Differential Proteomic Analysis of the Resistant Soybean Infected by Soybean Cyst Nematode, *Heterodera glycines* Race 3

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Abstract

Plant parasitic nematode, *Heterodera glycines* (Soybean cyst nematode, SCN) is the major pathogen of *Glycine max* (soybean). This pathogen is widely distributed, seriously disserving, and has a wide approach for spreading. Huipizhi Heidou (ZDD2315), a germplasm resource of soybean originated in China, has an excellent resistance to SCN. In this paper, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) were employed to separate the differentially expressed proteins from soybean induced by SCN. The F_4 separated populations from the cross between the resistant Huipizhi Heidou and the susceptible Liaodou 15 were used as test materials by bulked segregant analysis. The 2-DE gels analysis revealed 367 protein spots from the resistant samples and 372 protein spots from the sensitive samples. Among those protein spots, 23 protein spots from the resistant samples and 4 protein spots from the sensitive samples were differentially expressed, and then were selected for peptide mass fingerprinting (PMF) and sequencing assay by MS. Of the 27 differentially expressed proteins, 16 protein spots were identified by MALDI-TOF-MS and 11 were not identified due to low scores. Further analysis showed that the majority of these 16 proteins were involved in defense, energy and metabolism, suggesting that they might be related to the soybean resistance to SCN.

Keywords: Soybean, *Heterodera glycines*, Proteomic analysis, Resistance

1. Introduction

Plant parasitic nematodes are a major worldwide agronomic problem. *Heterodera glycines*, soybean cyst nematode (SCN), is one of the most devastating plant parasitic nematodes. SCN was first found in northeast China in 1899 (Dai, 1958), and was first reported in 1915 by Japanese. With an increasing demand for soybean (*Glycine max*), more attention has been paid to the identification of the genes involved in the process of resistance. For SCN management, the resistant soybean cultivar breeding is the most economical and effective

method. In Northeastern China, SCN race 3 is the most prevalent pathogen of soybean. This pathogen is widely distributed, seriously disserving, and has a wide approach for spreading.

Huipizhi Heidou (ZDD2315) is a germplasm resource of soybean originated in China. It has been shown that this cultivar has an excellent resistance to SCN race 3. The mechanism of resistance to SCN in this cultivar has been reported. Therefore, screening the differentially expressed proteins between the resistant Huipizhi Heidou and the susceptible Liaodou 15 can reveal valuable functional molecules and would aid in their use for genetically engineering resistance.

Two-dimensional gel electrophoresis (2-DE) is a powerful and high throughput tool for describing the changes in protein expression and modification, which involves separation of cellular proteins according to their isoelectric points (*p*I) and relative molecular masses (M_r). This technique was originally described by Klose (1975) and O'Farrell (1975). Two-dimensional gel electrophoresis had been used to identify differentially expressed proteins in response to fungal infection in maize embryos (Campo *et al*., 2004), identify proteins in resistant wheat cultivar induced by *Fusarium graminearum* infection (Wang *et al*., 2005), understand the molecular mechanism of interaction between *F. graminearum* and *Triticum aestivum* (Zhou *et al*., 2006), study the compatible and incompatible interactions between rice and bacteria (Mahmood *et al*., 2006), separate proteins extracted from leaf blades of rice plants infected with blast fungus *Magnaporthe grisea* (Konishi *et al*., 2001). Differential proteomics are popularly used to compare expression similarities and differences in plant cells under different physiological or pathological conditions, consequently the related proteins are classified and identified as functions (Shao *et al*., 2008). In this paper, we applied the high-resolution capacity of 2-DE coupled with tandem MS analysis of peptide profiles to screen and identify the differentially expressed proteins between the resistant and the susceptible soybean infected by SCN race 3. The identification of proteins differentially expressed in the resistant and sensitive samples would provide a resource of SCN resistant-related genes and new insights for SCN stress response in soybean.

2. Materials and Methods

2.1 Plant materials

The resistant cultivar Huipizhi Heidou and susceptible cultivar Liaodou 15 to SCN race 3 were used in this study. The hybrid filial generation was breed for two yeas in Shenyang, Liaoning Province, China and Sanya, Hainan Province, China. The F_4 separated populations from the cross between the resistant and the susceptible were used as test materials by bulked segregant analysis to identify the differentially expressed proteins induced by SCN race 3.

2.2 Nematode procurement

The SCN race 3 were collected from the soybean field of Nematology Institute of Northern China, Shenyang Agricultural University, Shenyang, China. Cysts were separated from the soil specimen with a set of sieves of 850 μm + 500 μm + 250 μm. The SCN inoculants were prepared according to the method described by Liu (2000).

2.3 Resistance identification

Seeds of the F_4 separated populations from the cross between Huipizhi Heidou and Liaodou 15 were germinated at 25℃ for 5 days, and then the soybean seedlings were transplanted into plastic pots containing a mixture of sand and soil sterilized (in a ratio of 1:2). One seedling was put into one plastic pot (16 × 16 cm). After 5 days, each seedling was inoculated with approximately 2000 eggs and juveniles of SCN race 3. The plastic pots were then maintained in the greenhouse at 25-28℃. The newly formed females were counted directly on the roots after inoculated for 30 days. Finally, the seedlings were divided into the resistant and sensitive samples for further bulked segregant analysis (BSA). The resistant index to SCN was divided into 0-4, 5 grades according to the number of females on the roots: 0 = no cyst (immune), 1 = 1-5 cysts (resistant), 2 = 6-10 cysts (moderate resistant), 3 = 11-30 cysts (susceptible), 4 = more than 30 cysts (high susceptible).

2.4 Root protein extraction

The total proteins from soybean roots were extracted by TCA/acetone method. About 2 g of root samples and 0.2 g polyvinylpyrrolidone (PVP) were ground to fine powder in a mortar and immersed in liquid nitrogen. The powder was put in a 50 ml centrifuge tube, and a tenfold volume of 10% TCA/acetone was dded. Dithiothreitol (DTT) was also added to a final concentration of 30 mM. When precipitated at -20℃ for 2 h, the mixture was then centrifuged at 20 000 r/min for 15 min at 4℃. After pouring out the top liquid, the precipitates were washed with a fourfold volume of precooled 80% acetone and a final concentration of 30 mM of DTT, precipitated at -20℃ for 30 min, and then centrifuged at 20 000 r/min for 15 min at 4℃. The supernatant was thrown away. The

precipitates were dried to pellets under vacuum condition, and the dried pellets were dissolved in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 4% NP-40, 20 mM Tris-HCl). PMSF and EDTA were then added to the dissolved pellets with a final concentration of 1 mM and 2 mM, respectively. After mixing for 5 min, DTT was added with a final concentration of 10 mM. By sonication for 10 min, the final mixture was centrifuged at 35 000 r/min for 30 min, and the protein liquid was collected and stored at -80℃ until use for proteomic analysis.

2.5 Two-dimensional gel electrophoresis

About 150 μg protein was loaded onto a 24 cm IPG strip (pH 4-7), then a step of isoelectric focusing procedure was carried out with hydration 8 h, 50 V for 4 h, 500 V for 1 h, 1000 V for 1 h, 10000 V for 1 h, 10000 V for 90000 VHr, 500 V for several hours. After isoelectric focusing, the strip was equilibrated in 15 ml equilibration buffer I (50 mM Tris, 6 M urea, 2% SDS, 30% glycerol, pH 8.8) containing 1% DTT for 15 min and washed by distilled water, then transferred to an equilibration buffer II (50 mM Tris, 6 M urea, 2% SDS, 30% glycerol, pH 8.8) containing 2.5% iodoacetamide (IAM) and equilibrated for another 15 min. Next, the strip was sealed with agarose containing 1% bromophenol blue and run on 12% SDS-PAGE gel, 10 mA/gel for 1 h, 20 mA/gel for 10-12 h. After electrophoresis, the SDS-PAGE gels were put in stationary liquid containing 10% glacial acetic acid and 40% ethanol for 30 min, sensitized in a sensitizer containing 30% ethanol, 0.2% sodium thiosulfate and 6.8% sodium acetic acid for 30 min, washed thrice in double braised water for 10 min, and then stained with silver nitrate liquid containing 0.25% silver nitrate and 0.04% formalin for 20 min under normal temperature. The gels were washed twice in double braised water for 1 min and developed with 2.5% sodium carbonate and 0.02% formalin, and then the development was stopped with 1.46% EDTA for 10 min. The gels were saved with 1% glacial acetic acid under 4℃.

2.6 Gel image analysis

Three 2-DE gels were prepared for each sample of total proteins from the resistant and sensitive soybeans. Gels were scanned using an image scanner (UMAX PowerLook 2100XL). The digitized images were analyzed using the ImageMaster 2D Platinum 5.0 image analysis software (Amersham, Sweden). Unmatched and twofold upper or lower protein spots were selected on the 2-DE maps as the differentially expressed proteins compared with the reference gel.

2.7 Matrix assisted laser desorption ionization time of flight mass spectrometry

The differentially expressed protein spots were excised and identified with MALDI-TOF-MS (Applied Biosystems Voyager DEPro, ABI, USA). Parameters were set as follows: reflection mode, nitrogen laser (337 nm, 0.5 ns pulse width, 20 Hz repetition rate), delayed ion extraction 100 ns, Grid voltage 70%, vacuum degree 4e-008, single scan of MS signals accumulating 200 times, positive spectra determination.

2.8 Database searching

Sequence similarity searches based on MALDI-TOF-MS/peptide mass fingerprinting (PMF) were performed with Mascot software using default parameters against NCBInr Database. Protein scores greater than 71 were significant ($p < 0.05$).

3. Results

3.1 Identification of the resistance to SCN of F_4 separated populations

After inoculated for 30 days, the newly formed females were counted directly on the roots. The F_4 separated populations from the cross between the resistant cultivar Huipizhi Heidou and the sensitive Liaodou 15 were divided into the resistant and sensitive samples for BSA. According to the number of females on the roots (Table 1), ten soybean individuals resistant to SCN were selected as the resistant samples, and ten soybean individuals susceptible to SCN were selected as the sensitive samples.

3.2 Proteomic analysis of proteins in the resistant and sensitive samples

Proteomes of soybean roots in the resistant and sensitive samples were analyzed using 2-DE assay. The reproducibility of the data for soybean proteomes were confirmed by analyzing the 2-DE gels using image analysis software. The proteins in the resolved gels of the resistant and sensitive samples were estimated and compared using an image analysis program. The 2-DE electrophoresis profiles of the total proteins of the resistant and sensitive samples are illustrated in Fig. 1. Image analysis of the silver nitrate stained 2-D gels revealed 367 protein spots from the resistant samples and 372 protein spots from the sensitive samples. Comparison of the proteomes of soybean roots between the resistant and sensitive samples revealed a handful of differences: there were 6 unmatched protein spots, 13 twofold upper protein spots and 4 twofold lower protein spots in the resistant samples, 4 unmatched protein spots in the sensitve samples (Table 2).

3.3 MS analysis of the differentially expressed proteins

The differentially expressed protein spots isolated with sufficient amount for PMF were excised from the gels and analyzed by mass spectrometry. Fingerprints were used for searching the GenBank nonredundant protein database containing all proteins from all species using Mascot software. Of the 27 differentially expressed protein spots, 16 protein spots were identified, and 11 protein spots were not identified due to low scores. As shown in Table 3, unknown (denoted as No. 53, 71 and 284), hypothetical protein SORBIDRAFT_04g012541 (denoted as No. 116), trypsin inhibitor p20 (denoted as No. 117), Xylem serine proteinase 1 precursor, putative (denoted as No. 118), triosephosphate isomerase (denoted as No. 173 and 187), and caffeoyl coenzyme A 3-*O*-methyltransferase 2 (denoted as No. 204) were up-expressed in the resistant samples, putative RNA polymerase III (denoted as No. 296) and ATP synthase beta chain (denoted as No. 340) were down-expressed in the resistant samples, hypothetical protein SORBIDRAFT_04g012541 (denoted as No. 7), predicted protein (denoted as No. 195), ATPase alpha subunit (denoted as No. 224), and homeobox-like protein (denoted as No. 243) were newly expressed in the resistant samples, whereas unknown (denoted as No. 14) was newly expressed in the sensitive samples. A large proportion of the identified proteins were involved in defense, energy and metabolism.

4. Discussion

Soybean cyst nematode disease is epidemic and destructive worldwide. As a soil-borne disease, it is very difficult to control (Duan *et al*., 2009). Development of the resistant soybean cultivars is an effective management to control SCN. But its shortcomings are still obvious, such as long period of breeding resistant cultivars, resistance being easily to lose (Yu, 1998). Among the most important of the plant-nematode interactions is that between *H. glycines* and *G. max*. The system *G. max*-*H. glycines* provides a powerful model to study plant parasitic nematodes because of the availability of 16 different *H. glycines* populations that vary in their ability to infect these resistant genotypes. Importantly, information learned through its study can be translated directly to improve resistance in an agriculturally relevant plant (Vincent *et al*., 2010). Soybean cultivar Huipizhi Heidou is one of the best Chinese black soybean cultivars with resistance to SCN race 1, 2, 3, 4, 5, 7 and 14. Research on the mechanism of resistance to SCN race 4 in Huipizhi Heidou had been reported. Sex ratio of SCN adults was about 10 on Huipizhi Heidou root, and was one and fewer on susceptible one. There were high mortalities from the second stage juvenile nematode (J_2) to J_3 and from J_3 to J_4 on Huipizhi Heidou root compared with susceptible control (Yan *et al*., 1996). The syncytium in Huipizhi Heidou root was with smaller syncytium component cells, and there was more ribosomes, less and smaller rough endoplasmic reticula, and more lipid-like droplets in cytoplasm after inoculated SCN race 4 (Yan *et al*., 1997).

Two-dimensional gel electrophoresis is currently the most widely used method for protein analysis in proteome research, by which powerful and high-throughput identification of proteins in cells and organs may be accomplished. In this study, we used 2-DE gel electrophoresis to explore protein expression profiles of the soybean roots between the resistant and sensitive samples. For MS analysis, PMF were acquired using MALDI-TOF-MS. NCBI database was searched using MASCOT software to identify these differentially expressed proteins. Of the 27 differentially expressed protein spots, 16 protein spots were identified.

By proteomic analysis, we identified a trypsin inhibitor p20 that is related to the resistance to SCN. Trypsin inhibitor p20 has GTP-binding activity, it might be a multifunctional protein, in some way required for the signal transduction pathway and seed development (Hirata *et al*., 1999; Mulligan *et al*., 1997; Welham *et al*., 1998). It has been demonstrated that most plants naturally accumulate protease inhibitors to defend against insect attacks (Ryan, 1990). There were many kinds of protease inhibitors in the seeds of soybean, and Kunitz trypsin inhibitor (KTI) was the dominant type. The expression of trypsin inhibitor can provide an efficient method for crop protection. The trypsin inhibitor proteins produced in the transgenic cauliflower plants were functionally active in planta resistance to *Pieris conidia* and *Plutella xylostella* (Ding *et al*., 1998). The barley trypsin inhibitor expressed in transgenic rice seeds provided significant protection against the coleopteran rice weevil *Sitophilus oryzae*, one of the most important insect storage pests of rice (Julio *et al*., 2003). It has also been shown that soybean trypsin inhibitor can attend the resistance of soybean to SCN (Yang *et al*., 2008).

Lignin content and its related enzyme activity were highly correlated with plant growth, anti-disease ability and adverse resistance (Li *et al*., 2003). CCoAOMT (Caffeoyl coenzyme A 3-*O*-methyltransferase) is the key enzyme of lignin metabolism (Zhao *et al*., 2004). CCoAOMT deficiency has a small effect on lignin content causing an increase in the S/G ratio due to decreased formation of G units, and thus implicating CCoAOMT in the biosynthesis of the G precursors (Cao *et al*., 2007). The accumulation of lignin for cell wall reinforcement was correlated with resistance in several host-pathogen interactions, and the physiological significance of

CCoAOMT for cell wall reinforcement has been outlined in other plant systems (Hammerschmidt *et al*., 1985; Tiburzy *et al*., 1990; Reimers *et al*., 1991; Schmitt *et al*., 1991; Matern *et al*., 1995). In this study, we also identified a caffeoyl coenzyme A 3-*O*-methyltransferase 2 that is related to the resistance to SCN.

Further study will be focused on the cloning of the cDNA sequences encoding these differentially expressed proteins and the potential function of these proteins. Functional genomic studies on these proteins will reveal their regulated mechanisms of expression and find the new control strategy for SCN.

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Table 1. Identification of F_4 separated populations of the resistance to SCN race 3

Samples	Code of plants	No. of cysts	Samples	Code of plants	No. of cysts
Resistant	4	2	Sensitive	2	30
	7	2		3	29
	16	4		6	43
	19	5		12	32
	25	2		13	45
	33	2		35	80
	36	2		43	32
	52	1		47	36
	54	0		49	70
	61	2		58	44

Table 2. General information of proteomes of soybean roots in the resistant and sensitive samples

Samples	Total spots	Unmatched spots	Twofold upper protein	Twofold lower protein
Resistant	367	6	13	4
Sensitive	372	4	-	-

Table 3. Differentially expressed soybean proteins in the resistant and sensitive samples identified by MALDI-TOF-MS

Spot	Protein name	GenBank No.	Score	*p*I	MW(Da)	Species
7	hypothetical protein SORBIDRAFT_04g012541	XP_002453742	79	5.27	69535	Sorghum bicolor
14	unknown	ACU21370	85	6.07	32549	Glycine max
53	unknown	ACU13903	78	4.11	16964	Glycine max
71	unknown	ACU13563	122	6.06	19846	Glycine max
116	hypothetical protein SORBIDRAFT_04g012541	XP_002453742	77	6.10	26766	Sorghum bicolor
117	trypsin inhibitor p20	BAA82254	127	5.78	26807	Glycine max
118	Xylem serine proteinase 1 precursor, putative	XP_002522297	84	5.58	26807	Ricinus communis
173	triosephosphate isomerase	ABA86966	200	6.72	30928	Glycine max
187	triosephosphate isomerase	ABA86966	130	6.06	32525	Glycine max
195	predicted protein	XP_001779411	72	5.38	33196	Physcomitrella patens
204	caffeoyl coenzyme A 3-*O*-methyltransferase 2	ABE60812	82	4.73	33658	Leucaena leucocephala
224	ATPase alpha subunit	ABI54654	88	5.56	34892	Scapania nemorea
243	homeobox-like protein	AAV33463	87	5.16	37476	Fragaria ananassa
284	unknown	ACU23916	150	5.66	40470	Glycine max
296	putative RNA polymerase III	AAP03366	96	5.58	41329	Oryza sative Japonica
340	ATP synthase beta chain	NP_001151807	114	5.34	57681	Zea mays

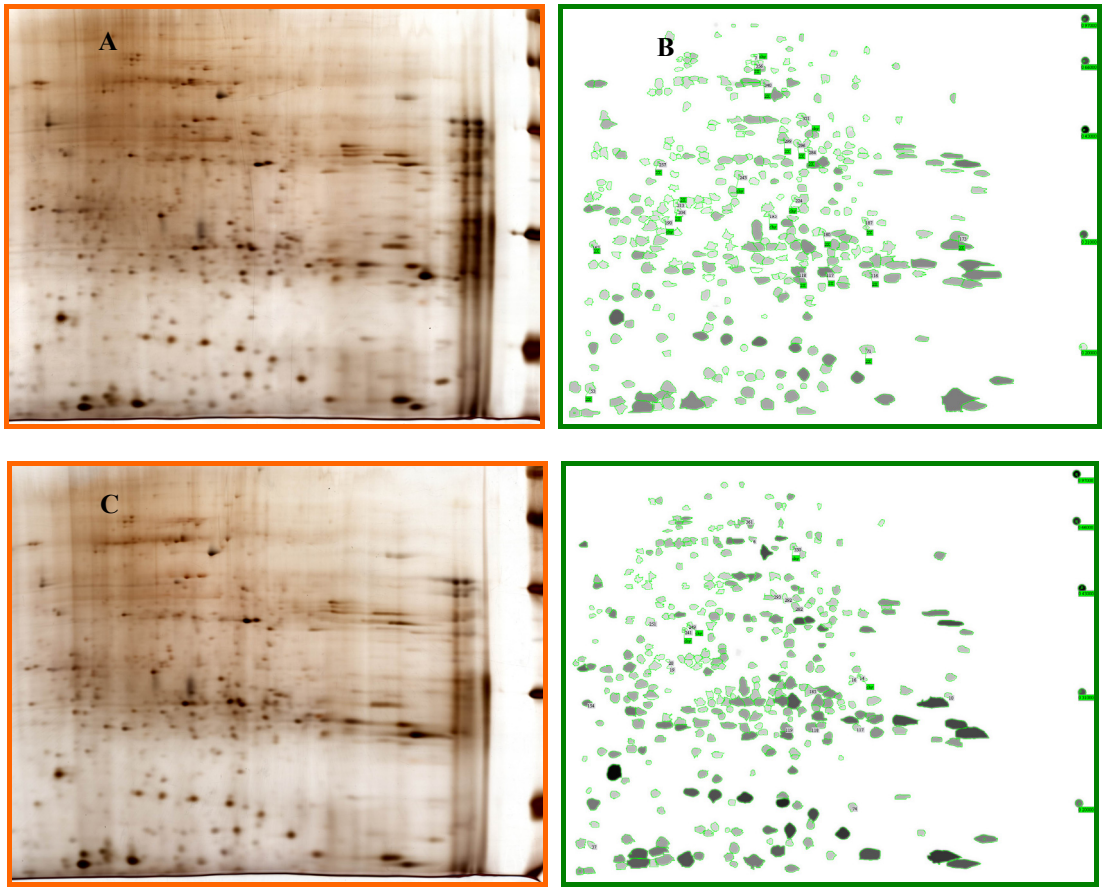

Figure 1. 2-DE images of total proteins from soybean roots in the resistant and sensitive samples
A, B: proteins in the resistant samples; C, D: proteins in the sensitive samples