RESEARCH ARTICLE

Identification of *Phytophthora sojae* genes involved in asexual sporogenesis

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Abstract

To explore the molecular mechanisms involved in asexual spore development in *Phytophthora sojae*, the zoospores of strain PS26 were treated with ultraviolet (UV) irradiation. After selection, a mutant progeny, termed PS26-U03, was obtained and demonstrated to exhibit no oospore production. A suppression subtractive hybridization (SSH) approach was developed to investigate differences in gene expression between PS26 and PS26-U03 during asexual sporogenesis. Of the 126 sequences chosen for examination, 39 putative unigenes were identified that exhibit high expression in PS26. These sequences are predicted to encode proteins involved in metabolism, cell cycle, protein biosynthesis, cell signalling, cell defence, and transcription regulation. Seven clones were selected for temporal expression analysis using RT-PCR based on the results of the dot-blot screens. Three of the selected genes, developmental protein DG1037 (*UB88*), glycoside hydrolase (*UB149*) and a hypothetical protein (*UB145*), were expressed only in PS26, whereas the transcripts of phosphatidylinositol-4-phosphate 5-kinase (*UB36*), FAD-dependent pyridine nucleotide-disulphide oxidoreductase (*UB226*) and sugar transporter (*UB256*) were expressed at very low levels in PS26-U03 but at high levels in PS26.

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Introduction

Phytophthora sojae is an oomycete pathogen of soybean, classified in the kingdom Stramenopiles. It causes 'damping off' of seedlings and root-rot of older plants, with an annual cost worldwide of $ 1–2 billion (Tyler 2007). Central to the disease cycles are spores, which disseminate the species spatially and germinate to form infective structures. The primary asexual spore of the pathogen is the multinucleate sporangium. The induction of asexual sporulation occurs in non-mating aerial hyphae upon ageing or nutrient limitation (Fabritius *et al*. 2002), and involves the differentiation of hyphal tips into sporangiophores (Bartnicki-Garcia and Wang 1983; Ribeiro 1983). Terminal swellings then form and become the sporangia, each containing several diploid nuclei. Mitosis stops within sporangia, even though their cytoplasm remains active and undesiccated, unlike the conidia of true fungi (Hemmes 1983). Asexual sporangia are transported between plants by wind or water. At cool temperatures, the sporangia release motile zoospores that can home in and encyst on a host and then breach its barriers using a germ tube or appressorium.

Most studies of asexual sporulation in *P. sojae* have been limited to physiological and cytological analyses (Bartnicki-Garcia and Wang 1983; Deacon and Donaldson 1993; Hardham 2001). However, understanding the molecular basis of asexual sporulation in *P. sojae* may hasten the development of new strategies for controlling disease, for example by identifying targets for chemicals interfering with asexual sporulation or germination. But molecular details about asexual spore development in *P. sojae* are limited (Tyler *et al*. 2006).

In the present study, to understand the molecular basis of asexual spore development in *P. sojae*, we investigated gene expression changes involved in asexual sporulation after ultraviolet (UV) irradiation in *P. sojae* using a suppression subtractive hybridization (SSH) approach that is an extremely efficient method in a number of different systems to identify novel or specific genes (Lisitsyn *et al*. 1993; Wei *et al*.

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2001; Shim and Dunkle 2002; Cramer and Lawrence 2004; Bulman *et al*. 2006). Of the 126 sequences chosen for examination, 39 putative unigenes were identified that exhibit high expression in oosporogenesis stage. These sequences are predicted to encode proteins involved in metabolism, cell cycle, protein biosynthesis, cell signalling, cell defense, and transcription regulation.

Materials and methods

Pathogens and growth conditions

Isolate of P. sojae: PS26, was isolated from Heilongjiang province of China; by hyphal tip isolate from soybean stem in 2005, and purified by single zoospore isolation. Isolates were maintained in culture in 9-cm diametre petri dishes on V8-juice agar at 25°C in dark. Cultures were subcultured onto fresh V8-juice agar medium every seven days.

UV exposure and comparision of morphological characteristics

To obtain a mutant that is unable to form sporangia, the zoospores of PS26 were irradiated in 9-cm diametre petri dishes on V8-juice agar from above at a distance of 30 cm from a 20 W UV-lamp for 10 s, 20 s, 30 s and 40 s, respectively. Control zoospores were subjected to similar procedures without UV exposure, and then the zoospores were incubated at 25°C in dark for two to seven days.

Colony appearance (shape and color), growth rate, yields of sporangia, and survival of oospores were compared with PS26. Growth rate was measured according to the procedures described previously (Kaufmann and Gerdemann 1958). To measure the number of oospores, the cultures were grown in 9-cm diametre petri dishes contained 15-ml V8-juice agar for 30 days at 25°C, according to Bhat and Schmitthenner (1993). A mycelial mass (40×40 mm) with V8-juice agar was excised from the middle of plate, and it was put into a bottle with 20 ml sterile distilled water to produce suspension of oospores as described by Bhat and Schmitthenner (1992). The oospores in the suspension were counted under a microscope. To count the number of sporangia, the cultures were grown in 9-cm diametre petri dishes containing 15-ml V8-juice agar for seven days at 25°C, according to Rutherford *et al*. (1985). Ten mycelial masses (10×10 mm) were transferred to petri dishes containing 30 ml sterile distilled water to induce sporangia as described by Bumbieris (1974). Every mass was observed to count the amount of sporangia using a microscope at 150× magnification. Average sporangial production by PS26 was compared with isolates subjected to UV exposure and the experiment was repeated thrice.

RNA isolation and cDNA synthesis

Total RNA of the mycelia was extracted with TRIzol Reagent kit (Invitrogen, Carlsbad, USA) and the cDNA populations were created using the SMART cDNA synthesis system (BD Biosciences-Clontech, Palo Alto, USA). The SMART approach is a PCR-based amplification system that allows the creation of cDNA from very small amounts of total RNA. In this study, approximately 1.0 μg of total RNA was used to generate each cDNA population for use in the subtraction procedure. The manufacturer's recommendations were used throughout the cDNA synthesis procedure.

Suppression subtractive hybridization

Suppression subtractive hybridization (SSH) was performed between the driver (mixed mycelia of PS26-U03 inoculated for 6, 12, 18, 24, or 36 h) and tester (mixed mycelia of PS26 inoculated for 6, 12, 18, 24, or 36 h) cDNA populations. The SSH procedure was performed with the PCR-Select cDNA Subtraction kit according to the manufacturer's directions (BD Biosciences-Clontech, Palo Alto, USA), with slight modifications as noted below. Briefly, the cDNA generated from the SMART procedure was digested with 15 U of *Rsa*I. The digestion was run through a Qiagen Qiaquick column (Qiagen, Valencia, USA) to recover purified digested cDNA. Two aliquots of the tester cDNA were ligated to adaptors 1 and 2R, respectively, with 400 U of T4 DNA ligase. The ligation proceeded overnight at 16°C and was stopped by adding 1 μl of EDTA/glycogen and incubating the ligations at 72°C for 5 min. The first hybridization was performed at 68°C for 8 h. During this hybridization, excess driver cDNA was added to each tester population (with different adaptors) to further enrich for differentially expressed genes.

The second hybridization involved mixing the two samples from the first hybridization and adding fresh denatured driver cDNA to further enrich for differentially expressed sequences. Second hybridization reactions were incubated overnight at 68°C. PCR was used to selectively amplify differentially expressed sequences. During the first step in PCR, only ds cDNAs with different adaptor sequences on each end were exponentially amplified. The conditions for the first PCR were as follows: 5 min at 75°C, 25 s at 94°C, and 30 cycles of 10 s at 94°C, 30 s at 66°C, and 1.5 min at 72°C. Finally, a nested PCR (using nested primers 1 and 2R included in the SSH kit) was used to reduce background and further enrich for differentially expressed sequences in the tester population. The conditions for this secondary nested PCR were 12 cycles of 10 s at 94°C, 30 s at 68°C, and 1.5 min at 72°C. All PCRs during the SSH procedure were performed in an MJ-PTC 200 thermocycler (Bio-Rad Laboratories, San Francisco, USA). The PCR mixture enriched for differentially expressed sequences from the tester population was cloned using the Pmd18 T-vector Cloning kit (TaKaRa, Dalian, China) and transformed into JM109 *Escherichia coli* cells for blue–white selection. The forward subtracted library (PS26) contained approximately 4000 white clones and 1806 white colonies were randomly picked for identification and characterization.

cDNA insert analysis by PCR

The forward subtracted cDNA library was enriched for genes specific to oosporogenesis. Randomly picked white colonies were grown in 96-well plates with 100 μl LB containing ampicillin at the concentration of 50 ngl^{-1} at 37°C. The cDNA inserts in individual *E. coli* colonies were analysed by nested PCR. One μl of overnight culture was used in a 19 μl-reaction volume with nested primer1 and 2R. PCR was performed under the following parameters: 94°C for 30 s, 23 cycles of 95° for 30 s and 68° for 3 min. PCR product (8 μl) was checked on 2% agarose gel and the rest was used for differential screening.

cDNA macro-arrary dot-blot differential screening of clones

We used cDNA macro-array dot-blots to confirm differential expression of the clones, following the manufacturer's protocol in the PCR-Select Differential Screening kit (BD-Biosciences-Clontech, Palo Alto, USA), with minor modifications. Briefly, selected white colonies were grown in LB medium containing ampicillin (50 ngl^{-1}) overnight at 37°C and plasmids were extracted by alkaline method. Plasmids were denatured by suspension in 0.3 M NaOH and blotted in duplicate on Hybond-N+ nylon membranes. Both the forward subtracted and reverse subtracted probes were labelled with DIG using the random primer labelling of cDNA protocol in the PCR-Select Differential Screening kit (BD-Biosciences-Clontech, Palo Alto, USA). Hybridizations were conducted at 72°C overnight with continuous agitation. Following hybridization, membranes were washed in a low stringency solution (2× SSC and 0.5% SDS) at 68°C and then a high stringency solution (0.2× SSC and 0.5% SDS) at 68°C for 30 min each. The procedures of washing and colouring were operated according to the kit.

Clone sequencing and analysis

DNA sequencing was performed by Beijing Sunbiotech (http://www.sunbiotech.com.cn), using an ABI 3730XL DNA sequencer. M13 forward or M13 reverse primers were used in all sequencing reactions. The cDNA sequences were edited manually to remove vector sequences and to correct ambiguous base calls by comparison with the electropherograms to enhance the quality and reliability of the data.

The redundancy of cDNA sequences was determined by comparing all sequences with one another using the BlastN algorithm on the JGI local nucleotide database (http://genome.jgi-psf.org/sojae1.home.html) generated with the Formatdb program (*P. Sojae* Genome Database release v1.0). The self-score values were used to normalize the score values of matches to other sequences as described by Kamoun *et al*. (1999). Normalized score values greater than 10% were taken as an indication that the sequences represented the same gene.

cDNA sequences were compared with the international databases at the protein level using the gapped BlastX algorithm (Altschul *et al*. 1997). *P. sojae* sequences were considered to be homologous to previously reported genes with the prerequisite that the *E* value was less than 10^{-5}. Database searches were performed with the cDNA clones using the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), the Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and Oomycete EST Database version 1.5 (http://cbr-rbc.nrc-cnrc.gc.ca/service/cogeme) and Joint Genome Initiative (JGI) draft, the *Phytophthora sojae* Genome Database release v1.0 (http://genome.jgi-psf.org/sojae1/sojae1.home.html).

Sequences were grouped into MIPS (Munich Information Center for Protein Sequences) functional categories according to their putative BlastX identification (Mewes *et al*. 2002). All these sequences have been submitted to EST database of NCBI GenBank. The GenBank_Access numbers are listed in table 1.

RT-PCR analysis

After *P. sojae* was cultured in V8-juice at 25°C in darkness for five days, the mycelia were cultured in water at 25°C under light for 6, 12, 18, 24, or 36 h respectively (refresh water every 6 h), and the RNA was extracted. All RNA used for RT-PCR were treated with DNase I (TaKaRa, Dalian, China) prior to cDNA synthesis, to remove DNA contamination. A total of 1.5 μg of total RNA from each sample was used to create first strand cDNA according to the manufacturer's protocol. We used 1 μl of first strand cDNA in 100 ml PCR according to the LA PCR kit from TaKaRa (TaKaRa, Dalian, China). RT-PCR primers were designed based on the cloned sequence or the EST sequence if the clone had a corresponding EST. Primer lengths were between 18 and 22 bp with melting temperatures higher than 55°C in all cases. The primer sequences are available from the authors upon request.

PCRs were conducted in an MJ-PTC 200 thermocycler (Bio-Rad Laboratories, San Francisco, USA) with the following program: actinA, 95°C for 1 min followed by 28 cycles of 95°C 15 s, 64°C 30s, 72°C 40 s, and a final extension for 7 min at 72°C. Other genes examined in this study, 95°C for 1 min followed by 28 to 33 cycles depending on each gene, 56–61°C 30 s depending on each gene, 72°C 30 s, 1 min, or 1 min 30 s depending on the gene, and a final extension for 7 min at 72°C. PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized with Gel Doc 2000 (BIO-RAD, Segrate, Italy). All RT-PCRs were repeated at least twice.

Results

Morphological characteristics of survival

After irradiation and incubation, the colony appearance, growth rate, yields of sporangia and oospores of survival were compared with those of PS26. We found there was no

Table 1. Summary of 39 sequenced inserts from the SSH library enriched with *Phytophthora sojae* genes expressed in PS26 during sporogenesis.

Clone	Accession no.	Size (bp)	Category and putative function[a]	*E*-value[b]	Protein ID[c]	Redundancy
UB9	GE632025	592	Unknown	0	127051	7
UB12	GE632026	329	Mitochondrial substrate carrier	0	141471	2
UB13	GE632027	442	Unknown	0	142633	2
UB16	GE632028	304	Unknown	0	125130	2
UB36	GE632029	700	Phosphatidylinositol-4-phosphate 5-kinase	0	139746	5
UB39	GE632030	620	Ribosomal protein S2	0	136075	3
UB55	GE632031	546	Unknown	0	127792	3
UB73	GE632032	529	Hypothetical protein	0	108136	3
UB74	GE632033	621	Unknown	0	129763	5
UB84	GE632034	763	G-protein coupled receptors family 3	0	140820	2
UB88	GE632035	727	Developmental protein DG1037	0	140736	6
UB93	GE632036	444	Unknown	0	142841	3
UB95	GE632037	700	Unknown	0	141553	1
UB98	GE632038	606	Hypothetical protein	0	135537	1
UB110	GE632039	593	Unknown	0	141179	2
UB118	GE632040	601	Phosphatidyl serine synthase	2e−26	127041	2
UB121	GE632041	498	Kazal-like serine protease inhibitor	2*e*−26	128852	1
UB126	GE632042	597	ABC transporter	0	128854	3
UB127	GE632043	517	Eukaryotic thiol (cysteine) protease	0	130582	1
UB128	GE632044	514	Hypothetical protein	0	139685	5
UB143	GE632045	453	Hypothetical protein	0	142286	3
UB145	GE632046	270	Hypothetical protein	0	139830	7
UB149	GE632047	531	Glycoside hydrolase	0	131015	8
UB160	GE632048	376	Unknown	0	136723	2
UB168	GE632049	591	Phospholipase D/Transphosphatidylase	0	138727	3
UB169	GE632050	487	Hypothetical protein	0	130359	2
UB171	GE632051	485	Eukaryotic thiol (cysteine) protease	0	129496	1
UB180	GE632052	602	Phosphatidylinositol 3- and 4-kinase	0	140044	5
UB182	GE632053	303	Glutathione transferase	2*e*−26	133628	1
UB183	GE632054	367	Unknown	0	141692	4
UB220	GE632055	587	Glutamyl-tRNA synthetase	0	133964	1
UB223	GE632056	660	Acetyl-coenzyme A synthetase	2*e*−26	109048	2
UB226	GE632057	698	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	0	118708 6	
UB232	GE632058	527	Protein kinase	0	132069	3
UB234	GE632059	513	Metallo-phosphoesterase	0	139431	2
UB256	GE632061	590	Sugar transporter	0	135543	7
UB258	GE632062	425	Eukaryotic aspartic protease	0	134291	3
UB259	GE632063	388	Unknown	0	139197	4
UB262	GE632064	803	Integrase, catalytic domain	0	141588	3

[a] Putative identification is based on tBlastx searches of the *P. sojae* genome database.

[b] Only scores with an *E*-value of $< 10^{-5}$ were considered significant and the hits with highest significance are reported in this table.

[c] The protein ID number according to the *P. sojae* genome database.

sporangia in a survival isolate, PS26-U03, and there were no differences in the colony growth rates or the yield of oospores. In addition, the colony shapes and colours were nearly identical.

For the wild strain PS26, no asexual sporangia were found in any disc for 6 h after incubation; 12 h later, a mean of 4.0 ± 1.3 sporangia per eyeshot (40×) was detected in the same sample and at 18 and 24-h sporangia formation

was enhanced, respectively reaching 12.5 ± 3.0 and 31 ± 5.2 oospores per eyeshot. At 36 h and more, there were no significant differences in the yield, with a mean of 35.0 ± 3.1 oospores per eyeshot.

Generation of cDNA fragments of differentially expressed genes

The suppression subtractive hybridization technique (Diatchenko *et al*. 1996) was used to construct an SSH cDNA library enriched for sequences differentially expressed in the 'tester' sample (the mixture mycelia of PS26 cultured for 6, 12, 18, 24, or 36 h in water). RNA from the tester was subtracted against RNA from the 'driver' sample (the mixture mycelia of PS26-U03 cultured for 6, 12, 18, 24, or 36 h in water). To identify the bacterial colonies that possessed cloning vectors with inserts, the cDNA library was converted into plasmid clones by mass excision for library screening and analysis. Examination of 96 random cDNA clones indicated that 96% of the recombinant clones had inserts ranging from 0.15 to 2.1 kb, with an average insert size of 0.50 kb. A total of 1500 plasmid cDNA clones were transferred to 384-well microtiter plates that were then stored at −70°C. These clones were candidates for transcripts specific to, or upregulated in, the tester.

Expression screening of forward-subtracted clones by macroarray dot blots

To identify differentially expressed clones, the plasmids of the above 1500 clones were arrayed on 16 nylon membranes in duplicate, for a total of 32 membranes. Forward-subtracted (tester minus driver) and reverse-subtracted (driver minus tester) cDNA populations were DIG-labelled and used as individual probes for identical blots. Differences in hybridization signal intensities were apparent on the membranes probed with the two different types of probes, demonstrating the success of the SSH procedure in identifying differentially expressed sequences in the two populations.

A subset of the clones subjected to the macroarray dot-blot screening is presented in figure 1. Differences in hybridization signal intensities are apparent on membranes probed with the forward subtracted probe (figure 1,a) and the reverse subtracted probe (figure 1,b). A wide range of signal intensities, from very strong to faint, are evident in figure 1,a. Faint signals are presumed to represent rarely transcribed differentially expressed genes. Clones that hybridized only to the forward-subtracted probe were strong candidates for differential expression. Figure 1,a shows that almost 15% of the clones fell into this category. The clones that failed to hybridize may lie below the sensitivity limit and therefore represent extremely rare transcripts (figure 1,a; e.g. A3, C11 and G12). These clones typically correspond to low-abundance transcripts that are enriched during the subtraction. Of the 1500 PS26 clones examined, 126 passed this screening test and were strong candidates for differential expression; the identified clones were denoted sporangia-specific clones.

Sequence analysis of differentially expressed clones

A randomly chosen subset of the 126 sporangia-specific clones were sequenced. Given that each cDNA clone obtained using subtraction was digested with a restriction enzyme before subtractive hybridization (Diatchenko *et al*. 1996), certain ESTs correspond to partial regions of the same full-length cDNAs. Therefore, the sequences of 126 cDNAs were compared to the *P. sojae* Genome Database release v1.0. Self-BLAST analysis suggested that the 126 ESTs formed 39 clusters, representing 39 different putative genes (table 1). Among the 39 genes, 32 were represented by two or more ESTs. BLAST searches against the *P. sojae* Genome Database indicated that all of the above genes correspond to known *P. sojae* genes (table 1).

The 39 unique cDNAs were grouped into MIPS (Munich Information Center for Protein Sequences) functional

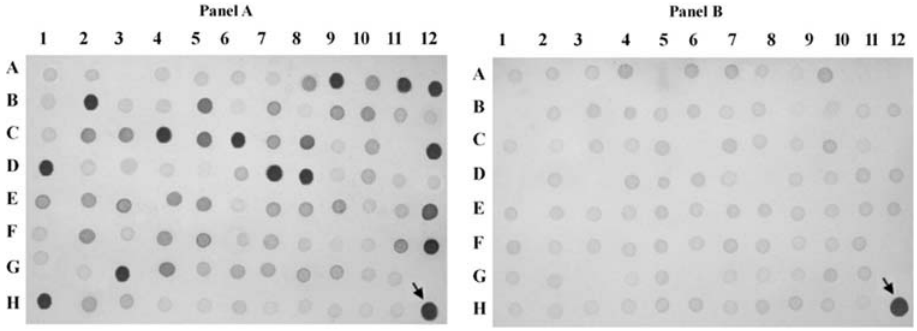

Figure 1. Comparison of macro-array dot-blots of PS26 and PS26-U03 subtracted cDNAs. Equal amounts of amplified PS26 cDNAs were arrayed onto two sets of membranes and hybridized with two different probes. (a) Blots hybridized with forward (PS26) subtracted cDNA probe. (b) Blots hybridized with reverse (PS26-U03) subtracted cDNA probe. Differentially expressed PS26 transcripts were shown as clones with dark signals in panel A. On each membrane, positive hybridization control is shown with an arrow.

categories, according to their putative identification in BLAST searches against the *P. sojae* Genome Database (Mewes *et al*. 2002). Approximately 12% of the annotated proteins are involved in signal transduction, 10% are involved in some aspect of metabolism, 10% are involved in cell defense, and 23% are associated with cell cycle, transport facilitator, protein synthesis and transcription regulation (figure 2). Approximately 41% are similar to hypothetical proteins or to proteins for which a function is yet to be determined; these were designated as unclassified. Of the unique ESTs, about 47% correspond to cDNAs whose resultant proteins perform other functions. This proportion is consistent with that reported for other fungal EST databases (Kamoun *et al*. 1999; Neumann and Dobinson 2003; Trail *et al*. 2003) and is dependent upon the organism, the experimental design, and the development stage.

RT-PCR analysis

Among the 39 genes, 10 were represented by five or more ESTs, and these genes may be important to asexual sporulation. To determine the detailed expression patterns of the genes, seven clones were selected to examine the mRNA levels by RT-PCR (figure 3). We examined the presence of the transcripts of these genes at five time points over 36 h during the period of sporulation.

The PCR special normalization of gene expression showed the expression of all genes examined to be low or none in PS26-U03, high in PS26 (figure 3). The transcripts of the UB88, UB149 and UB149 were not found in PS26-U03, they were found only in PS26; the transcripts of the UB36, UB226 and UB256 were found to be very low in PS26-U03, high in PS26, strengthening the conclusion that these genes are dramatically upregulated in PS26.

Discussion

This study represents a first step in elucidating the molecular basis of asexual sporogenesis in *P. sojae*. To obtain isolate of lost asexual spores reproduction, the zoospores of *P. sojae* were irradiated with UV. Previous genetic studies have indicated that it is highly probable that the differences in gene expression caused by UV irradiation resulted from mutations (Eisen *et al*. 1998; Mone *et al*. 2001). In the broader sense, mutations involve any changes in the nuclear genome that might take place during DNA replication, and can have a signifcant impact on the expression of certain genes (Fabritius *et al*. 2002). We identified 39 unigenes with most of the genes upregulated between PS26-U03 and PS26. It seems that genes highly expressed play an important role during asexual sporulation.

Phytophthora zoospores are chemotactically attracted to favourable infection sites on potential host plants. On reaching these sites, the zoospores encyst, rapidly detaching the flagella and secreting adhesive material onto the host surface (Robold and Hardham 2005). The adhesive material is synthesized during asexual sporulation and stored in secretory vesicles targeted to the ventral surface of the zoospores (Hardham and Gubler 1990; Dearnaley *et al*. 1996). During the first 2 min of plant infection, the adhesive material is secreted from the ventral vesicles and forms an adhesive pad that glues the spore to the plant surface (Gubler *et al*. 1989; Hardham and Gubler 1990). A gene encoding a 34-kDa glycoprotein, CBEL, that is expressed in hyphae and binds to cellulose fibres and cellulose in plant cell walls, has been cloned from *P. nicotianae* (Mateos *et al*. 1997) but silencing this gene does not affect pathogenicity (Gaulin *et al*. 2002) and its role in pathogen attachment to host plants remains to be determined. In our study, sequence information also provides insight into the function of many genes correlated to adhesin synthesis, for example, sugar transporter gene (*UB256*), that was represented by seven redundancy

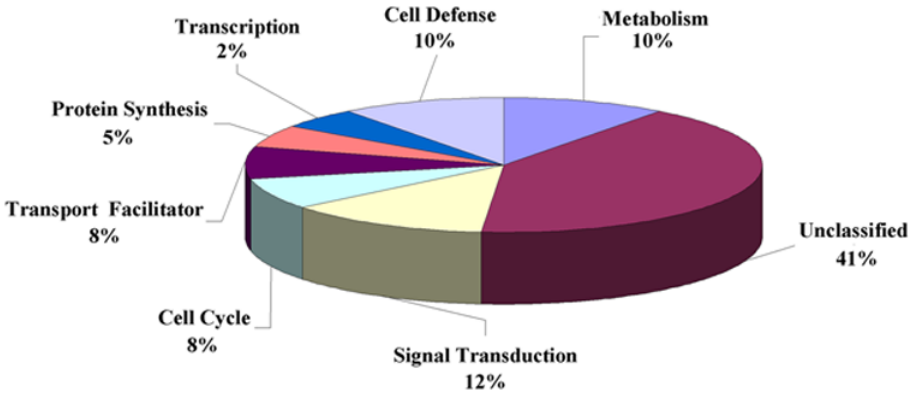

Figure 2. Functional classification of *P. sojae* cDNAs derived PS26 asexual sporogenesis library. The percentage (n = 40) in each of nine functional categories is shown (refer to table 1).

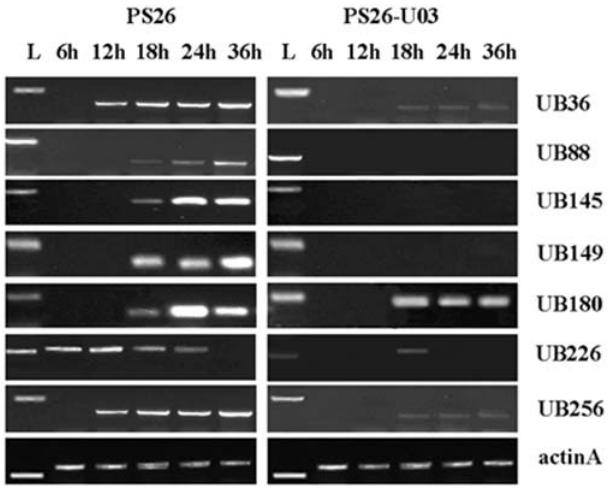

Figure 3. RT-PCR analysis of the expression of the seven genes up-regulated in PS26 and PS26-U03. *P. sojae* was cultured in V8-juice at 25°C in the darkness for five days, then the mycelia were transfer into water and cultured at 25°C under light for 6, 12, 18, 24, or 36 h. Lane L, 250-bp ladder; 6 h, culture 6 h; 12 h, culture 12 h; 24 h, culture 24 h; 36 h, culture 36 h.

ESTs, may play an important role in the adhesive material transport during asexual sporulation. The RT-PCR also show that UB256 was found very low in PS26-U03 and high in PS26.

We also discovered a gene of phosphagen kinases (phosphatidylinositol-4-phosphate 5-kinase, *UB36*), known for their roles in buffering ATP levels or metabolic channelling in animal muscle (Ellington 2001). Until the recent discovery of a phosphagen kinase in trypanosomes (Pereira *et al*. 2000), such proteins were believed to be restricted to metazoans. Flagellated life-stages are common to oomycetes, animals, and trypanosomes; therefore, it is tempting to speculate that flagella and phosphagen kinases have coevolved, possibly to maintain ATP concentrations in their highly active motile stages. Such kinases may reside in the cytoplasm or mitochondria, or be integral to the flagellar apparatus (Randall *et al*. 2005).

Several sporulation-induced genes (e.g. *UB88, UB171* and *UB258*) encode proteins associated with wall and matrix protein synthesis and thus contribute to the asexual structures such as the adhesive between antheridia and spore wall (Cassab 1998). Some genes appear to participate in various aspects of metabolism, especially transport or lipid modification. These include *UB118, UB168* and *UB234* which resemble phosphatidyl serine synthase, phospholipase, and phosphoesterase, respectively. These are of interest since oxidative lipid metabolism was shown necessary for antheridial induction, gametangial fusion, meiosis, and oospore maturation in *Lagenidium* (Kerwin and Washino 1986). The ABC transporter encoded by *UB126* might be involved in efflux of the mating hormones as in true fungi, and also might be required for tolerance to phytoalexins (Fleissner *et al*. 2002).

This study identified several genes whose products encoded regulators such as cell cycle, protein biosynthesis, cell signalling, cell defence and transcription regulation activities. However, more than 40% of the ESTs are yet to be assigned to a putative function, indicating that many of the mechanisms underlying pathogenesis remain unexamined. For example, the three clones *UB9*, *UB74* and *UB128* were the most abundant cDNAs in the sporogenesis-specific libraries, but their functions are unknown. With the development of suitable *Phytophthora* transformation and RNA-interference systems (Fire *et al*. 1998; Hannon 2002), the specific roles of such genes in pathogen pathogenicity may be determined.

Acknowledgements

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