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Differential Gene and Protein Expression in Soybean at Early Stages of Incompatible Interaction with *Phytophthora sojae*

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Abstract

Soybean root and stem rot caused by *Phytophthora sojae* is a destructive disease worldwide. Using genetic resistance is an important and major component in the integrated pest management of this disease. To understand molecular mechanisms of root and stem rot resistance in soybeans, the gene and protein expression in hypocotyls and stems of variety Suinong 10 carrying resistance genes *Rps*1a and *Rps*2 was investigated by using mRNA differential display reverse transcription PCR and two-dimensional electrophoresis at 0, 0.5, 1, 2, and 4 h after inoculation with *P. sojae* race 1. The results of the comparison of gene and protein expression showed that at least eight differential fragments at the transcriptional level were related to metabolic pathway, phytoalexin, and signal transduction in defense responses. Sequence analyses indicated that these fragments represented cinnamic acid 4-hydroxylase gene, ATP β gene coding ATP synthase β subunit and ubiquitin-conjugating enzyme gene which upregulated at 0.5 h post inoculation, blue copper protein gene and UDP-N-acetyl-α-D-galactosamine gene which upregulated at 2 h post inoculation, TGA-type basic leucine zipper protein TGA1.1 gene, cyclophilin gene, and 14-3-3 protein gene which upregulated at 4 h post inoculation. Three resistance-related proteins, α-subunit and β-subunit of ATP synthase, and cytochrome P450-like protein, were upregulated at 2 h post inoculation. The results suggested that resistance-related multiple proteins and genes were expressed in the recognition between soybean and *P. sojae* during zoospore germination, penetration and mycelium growth of *P. sojae* in soybean.

Key words: *Phytophthora sojae*, resistance mechanism, incompatible interaction, mRNA differential display reverse transcription PCR, two-dimensional electrophoresis

INTRODUCTION

Phytophthora root and stem rot disease caused by *Phytophthora sojae* Kaufmann and Gerdemann was first observed in Indiana and Ohio in 1951 (Kaufmann and Gerdemann 1958) and recently became one of the most important diseases in soybean [*Glycine max* (L.) Merr.] production all around the world. Annual soybean pro-

duction losses resulting from *P. sojae* infestations are serious. Using race-specific resistance has been the best measure to control Phytophthora root and stem rot (Burnham *et al*. 2003; Jia and Kurle 2008). Fifteen resistance genes (*Rps*) have been providing soybean with reasonable protection against this pathogen. In recent years, however, isolates with the novel virulence have been identified in field surveys (Dorrance *et al*. 2003), which has being threatened the currently ap-

plied resistant soybean varieties. *P. sojae* is known to produce free-swimming zoospores that are attracted to isoflavonoids secreted from soybean roots (Morris *et al.* 1998). After chemotactically attracted to favorable infection sites, zoospores become immobile and secrete cell wall to form cysts (Hua *et al.* 2008). These cysts germinate and attempt to penetrate soybean roots and hypcotyles (Carlile 1986; Deacon and Saxena 1997; Callow *et al.* 2001).

Currently, most studies on the interaction between soybean and *P. sojae* are focused on pathogenesis-related proteins, phytoalexin, soybean agglutinin, regulator of G protein signaling, and cell wall appositions (Gibson *et al.* 1982; Stossel and Leuba 1984; Moy *et al.* 2004; Hua *et al.* 2009). The infection process of *P. sojae* to soybean hypocotyls consisted of four stages, i.e., pre-infection, penetration into epidermal cell, spreading in the cortex, and vascular tissue colonization. Zoospore encystment and germination penetration into epidermal cells, entering root cortex, and growth in vascular tissues were observed on 1.5, 3.6, and 24 h post inoculation, respectively (Zuo *et al.* 2005). So, understanding molecular recognition and biochemical reactions in soybean root system at the early stage of incompatible interaction between soybean and *P. sojae* is important to reveal the nature of soybean resistance to Phytophthora root and stem rot and use genetic resistance against the disease (Melgar *et al.* 2006). However, molecular mechanisms of soybean resistance to Phytophthora root and stem rot at the very early stages (0-4 h) of the host-pathogen recognition are unclear. Therefore, the objective of the present study was to investigate the gene and protein expression in soybean hypocotyls and stems at early stages of incompatible interaction between soybean and *P. sojae* using mRNA differential display reverse transcription PCR (DDRT-PCR) and two-dimensional electrophoresis (2-DE).

MATERIALS AND METHODS

Plant materials and inoculation

Soybean variety Suinong 10 carrying resistance genes *Rps*1a and *Rps*2 and the isolate H_2-4 of *P. sojae* race 1 that was originally isolated from a diseased soybean plant in Heilongjiang Province of China were used for all experiments in the present study. Soybean seeds were surface-sterilized in 70% (v/v) ethanol for 5 min, rinsed with double-distilled water and incubated on 4-8 layers of moist gauze at 24°C for 4 d. Seedlings with 20 mm long radicles were placed into Ampoules bottles filled with 10 mL sterile water per bottle. There were four seedlings in each bottle. Only cotyledons were remained outside the bottles and rest parts of seedlings were immersed in water. When radicles reached 80 mm in length, seedlings were inoculated with *P. sojae* zoospores at the rate of 250 zoospores per seedling by adding zoospore suspensions into Ampoules bottles. After inoculation, the seedlings were incubated at room temperature (22°C) with a 24-h photoperiod of 270 μmol m^{-2} s^{-1} illumination (Li *et al.* 2008).

Total RNA and protein extraction

Soybean hypocotyls and stems were collected at 0 (as control), 0.5, 1, 2, and 4 h after inoculation and ground to a fine powder in a sterilized mortar containing liquid nitrogen. Total RNA was extracted by using the Trizol method (Gibco BRL, USA) and purified using the method described by Skalamera *et al.* (2004) with some modifications for cDNA synthesization and mRNA differential display reverse transcription PCR. Proteins were extracted from 2 g of inoculated soybean hypocotyls and stems and purified by using the improved method described by Bradford (1976). The 1 g of the extracted protein powder was suspended in 10 mL of chilled (4°C) acetone containing 10% TCA and 0.14% mercaptoethanol and homogenized at -20°C for 2 h. The lysis solution was clarified by centrifugation at 14 000 r/min in an Eppendorf microcentrifuge at 4°C for 40 min. The above procedures of suspension and precipitation were repeated once for 1 h. The freeze-dried protein powder was dissolved at the rate of 20 mg μL^{-1} with the extracting solution containing 5 mol L^{-1} urea, 2 mol L^{-1} thiocarbamide, 35 mmol L^{-1} Tris, 2% CHAPS, 1% ASB-14, 65 mmol L^{-1} DTT and 1% IPG buffer (pH 3-10). The solution was incubated on a shaker at 29°C for 1 h to obtain completely dissolved protein. Finally, the sample was centrifuged at 14 000 r/min for 20 min at room temperature. Successively, the supernatant was collected, mixed with five times volume of cold (-20°C) acetone containing 0.14%

mercaptoethanol, and incubated at -20°C for 1 h to allow protein precipitation. Then, precipitated proteins were divided into 1.5 mL Eppendorf tubes and centrifuged at 14 000 r/min at 4°C for 30 min. The pellet was washed three times with a cold (-20°C) acetone solution containing 0.14% mercaptoethanol and centrifuged at 14 000 r/min for 30 min. Precipitated proteins were dissolved in 20 μL deionized water.

Reverse transcription of RNA into first strand cDNA and PCR amplification

Three arbitrary primers (AAGCT$_{11}$A/C/G) and 26 random primers [AP1-26 (5′→3′): AAGCTACAACGAGG; AAGCTGGATTGGTC; AAGCCTTTCTACCC; AAGCTTTTGGCTCC; AAGCGGAACCAATC; AAGCAAACTCCGTC; AAGCTCGATACAGG; AAGCTGGTAAAGGG; AAGCTCGGTCATAG; AAGCGGTACATTGG; AAGCTACCTAAGCG; AAGCCTGCTTGATG; AAGCGTTTTCGCAG; AAGCGATCAAGTCC; AAGCGATCCAGTAC; AAGCGATCACGTAC; AAGCGATCTGACAC; AAGCGATCTCAGAC; AAGCGATCATAGCC; AAGCGATCAATCGC; AAGCGATCTAACCG; AAGCGATCGCATTG; AAGCGATCTGACTG; AAGCGATCATGGTC; AAGCGATCATAGCG; AAGCGATCATAGGC] were used for PCR amplification (Ye *et al.* 2008). The gel was silver-stained for testing differential fragments (Morris *et al.* 1998; Connolly *et al.* 2005). The reverse Northern hybridization method was adopted to identify and analyze the positive differential fragments according to the instruction of DIG DNA Labeling and Detection Kit (Roche, Mannheim, Germany).

Cloning, sequencing, and analysis of the differential genes

The positive fragments identified by Northern blotting were cloned into the vector pGEM-T according to the instructions of manufacturer (http://www.promega.com.cn/techserv/ctbs/pGEM-T.pdf), and recombinant vectors were further transformed into *Escherichia coli* DH5α using the calcium chloride transformation method. The recombinants were identified by using PCR analysis and sequencing.

Two-dimensional gel electrophoresis for analysis of differential proteins

This experiment was conducted using the Bio-Rad two-dimensional electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA) and the method described by Liu *et al.* (2010). Gel images were scanned using the UMAX Power Look 2100XL (UMAX Company, Taiwan, China). Noise reduction, background subtraction, spot detection, quantification, gel-to-gel matching, and differential protein display analysis were carried out by using the PDQuest 8.0.1 software (Bio-Rad Laboratories, Hercules, CA). Differentially expressed proteins over 2.5-fold of up-regulated expression in abundance, with good quality and with high reproducibility were selected for further identification by using Applied Biosystems Voyager DE Pro (Applied Biosystems Inc., Foster City, CA). The results were compared against the NCBI (National Center for Biotechnology Information, USA) databases.

RESULTS

Differential expression of genes in soybean induced by *P. sojae*

To assess the extent of transcriptional changes, differential fragments were isolated (Fig. 1). At last 38 differential expression sequences with about 250 bp in length were found in the NCBI database after positive fragments (Fig. 2) were identified by reverse Northern-blotting (Fig. 3). Sequencing results involved in disease resistance are summarized in Table 1. Sequence analysis of BLAST search indicated that at least 10 differential fragments at the transcriptional level were related to disease resistance at early stages of incompatible interaction between *P. sojae* race 1 and soybean variety Suinong 10.

The results of gene expression comparison showed that at least eight differential fragments at the transcriptional level were related to metabolic pathway, phytoalexin, and signal transduction of defense responses. Sequence analyses indicated that these fragments represented cinnamic acid 4-hydroxylase gene (C4H) (A1, A2), ATP β gene coding ATP synthase
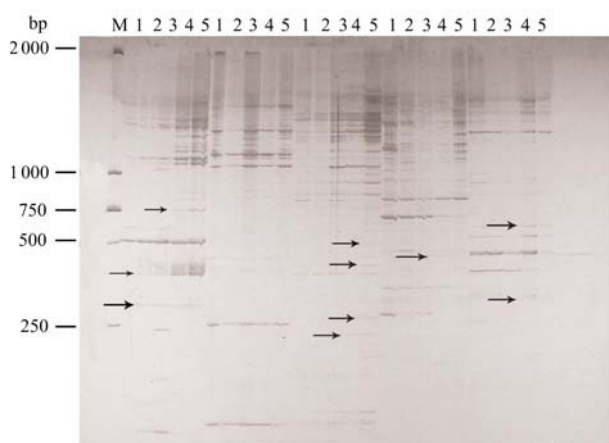
Fig. 1 Results of partial DDRT-PCR. Amplified products of cDNA by using primers AP2/RP4, AP1/RP7, AP3/RP10, AP1/RP11, and AP2/RP7, respectively. 1-5 represent amplified products of cDNA for each set of primers at 0, 0.5, 1, 2, and 4 h, respectively, after inoculation with *P. sojae*. M is marker. Arrows indicate differential fragments.
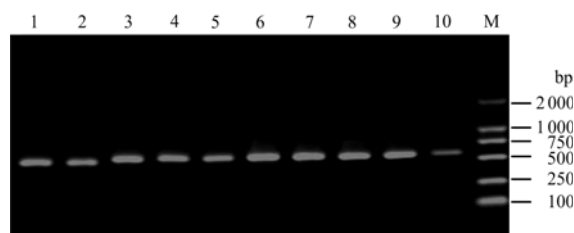

Fig. 2 Results of partial positive recombinant plasmids by PCR test. 1-10 represent amplified products of L8, L7, L1, L33, L2, L3, L4, A2, L5, and A1, respectively.

β subunit (L8) and ubiquitin-conjugating enzyme gene (L9) at 0.5 h post inoculation, blue copper protein gene and UDP-N-acetyl-α-D-galactosamine gene at 2 h post inoculation, TGA-type basic leucine zipper protein TGA1.1 gene (L1), cyclophilin gene (L31), and 14-3-3 protein gene (L33) at 4 h post inoculation, other differential expression sequences were analyzed by BLAST in the NCBI database.

Differential proteins at early stages of incompatible interaction

In order to investigate the changes of proteins in response of soybean to *P. sojae*, 2-DE analysis was conducted for total proteins in soybean hypocotyls and stems. The protein spots were broadly distributed in the pH ranging from 4.0 to 7.0.

Fig. 3 Results of partial screening positive clones of inserted fragment with reverse Northern blot. a, for positive clones; b, for false positive clones.

Peptide mass fingerprints successfully showed six protein spots (Y1-Y6) that enabled a search for matches in protein mass databases (Fig. 4). These protein spots represented five differential proteins including four known proteins and an unknown protein. Meanwhile, Y1 and Y6 were the same protein (Table 2). The gene sequence search in NCBI database by using the Mascot software showed that three differential proteins, α-subunit (spot Y6) and β-subunit (spots Y1 and Y6) of ATP synthase cytochrome, and P450-like protein (spot Y3) were related to disease resistance at translational and post-transcriptional levels at 2 h after inoculation.

The results showed that abundance of proteins changed at 0.5 h after inoculation and had certain representativeness of the abundance at 4 h after inoculation. After comparing the protein spots on two 2-DE maps at 0 and 4 h after inoculation, seven protein spots were identified by using Applied Biosystems Voyager DE Pro.

DISCUSSION

Enkerli *et al*. (1997) found differences between compatible and incompatible interactions of two soybean isolines containing either *Rps*1a or *Rps*1b resistance genes with races 2 and 8 of *P. sojae* as early as 4 h after inoculation. However, molecular mechanisms of soybean resistance to Phytophthora root and stem rot at early stages, during 0 and 4 h after inoculation, remain unclear.

The results of the present study indicated that up-regulated genes including C4H gene, ATP β gene and

Table 1 Features of partial sequenced clones and results of BLAST search

No.	Accession	Description	Max score	Query coverage (%)	E value	Identities
A1	X92437.1	*Glycine max* mRNA for cinnamic acid 4-hydroxylase (CYP73)	593	99	1e-166	323/324 (99%)
A2	X92437.1	*Glycine max* mRNA for cinnamic acid 4-hydroxylase (CYP73)	555	99	5e-155	304/306 (99%)
L1	AF402607.1	*Phaseolus vulgaris* TGA-type basic leucine zipper protein TGA1.1 mRNA, complete CDS	359	97	4e-96	250/276 (90%)
L2	AK286797.1	*Glycine max* cDNA, clone no. GMFL01-36-P08	477	97	2e-131	269/271 (99%)
L4	AY742749.1	*Phytophthora sojae* isolate S317 18S ribosomal RNA gene, partial sequence	571	81	6e-160	309/309 (100%)
L5	AY742749.1	*Phytophthora sojae* isolate S317 18S ribosomal RNA gene, partial sequence	569	98	2e-159	308/308 (100%)
L7	AK245285.1	*Glycine max* cDNA, clone no. GMFL01-25-P24	318	94	9e-84	229/261 (88%)
L8	AY935856.1	*Glycine max* isolate 34 ATP synthase β subunit (ATP β) gene, partial CDS; plastid	311	100	6e-82	170/171 (99%)
L9	DQ887088.1	*Arachis hypogaea* ubiquitin-conjugating enzyme 1 like proteinmRNA, complete CDS	78.8	65	1e-11	139/193 (72%)
L10	AC141866.11	*Medicago truncatula*, clone no. mth2-12p22, complete sequence	80.6	94	3e-12	140/201 (70%)
L11	AP006074.1	*Lotus japonicus* genomic DNA, chromosome 3, clone no. LjT40002, TM0111b, complete sequence	86.0	23	9e-14	72/85 (84%)
L12	AK062948.1	*Oryza sativa* japonica group cDNA, clone no. 001-109-C05, full insert sequence	178	44	2e-41	163/194 (84%)
L14	AP006074.1	*Lotus japonicus* genomic DNA, chromosome 3, clone no. LjT40002, TM0111b, complete sequence	80.5	17	5e-12	69/80 (86%)
L15	HP007959.1	*Arachis duranensis* DurSNP_c7969.Ardu mRNA sequence	275	89	6e-71	160/165 (97%)
L17	AC235190.1	*Glycine max* strain Williams 82 clone no. GM_WBb0011N22, complete sequence	95.1	44	7e-17	54/55 (99%)
L18	X69639.1	*Glycine max* ADR6 mRNA	204	100	6e-50	114/116 (98%)
L19	EU079374.1	*Cocculus orbiculatus* internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	126	59	1e-26	68/68 (100%)
L20	AF536226.1	*Vigna angularis* chloroplast S10A operon, partial sequence	178	99	3e-42	98/99 (98%)
L22	BT089123.1	Soybean clone JCVI-FLGm-1M1 unknown mRNA	228	89	6e-57	128/129 (99%)
L24	DQ317523.1	*Glycine max* cultivar PI 437654 chloroplast, complete genome	183	99	7e-44	99/99 (100%)
L27	AF456323.1	*Glycine max* cyclophilin (Cyp) mRNA, complete CDS	115	90	2e-23	77/84 (91%)
L28	BT089756.1	Soybean clone JCVI-FLGm -2D6 unknown mRNA	338	93	7e-90	190/192 (99%)
L30	Z47790.1	*P. sativum* mRNA for gibberellin-responsive ovary protein	62.6	50	4e-07	64/82 (78%)
L31	AF456323.1	*Glycine max* cyclophilin (Cyp) mRNA, complete CDS	120	88	9e-25	79/85 (92%)
L33	AB042299.1	*Vigna angularis* mRNA for 14-3-3 protein, complete CDS, clone no. VaM41	350	96	2e-93	236/257 (91%)
L34	Z25471.1	*P. sativum* blue copper protein mRNA, complete CDS	50.0	63	0.003	87/124 (70%)
L36	AL844865.6	UDP-N-acetyl-α−D-galactosamine: polypeptide N-acetylgalacto saminylt ransferase 13	41.0	34	1.3	40/52 (76%)

ubiquitin-conjugating enzyme gene began to express in soybean hypocotyls and stems as early as 0.5 h after inoculation with *P. sojae* race 1 that is incompatible to variety Suinong 10. C4H is reported as a critical enzyme to catalyze hydroxylation of cinnamic acid that is involved in the phenylpropanoid pathway (Shen *et al.*
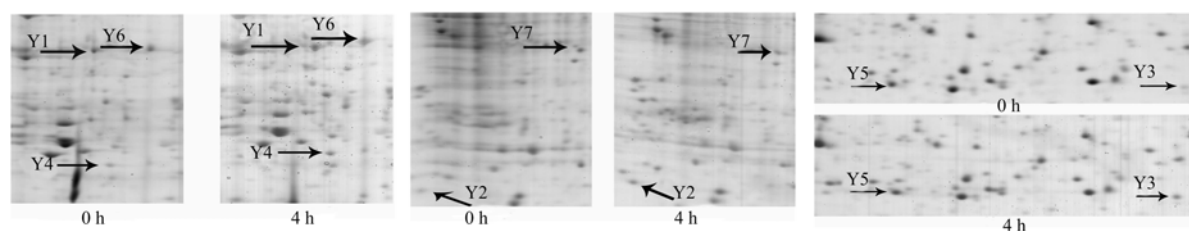
Fig. 4 Examples of enlargemental protein spots showing significant differences in abundance (up) between control and *P. sojae*-treated soybean hypocotyls and stems at 0 (A) and 4 (B) h after inoculation.

Table 2 Differential proteins identified by using MALDI-TOF MS

Spot no.	Score	Peptides match	Sequence coverage (%)	Accession	Regulation	Protein name
Y1	125	12	28	gi|62902819	Up	ATP synthase β subunit
Y2	149	42	11	gi|30580468	Up	Dynein-1-α heavy chain, flagellar inner arm I1 complex (1-α DHC)
Y3	89	12	33	gi|14030557	Up	Cytochrome P450-like protein
Y4	77	12	12	gi|162458104	Up	SET domain-containing protein SET104
Y5	72	6	42	gi|18643	Up	Unnamed protein product
Y6	263	35	57	gi|231585	Up	ATP synthase subunit α, mitochondrial

1991; McDonald and David 1999). ATP β gene coding ATP synthase β subunit is a significant gene in photosynthesis. Ubiquitin-conjugating enzyme gene participates in ubiquitin-dependent proteolytic pathway, which plays a role in signal transduction, differentiation, malignant transformation, and apoptosis (Shang *et al*. 1997). Therefore, expressions of C4H gene and ATP synthase gene at 0.5 h after inoculation in the present study might play a pivotal role in soybean resistance and energetic supersession in organism and participate in oxidative phosphorylation and photophosphorylation to *P. sojae*, which brought the time of initiation of resistance-related gene expression earlier 2.5 h compared to the report by Moy *et al*. (2004).

The expression of blue copper protein gene and UDP-N-acetyl-α-D-galactosamine gene were detected in soybean hypocotyls and stems at 1 and 2 h after inoculation, respectively. Blue copper proteins are found in the electron transport chain of prokaryotes and eukaryotes (Giri *et al*. 2004). UDP-N-acetyl-α-D-galactosamine gene is related to an agglutinin pathway. Compared to susceptible cultivars, agglutinin was detected in soybean seeds earlier and two times higher in amount in resistant cultivars to *P. megasperma* var. *sojae* (Gibson *et al*. 1982). Therefore, the expression of UDP-N-acetyl-α-D-galactosamine gene at 2 h after inoculation in the present study suggested that agglutinin might

be involved in defense reactions of soybean to *P. sojae*. But all these conclusions need further validation.

The up-regulation of three signal transduction related genes TGA-type basic leucine zipper protein TGA1.1 gene, cyclophilin gene, and 14-3-3 protein gene indicated host-pathogen recognition and resistance-triggering stated in 4 h after inoculation. TGA-type basic leucine zipper protein TGA1.1 gene was reported to play a role in signal transduction in bean abscission formation (Tucker *et al*. 2002). Cyclophilin gene encodes cyclophilin with functions in protein folding, protein degradation, stress response and signal transduction (Laxa *et al*. 2007). The 14-3-3 proteins are highly conserved molecules that function as signal transduction, cell cycle control and apoptosis (Yano *et al*. 2006). Original function of 14-3-3 proteins was described as activator of neurotransmitter synthesis (Ichimura *et al*. 1988) and subsequently as regulators of signaling proteins (Mori *et al*. 2000). The defense response genes and regulation factors identified in the present study could provide key genes, molecular markers and regulative factors to the soybean molecular breeding program for resistance to Phytophthora root and stem rot.

The results of present study suggested that three differential proteins, α-subunit (spot Y6) and β-subunit (spots Y1 and Y6) of ATP synthase and cytochrome P450-like protein (spot Y3), were related to disease re-

sistance of soybean to *P. sojae* (Table 2). The separation of the α-subunit and β-subunit of ATP synthetase at translational and post-translational periods indicated that soybean plants might increase energy metabolism rates to defend against the invasion of pathogens (O'Brien *et al*. 1999). Cytochrome P450-dependent hydroxylases involves in skeleton modification can modify basic flavonoids skeletons (Latunde *et al*. 2001). Flavonoids are in a diverse group of natural products which play important roles in plants against microorganisms (Harborne 1994; Dixon and Steele 1999). Therefore, the increase of P450 protein (spot Y3) in the present study could be related with a defense function or a defensive signaling molecule in response to the infection of *P. sojae*. Among the six disease resistance-related proteins identified in the present study, none of them was included in the 19 differential expression proteins that were reported in soybean variety Suinong 10 (Qiu *et al*. 2009).

The results of the present study showed that the ATP synthetase gene was separated during transcriptional periods at early stage of incompatible interaction, and α-subunit and β-subunit of ATP synthase were also separated during translational and post-translational periods, which suggested that a high level of energy was needed for soybean plants against the invasion of *P. sojae* during early infection stages.

C4H (CYP73) gene and P450 protein were separated at transcriptional as well as translational and post-translational levels, respectively. Cytochromes P450 of the CYP73 family catalyze the 4-hydroxylation of cinnamic acid, which is an early and obligatory step in the biosynthesis of most phenolic compounds such as lignin monomers, flavonoids, coumarins, stilbenes, lignans, and tannins that are related to disease resistance in plants (Dixon 2001). Therefore, the two products are very important to the metabolic pathway of plant disease resistance. On the other hand, functions of the P450 protein family are more extensive in disease resistance than C4H (Shen *et al*. 1991; Dixon 2001). In the present study, expression of ubiquitin-conjugating enzyme gene and blue copper protein gene at the transcriptional level, but not at translational and post-translational levels suggested that expression of these genes may be lag or not separated. The possible reason was either the genes expressed at a lower level, or these genes encoded the

key substances for some other synthesis pathways during the early stages of soybean and *P. sojae* interactions.

In summary, the incompatible interaction between soybean and *P. sojae* resulted in the joint action of transcriptional, translational, and post-translational levels. The results of the present study could give new insights into *P. sojae*-stress responses in soybean hypocotyls and stems.

Acknowledgements

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