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Differential screening reveals genes differentially expressed in low- and high-virulence near-isogenic *Phytophthora sojae* lines ☆

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Abstract

To explore the molecular mechanisms involved in virulence variations in *Phytophthora sojae*, the low-virulence isolate PS2 was inoculated successively on a resistant soybean (*Glycine max*) cultivar. After 14 successive inoculations, a high-virulence progeny, termed PS2-vir, was obtained and demonstrated to exhibit lower oospore production. DNA fingerprinting revealed no large-scale DNA differences in PS2 and PS2-vir. A suppression subtractive hybridization (SSH) approach was developed to investigate differences in gene expression between PS2 and PS2-vir in the early stages of soybean infection. Of the 323 sequences chosen for examination, 74 putative unigenes were identified that exhibit high expression in PS2-vir. These sequences are predicted to encode proteins involved in energy production, protein biosynthesis, cell signaling, cell-wall biogenesis, and transcription regulation. Ten clones were selected for temporal expression analysis using RT-PCR based on the results of the dot-blot screens. The possible genetic mechanisms involved in these phenomena are discussed.

Keywords: *Phytophthora sojae*; Transcription regulation; Virulence variation; Oospore production

1. Introduction

The genus *Phytophthora* consists of approximately 60 species, which together cause many of the most devastating plant diseases worldwide. These species belong to the oomycetes, a class of inferior eukaryotes that is evolutionarily far from fungi and is, instead, clustered with brown algae and diatoms, although these organisms exhibit a fungus-like morphology (Baldauf et al., 2000). The species include *Phytophthora infestans*, which caused the potato famine in Ireland in the 1840s and is still a worldwide problem in potato-growing regions (Birch and Whisson, 2001); *P. ramorum*, which causes Sudden Oak Death in California (Rizzo et al., 2002); and *P. cinnamomi*, which causes severe damage to forests and heathlands in Australia (Podger, 1972). In addition, many other *Phytophthora* species cause serious diseases in many dicotyledonous plants and forest ecosystems.

P. sojae, which causes major problems for soybean production throughout the world (Hansen and Maxwell, 1991; Tyler, 2001), is especially damaging when conditions are wet during the early stages of plant development, and can significantly reduce both establishment and yield (Teutsch and Sulc, 1997). Introgression of single dominant resistance genes has been used to minimize crop losses but the use of resistant cultivars also promotes the buildup of new virulent populations and this is particularly evident in fields under continuous soybean productions (Tooley and Bergstrom, 1984; Anderson and Buzzell, 1992; Ryley and Obst, 1992; Yang et al., 1996; Abney et al., 1997; Dorrance et al., 2003). The mechanisms involved in this phenomenon are unclear, but could be the result of mutation, mitotic

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recombination, loss of extra chromosomes, or genetic recombination at the sexual oospore stage (Ward, 1990; Bhat and Schmitthenner, 1992; Schmitthenner et al., 1994).

P. sojae is homothallic (Walker and Schmitthenner, 1984). Reproduction of *P. sojae* by rare outcrosses, associated with genetic recombination during meiosis in the antheridium or the oogonium, is a major mechanism of genetic variation in this diploid organism (Förster et al., 1994). However, other mechanisms of genetic variability may also have a significant role in creating new variants of this pathogen. Mutation, mitotic recombination, and parasexual recombination are the most common mechanisms of genetic variability in the absence of sexual reproduction (Goodwin et al., 1995; Goodwin, 1997). Several authors have observed variability in virulence among single-zoospore isolates derived from a common ancestor (Hilty and Schmitthenner, 1962; Rutherford et al., 1985; Bhat and Schmitthenner, 1992). Variation among hybrid progeny of *P. sojae* has also been reported (Layton and Kuhn, 1990; Bhat and Schmitthenner, 1993; Förster et al., 1994). However, variation in virulence among isolates generated by successive mass mycelial transfers on resistant soybean cultivars has received much less attention. To our knowledge, no investigations, to date, have demonstrated that virulent strains differ in gene expression from avirulent or low-virulence isolates during the plant–pathogen interaction. It is also not known what genes are involved in the increase in virulence under the pressure of the resistant cultivar. This information would have practical and theoretical implications.

Several researchers have utilized near-isogenic avirulent and virulent lines, to investigate genes that are differentially regulated in avirulent and virulent nematodes (Dalmasso et al., 1991; Jarquin–Barberena et al., 1991; Neveu et al., 2003). In these experiments, virulent strains were obtained by selection from avirulent lines continuously bred on resistant cultivars so that the near-isogenic avirulent and virulent lines should differ only in their ability to overcome the resistance genes of the cultivar. Therefore, it can be hypothesized that any molecular difference between the lines is related to the character of interest and, thus, such near-isogenic lines (NIL) constitute an ideal starting point for the identification of virulence or avirulence genes by means of differential analysis. Recently, Neveu et al. (2003) used a cDNA-amplification fragment length polymorphism (AFLP)-based strategy to identify genes differentially expressed between two pairs of NILs of two strains of the root-knot nematode *Meloidogyne incognita* that are avirulent and virulent against the tomato *Mi* resistance gene. Gene expression profiles were compared, and 22 of the 24,025 transcript-derived fragments (TDF) generated proved to be differentially expressed.

Therefore, to understand the molecular basis of virulence or pathogenicity in response to the selective pressure of a resistant host, we established near-isogenic low-virulence and virulent lines of *P. sojae*, derived from a low-virulence single-oospore line, continuously cultured on a resistant soybean cultivar. We investigated the differences in gene expression between such avirulent and virulent *P. sojae* NILs during the plant–pathogen interaction using a suppression subtractive hybridization (SSH) approach that is an extremely efficient method for the isolation of genes differentially expressed in plant–microbe interactions (Lisitsyn et al., 1993; Wei et al., 2001; Shim and Dunkle, 2002; Cramer and Lawrence, 2004).

2. Materials and methods

2.1. Pathogens, soybean cultivar and growth conditions

Isolate of *P. sojae*, PS2, was isolated from Heilongjiang province of China by hyphal tip isolate from soybean stem in 2002, and purified by single zoospore isolation. The pathotype of PS2 was determined by the hypocotyl injection technique on the following differentials: Williams (universal suscept), Harlon (*Rps*1a), Harosoy13XX (*Rps*1b), Williams 79 (*Rps*1c), PI103091 (*Rps*1d), Williams 82 (*Rps*1k), L76-1988 (*Rps*2), L83-570 (*Rps*3), PRX 146-36 (*Rps*3b), PRX 145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Harosoy 62XX (*Rps*6), and Harosoy (*Rps*7). Its virulence formula is 1b, 1d, 2, 3a, 3b, 4, 5, 6, and 7 (virulences to *Rps*1b, *Rps*1d, *Rps*2, *Rps*3a, *Rps*3b,*Rps*4, *Rps*5, *Rps*6, and *Rps*7, respectively). Isolates were maintained in culture in 9 cm diameter Petri dishes on lima bean agar (LBA) (extract of 60 g/l frozen lima beans solidified with 1.5% Bacto-agar) at 25 °C in dark. Cultures were subcultured onto fresh LBA medium every 7 days.

Seeds of *Glycine max* cultivar Nannong 88-48 were a gift of Dr. H. Xing, National Center for Soybean Improvement, Nanjing, Jiangsu, China and were used to select virulent *P. sojae* isolates. Seeds were sown in sterilized soil in 15 cm plastic pots (5 seeds per pot). Seedlings were allowed to grown for 10 days in a greenhouse with a 14 h photoperiod, a 26/21 °C day/night temperature before inoculation, and daily watering.

2.2. Selection of virulent isolates

Virulent *P. sojae* isolates was selected by successive mass mycelial transfers of PS2 on the resistant soybean cultivar Nannong 88-48. Twenty seedlings were inoculated in each selection with another five seedlings per origin line serving as controls.

The cultures were grown on LBA for 7 days at 25 °C. Inoculations were performed by the standard hypocotyl method (Laviolette and Athow, 1981), using 2×2 mm pieces of mycelia, and the wound was covered with petrolatum to prevent desiccation of the inoculum and host tissue. After 5 days, measurements (cm) were taken on the length of the lesion from the inoculation point upward the stem. In these experiments, virulence of isolates was evaluated by measuring the length of the lesion. All observations and measurements were obtained from at least 15, and usually 20, seedlings. At each stage in the virulence assays, hypo-

cotyl tissue from the longest lesions were used to re-isolate hyphae to be used in the next selection round.

2.3. Checking the stability of acquired virulence

PS2-vir, a virulent line selected from PS2 by successive mass transfers on a resistant soybean cultivar Nannong 88-48 for 14 times, was maintained in culture in 9 cm diameter Petri dishes on LBA at 25 °C in the dark for six months. Cultures were subcultured every 7 days in the course of the treatment. At intervals of 1–6 months the subcultures on the LBA were inoculated on Nannong 88-48 again and the length of the lesions on the resistant cultivar were measured at 5 days post-inoculation as described above.

2.4. Compare of morphological characteristics

Colony appearance (shape and color), growth rate, yields of sporangia, and oospores of PS2-vir were compared with PS2. Growth rate was measured according to procedures described previously (Kaufmann and Gerdemann, 1958). To measure the number of oospores, the cultures were grown in 9 cm diameter Petri dishes contained 15 ml LBA for 30 days at 25 °C according to Bhat and Schmitthenner (1993). A mycelial mass (40 × 40 mm) with LBA 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 using a Nikon microscope. To count the number of sporangia, the cultures were grown in 9 cm diameter Petri dishes contained 15 ml LBA for 7 days at 25 °C according to Rutherford et al. (1985). Ten mycelial masses (10 × 10 mm) were transferred to Petri dishes contained 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 Nikon microscope at 150 × magnification (Zheng, 1995). Average sporangial production by PS2 were compared with that by PS2-vir and the experiment were repeated for three times.

2.5. DNA extraction and RAPD analysis

In the present study, differences of genetic characters were analyzed between PS2 and PS2-vir using random amplified polymorphic DNA (RAPD) fingerprints. Genomic DNA was extracted from mycelia, following the CTAB (cetyltrimethylammonium bromide) protocol of Winnepenninckx et al. (1996), modified so that mycelia were not ground under liquid nitrogen, but instead, directly incubated in CTAB buffer. PCR amplification took place in an MJ-PTC 200 thermocycler (Bio-Rad Laboratories, South San Francisco, CA) using the following profile: an initial 3 min denaturation at 94 °C, 35 cycles of 15 s denaturation at 94 °C, 25 s annealing at 36 °C and 60 s extension at 72 °C, followed by a 10 min final extension at 72 °C. Reaction were carried out in 25 μl volume with 10 ng of template DNA, 2.5 μl of 10× reaction buffer, 1.5 μl of $MgCl_2$ buffer (10 mM), 3.5 μl of dNTP (2.5 mM), 0.1 μM oligoprimer, and 0.5 U of *Taq* polymerase (Promega). Two hundred 10-base oligonucleotide primers (nos. S101–S300) obtained from the TaKaRa Biotechnology (Dalian) Co. Ltd. (http:www.takara.com.cn) were used. The amplified products were electrophoresed on 2% agarose gels (FMC Bio Products) containing 0.5 μg/ml ethidium bromide, which were run in 1 × TAE running buffer. A 2 kbp DNA ladder (TaKaRa, Japan) was included on each gel as a molecular size standard.

2.6. RNA isolation and cDNA synthesis

P. sojae was grown in 20% tomato juice in petri plates at 25 °C for 4 days in incubator. The mycelia were inoculated on the surface of hypocotyl of Nannong 88-48 with 10 days growth in greenhouse. Mycelia were cultured on hypocotyl for 12, 24, and 36 h, respectively, and then, the film of hyphae were peeled from surface of hypocotyl and were mixed to extract RNA.

Total RNAs of the mycelia were extracted with TRIzol Reagent kit (Invitrogen, Carlsbad, CA) and the cDNA populations were created using the SMART cDNA synthesis system (BD Biosciences–Clontech, Palo Alto, CA). 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.

2.7. Suppression subtractive hybridization

Suppression subtractive hybridization was performed between the driver (mixed mycelia of PS2 inoculated for 12, 24, and 36 h) and tester (mixed mycelia of PS2-vir inoculated for 12, 24, and 36 h) cDNA populations. The SSH procedure was performed with the PCR-Select cDNA Subtraction kit according to the manufacturer's directions with slight modifications noted below (BD Biosciences–Clontech, Palo Alto, CA). 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 USA, Valencia, CA) 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 dena-

tured 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 are 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, South San Francisco, CA). The PCR mixture enriched for differentially expressed sequences from the tester population was cloned using the Pmd18 T-vector Cloning kit (TaKaRa, Japan) and transformed into JM109 *Escherichia coli* cells for blue–white selection. The forward subtracted library (PS2-vir) contained approximately 5000 white clones and 2112 white colonies were randomly picked for identification and characterization.

2.8. cDNA insert analysis by PCR

The forward subtracted cDNA library productions were enriched for genes specific to PS2-vir in *E. coli*. Randomly picked white colonies were grown in 96-well plates with 100 μl LB containing ampicillin at the concentration of 50 ng ml^{-1} at 37 °C. The cDNA inserts in individual *E. coli* colonies were analyzed by nested PCR using (MJ Research multiple 96 plate). One microliter 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 °C for 30 s and 68 °C for 3 min. PCR product (8 μl) was checked on 2% agarose gel and the rest was used for differential screening.

2.9. cDNA macro-array dot-blot differential screening of clones

cDNA macro-array dot-blots were used to confirm differential expression of the clones and performed according to the modified manufacturer's protocol in the PCR-Select Differential Screening kit (BD-Biosciences–Clontech, Palo Alto, CA). Briefly, selected white colonies were grown in LB medium containing ampicillin (50 ng ml^{-1}) overnight at 37 °C and plasmids were extracted by alkaline method. Plasmid 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 labeled with DIG using the random primer labeling of cDNA protocol in the PCR-Select Differential Screening kit. 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 coloring were operated according to the kit.

2.10. Clone sequencing and analysis

All DNA sequencing was performed by Beijing Sunbiotech Co., Ltd. (http://www.sunbiotech.com.cn). The company uses 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 generated with the Formatdb program. 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 *P. 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., 1997, 2002). All these sequences have been submitted to EST database of NCBI GenBank. The Ids of dbEST_and GenBank_Access Nos. are listed in Table 2.

2.11. RT-PCR analysis

RT-PCR analysis was conducted using the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA). Two-week-old soybean leaves were detached and placed in Petri plates for inoculation with *P. sojae* strains PS2-vir and PS2. Mycelia were cultured in 20% tomato juice for 4 d. Prior to inoculation, the mycelia were washed in autoclaved sterile water. Leaves inoculated with washed mycelia were incubated at 25 °C for 6, 12, 24, 36, and 48 h, respectively, and the RNA was extracted as described above. All RNA used

for RT-PCR was treated with DNase I (Qiagen, Valencia, CA) 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. One microliter of first strand cDNA was used in 100 μl PCRs according to the LA PCR kit from TaKaRa (TaKaRa, Japan). RT-PCR primers were designed based on the cloned sequence or the EST sequence if the clone has 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, South San Francisco, CA) with the following program: actinA, 95 °C for 1 min followed by 28 cycles of 95 °C 15 s, 64 °C 30 s, 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 28–33 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 each 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 two times.

3. Results

3.1. Variability in virulence and stability of acquired virulence

PS2 was reared for 14 generations on the resistant soybean cultivar Nannong 88-48 to select for virulence. The length of hypocotyl lesions at 5 days post-inoculation are shown in Fig. 1A. Fig. 1A shows several trends that were observed. The lesion length gradually increased before the ninth inoculation, and there was a significant increase in lesion length between the ninth and tenth inoculation. The length continued to increase between the eleventh and twelfth inoculations, finally stablizing at 3.1 cm. In fact, the hypocotyls of the seedlings had collapsed in the condition. These results suggest that the virulence of the pathogen increased significantly after 14 successive mass mycelial transfers on the Nannong 88-48.

To examine the stability of the induced virulence in the absence of the selection pressure, subcultures of the selected strain grown on LBA were reinoculated on the resistant soybean cultivar at intervals of one to six months, and the virulence was assayed by measuring the length of the lesions caused by PS2-vir at 5 days after inoculation. The results show that a slight decrease in virulence occurred, but that the overall virulence of the strain was maintained (Fig. 1B and Fig. 2).

The virulence formula of PS2-vir was also measured by inoculation on identification hosts. We found that virulences were identical between PS2 and PS2-vir (virulence for *Rps*1b, *Rps*1d, *Rps*2, *Rps*3a, *Rps*3b, *Rps*4, *Rps*5, *Rps*6, and *Rps*7). The result indicated there was no change in known avirulence genes.

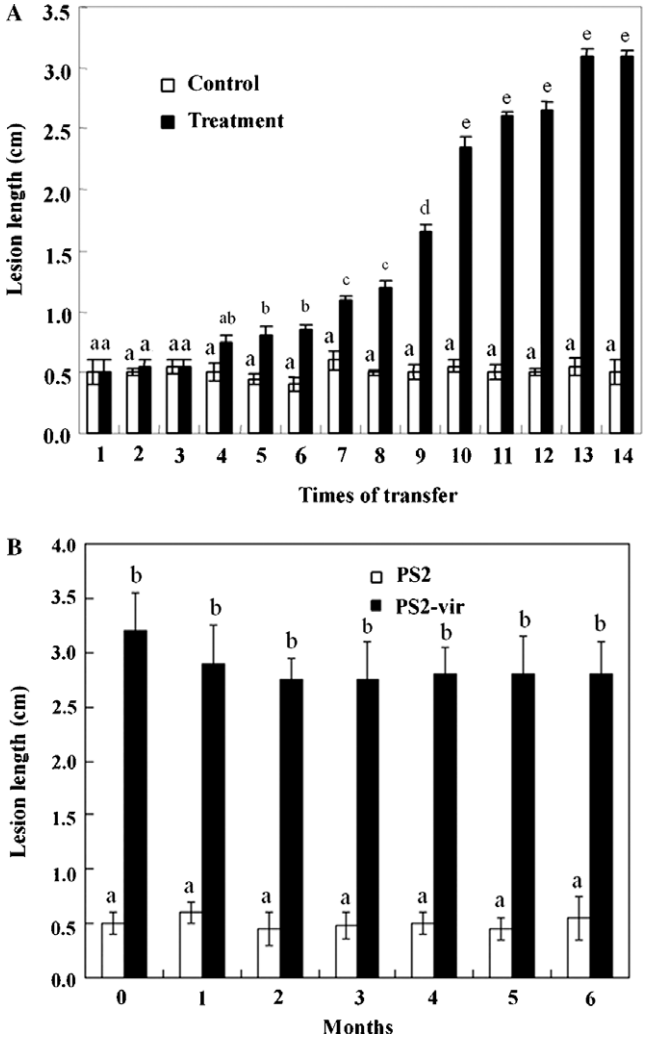

Fig. 1. (A) Changes in lesion size at 5 days post inoculation following successive re-inoculations on the cvs Nannong 88-48. Length of lesions induced by PS2 increased following a stepwise process. There was significant change between treatment of 14 times and the control. (B) Pathogenicity of PS2-vir while maintained on LBA media. PS2-vir, a virulent isolate selected from PS2 by successive inoculating PS2 on a resistant Nannong 88-48 for 14 times. It was reinoculated after maintaining on LBA for 1–6 months and the virulence was checked by measuring length of lesions 5 days later. The little bars represent standard errors (SE) of means. According to Duncan's new multiple range test, the values with different letters are significantly different at the 0.05 level.

3.2. Morphological characteristics and DNA-fingerprinting analysis

The colony appearance, growth rate, and yields of sporangia and oospores of PS2-vir were compared with those of PS2 (Table 1). The yields of oospores of PS2 and PS2-vir were significantly different, with the yield of PS2-vir only one-third that of PS2, but there were no significant differences in the colony growth rates or the yield of sporangia. In addition, the colony shapes and colors were nearly identical.

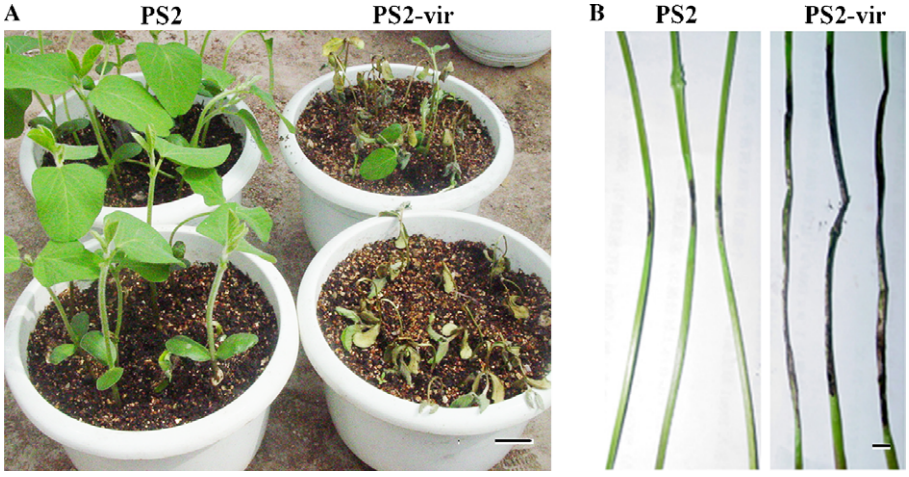

Fig. 2. Pathogenicity of *P. sojae* isolates. (A) PS2 could not kill the plants, and PS2-vir killed 95% or more of the plants. Bar = 20 mm. (B) The changes of lesion Length between PS2-vir and PS2 inoculated on the cvs Nannong 88-48 for 5 day. PS2, a low virulence line for Nannong 88-48; PS2-vir, a virulent line selected from PS2 by successive mass transfers on a resistant soybean cultivar Nannong 88-48 for 14 times. Bar = 5 mm.

Table 1
Growth rate, sporangium and oospore production of PS2 and PS2-vir

Isolates	Mean ± SD growth rate[a] (mm per day)	Mean ± SD no. of oospores[b] (10^4)	Mean ± SD no. of sporangia[c] (per mycelial mass)
PS2-vir	3.2 ± 0.4a[d]	17 ± 1.3b	23 ± 5.3a
PS2	3.2 ± 0.5a	52 ± 5.0a	22 ± 4.5a

[a] The cultures were grown in 9 cm diameter Petri dishes contained 15 ml LBA for 30 days in the dark at 25 °C.
[b] From mycelial masses (40 × 40 mm) grown on LBA for 30 days at 25 °C.
[c] Sporangia were counted after mycelial masses (10 × 10 mm) had been cultured in sterile distilled water for 36 hrs.
[d] The mean number followed by the same letter are not significantly different at 0.01 level according to Duncan's new multiple range test.

To check whether the difference in virulence was induced by large-scale changes in the genome, the DNA fingerprints of PS2 and PS2-vir were compared using RAPD analysis. Two-hundred primers were used, and a total of 912 bands were generated. In all cases, there were no polymorphisms in band numbers or sizes between PS2 and PS2-vir (Fig. 3). There is no evidence that the changes were due to a mix of isolates, heterokaryosis or parasexuality.

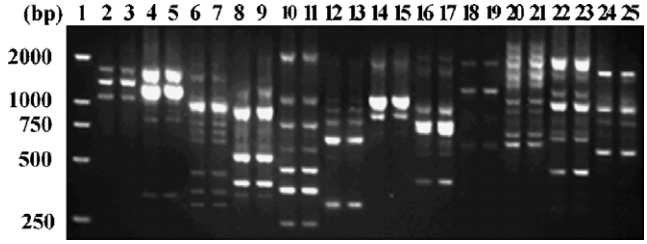

Fig. 3. The fingerprints generated by random amplified polymorphic DNA (RAPD) primers. PCR amplification products of two isolates, PS2 and PS2-vir, using 12 RAPD primers (nos. S173-S184, they were produced by the TaKaRa Biotechnology (Dalian) Co. Ltd. (http:www.takara.com.cn)). Lane 1: 2000 bp DNA ladder, the left numbers indicate the size of the two DNA bands (bp); lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24: PS2 and primers S173-S184 respectively; lanes 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25: PS2-vir and primers S173-S184 respectively. These results indicate that there may be no change of DNA on a big scale in PS2-vir.

3.3. 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 PS2-vir. RNA from PS2-vir was subtracted against RNA from PS2. 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 98% of the recombinant clones had inserts ranging from 0.12 to 2.0 kb, with an average insert size of 0.50 kb. A total of 2112 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, PS2-vir.

3.4. Expression screening of forward subtracted clones by macroarray dot-blots

To identify differentially expressed clones, the plasmids of the above 2112 clones were arrayed on 22 nylon membranes in duplicate, for a total of 44 membranes. Forward subtracted (tester minus driver) and reverse subtracted (driver minus tester) cDNA populations were DIG-labeled

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 Fig. 4. Differences in hybridization signal intensities are apparent on membranes probed with the forward subtracted probe (Fig. 4; Panel A) and the reverse subtracted probe (Fig. 4; Panel B). A wide range of signal intensities, from very strong to faint, are evident in Panel A of Fig. 4. 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. Panel A in Fig. 4 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 (Fig. 4; Panel A; e.g., B2, G1, G4, G5, G7, H1, H10). These clones typically correspond to low-abundance transcripts that are enriched during the subtraction. Of the 2112 PS2-vir clones examined, 323 passed this screening test and were strong candidates for differential expression; the identified clones were denoted PS2-vir-specific clones.

3.5. Sequence analysis of differentially expressed clones

A randomly chosen subset of the 323 PS2-vir-specific clones was 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 308 cDNAs were compared to the *P. sojae* Genome Database release v. 1.0. Self-BLAST analysis suggested that the 308 ESTs formed 74 clusters, representing 74 different putative genes. Among the 74 genes, 52 were represented by two or more ESTs. Three genes, whose transcripts are highly represented among the ESTs selected by differential hybridization, are GARP_PLAFF glutamic acid-rich protein precursor (WE69), Myb DNA-binding domain (WB20), and nitrilase (WD21) (Table 2).

BLAST searches against the *P. sojae* Genome Database indicated that 73 of the above genes correspond to known *P. sojae* genes, and only one gene is categorized as having no match in the database. All of the *P. sojae* ESTs in the present study have homologous sequences in *P. ramorum*. Partial sequence alignment revealed different levels of similarity (from about 40% to over 90% identity) between *P. sojae* and *P. ramorum* or *P. infestans* genes, suggesting varying degrees of conservation of these genes. A BLASTX search performed against the NCBI database revealed that 26 ESTs had matches in this database (data not shown).

A further search against the COGEME database identified 52 genes corresponding to known *P. sojae* genes and one gene corresponding to a known *P. infestans* gene. Three genes, WD6, WS26, and YD82, correspond to known *Mycosphaerella graminicola*, *Fusarium sporotrichioides*, and *Cladosporium fulvum* genes, respectively (*E* value less than 10^{-20}). Four genes correspond to homologs of known genes (*E* value between 10^{-5} and 10^{-10}) from *Magnaporthe grisea*, *Verticillium dahliae*, and *Ustilago maydis*. Five genes showed a weak match (*E* value less than 10^{-4}) with hypothetical proteins in the COGEME database, and nine genes were categorized as having no match in the COGEME database (data not shown).

The 74 unique cDNAs were grouped into MIPS (Munich Information Center for Protein Sequences) functional categories, according to their putative identification in BLAST searches against the *P. sojae* Genome Database (Mewes et al., 1997). Approximately 19% of the annotated proteins are involved in some aspect of primary metabolism or energy production, and 9% are involved in protein synthesis or protein fate (Fig. 5). Approximately 32% are similar to hypothetical proteins or to proteins for which a function has yet to be determined; these were designated as unclassified. Of the unique ESTs, 40% correspond to cDNAs whose resultant proteins perform other functions.

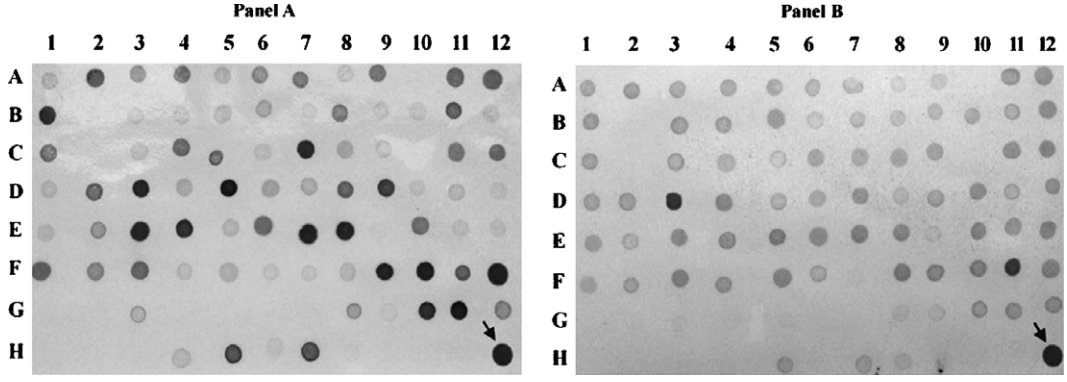

Fig. 4. Comparison of Macro-array dot-blots of PS2-vir and PS2 subtracted cDNAs. Equal amounts of amplified PS2-vir cDNAs were arrayed onto two sets of membranes and hybridized with two different probes. Panel A: Blots hybridized with forward (PS2-vir) subtracted cDNA probe. Panel B: Blots hybridized with reverse (PS2) subtracted cDNA probe. Differentially expressed PS2-vir transcripts were shown as clones with dark signals in Panel A. On each membrane, positive hybridization control (shown with arrow).

Table 2
Summary of 74 sequenced inserts from the SSH library enriched with *P. sojae* genes expressed in PS2-vir following a compatible interaction with its host

Clone	Accession No.	Size (bP)	Category and putative function[a]	E-value[b]	Protein ID[c]	Redundancy
WA14	DW005274	200	Hypothetical 99.7 kDa protein in SDL1 5′region precursor	2e−29	127573	1
WA17	DW005275	217	Unknown	6e−43	139980	2
WA22	DW005276	593	Alpha-latrotoxin precursor	0	134563	6
WA45	DW005277	305	Serine carboxypeptidase III precursor	0	137325	3
WB20	DW005278	463	Myb DNA-binding domain	0	135813	17
WB26	DW005279	155	Trichohyalin	1e−29	142023	4
WB57	DW005280	148	MRG family protein	1e−27	144750	4
WC8	DW005281	489	Peptidyl-prolyl cis-trans isomerase	0	132411	5
WD6	DW005282	399	Acetoacetyl CoA thiolase	0	121018	7
WD21	DW005283	576	Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	0	123641	13
WE7	DW005284	1081	Amino acid permease	0	136938	7
WE69	DW005285	424	Glutamic acid-rich protein	0	139977	26
WG27	DW005286	637	Putative amino acid transporter	2e−30	136938	2
WG85	DW005287	210	Hypothetical protein	9e−24	139685	10
WH43	DW005288	131	Aminotransferase, class-II	9e−24	131314	3
WH81	DW005289	602	Serine C-palmitoyltransferase	0	110200	4
WI8	DW005290	106	60S ribosomal protein L39	5e−18	120395	3
WI9	DW005291	262	Unknown	1e−41	140246	4
WI36	DW005292	283	Delta tubulin	0	157300	1
WI37	DW005293	291	Ribosomal L23 protein	2e−43	157934	1
WI43	DW005294	190	Hypothetical protein	5e−38	132912	1
WI45	DW005295	138	Cytochrome *c* heme-binding site	3e−28	141622	2
WJ12	DW005296	152	Unknown	1e−30	140247	1
WK2	DW005297	345	Cytochrome *c*	0	109170	1
WK61	DW005298	369	Beta-glucan synthesis-associated protein KRE6 (Killer toxin-resistance protein 6)	0	134704	5
WK77	DW005299	369	Phosphatase 5 (47.7 kD)	0	130258	4
WK84	DW005300	239	Ornithine carbamoyltransferase	3e−44	142096	2
WO2	DW005301	138	Unknown	8e−18	127372	1
WO4	DW005302	259	Unknown	0	156721	6
WO41	DW005303	275	Vesicle-fusing ATPase	6e−39	132189	4
WO63	DW005304	778	TATA-binding protein-associated factor TAFII55 family protein	0	120639	6
WO75	DW005305	214	Unknown	2e−19	119130	4
WP24	DW005306	212	CG12090 protein	1e−32	158528	5
WP30	DW005307	126	Putative oxidoreductase	0	133634	1
WP37	DW005308	276	Hypothetical protein	0	155900	2
WP48	DW005309	125	Hypothetical protein	2e−15	132382	1
WP49	DW005310	688	TonB-dependent receptor protein	0	156324	5
WP53	DW005311	819	Glycoprotein gp2	0	129547	5
WP59	DW005312	228	Polygalacturonase	3e−40	118151	9
WP60	DW005313	333	Ribosomal protein S16	0	109109	2
WP64	DW005314	182	Acyltransferase ChoActase/COT/CPT	7e−26	129971	8
WP68	DW005315	133	Hypothetical protein	8e−15	145178	1
WP71	DW005316	572	Ribosomal protein S15 isoform	0	111343	6
WR14	DW005317	460	DNA mismatch repair protein mutL	0	143988	3
WR31	DW005318	113	RNH1 protein	3e−19	132909	2
WR39	DW005319	568	CGI-90 protein	0	136613	4
WR58	DW005320	273	GABA-B R2 receptor	2e−37	140815	1
WR60	DW005321	195	E-class P450	6e−26	136346	1
WR62	DW005322	390	Ubiquitin-protein ligase	0	129180	4
WR71	DW005323	237	Ca^{2+}-transporting ATPase	0	129570	3
WR91	DW005324	123	Unknown	7e−20	141586	7
WR96	DW005325	448	ATP/GTP-binding site motif A	0	130298	6
WS2	DW005326	392	Nucleoporin 205kDa	0	133412	1
WS21	DW005327	177	Sexually induced protein 2	8e−36	141162	3
WS26	DW005328	305	Salt-induced AAA-Type ATPase	0	110444	9
WS39	DW005329	290	Ubiquitin thiolesterase	0	139592	7
YA11	DW005330	274	Hypothetical protein LOC63929	0	108328	1
YA53	DW005331	667	Serine/threonine-specific MAP kinase	0	136672	4
YA57	DW005332	562	Unknown	0	138797	3

(*continued on next page*)

Table 2 (*continued*)

Clone	Accession No.	Size (bP)	Category and putative function[a]	E-value[b]	Protein ID[c]	Redundancy
YA61	DW005333	160	Unknown	2e−31		1
YB95	DW005334	298	Transcription factor C-MYB	1e−40	132728	4
YC12	DW005335	499	Hypothetical protein	0	127894	11
YC30	DW005336	635	No Significant Match	NA	NA	1
YC56	DW005337	477	Unknown	0	130576	5
YC58	DW005338	345	Unknown	1e−12	131575	1
YD11	DW005339	177	ABC transporter	0	136643	2
YD19	DW005340	609	Unknown	0	158533	1
YD24	DW005341	603	Acyl-CoA-binding protein	0	130712	1
YD27	DW005342	544	Tubulin-tyrosine ligase	0	131864	1
YD36	DW005343	242	Cathepsin L-like protease	8e−31	140951	1
YD44	DW005344	232	Synaptotagmin VII	7e−45	139331	3
YD59	DW005345	203	Putative cinnamyl-alcohol dehydrogenase	9e−37	130176	2
YD72	DW005346	544	Diphosphonucleotide phosphatase	0	133696	6
YD82	DW005347	258	Histone-lysine N-methyltransferase	0	108880	4

[a] Putative identification is based on tBlastx searches of the *P. sojae* Genome database.
[b] Only scores with an *E*-value of <10e−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.

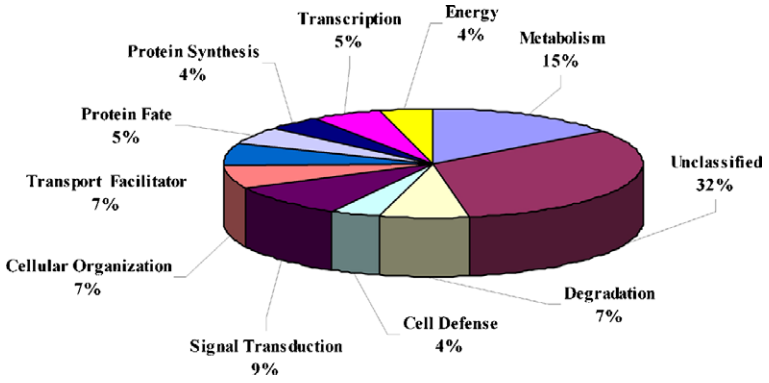

Fig. 5. Functional classification of *P. sojae* cDNAs derived from PS2-vir interacted with its host. The percentage ($n = 74$) in each of 11 functional categories is shown (refer to Table 1).

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.

Among the 74 genes, 15 were represented by six or more ESTs, and these genes may be more important to the variation in virulence. So these genes were taken for further data mining and bioinformatics analysis, such as in silico expression analysis, signal peptide prediction, and gene copy number in *P. sojae* and *P. ramorum* (Table 3). In *P. sojae* and *P. infestans* EST databases (Randall et al., 2005), we analyzed the distribution of ESTs representing the clones over the various libraries and, based on these numbers, we predicted stage specific expression patterns and expression levels. For example, for WS26 many ESTs of *P. infestans* are found in the germinating cysts library, zoospore library and starvation library but none in a sporangia library. This indicates that the WE69 gene is specifically expressed at a relatively high level in zoospores, germinating cysts and starvation. In contrast, for WD21 there is only one EST in *P. sojae* and *P. infestans* EST databases, respectively, which indicates that this gene is transcribed at a low level in infection or starvation. However, for WS26 nine ESTs of *P. sojae* are found in the infection library, which indicates that this gene is transcribed at a high level in infection. For the virulence-associated genes, the presence of a signal peptide can be indicative for infection function. Six proteins are predicted to be secreted by the program SignalPv2.0 (Nielsen et al., 1997; Nielsen and Krogh, 1998) (Table 3). To evaluate the likelihood we analyzed the phylogenetic distribution and we investigated whether the cloned genes belong to a gene family. Of the 15 genes that have homologues in *P. ramorum*, only one seem to be unique for *Phytophthora* (Table 3).

3.6. RT-PCR analysis

Ten clones were selected for temporal expression analysis based on the results of the dot-blot screens and the bioinformatics analyzes. RT-PCR was used to examine the expression patterns of these genes. We examined the pres-

Table 3
Analysis of clone using data mining and bioinformatics

Clone	Protein ID[a]	Protein size	Signal peptide[b]	Transcripts in *P. sojae* EST database[c]	Transcripts in *P. infestans* EST database[c]	SwissProt BLAST hit of *P. sojae*	Phylogenetic distribution[d]	Genes in *P. sojae*[e]	Genes in *P. ramorum*[e]
WA22	134563	549	—	MY(1)	MY(2) IN(1) ST(1)	LATA_LATMA (P23631)	Other species	1	1
WB20	135813	129	—	IN(1)	ST(1)	SFR4_MOUSE(p23524)	Other species	3	3
WD6	121018	134	—	IN(2)	ZO(1)IN(2)	THIL_YEAST(P41338)	Other species	2	2
WD21	123641	292	SP	ST(1)	IN(1)	NAE2_THEMA(Q9X0Y0)	Other species	1	1
WE7	136938	292	—	ZO(1) IN(3) MY(1)ST(1)	ZO(1) SP(1) ST(1)	NAE2_THEMA(Q9X0Y0)	Other species	2	1
WE69	139977	678	SP	IN(1)	ST(1)	GARP_PLAFF (P13816)	Other species	1	1
WG85	139685	502	SP	ZO(1)IN(1) MY(2)	ST(1)	ITN2_HUMAN(Q9NZM3)	Other species	4	1
WO63	120639	119	—	ZO(3)IN(1)	ST(2)	TF2D_DROME (P20227)	Other species	1	1
WP59	118151	341	SP	MY(2)	CY(1)	POLX_TOBAC(P10978)	Other species	13	7
WP64	129971	914	—	—	—	CPT2_MOUSE (P52825)	Other species	1	1
WR96	130298	293	—		ZO(1)		Only in *Phytophthora*	1	1
WS26	110444	490	SP	ZO(1) IN(9)	ZO(2) CY(1) ST(5)	VPS4_YEAST(P52917)	Other species	1	1
WS39	139592	977	—	—	ZO(1) SP(1) MY(1)	UB24_HUMAN(Q9UPU5)	Other species	2	1
YC12	127894	561	SP	MY(1)	—	DSPP_HUMAN(Q9NZW4)	Other species	2	2
YD72	133696	694	—	—	IN(2)ST(2)	PPA_ASPFI (Q12546)	Other species	11	10

[a] *P. sojae* hits with *E* value < 1e−50 and identity > 99% are listed.
[b] SP indicates that a signal peptide is predicted at the N-terminus by the program SignalP (Nielsen et al., 1997; Nielsen and Krogh, 1998).
[c] The tissue types from which the EST libraries are derived are zoospores (ZO), germinated cysts (CY), sporangia (SP), mycelia (MY), infection(IN) and starvation(ST). The numbers in brackets indicate the number of ESTs present in the various libraries (Randall et al., 2005).
[d] Homologues in species other than *Phytophthora* were considered as homologues when the BLAST *E* value was less than 1e−3 and the similarity >30%.
[e] *P. sojae* and *P. ramorum* whole genome sequences and gene annotation at the JGI website (http://www.jgi.doe.gov/genomes) 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.

ence of the transcripts of these genes at six time points over 48 h during the period of interaction (Fig. 6). In both PS2-vir and PS2, two transcripts from these genes were detected at the zero time point of the plant–pathogen interaction. In PS2, no WD21, WS26, YC12 or WE69 transcript was detected at any time point, and WB20 and WP59 were transcribed only at the 6-h time point, strengthening the conclusion that these genes are dramatically upregulated in PS2-vir. The control, actinA derived from *P. sojae*, does not allow determination of the amounts of fungal biomass present at each infection time point. The level of transcript from clone WB20, identified as encoding a Myb DNA-binding domain, reached a peak value at the 24-h time point. Clone WD21, encoding nitrilase, was expressed

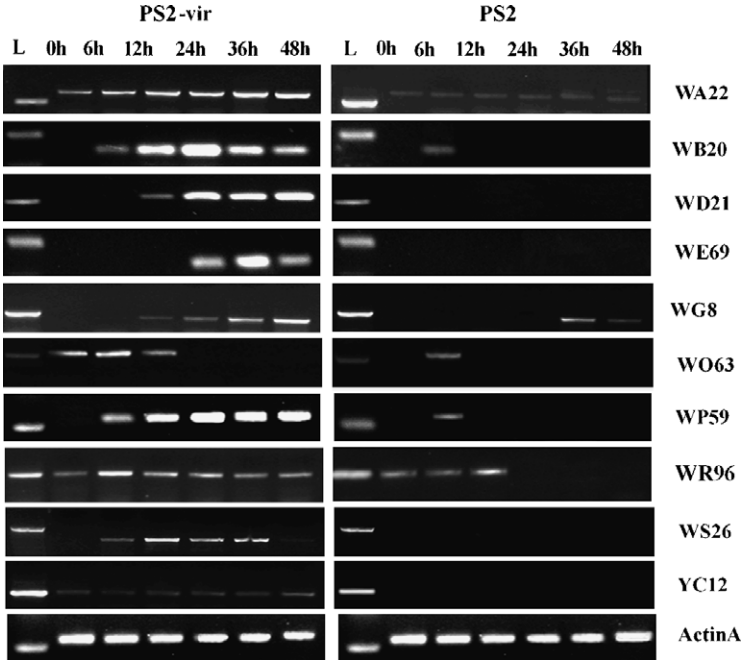

Fig. 6. RT-PCR analysis of the expression of the 10 genes upregulated in PS2-vir and PS2. Lane L, 250 bp ladder; 0h, uninteract; 6 h, interact 6 h; 12 h, interact 12 h; 24 h, interact 24 h; 36 h, interact 36 h; and 48 h, interact 48 h.

beginning at 12 h after infection and increasing thereafter. Clone WP59, encoding polygalacturonase, was expressed at 24 h and reached a peak value at the 36-h time point.

4. Discussion

The results of this study support the hypothesis that the virulence of *P. sojae* can change continuously during asexual reproduction. This phenomenon has been reported previously, as has been variation in other traits, such as the growth rate and colony morphology (Hilty and Schmitthenner, 1962; Rutherford et al., 1985; Dorrance et al., 2003). The latter studies, performed with isolates generated by mass mycelial transfers in Petri dishes on agar, suggested highly stable virulence phenotypes following successively mass mycelial transfers, which contrasts with the results of the present study. The reports of previous studies presented limited conclusions and were confused by heterokaryotic isolates, which probably led to findings that differ from the present results (Rutherford et al., 1985; Bhat and Schmitthenner, 1992). In our study, this possibility was excluded by using hyphal-tip isolates and RAPD markers, which revealed identical fingerprinting patterns between PS2 and PS2-vir.

Previous genetic studies have indicated that *P. sojae* is vegetatively diploid (Walker and Schmitthenner, 1984). The diploid chromosomal system, similar to that of higher plants, provides for classical allelic interactions of dominance and recessiveness at each locus, as well as the effects of homozygosity and heterozygosity on complex gene expression. Shaw (1983) discussed the possible mechanisms of genetic variability during asexual reproduction in *Phytophthora* spp. and proposed three major mechanisms for this variability: mutation, mitotic crossing-over, and extrachromosomal elements.

Mutations, in the broader sense, involve any changes in the nuclear genome that might take place during DNA replication, and can have a significant impact on the expression of certain genes. Mitotic crossing-over is a mechanism that results in variation during asexual reproduction and provides an alternative to the mutation theory. Crossing-over during the mitotic prophase in heterozygous diploid nuclei in organisms such as *Saccharomyces cerevisiae* Hansen (Hurst and Fogel, 1969) is well understood. This mechanism of genetic change does not generate new variations, but can reveal recessive variations that are hidden in heterozygotes. It is possible that this mechanism has evolved in *P. sojae* (Förster et al., 1994; Goodwin, 1997). The third possible cause of genetic variability during vegetative growth and asexual reproduction in *P. sojae* is the influence of extrachromosomal genes (Caten, 1971; Shaw, 1983; Goodwin, 1997). Mitochondrial DNA is a strong candidate for the location of mutations showing nonchromosomal behavior. If some of the genes involved in pathogenicity and virulence are of cytoplasmic origin, mutations in these genes may create new variants (Fabritius et al., 2002).

It is highly probable that the variations in virulence in PS2 in the present study resulted from mutations and differences in gene expression, because the acquired virulence was stable, the change occurred suddenly, and there was no difference in the DNA fingerprints of PS2 and PS2-vir as determined by RAPD analysis.

Thrall and Burdon (2003) reported variations in populations of wild flax (*Linum marginale*). They showed that the increased virulence of a parasite of wild flax, rust fungus (*Melampsora lini*), is associated with reduced spore production, a clear fitness cost. Virulent isolates of rust fungus were found more frequently in highly resistant populations of wild flax, and, intriguingly, showed reduced reproductive fitness. Meanwhile, avirulent forms of the parasite dominated susceptible wild flax populations and often exhibited greater reproductive fitness. These observations appear to be supported by the present study, as evidenced by the difference in oospore yields in PS2 and PS2-vir. It is possible that the increase in virulence is associated with the decrease in oospore yield, and that these functions may be regulated by the same genes (Brown, 2003). Alternatively, it is also possible that the change is not associated with the variation in virulence; these phenomena may be under separate control by different genes. This variability is difficult to explain by a single mechanism, and more than one mechanism may be involved. This phenomenon is important in soybean root- and stem-rot management efforts and should be studied in more depth, in order to understand the causes and mechanisms involved. The availability of molecular tools should make this task possible.

The molecular events associated with variations in virulence in *Phytophthora* are poorly understood and, therefore, the purpose of this study was to identify *P. sojae* virulence factor genes. This study represents a first step in elucidating the molecular basis of variations in *Phytophthora* virulence. We identified 74 unigenes from a virulence-specific cDNA library, with most of the genes upregulated from incompatible to compatible interactions of *P. sojae* with its host. It seems likely that genes that are highly expressed in PS2-vir play an important role in virulence and are involved in plant infection. Sequencing of the selected cDNAs and database searches for similar genes revealed relevant information (Table 3). For example, WB20, WD6, WE7, WE69, WG85, WO63, and WS26 all were found in the infection library of *P. sojae* ESTs database.

Polygalacturonase (PG) is an enzyme that facilitates the invasion of host tissue (Walton, 1994). In *Aspergillus flavus*, PGs exist in multiple forms, and the virulence of pathogens is related to specific PG isozymes (Brown et al., 1992; Cleveland and Cotty, 1991). In *Mycocentrospora acerina*, the virulence of pathogen strains toward their hosts is correlated with the total PG activity secreted by the fungus (Le-Cam et al., 1994). *Phomopsis cucurbitae* contains a set of PG isozymes that plays an important role in the postharvest decay of cantaloupe fruit caused by this fungus (Zhang et al., 1997). Furthermore, molecular genetic studies have demonstrated that PG is a virulence factor for several

plant pathogenic fungi. Disruption of one of the six PG genes of *Botrytis cinerea* caused a reduction in the virulence of this organism on host plants (Ten Have et al., 1998). Gene-replacement mutants of *Claviceps purpurea* lacking PG activity are nearly nonpathogenic on rye (Oeser et al., 2002). However, genetic evidence obtained by gene disruption or replacement experiments does not always support an essential role for PGs in fungal pathogenicity. Targeted inactivation of PG genes had no detectable effect on the pathogenicity of *Cochliobolus carbonum* on maize (Scott–Craig et al., 1990, 1998). Inactivation of a PG gene did not result in reduced pathogenicity of *Cryphonectria parasitica* on American chestnut stems (Gao et al., 1996). Disruption of the *Fusarium oxysporum* PG gene *pg5* did not alter the virulence of the fungus toward tomato plants (Garcia–Maceira et al., 2001). *Phytophthora* has been shown to secrete cell-wall-degrading enzymes such as exo- and endo-polygalacturonases (Götesson et al., 2002; Torto et al., 2002; Yan and Liou, 2005). Indeed, the cDNA WP59, which encodes polygalacturonase, was represented by nine ESTs in the present study.

In other phytopathogen systems, mitogen-activated protein kinases (MAPK) are involved in the transmission of inductive signals derived from contact with plant surfaces, leading to the expression of infection-related genes. MAPK pathways have also been implicated in the induction of cytokinesis and cytoskeletal reorganization required for the formation of appressoria (Xu and Hamer, 1996; Kim et al., 2000). In *Cochliobolus carbonum*, the serine-threonine protein kinase SNF1 is required for the release of catabolite repression and regulates the expression of hydrolytic enzymes required for infection of maize (Tonukari et al., 2000). Recently, a SNF1 homolog in *S. sclerotiorum* was described (Vacher et al., 2003), suggesting that conserved regulatory networks may be involved in regulating the expression of pathogenicity determinants. In the present study, several cDNAs were found that encode a factor that may be involved in such signaling pathways, namely, serine/threonine-specific MAP kinase (YA53), which was represented by four ESTs.

Three of the identified cDNAs encode proteins similar to those previously implicated in resistance to metabolites: an ATP-binding cassette (ABC) transporter (YD11), nitrilase (WD21), and oxidoreductase (WP30). ABC transporters are required for tolerance to phytoalexins and pathogenicity in potato tubers infected with the potato fungal pathogen *Gibberella pulicaris* (Fleissner et al., 2002). Nitrilase is a member of a novel major facilitator superfamily from *B. cinerea* that provides tolerance to toxic compounds such as nitriles, cyanide, and certain fungicides (Sexton and Howlett, 2000; Barclay et al., 2002). Oxidoreductase is required by *C. albicans* for resistance to oxidative stress and full virulence (Hwang et al., 2002).

Myb genes constitute the largest gene family of transcription factors that regulate temporal and spatial expression patterns of specific stress genes at the transcriptional level, and are an important part of the stress response (Rushton and Somssich, 1998). Two genes encoding Myb transcription factors, WB20 and WB95, were identified in the present study. WB20, represented by 17 ESTs, is a Myb DNA-binding domain, and WB95, represented by four ESTs, is a c-MYB gene. Plant MYB genes are involved in a variety of biological functions and have been shown to play important roles, including the regulation of phenylpropanoid metabolism, the control of development, the determination of cell fate and identity, and responses to hormones and environmental factors (Martin and Paz-Ares, 1997; Stracke et al., 2001).

The information acquired through this project has identified several genes whose products are likely to be involved in the destruction of plant tissues, the disarming of plant defenses, or the expression of pathogenic factors. However, fully one-third of the ESTs have yet to be assigned to a putative function, indicating that many of the mechanisms underlying pathogenesis remain unexamined. For example, two clones, WG85 and YC12, were the most abundant cDNAs in the PS2-vir-specific libraries, but their functions are unknown. With the recent development of suitable *Phytophthora* transformation and RNA-interference systems (Fire et al., 1998; Hannon, 2002), the specific roles of such genes in pathgen pathogenicity may be examined.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2006.06.001.

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