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Full Length Research Paper

Gene differential expression of *Phytophthora sojae* during sporangium formation process

WEN Jing-zhi[1]*, LI Yan[1,2], LI Yong-gang[1], YANG Ming-xiu[1], SHI Na[2] and LIU Wei-ting[3]

[1]Department of Plant Protection, College of Agriculture, Northeast Agricultural University, Harbin 150030, People's Republic of China.
[2]Pesticide Management and Test Station of Heilongjiang Province, Harbin 150090, People's Republic of China.
[3]Heilongjiang Academy of Agricultural Sciences, Harbin 150086, People's Republic of China.

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The gene differential expression of *Phytophthora sojae* during early stage (0 to 7 h) of sporangium formation was studied by differential display reverse transcription polymerase chain reaction (DDRT-PCR) in the present study. Thirty out of 78 primer combinations were selected and 90 stable differential bands were obtained. Thirty-nine positive differential fragments were found in the experiments by using the reverse northern blot, in which 36 sequences of differential fragments were obtained after sequencing. Homology search and analysis were tested by BLASTX in the *P. sojae* genome database. The results showed that 23 fragments had significant similarities with proteins in *P. sojae* genome database, 15 of which matched with the known functional proteins involved in metabolism, cellular processes, and signal transduction processes. Data mining and bioinformatics analysis on 4 fragments which redundancy was greater than 5 showed that sequence AR_{15}-258 had a very high expression level in *Phytophthora* mycelium, which may be related to mycelium infection or hunger; AR_{10}-263 and GR_1-304 were only found in *Phytophthora*, which might be unique expression in this genus. The analysis and research on important regulatory genes controlling sporangium formation of *P. sojae* was a foundation for studying of key genes controlling growth and development of *P. sojae*.

Key words: *Phytophthora sojae*, sporangium formation, mRNA differential display, differential fragments.

INTRODUCTION

Phytophthora root rot of soybean, caused by infection of *Phytophthora sojae*, is a worldwide recognized soybean disease due to its large infection area and severity (Tyler, 2007). It has also been listed as one of the destructive diseases of soybean (Tyler et al., 2006). It is basically a kind of single cycle soil-borne disease; therefore, the infection mainly depends on whether the oospores survived in the soil could germinate to form sporangia and whether the zoospores could be released from sporangia or not. With the understanding and knowledge of molecular mechanism of these key aspects, we can control the production of inoculums and thus control the infection and harm fundamentally.

*Corresponding author. E-mail: jzhwen2000@yahoo.com.cn.

Previous studies indicated that two genes are important in formation of sporangium of *Phytophthora infestans*, one of which is *Pigpb1*, which codes G-protein β-subunit, *Pigpb1*-silenced mutants form very few sporangia (Latijnhouwers and Govers, 2003). Another is *piCdc14*, which expresses in sporangiophore initials, coding Cdc14 proteins to regulate mitosis and the cell cycle (Ah-Fong and Judelson, 2003). In addition, Blanco and Judelson (2005) identified an interactor of the zoosporogenesis-induced kinase *Pipkz1*. *Pibzp1* is necessary for normal zoospore movement, essential for appressoria formation and plant infection. *PsGPR11*-silenced transformants in *P. sojae* exhibited no differences in sporangium production or size; however, the release of zoospores from sporangia was severely impaired, and about 50% of the sporangia did not completely release their zoospores (Wang et al., 2010). So far, no results on genes relating

to sporangium formation of *P. sojae* have been reported. The recent completion of *P. sojae* genome sequencing and the development of effective molecular genetic tools offer new opportunities for examining the genetic basis of *P. sojae* biology, physiology and pathogenicity (Tyler et al., 2006; Lamour et al., 2007).

Differential display reverse transcription PCR (DDRT-PCR) is an effective method on screening of differentially expressed genes from organism's cells and tissues (Liang and Arthur, 1992). This method has also been widely applied in the study of differentially expressed genes of fungi (Takemoto et al., 2000; Nagee et al., 2008). The purpose of this research was to study gene differential expression in formation (0 to 7 h) of *P. sojae* sporangium with DDRT-PCR technique, analyze important regulatory genes controlling sporangium formation, in order to lay a foundation for study of the key genes relating to growth and development of *P. sojae*.

MATERIALS AND METHODS

Strain of *P. sojae* and induction of sporangia

P. sojae Ps-597-3 with a pathotype of 1a3c7 isolated from soybean plant infected with Phytophthora root rot disease was provided by the Plant Pathology laboratory of Northeast Agricultural University (China).

Pure vegetative mycelia of *Ps*-597-3 were produced by the method of inoculating it on a layer of sterile cellophane on surface of carrot agar medium and cultivated for several days under 25°C. A mycelia disc was taken with a sterile hole puncher and placed in a sterile Petri-dish, then added with sterile distilled water to induce the formation of sporangia under 25°C for 6 days, changing the water every 3 h. The mycelia disc was taken respectively at 0, 0.5, 1, 3, 4, 6 and 7 h during the process of induction and quickly dried with sterile filter paper. The samples were preserved at -80°C after frozen in liquid nitrogen for extraction of total RNA.

Extraction, purification and reverse transcription of total RNA into first strand cDNA

RNAprep Tissue / Bacteria Kit (TIANGEN, Beijing, China) were used for extraction and purification of total RNA. Reverse transcription was done with RevertAid^{TM} M-MμLV Reverse Transcriptase (Fermentas, MBI, USA), and anchor primers were P_A (5'-AAGCT$_{11}$A-3'), P_C (5'-AAGCT$_{11}$C-3') and P_G (5'-AAGCT$_{11}$G-3') (Shanghai Sangon, Shanghai, China).

Differential PCR amplification and primer screening

The anchor primers used in the aforementioned reverse transcription and 26 random primers RP_{1-26} (Li et al., 2011) (Shanghai Sangon, Shanghai, China) were also used in PCR amplification. Reactions were carried out in 20.0 μL volume containing PCR buffer (10 ×) 2.0 μL, cDNA first strand (200 ng/μL) 1.0 μL, anchor primer (10 μmol/L) 1.5 μL, random primer (10 μmol/L) 1.0 μL, Mg^{2+} (25 mmol/L) 1.6 μL, dNTPs (10 mmol/L each) 0.4 μL, Taq DNA polymerase (5 U/μL) 0.2 μL, ddH_2O 12.3 μL (Shanghai Sangon, Shanghai, China). PCR amplification program: 94°C denaturation for 5 min; 94°C denaturation for 30 s, 40°C renaturation for 1 min, 72°C extension for 1 min, 5 cycles; 94°C denaturation for 30 s, 45°C renaturation for 1 min, 72°C extension for 1 min, 33 cycles; 72°C extension for 10 min; preserve at 4°C. Amplification products were separated on 1% agarose gel to choose primer pairs with multiple amplification and clear bands for polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis and achievement of differential bands

In brief, 5 μL of PCR product added with 2 μL of loading buffer was denaturated and placed on ice before 6% polyacrylamide gel electrophoresis. Differential bands produced by 7 samples amplified with the same pair of primer were directly cut from the gel after silver staining and recovered by boiling. Then, 5 μL of supernatant was taken as template for secondary amplification with the same PCR system and procedures as the aforementioned differential PCR amplification. PCR amplification products were run by agarose gel electrophoresis and bands of the similar size as the original band were purified and recovered with UNIQ-10 column DNA gel extraction kit (Sangon, Shanghai, China).

Differential fragments test with reverse northern blot

Extraction, purification of total RNA of *P. sojae* mycelia and reverse transcription synthesis of cDNA were repeated and the purified cDNA was labeled with Digoxigenin (Roch) and hybridized with the purified recovered differential fragments and chromatography. The specific method was referred to instructions of DIG High Prime DNA Labeling and Detection Starter Kit II (TaKaRa, Dalian, China).

Cloning and sequencing of differential fragments and sequence analysis

Positive differential fragments after hybridization was connected with pGM-T vector (Beijing TIANGEN, Beijing, China) and cultivated in SOC medium containing IPTG, X-gal and ampicillin at 37°C after competent cells of *Escherichia coli* DH5α hot-striked with DNA recombinant plasmid. White spot was inoculated to LB liquid culture medium at 37°C overnight and PCR reaction was done with 1 μL liquid according to the procedures of differential PCR amplification. Positive clones from electrophoresis test were sent to Harbin Boshi Biotech Co., Ltd. for sequencing.

NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) BLASTX and the *P. sojae* Genome Database release version 1.1 (http://genome.jgi-psf.org/sojae1/sojae1.home.html) BLASTX were used for homology sequence analysis of the results and the results were only considered as important when Expectation values <1e-4 (Trudy et al., 2007). Both E value <1e-4 and the BLOSUM62 scoring matrix were used in Blast Algorithms. Finally, fragments with a redundancy greater than 5 were used for data mining and bioinformatics analysis (Chen et al., 2007).

RESULTS

Screening of primer combinations

In the present study, three kinds of anchor primers and 26 differential displayed random primers, which were 78 primer combinations in total, were used for DDRT-PCR amplification of 21 cDNAs. Among them, 30 primer combinations with anchor primer P_A, P_C, P_G and random primer including R_1, R_2, R_4, R_7, R_{10}, R_{15}, R_{19}, R_{22}, R_{24} and

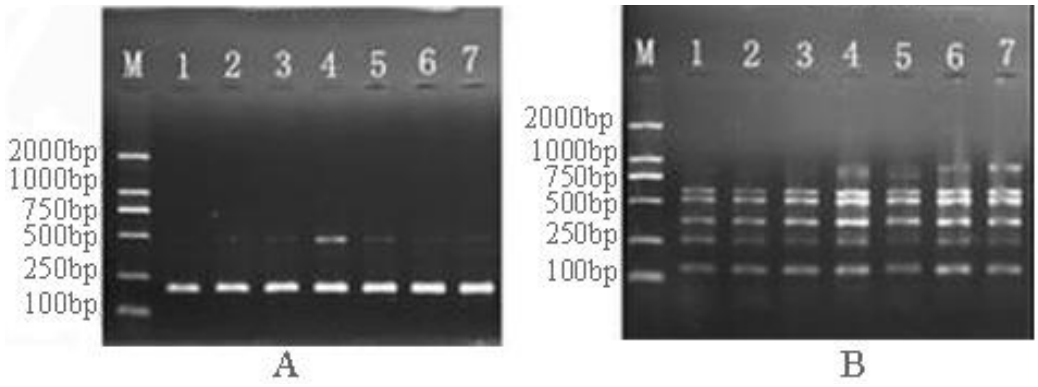

Figure 1. Electrophoresis profiles of the PCR products amplified by primer combination P_AR_{17} (A) and P_CR_{10} (B) from the single strand cDNA of *P. sojae* mycelia induced with sterile water. M: Marker DL2000; A: Combination of primer P_A and R_{17}; B: Combination of primer P_C and R_{10}; lanes 1 to 7: PCR amplification products from the single strand cDNA of *P. sojae* mycelia at 0, 0.5, 1, 3, 4, 6 and 7 h after induced with sterile water.

R_{26} had clear-band of PCR product. The number of bands amplified with different primer combinations was varied, and the size of DNA fragments amplified ranged from 100 to 750 bp. Amplification results of primers P_AR_{17} and P_CR_{10} are shown in Figure 1.

Differential display PCR amplification

The PCR products from the 30 primer combinations were tested with 6% denatured polyacrylamide gel electrophoresis (Figure 2). In total, 101 differential bands ranging from 100 to 400 bp were amplified and could be divided into four categories: (1) fragments (band a) that appeared in the control group, but not shown in the induction group; (2) fragments (band b) that appeared in the control group, but gradually disappeared in the induction group; (3) fragments (band c) that did not appear in the control group, but shown instantly in the induction group; (4) fragments (band d) that did not appear in the control group, but shown in the induction group in a certain time and expressed from beginning to end.

These 101 differential fragments were cut for another PCR amplification and purification, and 90 stable differential bands were obtained, in which 24 differential bands contained anchor primer P_A, 45 contained anchor primer P_G, and 21 contained anchor primer P_C.

Reverse northern blot

Reverse northern blot was applied in this study to identify positive differential bands. Digoxigenin (Roch) was used to label cDNA synthesized from reverse transcription, which was used as a probe hybrid with differential bands on nylon membrane for a secondary amplification and screened for the real differential fragments. The results of reverse northern blot showed that 13 out of 24 differential

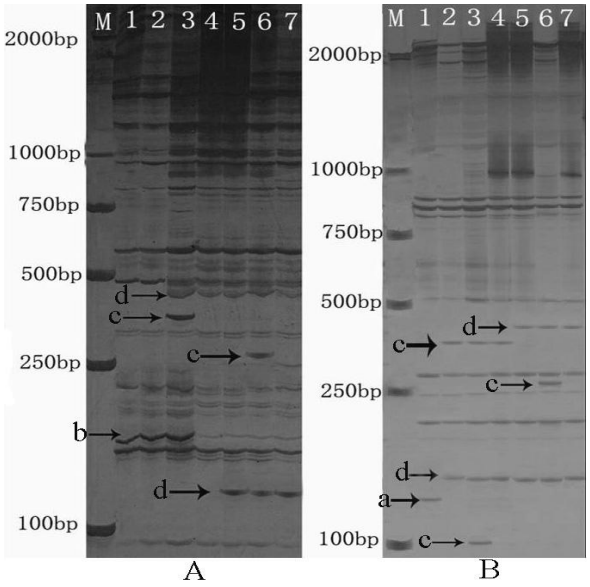

Figure 2. Partial mRNA differential display profiles of *P. sojae* during sporangium formation process. M: Marker DL2000; A: Combination of primer P_A and R_1; B: Combination of primer P_G and R_{19}; lane 1 to 7: PCR amplification products from single strand cDNA of *P. sojae* mycelia at 0, 0.5, 1, 3, 4, 6 and 7 h after induced with sterile water. Differential fragments are pointed by arrows.

fragments with anchor primer P_A, 18 out of 45 with anchor primer P_G and 8 out of 21 with anchor P_C were positive. Thus, in total, there were 39 positive differential fragments, and the remaining 51 differential fragments were false positive and false-positive rate was 56.7% (Figure 3).

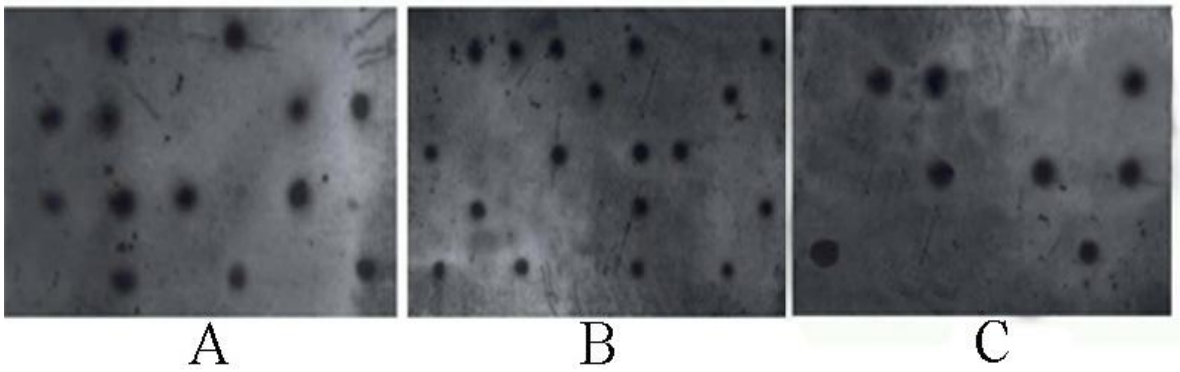

Figure 3. Reverse northern blot for the differential fragments. A: 24 differential fragments with anchor primer P_A; B: 45 with anchor primer P_G; C: 21 with anchor primer P_C.

Sequencing and analysis of differential fragment

Thirty-nine differential fragments were cloned, verified, and delivered to Harbin Boshi Biotech Co., Ltd., and a total of 36 differential fragments were sequenced. Fragments were named as anchor primers (A / C / G) + random primers (R_1 to R_{26}) + (-) + fragment length, such as AR_1-163.

Sequences of 36 differential fragments were compared in the *P. sojae* Genome Database with BLASTX (Table 1). The results suggested that 23 differential fragments significantly matched the proteins in genome database of *P. sojae*, and the matching rate was 63.9%, in which 15 matches were known functional proteins involved in metabolism, cellular processes and signal transduction process; 13 proteins had no significant match; four fragments, which redundancies were greater than 5, were respectively GR_1-304, GR_1-373, AR_{10}-263 and AR_{15}-258 (mRNA differential display pattern was shown in Figure 4), and the function of comparison proteins in genome database of *P. sojae* were respectively cytokinesis regulating protein-like, diacylglycerol acyltransferase (DAGAT), cellulose-binding elicitor and lectin-like(CBEL) protein and putative ABC transporter protein.

Data mining and bioinformatics analysis of 4 fragments with a redundancy greater than 5 (Table 2) showed that 26 EST matched with AR_{15}-258 in *Phytophthora* mycelium database, AR_{15}-258 also matched with one EST in *P. sojae* infection database and hunger database of *Phytophthora*, respectively. The results indicated that AR_{15}-258 was highly expressed in *Phytophthora* mycelium, which could be possibly related to mycelium infection or hunger; no EST matched with AR_{10}-263 was found in *Phytophthora* database, which indicated that AR_{10}-263 was poorly expressed in *Phytophthora*. Homologous genes were found in GR_1-304, GR_1-373 and AR_{15}-258 as in *P. ramorum* and *P. capsici* in 4 fragments with redundancy greater than 5, no homologous genes were found in AR_{10}-263 as in *P. ramorum* and *P. capsici*; at the mean time, AR_{10}-263 and GR_1-304, which were unique expressions, were only found in *Phytophthora*.

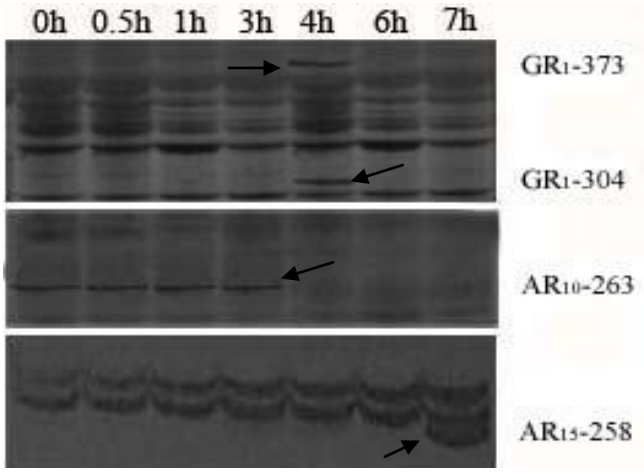

Figure 4. mRNA differential display profiles of 4 differential fragments (Redundancy >5). 0, 0.5, 1, 3, 4, 6 and 7 h represented PCR amplification products from single strand cDNA of *P. sojae* mycelia at 0, 0.5, 1, 3, 4, 6 and 7 h after induced with sterile water. Differential fragments are pointed by arrows.

DISCUSSION

Material selection

One of the characteristics of DDRT-PCR technique is high sensitivity, which means a smaller difference on genetic background of the test material leads to lower false positive rate. Differential expressed genes induced by sterile water in formation of *P. sojae* sporangium were studied in this experiment. Pure vegetative mycelia free from sporangium and oospore were required in order to reduce the differences between test materials. Cellophane has been applied on the fungal culture before, including *Phytophthora parasitica* (Gaulin et al., 2002) and *Magnaporthe grisea* (Sun et al., 2006; Mehrabi et al., 2008). However, those applications were only for an easier observation of the infection and movement of the fungus. Carrot agar medium with a layer of sterile

Table 1. Results of BLASTX comparison of 36 differential expressed sequences in genome database (version1.1) of *P. sojae* during the early stage (0 to 7 h) of sporangium formation under sterile water stress.

Sequence code	Category and putative function	E value	Protein ID[1]	Redundancy[2]
AR_1-157	Hypothetical protein (*Arabidopsis thaliana*)	8e-10	130120	1
AR_1-163	No significant matches			
AR_7-92	No significant matches			
AR_7-178	No significant matches			
AR_7-296	Dolichol-phosphate mannosyltransferase	3e-28	108591	1
AR_{10}-158	Putative phosphoinositide phosphatase	3e-06	155514	1
AR_{10}-263	CBEL (cellulose-binding elicitor and lectin-like) protein	2e-16	135818	11
AR_{10}-329	Decarboxylase/sphingosine phosphate lyase	3e-33	130206	1
AR_{15}-258	Putative ABC transporter protein	4e-48	140011	54
AR_{26}-147	No significant matches			
AR_{26}-360	unknown protein	9e-25	129960	1
CR_1-182	No significant matches			
CR_1-298	No significant matches			
CR_4-289	ENSANGP00000020783	6e-36	142830	1
CR_{10}-284	expressed protein	2e-32	134388	1
CR_{10}-409	expressed protein	4e-32	134388	1
CR_{15}-282	GTPase Rab5/YPT51 and related small G protein superfamily GTPases	1e-35	108345	2
CR_{15}-290	No significant matches			
GR_1-167	No significant matches			
GR_1-304	Cytokinesis regulating protein-like	2e-26	134372	75
GR_1-373	Acyl-CoA:diacylglycerol acyltransferase (DGAT)	7e-45	136565	12
GR_2-276	No significant matches			
GR_7-256	No significant matches			
GR_7-292	Dolichyl-phosphate mannosyltransferase polypeptide 1	1e-27	108591	1
GR_7-320	Crinkling and necrosis-inducing protein CRN1	7e-05	134182	1
GR_7-332	Triacylglycerol lipase and aldehyde dehydrogenase	2e-45	132443	2
GR_7-397	Related to histone-lysine N-methyltransferase [MIPS]	2e-54	128330	1
GR_{15}-166	No significant matches			
GR_{15}-200	No significant matches			
GR_{15}-241	Import in beta-2 subunit family protein	7e-17	134304	1
GR_{19}-312	Autophagocytosis protein AUT1-like	1e-25	110627	1
GR_{19}-338	unknown protein	8e-30	136983	1
GR_{19}-366	Putative senescence-associated protein	1e-14	125126	3
GR_{22}-343	No significant matches			
GR_{26}-366	unknown protein	9e-25	129960	1
GR_{26}-369	Hypothetical protein FG02288.1	4e-31	141804	2

[1]The protein ID numbers according to the *P. sojae* Genome database. [2]Redundancy according to protein number by BLASTX searches of the *P. sojae* genome database version 1.1.

cellophane was used in this experiment for cultivation of *P. sojae.* The mycelium grew slower on the cellophane than directly on carrot agar medium; however, oospores and sporangia were not found in 10 days, which means difference among material was reduced with no affection from oospores and sporangia. Meanwhile, medium would not be attached to mycelium either, which was better for RNA extraction. The sterile water was used in this research for sporangium induction, as the sporangium of *P. sojae* could not be induced on solid medium.

Researches on growth and development of *P. sojae* are still inadequate although the economic losses caused by this pathogen were well focused. The purpose of this test was to study genes associated with formation of *P. sojae* sporangium. Samples were continuously taken at early stage of sporangium formation to extract total RNA for differential display reverse transcription polymerase chain reaction (DDRT-PCR) test, in order to find the key genes regulating the formation of sporangium. Mycelia at 0 h, instead of non-induced mycelia at the same stage, were

Table 2. Data mining and bioinformatics analysis of differential fragments (redundancy >5).

Sequence code	Expression time (h)	Expression symbol	Protein ID in *P. sojae* genome database	Protein size	Transcripts in *P. sojae* EST database[1]	Transcripts in *Phytophthora* EST database[1]	Phylogenetic Distribution[2]	Genes in *P. sojae*[3]	Genes in *P. ramorum*[3]	Genes in *P. capsici*[3]
AR_{10}-263	0 - 3	b	135818	325			Only in *Phytophthora*	1	0	0
GR_1-373	4	c	136565	809		AP (1) MY (1) ZO (1)	Other species	2	1	11
GR_1-304	4	c	134372	463	IN(1)	IN (1) ST (1)	Only in *Phytophthora*	7	1	3
AR_{15}-258	7	d	140011	1118	IN(1)	MY (26) IN (1) ST (1)	Other species	13	19	30

[1]The tissue types from which the EST libraries are derived are zoospores (ZO), mycelia (MY), infection (IN), starvation (ST), and appressorium (AP). The numbers in brackets indicate the number of ESTs present in the various libraries (Randall et al., 2005). [2]Homologues in species other than *Phytophthora* were considered as homologues when the BLAST E value was less than 1e-3 and the similarity > 30%. [3]*Phytophthora sojae*, *Phytophthora ramorum*, *Phytophthora capsici* whole-genome sequences and gene annotation at the JGI website (http://genome.jgi-psf.org/) were used for analysis. Genes with BLAST similarity higher than 50% were considered to be members of the same gene family. Numbers indicate the size of the family.

used as control in screening differential fragments; this not only saved time, but also allowed comparison of mycelia induced by sterile water at various stages.

Function analysis of differential fragments

Cellulose binding elicitor lectin (CBEL) can recognize cellulose; it is related with natural cell wall sedimentation of *Phytophthora* and can thicken the mycelium (Gaulin et al., 2002). Differential fragments AR_{10}-263 and CBEL protein (Mateos et al., 1997) have important similarities, which included expression at 0 to 3 h in *P. sojae* mycelium, causing cell wall of *P. sojae* mycelium settled normally, could lead to cell wall thickening and thus provides condition for formation of sporangium.

Diacylglycerol acyltransferase (DAGAT) was strongly induced at the beginning of fat synthesis (Shockey et al., 2006); triacylglycerol was catalyzed into glycerol and fatty acyl-S-CoA (Zou et al., 1999), which regulates the content of triglyceride in seed development (Jako et al., 2001). Triacylglycerol were stored as energy in the rapidly growing cells, spores and various regenerating structures in fungi. Differential fragment GR_1-373, which was highly expressed at 4 h in *P. sojae* mycelium induced by sterile water shared essential similarity with DAGAT. It could also be involved in triacylglycerol synthesis and provide energy during tip growth of *P. sojae* mycelium and sporangium formation.

In addition, differential fragment GR_1-304 sharing great similarity with cytokines is regulatory protein (Sasaki et al., 2002), which showed high expression in *P. sojae* mycelium at 4 h induced by sterile water. It could be related to cytokinesis regulation of *P. sojae*, promoting the formation of zoospores by protoplasm dissever of enlarged cytoplasm inside the hypha.

PDR5 located in plasma membrane of the yeast can evacuate a large variety of anti-cancer drugs from cells (Sasaki et al., 2002). ABC transporter protein, similar to PDR5 and coded by TUR2, could possibly eliminate toxic metabolites out of cells under stress conditions Smart and Fleeting, 1996). Differential fragment AR_{15}-258 and assumed ABC (ATP-binding cassette) carrier PDR protein have an important similarity. It was highly

(expressed in *P. sojae* mycelium at 7 h induced by sterile water, which was probably due to lack of nutrition when *P. sojae* cells were under water stress and thus resulted in certain toxic metabolites requiring discharge by ABC transporter protein.

In summary, when *P. sojae* mycelia was induced by sterile water, CBEL gene was first regulated and 3 h later CBEL silencing led to mycelia cell wall thickening, which provided material basis for sporangium formation; and DAGAT gene was regulated to synthesize triacylglycerol and to provide energy for development of hypha tip of *P. sojae* as well as formation of sporangium. At the same time, cytokinesis regulatory proteins were controlled to promote the formation of zoospores by protoplasm dissever of enlarged cytoplasm inside the hypha and enhance sporangium formation; and some toxic metabolites were excreted in the process of sporangium formation. The aforementioned was only a speculation of function of each cDNA clone sequence of *P. sojae* sporangium formation induced by sterile water and a large number of tests were still to be done as proof. The functions of many differential fragments were still unknown in this study. Complete sequenced genes are essential to discover function and regulation mechanism of differential expressed genes induced by sterile water in formation of *P. sojae* sporangium and much further studies are needed.

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