and immediately frozen in liquid nitrogen for RNA extraction.

### Sequencing of cDNA Libraries of Huipizhiheidou and Liaodou 15

The total RNA was synthesized using the Agilent 2100 analysis system and the mRNA was enriched using magnetic beads with Oligo (dT). Construction of the soybean digitized gene expression library was conducted according to manufacturer's procedures for the NlaIII Preparation of Gene Expression Library (Illumina). The constructed library was sequenced using Illumina HiSeq™ 2000.

### Analysis of Differential Gene Expression

Differentially expressed genes were screened from the digital expression profile based on the method described by Audic and Claverie (1997). The differentially expressed genes were those with a False Discovery Rate (FDR) ≤ 0.001 and an expression fold change of no less than 2 (i.e., |log<sub>2</sub> | ≥ 1).

### KEGG Metabolic Pathway Analysis and GO Differential Gene Annotation

Gene ontology (GO) functional enrichment analysis was used to map the differentially expressed genes to the Gene Ontology database (<http://www.geneontology.org>). The number of genes for each GO term was calculated, and a *P*-value ≤ 0.05 indicated a highly enriched GO term. A total of 13,175 soybean gene sequences were annotated in the KEGG metabolic database (<http://www.kegg.org>), and these sequences were distributed in 101 metabolic pathways. In the digital expression profile of Huipizhiheidou after 10 days of SCN infection, 336 sequences were distributed in 87 metabolic pathways. The metabolic pathway threshold was defined at a *P*-value ≤ 0.05. We used the KEGG database to conduct an enrichment analysis of metabolic pathways. The differentially expressed genes were mapped to the KEGG database and the degree of enrichment was calculated for the genes in each pathway.

### Results

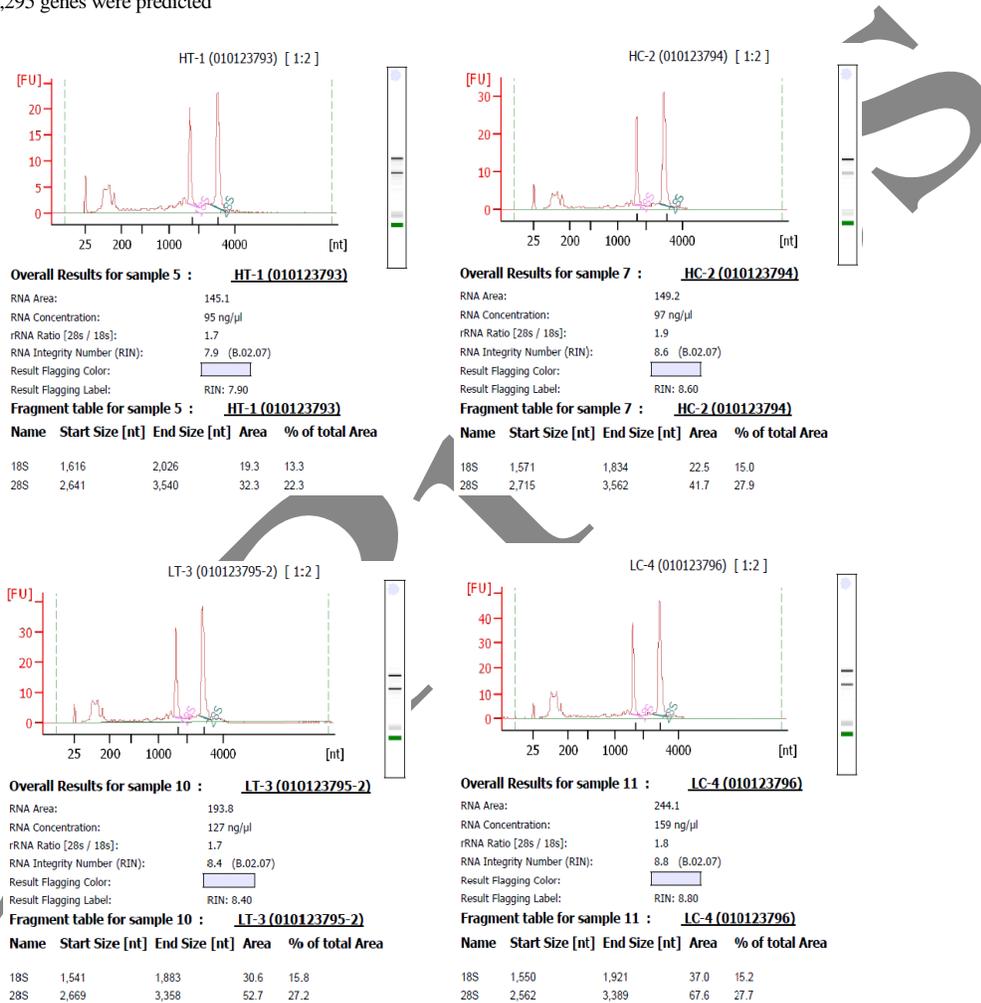
#### Sequencing Analysis of cDNA Libraries of Huipizhiheidou and Liaodou 15

The Agilent 2100 test results of the RNA samples showed that the four treatment samples all had RNA 28S:18S ratios of greater than 1.0 and RNA integrity number (RIN) values of greater than 7, which indicated good RNA integrity (Fig. 1). The expression libraries of the four samples in this study were sequenced by Illumina, and the original data underwent processing steps, including removal of the 3' adaptor sequence and contamination, to obtain clean tags for each sample (Table 1). Sequencing saturation analysis was performed to check whether the number of detected genes increased with an increase in sequencing volume. In this analysis, when the sequencing volume reaches more than three million, the number of detected genes tends to be saturated. Heterogeneity is a significant feature of cellular mRNA expression, with a small number of mRNA showing very high expression abundance; most other types of mRNA showed low or extremely low expression. In the clean tags data, the copy number of tags reflected the expression of the corresponding gene as well as its distribution statistics.

**Table 1:** Summary of output data and mapping work

Tag or gene name	HT1	HC2	LT3	LC4
Raw tags (Total)	6,096,997	5,988,158	6,111,097	5,761,219
Raw tags (Distinct)	343,916	329,333	323,384	314,418
Clean tags (Total)	5,920,146	5,808,654	5,937,084	5,537,523
Clean tags (Distinct)	170,290	153,380	153,024	148,071
All tag-mapped genes	23,292	22,988	23,250	23,411
% of 36,295	64.18%	63.34%	64.06%	64.50%

a: Raw tags sequence data prior to trimming and processing; clean tags, rimmed and processed 21-bp sequences. b: Distinct tags are classified according to their sequence. c: HT1 = Huipizhiheidou infected with SCN, HC2 = untreated Huipizhiheidou (control), LT3 = Liaodou 15 infected with SCN, and LC4 = untreated Liaodou 15. 36,295 genes were predicted



**Fig. 1:** The results of RNA samples detected by Agilent 2100

In this study, the reference genome consisted of 36,295 gene sequences, which included 32,608 sequences of CATG loci and accounted for 89.84% of the total number of genes. We compared all clean tags with the reference tag database, allowing up to one base mismatch, and conducted gene annotation for the unambiguous tag, which uniquely aligns to one gene. The number of raw clean tags corresponding to each gene was counted, and the original clean tag was normalized to obtain the gene expression levels.

### Analysis of Gene Expression of Huipizhiheidou Inoculated with SCN for 10 Days

A total of 389 up-regulated genes (1.19% of the total reference genome) were detected in the digital expression profile of Huipizhiheidou infected with SCN race 3 for 10 days (Fig. 2). Genes those were up-regulated by more than fivefold included lectin-like receptor kinase, protein kinase family, cytokinin-induced messenger and D-lactose dehydrogenase. In addition, genes that were up-regulated by

more than twofold included proline-rich protein,  $\beta$ -1,3-glucanase, autophagy-related protein 9, calmodulin and polyphenol oxidase. The results of the metabolic pathway analysis showed the up-regulation of metabolic pathway genes in the digital expression profile of Huipizhiheidou infected with SCN race 3.

The expression of some genes in Huipizhiheidou was inhibited by SCN infection. A total of 344 down-regulated genes (1.05% of the total reference genome) were detected in the expression profile of Huipizhiheidou infected with SCN race 3 for 10 days (Fig. 2), and genes that were down-regulated by more than fivefold encode unknown proteins. Genes those were down-regulated by two to fivefold included cytochrome P450 monooxygenase, membrane protein, ornithine decarboxylase and peroxidase. The inhibited metabolic pathways included phenylpropane biosynthesis, phenylalanine metabolic pathways, oxidative phosphorylation and arginine and proline metabolism.

### Gene Expression Analysis of Liaodou 15 Inoculated with SCN for Ten Days

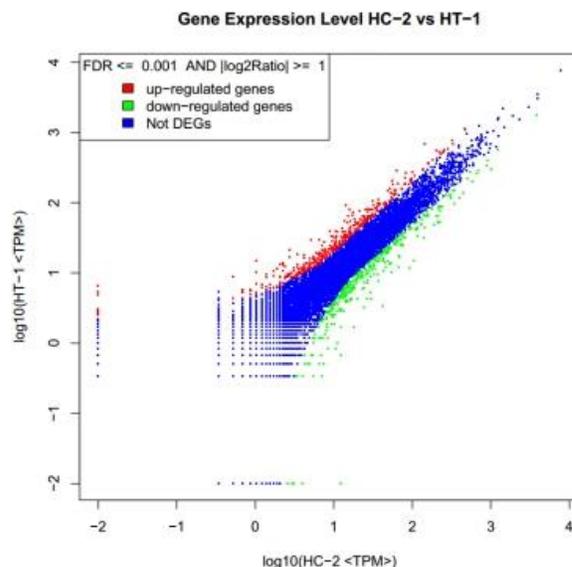
In the digital expression profile of Liaodou 15, 222 genes were up-regulated (0.68% of the total genes) (Fig. 3), and genes that were up-regulated by fivefold included lectin-like receptor kinase, heat-sensitive uronic acid kinase, cellulase synthase and phospholipid-transporting ATPase. Genes those were up-regulated by two- to fivefold included chitinase I and plant homologous protein. Genes with expression levels that more than doubled included amino acid transport carrier, ATP binding protein and peroxidase.

A total of 691 genes were down-regulated (2.12% of the total reference genome) (Fig. 3). Genes with inhibited expression included germin-like proteins (down-regulated by fivefold or more), thaumatin-like protein, cytochrome P450, cytochrome P450 monooxygenase and heat shock protein 70, and genes that were down-regulated to 50% or less of their normal expression levels included elongation factor proteins, spermine synthase, zinc transporter and inositol phosphokinase.

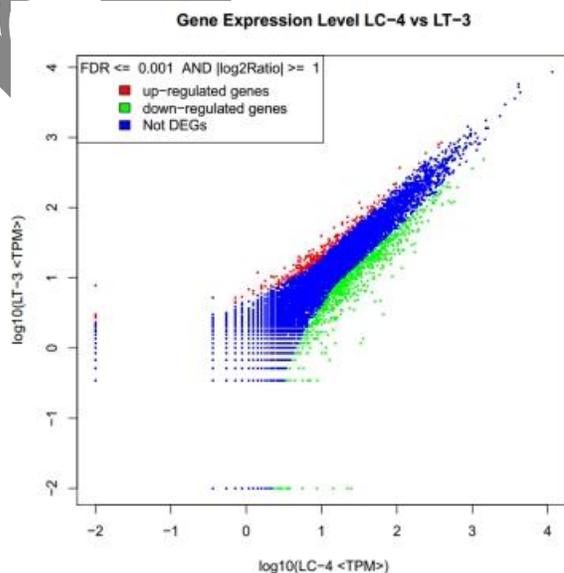
A total of 402 sequences of Liaodou 15 were distributed in 90 metabolic pathways. The up-regulated metabolic pathways included pathways for pyrimidine metabolism, alanine, aspartic acid and glutamic acid metabolism and pantothenic acid and coenzyme biosynthesis. The down-regulated metabolic pathways, including those for phenylpropane synthesis, phenylalanine metabolism and flavonoid biosynthesis, were more frequently annotated in the digital expression profile of Liaodou 15.

### Differential Gene Expression Analysis of Huipizhiheidou and Liaodou 15 at 10 Days after Inoculation

The criteria for differentially expressed genes were defined as a false discovery rate (FDR)  $\leq 0.001$  and a  $|\log_2 \text{ratio}| \geq 1$ .

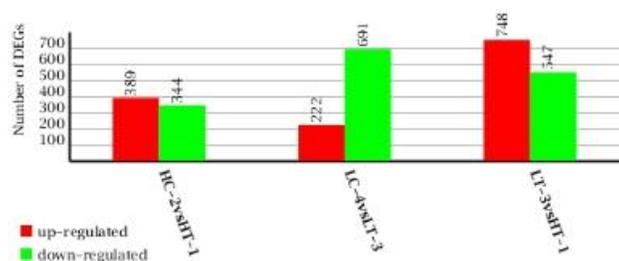


**Fig. 2:** Distribution of differentially expressed genes in the digital expression profile of Huipizhiheidou infected with SCN for 10 days



**Fig. 3:** Distribution of differentially expressed genes in the digital expression profile of Liaodou 15 infected with SCN for 10 days

At 10 days after inoculation with nematodes, a total of 748 genes were up-regulated and 547 genes were down-regulated in the digital profiles of Huipizhiheidou and Liaodou 15 (Fig. 4). We compared the digital profiles of Huipizhiheidou and Liaodou 15 after SCN infection and identified some significantly differentially expressed genes. The results revealed 748 up-regulated gene sequences, and the up-regulated genes were related to plant disease resistance; these genes included the disease resistance



**Fig. 4:** Differentially expressed genes in the treated vs. untreated (control) groups (HC vs. HT, LC vs. LT and LT vs. HT) and treated resistant (R) and susceptible (S) lines at 10 dpi. Left: Huipizhiheidou, uninfected compared with SCN-infected; up-regulated genes (red column) are compared to down-regulated genes (green column). Middle: Liaodou 15, uninfected compared with SCN-infected; up-regulated genes (red column) are compared to down-regulated genes (green column). Right: SCN-infected Liaodou 15 compared with SCN-infected Huipizhiheidou; up-regulated genes (red column) are compared to down-regulated genes (green column)

candidate protein KR1, Hs1<sup>pro-1</sup> protein, proteins with NBS-LRR structures, serine-threonine kinase, disease resistance protein KR3 and cell cycle-dependent kinase inhibitors. Genes those were up-regulated by two- to fivefold included heat-shock protein, receptor kinase, resistance proteins with TIR-NBS-LRR structure, resistance protein LM 17, elongation factors, calmodulin, cold protein, glutathione-S-transferase and resistance proteins with LRR structures. The up-regulated metabolic pathways in the digital expression profile of resistant soybeans included flavonoid biosynthesis, phenylalanine metabolism,  $\alpha$ -linolenic acid metabolism, cysteine and methionine metabolism, tryptophan metabolism and pentose phosphate metabolism.

A comparison of the expression profiles showed that genes that were down-regulated by more than fivefold included ADP-ribosylation factor, ATP binding protein, chitin-associated lectin, resistance proteins with PK-LRR-TM structures, the transcription factor Bzip102 and the transcription factors WRKY9 and WRKY45. Genes with down-regulated expression of two- to fivefold included nodule-enhanced protein phosphatase, LRR receptor-like proteins, stress-induced receptor kinases and the transcription factor Bzip47. The down-regulated metabolic pathways containing these differentially expressed genes included phenylpropane biosynthesis, oxidative phosphorylation and glutathione metabolism.

After inoculation with nematodes, most of the up-regulated genes in the resistant strain were those associated with resistance. In contrast, in susceptible varieties, the down-regulated genes may be those associated with plant resistance. Based on this idea, we compared the up-regulated genes in the Huipizhiheidou expression profile with the down-regulated genes in the Liaodou 15 expression profile and identified some genes related to resistance, including cytokinin induction messenger (ACU20960), polyphenol oxidase (ACB45218), LRR family protein (ACM8949.1) and transcription factor WRKY (ACU2360.1) and CDPK. These genes are directly or indirectly related to plant disease resistance. For example,

polyphenol oxidase is a plant defense enzyme involved in the synthesis of L-dopa in the secondary metabolic pathway.

## Discussion

Though the Huipizhiheidou variety is used as an SCN resistance source material in breeding resistance in soybeans, an in-depth investigation of the resistance mechanism of this strain has not yet been conducted. In this study, we used a high-throughput method (Digital Gene Expression Profiling, DGE) to perform this investigation. Due to the limitation of the annotated data resources, there were many data that were not functionally annotated in this study. However, the digital gene expression profiling results for Huipizhiheidou and Liaodou 15 still included some very meaningful data. In the plant defense enzyme system, phenylalanine ammonia-lyase, peroxidase and superoxide dismutase were up-regulated in the digital expression profile of Huipizhiheidou infected with SCN, and the caffeoyl coenzyme A methyltransferase in the lignin synthesis pathway was also up-regulated. These up-regulation data suggest that the synthesis of lignin is the main factor affecting the nematode larvae in the host root of SCN-resistant Huipizhiheidou. Wuyts *et al.* (2006) revealed that changes in the phenylpropane metabolic pathway led to changes in lignin, affecting the reproduction of female *Meloidogyne incognita*. Under nematode-induced stress, the metabolism of soybeans undergoes tremendous changes, and a large number of genes show either induced expression (up-regulation) or inhibited expression (down-regulation).

## Conclusion

We found that 389 genes were up-regulated and 344 genes were down-regulated in the resistant soybean variety of Huipizhiheidou, whereas 222 genes were up-regulated and 691 genes were down-regulated in the susceptible soybean variety of Liaodou 15 infected with SCN after 10 days.

## Acknowledgements

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