

Identification of *Alternaria brassicicola* genes expressed in planta during pathogenesis of *Arabidopsis thaliana*

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Abstract

Alternaria brassicicola is a necrotrophic fungal pathogen that causes black spot disease on cruciferous plants including economically important *Brassica* species. The purpose of this study was to identify fungal genes expressed during infection of *Arabidopsis*. In order to identify candidate genes involved in pathogenicity, we employed suppression subtractive hybridization (SSH) between RNA isolated from *A. brassicicola* spores incubated in water and on the leaf surface of the *Arabidopsis* ecotype Landsberg. Two populations of cDNA were created from total RNA extracted after 24 h when approximately 80% of the spores had germinated either on the leaf surface or in water. Following SSH, expression of clones was examined using dot-blot macro-arrays and virtual Northern blots. 47 cDNA clones differentially expressed between *Alternaria* infected *Arabidopsis* leaves and spore germination in water were selected for sequencing. Seventy-seven percent (36) of the cDNAs had significant homology to fungal sequences from databases examined, including available fungal genomes, while 13% (11) had no homology to sequences in the databases. All 36 genes had significant matches with genes of fungal origin, while 11 genes did not have significant hits in the databases examined. Five sequences were expressed on the plant leaf surface but not during spore germination in water according to virtual Northern blots. These five cDNAs were predicted to encode a cyanide hydratase, arsenic ATPase, formate dehydrogenase, major *Alternaria* allergen, and one unknown. RT-PCR was used to examine the expression of these five genes during infection of *Brassica oleracea* var. *capitata* (cabbage), in vitro growth in nutrient rich media, and infection of *Arabidopsis thaliana*. Four of these genes are expressed in the nutrient rich medium, while the unknown gene P3F2 was only expressed during plant infection. The results of this study provide the first insight into genes expressed during *A. brassicicola* infection of *Brassica* species that may be involved in fungal pathogenesis. © 2003 Elsevier Inc. All rights reserved.

Keywords: *Alternaria brassicicola*; *Arabidopsis thaliana*; *Brassica oleracea*; Pathogenicity; Suppression subtractive hybridization

1. Introduction

Alternaria brassicicola (Schw.) Wilts. is a necrotrophic fungal pathogen that causes black spot in several economically important *Brassica* species. Black spot can cause considerable reduction in the quality of harvested *Brassica* products, and no known source of resistance has been identified among *Brassica* crop species (Sigarova and Earle, 1999). Studies examining pathogenesis mechanisms of *A. brassicicola* have been limited. Like many *Alternaria* species, *A. brassicicola* is reported to

produce a host specific toxin (HST), AB toxin, which was isolated from spore germination fluid (Otani et al., 1998). Unlike other *Alternaria* HSTs, which are low molecular weight secondary metabolites, AB toxin is reported to be a 35 kDa protein. To date, the gene(s) responsible for AB toxin production have not been identified or cloned in *A. brassicicola*, and in fact, only one putative pathogenicity gene (cutinase *cutab1*) has been cloned to date from *A. brassicicola* (Trail and Koller, 1993).

Despite our limited understanding of *A. brassicicola* pathogenesis mechanisms, a fair amount of work has been done to characterize resistance mechanisms to *A. brassicicola* using the model plant *Arabidopsis thaliana*. Natural differences in susceptibility and resistance to *A. brassicicola* have been shown to exist in various

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Arabidopsis ecotypes providing a useful situation to study resistance mechanisms (Kagan and Hammerschmidt, 2002; Otani et al., 2001). Peninckx et al. (1996) showed that jasmonic acid levels increased dramatically when *Arabidopsis* plants were challenged with *A. brassicicola*, which resulted in the expression of *PDF1.2*, an antifungal defensin-like peptide. Two other genes are activated coordinately with *PDF1.2* upon *A. brassicicola* challenge including, *PR-3*, a basic chitinase, and *PR-4*, a hevein-like protein (Thomma et al., 1998). Thomma et al. (1998) demonstrated that the methyl jasmonate insensitive mutant, *coi1-1*, and the phytoalexin camalexin deficient mutant, *pad-3*, were also more susceptible to *A. brassicicola* than wild type Columbia-0 (Col-0). This suggests that jasmonic acid signaling is required for resistance to *A. brassicicola*, and indeed, Thomma et al. (1998) also found that *NahG* plants remain resistant indicating that salicylic acid is not required for resistance. Camalexin also appears to have a major role in resistance, with different *Arabidopsis* ecotypes having varying levels of this important phytoalexin (Kagan and Hammerschmidt, 2002).

Several large-scale gene expression studies have been undertaken to dissect *Arabidopsis* resistance to *A. brassicicola*. Schenk et al. (2000) used microarray analysis, to identify 168 genes upregulated during an interaction between the *Arabidopsis* ecotype Col-0 and *A. brassicicola*, but the role of these genes in resistance has yet to be determined. Schenk et al. (2003) have also examined gene expression in distal uninoculated tissues finding 35 genes with altered gene expression. Van Wees et al. (2003) identified 645 induced genes by *A. brassicicola* in wild type Columbia and *pad3* plants indicating that *pad3* does not have a major effect on defense signaling. Interestingly, 265 of the 645 *A. brassicicola* induced genes required *COI1* for full expression. Narusaka et al. (2003) reported that the timing of defense related gene expression was altered in *pad3* plants. Thus, based on the research to date, it seems clear that jasmonic acid and camalexin both play critical roles in resistance to *A. brassicicola*. These results indicate that the *A. brassicicola*–*Arabidopsis* interaction has the potential to become a useful system to study necrotrophic fungal pathogenesis and jasmonic acid signaling pathways.

Although the amount of research on resistance mechanisms to *A. brassicicola* is growing, research is still lacking on the pathogen side of this interaction. In this study, we have used suppression subtractive hybridization (SSH) to identify fungal genes expressed in planta during the first 24 h of *A. brassicicola* infection of the *Arabidopsis* ecotype Landsberg erecta (Ler). Ler has previously been shown to be one of the more susceptible *Arabidopsis* ecotypes to *A. brassicicola* infection (Otani et al., 2001). SSH was developed by Diatchenko et al. (1996) to overcome some of the difficulties in previous subtractive hybridization techniques. Based on repre-

sentational difference analysis (RDA) technology (Lisitsyn et al., 1993), SSH overcomes the problem of differences in mRNA abundance by using a hybridization step during the subtraction that equalizes mRNA levels between the two populations being compared. We chose to use SSH to identify genes potentially involved in *A. brassicicola* pathogenesis given SSH's previous success in gene discovery. SSH has been successfully used in a number of different systems to identify novel or specific genes, including: in planta expressed genes of the wheat leaf rust pathogen, *Puccinia triticina* (Thara et al., 2003), identification and cloning of an *Aspergillus nidulans* β -1,3 glucanase expressed during sexual development (Wei et al., 2001), and identification of genes expressed during cercosporin biosynthesis in *Cercospora zea-maydis* (Shim and Dunkle, 2002).

The objective of this study was to identify *A. brassicicola* genes specifically expressed during pathogenesis of the *Arabidopsis* ecotype Ler. Identification of genes preferentially expressed during in planta fungal growth may potentially lead to the cloning and characterization of putative pathogenicity genes in *A. brassicicola*.

During the course of this study, we have identified five genes that are highly expressed during *A. brassicicola* pathogenesis and also are subsequently expressed during infection of *Brassica oleraceae* var. *capitata* (green cabbage). The results of this study will allow a greater understanding of the infection strategy of this economically important necrotroph, and perhaps lead to novel control strategies for black spot in important *Brassica* species.

2. Materials and methods

2.1. Experimental setup, RNA isolation, and cDNA synthesis

Spores from *A. brassicicola* strain ATCC 34622 were placed either in sterile water held in a petri plate, or in 20 μ l aliquots at a concentration of 5×10^5 spores/ml on the leaf surface of the *Arabidopsis* ecotype Landsberg Erecta (Ler). Detached leaves for this experiment were obtained from axenically grown Ler plants. Following incubation at 25 °C for 24 h, spores from both populations were collected with pipets and examined for germination by light microscopy. After it was determined that a sufficient number of spores had germinated (approximately 80%), the spore suspensions were centrifuged at 8000g for 5 min. The supernatant was removed, and germinating spores were then used for extraction of total RNA which was performed with the RNeasy Plant Kit according to the manufacturer's protocol (Qiagen USA, Valencia, CA).

Because we found that it was difficult to obtain large amounts of RNA using this protocol, 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 100 ng 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.

2.2. Suppression subtractive hybridization

Suppression subtractive hybridization was performed between the driver (water germinated spores) and tester (Ler leaf surface germinated spores) 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 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 are exponentially amplified. The conditions for the first PCR were as follows, 5 min at 72 °C, 25 s at 94 °C, and 27 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 Applied Biosystems GeneAmp 2400 thermocycler (Applied Biosystems, Foster City, CA).

The PCR mixture enriched for differentially expressed sequences from the tester population was cloned using the AdvanTage PCR Cloning kit (BD-Biosciences-Clontech, Palo Alto, CA) and transformed into TOP-10

Escherichia coli cells (Invitrogen, Carlsbad, CA) for blue-white selection. The subtracted library contained approximately 500 clones and 288 white colonies were randomly picked for identification and characterization.

2.3. 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 manufacturer's protocol in the PCR-Select Differential Screening kit (BD-Biosciences-Clontech, Palo Alto, CA). Briefly, selected white colonies were grown in a 96-well plate in LB medium containing carbenicillin (50 µg/ml) for 2 h at 37 °C. A master mix containing the nested primers 1 and 2R and *Taq* DNA polymerase (Promega, Madison, WI) was added to 1 µl of bacteria culture. Thermal cycling was conducted under the following conditions, 94 °C for 30 s, then 23 cycles of 95 °C for 10 s and 68 °C for 3 min. PCR products were combined with an equal volume of 0.6 N NaOH in a 96-well plate. Two microliters of this mixture, for each clone, was blotted onto a nylon membrane using a vacuum blotter (Biorad, Hercules, CA). Membranes were blotted in duplicate. Both the forward subtracted and reverse subtracted probes were labeled with ³²P 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. Membranes were then exposed to X-ray film overnight at –70 °C.

2.4. Virtual Northern analysis

Virtual Northern blots (also called a cDNA Southern) were prepared by using 2.5 µg of double stranded cDNA generated by the SMART procedure. Each blot consisted of three samples, the driver, the tester, and a population of cDNA derived from ungerminated *A. brassicicola* spores grown on potato dextrose agar plates. The cDNA was electrophoresed in a 1% agarose gel, processed (denatured and neutralized), and blotted onto positively charged nylon membranes (Roche Diagnostics, Mannheim, Germany) via a vacuum blotter (Bio Rad, Hercules, CA). Individual cDNA clones generated from the subtraction procedure were used as probes and labeled using the PCR DIG Probe Synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). The control 18S rRNA probe was generated from 10 ng of genomic *A. brassicicola* DNA using the primers 5' GCGGTAATTCAGCTCCAATAGC 3' and 5' GATGACACGCGCTTACTAGGC 3'. Nylon

membranes were then hybridized over night in DIG Easy Hybridization solution (Roche Molecular Biochemicals, Mannheim, Germany) at 50 °C. Probe concentration for all blots was 25 ng/ml. Nylon membranes were washed in a final solution of 0.1× SSC and 0.1% SDS at 68 °C. All virtual Northern blots were repeated at least two times.

2.5. Clone sequencing and analysis

Selected individual cDNA clones were obtained with the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). All DNA sequencing was performed by the University of California-Davis DBS DNA sequencing facility (<http://dnaseq.ucdavis.edu>). The facility uses an ABI 3100 capillary electrophoresis genetic analyzer and ABI Big dye v. 3 terminator cycle sequencing chemistry. M13 forward or M13 reverse primers were used in all sequencing reactions. The BLAST algorithms at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) were used to initially identify sequences in the non-redundant GenBank database (Altschul et al., 1997). A match was considered significant if the expected (E) value was less than $1e-3$, and for this analysis only the most significant matches were examined. Sequences were grouped into MIPS (Munich Information Center for Protein Sequences) functional categories according to their putative BLASTx identification (Mewes et al., 1997, 2002).

Additional database searches were performed with the cDNA clones using the Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and Oomycete EST Database version 1.3 <http://cogeme.ex.ac.uk/>, the *Magnaporthe grisea* Genome Database release 2.1 <http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/index.html>, and the *Neurospora crassa* Genome Database 3 <http://www-genome.wi.mit.edu/annotation/fungi/neurospora/index.html>. Searches were performed with the BLASTx algorithms available at the respective web-sites using a cutoff E-value of $1e-3$ and the BLOSUM62 scoring matrix.

2.6. RT-PCR analysis

RT-PCR analysis was conducted using the Thermo-script RT-PCR System (Invitrogen, Carlsbad, CA). Six week old green cabbage leaves were detached and placed in petri plates for inoculation with *A. brassicicola* strain ATCC 34622. A spore inoculum in sterile water was created at a concentration of 5×10^5 spores/ml, and sprayed until runoff occurred on the detached leaves. Petri plate tops were sprayed with sterile water and sealed with parafilm to maintain the high humidity required for *A. brassicicola* infection. Plates were incubated at 24 °C with a 12 h light/dark cycle. Leaves from three separate plants were used and pooled together at 0

(uninfected), 1, 2, 3, 4, 5, and 7 days post-inoculation. RNA was extracted with the RNeasy Plant Mini-Kit (Qiagen, Valencia, CA) as described in Section 2.1. 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 25 µl PCRs containing a 2× master mix from Promega, (Madison, WI). Primers used in the reactions were: P3D7, 5' CGG CAG CAG TTG CCT TCT GC 3' and 5' TGC TTC CGA CTT GGT CAC GC 3'; P3G2, 5' ATG CAG TTC ACC ACC ATC GC 3' and 5' GTA ACG AGG GTG ACG TAG GC 3'; P2A12, 5' CTC CCT CGC CAT CCA GCT TG 3' and 5' TGG GCG CGG TGT CGA AGA TG 3'; P3G8, 5' GCC AGG TTC GAC CAT TCT CTC CTG CTG C 3' and 5' CAT TGG TGC AAA CTG GTC GGT GTG C 3'; beta-tubulin, 5' GCC AGT CTG GTG CTG GTA ACA ACT GG 3' and 5' CGA ATC CGA CCA TGA AGA AGT GGA GAC G 3'; P3F2, 5' ACT CTC CGA AAT GCA TTT AGG TGC 3' and 5' GCT CCA CAA TAC CTC ACG ATA CTG 3'; P2C12, 5' TCA ACC AAC GCC AGT CTC CTT ATC 3' and 5' ATA TCA AAG CCC GTC TTG GGT AGG 3'; and 18s rRNA, 5' CTG TTA ATC ATT GCG GCG TCT CTA G 3' and 5' GCG GTA ATT CCA GCT CCA ATA GC 3'. Primer sequences for the beta-tubulin and 18s rRNA sequences were derived from *A. brassicicola* GenBank Accession Nos. Y17084 (beta-tubulin) and AF229506 (18s rRNA). PCRs were conducted in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following program: 94 °C for 2:00 min followed by 35 cycles of 94 °C 0:30 s, either 55 °C or 53 °C 0:30 s depending on each gene, 72 °C 1:00, and a final extension for 5:00 at 72 °C. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized with ultra-violet light. All RT-PCRs were repeated at least two times.

3. Results

3.1. Expression screening of forward subtracted clones by macro-array dot-blots

A SSH cDNA library containing approximately 288 clones was constructed as described in Section 2. In order to identify which bacterial colonies possessed cloning vectors with inserts, colonies were transferred to 96-well plates containing Luria broth (LB) and 50 µg/ml of carbenicillin. Bacteria were used as templates in a nested PCR (using nested primers 1 and 2R included with the SSH kit) (Fig. 1A). Of the 288 colonies examined, 222 (77%) had inserts. PCR amplified inserts representing 222 clones were arrayed on 3 nylon

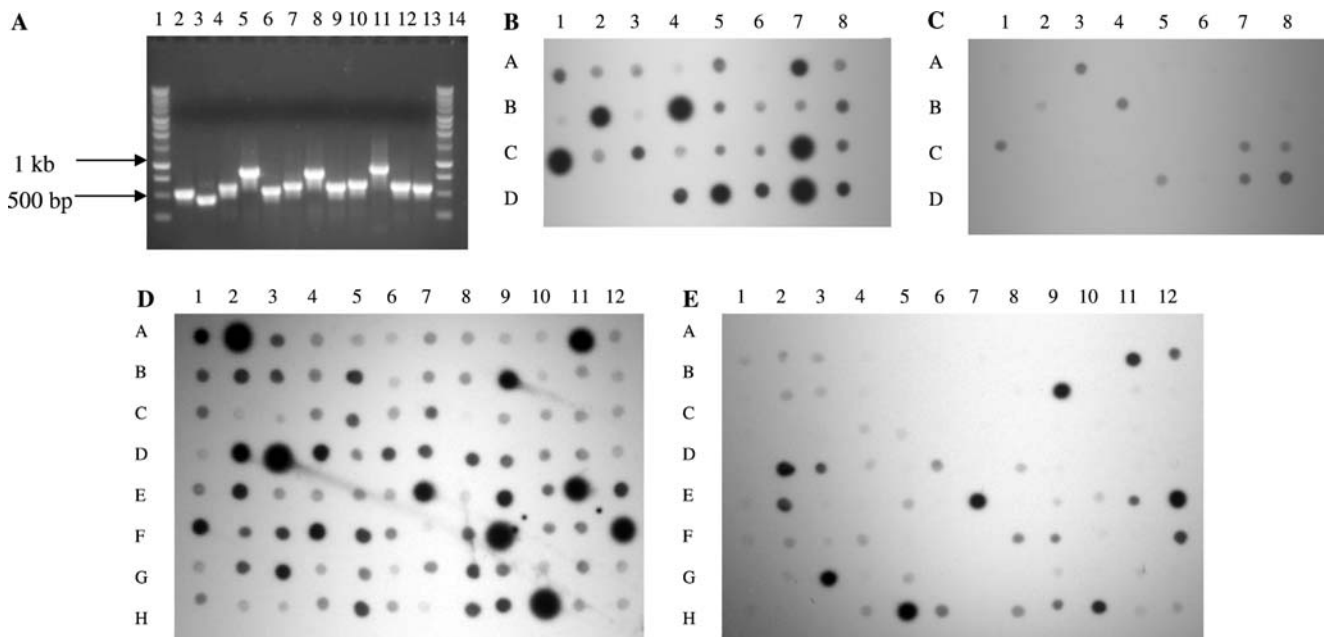


Fig. 1. PCR products and macro-array dot-blots. (A) Agarose gel showing cDNA inserts that subsequently are blotted onto nylon membranes for reverse Northern screens. Lanes: 1, 1 kb ladder; 2, P3C1; 3, P3D2; 4, P3E1; 5, P3H1; 6, P3F2; 8, P3C4; 10, P3D6; 11, P3C6; 12, P3C5; 13, P3F6; 14, 1 kb ladder. Lanes not reported were not sequenced in this study. (B and D) Membrane blotted with PCR amplified cDNA sequences from subtracted library and probed with the forward subtracted probe which is enriched for fungal genes expressed on the leaf surface of the plant. (C and E) Membrane blotted with PCR amplified cDNA sequences from subtracted library and probed with the reverse subtracted probe which is enriched for fungal genes expressed during spore germination in water. The blots in (B) and (C) and (D) and (E) are identical except for the probe with which they are screened. Clones sequenced in this study: blots B and C: A7, P2F2; B2, P2B5; B4, P2H6; B8, P2A8; C1, P2F9; D5, P2A12; D6, P2C12; and D7, P2D12. Blots D and E: clones correspond to all "P3" designated clones in this study, i.e., G2, clone P3G2.

membranes, in duplicate, for a total of 6 membranes. Both forward (tester minus driver) and reverse subtracted (driver minus tester) radioactively labeled cDNA populations were then used as individual probes for identical blots. Reverse subtracted cDNA probe is an ideal probe and negative control because this population is theoretically enriched for genes expressed only in spores germinated in water (driver cDNA population). A subset of clones subjected to this macro-array dot-blot screening are presented in Figs. 1B–E. Differences in hybridization signal intensities were apparent on the membranes probed with the forward subtracted probe (Figs. 1B and D) compared with the membranes probed with the reverse subtracted probe (Figs. 1C and E), which indicated the success of the SSH procedure in identifying differentially expressed sequences between the two populations being examined.

3.2. Virtual Northern analysis

After examination of the macro-array dot-blots, 47 clones (21%) were selected that had a hybridization signal clearly stronger on membranes probed with the forward subtracted probe population. These 47 clones were used in virtual Northern blots to further confirm their differential expression (Endege et al., 1999). Of the 47 clones examined, 35 (77%) were found to be signifi-

cantly upregulated during the first 24 h of *A. brassicicola* infection of the *Arabidopsis* ecotype Ler. Fig. 2 presents the virtual Northern blot results for 11 selected clones. Dramatic up-regulation on the plant leaf surface is seen in Fig. 2. Of particular interest are clones P3G2, P3D7, P3G8, P2A12, and P3F2 which appear to be expressed solely on the plant leaf surface during the first 24 h of infection. On some blots such as P3D8 and P3B5, multiple bands are present which may indicate the gene of interest is part of a multi-gene family. As expected, presence of the transcripts examined is low or non-existent in lane 1 (ungerminated spores) compared to lanes 2 (spore germination in water) and 3 (spore germination on plant leaf surface). Metabolic activity in ungerminated spores is low or non-existent compared with active growth, and thus genes required for spore germination and growth are unlikely to be transcribed unless they are preformed in the spore.

3.3. Bioinformatic analysis of SSH derived *Alternaria brassicicola* clones

Forty-seven clones were selected for sequence analysis based on results from the dot-blot screens and virtual Northern blots. Following sequencing of the 47 selected clones, the 47 cDNA sequences were used to search the GenBank non-redundant database

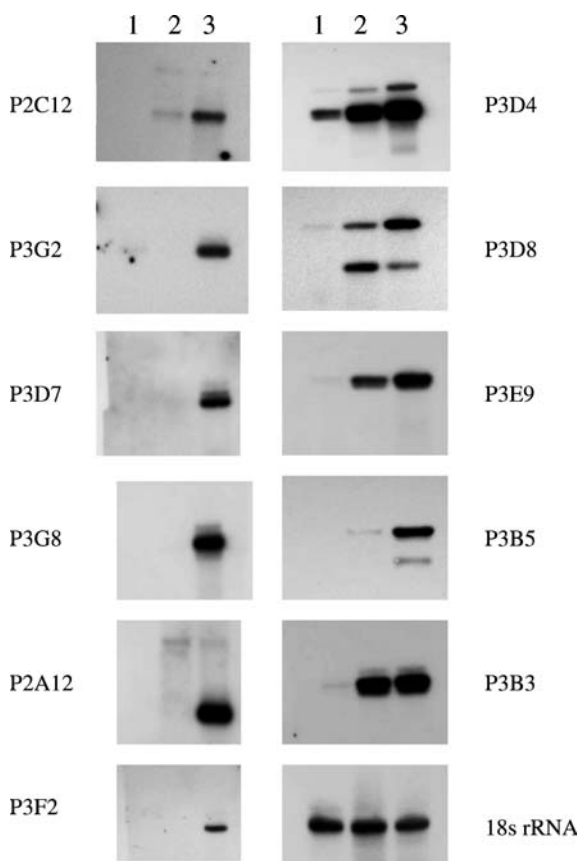


Fig. 2. Virtual Northern blots showing differential expression of selected cDNA clones. P2C12 (unknown), P3G2 (allergen Alt a 1), P3D7 (cyanide hydratase), P3G8 (formate dehydrogenase), P2A12 (ABC Transporter), P3F2 (unknown), P3D4 (mitochondrial matrix protein), P3D8 (Ketol acid reductoisomerase), P3E9 (ADP/ATP carrier protein), P3B5 (Translation initiation factor eIF-4a), P3B3 (ribosomal protein L12), 18s rRNA to show equal loading of lanes. On each blot lane 1, ungerminated spores cDNA; 2, water germinated spores 24 h cDNA; and 3, leaf surface germinated spores 24 h cDNA. A total of 2.5 μ g of cDNA was blotted to the membranes and probed with DIG labeled cDNA probes respective to each clone.

(Table 1), Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and Oomycete EST database (Table 2), *M. grisea* Genome Database release 2.1 (Table 3), and *N. crassa* Genome database 3 (Table 4) using the BLASTx algorithm. Thirty-six of the 47 sequences (77%) had at least 1 significant (E-value $<10e-3$) hit in the 4 databases examined.

Of the 31 genes that had significant hits in the GenBank non-redundant database, 94% (29) were homologous to sequences from other fungi. Two clones, P3F1 and P3G8, had significant hits with organisms other than fungi (P3F1 was a human match and P3G8 was a bacterial match). Four of the five genes that were solely expressed on the host leaf surface had significant BLASTx hits. The first, clone P3G2, had homology with the major *Alternaria* allergen, *Alt a 1*, clone P2A12 had

homology with an arsenite translocating ATPase (ABC transporter) involved in arsenic detoxification, clone P3D7 had homology with a cyanide hydratase gene from *Leptosphaeria maculans*, and clone P3G8 had homology with a formate dehydrogenase involved in formate metabolism. Functional grouping, according to the yeast protein sequence reference set from MIPS (Mewes et al., 1997, 2002), of the 47 sequences examined was based on results from BLASTx queries of the non-redundant GenBank database and are presented in Fig. 3. Forty-two percent (20) of the clones are unclassified proteins based on the results of the database searches performed in this study.

Thirty-one of the 47 clones (66%) had significant matches in the COGEME Phytopathogenic Fungi and Oomycete EST database. Clones P2F2, P3A11, P3D3, and P3B2 had no significant hits in the GenBank NR database, but significant hits in the COGEME EST database. Three clones, P3G8 (formate dehydrogenase), P3G2 (Alt a 1 allergen), and P3C5 had hits in the Genbank NR database but not the COGEME EST database.

BLASTx searches of the *M. grisea* Genome Database release 2.1 revealed that 57% (27) of the *A. brassicicola* expressed clones had potential homologs in the *M. grisea* genome. Clone P2C12, highly upregulated on the *Arabidopsis* leaf surface but of unknown function, had a significant hit with an unknown *M. grisea* protein. Interestingly, P2C12 was not present in the COGEME EST database, nor the *N. crassa* genome. Clone P3D6 also only had a significant hit with the *M. grisea* genome.

Twenty-six (55%) of the 47 clones had significant homologies with genes in the *N. crassa* Genome Database 3. Clone P3G8, the formate dehydrogenase, had a significant hit (E-value $1e-43$) with a gene in the *N. crassa* genome confirming its fungal origin. This gene was the only gene of the 47 to be present in the saprophytic *N. crassa* genome and not the phytopathogenic *M. grisea* genome.

Eleven (23%) of the 47 clones sequenced did not have significant hits with any of the 4 databases and may be unique to *Alternaria* or other Loculoascomycete species. Among these clones with no significant hits is P3F2 which is highly upregulated during *A. brassicicola* infection of *Arabidopsis*. The other 10 clones with no significant matches included P3B7, P3D2, P2B11, P1G5, P2B5, P1F1, P2F9, P2A8, P3D9, and P2D12.

3.4. RT-PCR analysis

We used RT-PCR to examine the expression patterns of the five genes expressed during plant infection and not in spores germinating in water according to the virtual Northern blots. We chose to examine the

Table 1

Summary of 47 sequenced inserts from the SSH library enriched with *Alternaria brassicicola* genes expressed during the first 24 h of a compatible interaction with the *Arabidopsis* ecotype Landsberg Erecta

GenBank	Clone ID	Size (bp)	Putative ID	Organism	E-value	MIPS functional category
CA405363	P2H6	486	Acetyl-coa acetyl transferase	<i>Schizosaccharomyces pombe</i>	3e – 39	1
CA405364	P3C4	525	Activator of Heat Shock Protein 90 ATPase	<i>S. cerevisiae</i>	1e – 17	11
CA405365	P3A1	296	ADP/ATP Carrier	<i>N. crassa</i>	3e – 38	1
CA405366	P3E9	285	ADP/ATP Carrier	<i>N. crassa</i>	3e – 38	1
CA405367	P3F4	344	ADP/ATP Carrier	<i>N. crassa</i>	4e – 38	1
AF499002	P3G2	464	Alt a 1 Allergen Precursor	<i>Alternaria alternata</i>	1e – 104	99
CA405368	P2A12	580	Arsenite translocating ATPase	<i>S. pombe</i>	4e – 13	7
CA405369	P3D7	621	Cyanide Hydratase	<i>Leptosphaeria maculans</i>	4e – 75	11
CA405370	P2F12	679	Conserved hypothetical protein	<i>N. crassa</i>	3e – 15	99
CA405371	P3F6	781	Delta (24)-Sterol C Methyltransferase	<i>C. albicans</i>	5e – 55	1
CA405372	P3G8	649	Formate Dehydrogenase	<i>M. formicicum</i>	6e – 25	1
CA405373	P3F5	805	Ketol Acid Reductoisomerase ilv-2	<i>N. crassa</i>	1e – 151	1
CA405408	P1A8	334	Ketol Acid Reductoisomerase	<i>N. crassa</i>	2e – 13	1
CA405374	P3D8	509	Ketol Acid Reductoisomerase	<i>N. crassa</i>	2e – 81	1
CA405375	P3D4	727	Mitochondrial Matrix Processing Peptide	<i>N. crassa</i>	6e – 35	1
CA405376	P3E1	525	NADH dehydrogenase	<i>A. nidulans</i>	4e – 10	2
CA405377	P3C1	440	Nbp35p Nucleotide binding ATPase	<i>S. cerevisiae</i>	2e – 11	99
CA405378	P3C6	544	Peptidyl Prolyl <i>cis-trans</i> isomerase FKBP-21	<i>N. crassa</i>	4e – 30	6
CA405379	P1D9	447	Heat Shock Protein MGE1 precursor	<i>N. crassa</i>	9e – 6	6
CA405380	P3H10	725	Prolidase	<i>A. nidulans</i>	2e – 17	6
CA405381	P1D10	640	<i>Botrytis cinerea</i> cDNA	<i>B. cinerea</i>	2e – 72	6
CA405382	P3F12	515	Ribosomal protein 30s	<i>C. vulgaris</i>	1e – 8	5
CA405383	P3B3	609	Ribosomal protein L12	<i>N. crassa</i>	1e – 64	5
CA405384	P3G9	641	Ribosomal protein L21	<i>S. cerevisiae</i>	3e – 24	5
CA405385	P1G1	484	Ribosomal protein L30 like	<i>Ophiostoma novo-ulmi</i>	5e – 7	5
CA405386	P1C7	460	Ribosomal protein L4	<i>S. pombe</i>	3e – 40	5
CA405387	P3F1	553	RNA Pol II Polypeptide H	<i>Homo sapiens</i>	1e – 7	4
CA405388	P3B5	328	TIF eIF-4a	<i>C. albicans</i>	1e – 42	5
CA405389	P3H1	478	Vacuolar membrane ATPase C	<i>Aspergillus oryzae</i>	3e – 5	7
CA405390	P3C5	500	Carbon catabolite repressor CreA	<i>Cochliobolus carbonum</i>	2e – 10	2
CA405391	P3B7	777	No Significant Matches	NA	NA	99
CA405392	P3D6	726	No Significant Matches	NA	NA	99
CA405393	P3D2	331	No Significant Matches	NA	NA	99
CA405394	P2F2	665	No Significant Matches	NA	NA	99
CA405395	P2B11	468	No Significant Matches	NA	NA	99
CA405396	P1G5	132	No Significant Matches	NA	NA	99
CA405397	P2B5	170	No Significant Matches	NA	NA	99
CA405398	P1F1	343	No Significant Matches	NA	NA	99
CA405399	P2F9	638	No Significant Matches	NA	NA	99
CA405400	P3A11	247	No Significant Matches	NA	NA	99
CA405401	P3D3	165	No Significant Matches	NA	NA	99
CA405402	P2A8	523	No Significant Matches	NA	NA	99
CA405403	P2C12	656	No Significant Matches	NA	NA	99
CA405404	P3B2	269	No Significant Matches	NA	NA	99
CA405405	P3F2	460	No Significant Matches	NA	NA	99
CA405406	P3D9	221	No Significant Matches	NA	NA	99
CA405407	P2D12	432	No Significant Matches	NA	NA	99

Putative identification is based on Blastx searches of the non-redundant GenBank database. Only scores with an E-value of $<10e-3$ were considered significant and reported in this table. MIPS Functional Category Nos.: 1, Metabolism; 2, Energy; 4, Transcription; 5, Protein Synthesis; 6, Protein Destination; 7, Transport Facilitation; 11, Cell Rescue/Defense/Stress; 99, Unclassified.

presence of transcripts from these genes for 5 days during an infection time course of the economically important *B. oleraceae* var. *capitata* (green cabbage), and at two times points during *Arabidopsis* infection (48 h post-inoculation when symptoms first appeared and 7 days after inoculation when no healthy tissue

remained). No visible symptoms could be seen on infected cabbage leaves for the first 48 h of infection. However, visible necrosis and chlorosis were visible on the *Arabidopsis* detached leaves at 48 h. At day 3, the cabbage leaves began to exhibit typical black spot symptoms characterized by dark brown to black lesions

Table 2

TBlastX searches of the Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and EST Database version 1.3

Genbank	Clone ID	Putative EST ID	Organism	E-value	Uni-sequence ID
CA405363	P2H6	Acetyl-coa acetyl transferase	<i>Mycosphaerella graminicola</i>	3e – 39	mg[1476]
CA405364	P3C4	Activator of Heat Shock Protein 90	<i>Botryotinia fuckeliana</i>	1e – 46	BfCon[1598]
CA405365	P3A1	ADP/ATP Carrier Protein	<i>Gibberella zeae</i>	7e – 22	GzCon[0018]
CA405366	P3E9	ADP/ATP Carrier Protein	<i>F. sporotrichiodes</i>	5e – 22	FsCon[0406]
CA405367	P3F4	ADP/ATP Carrier Protein	<i>G. zeae</i>	2e – 22	GzCon[0018]
AF499002	P3G2	No Significant Match	NA	NA	NA
CA405368	P2A12	Aresnite translocationg ATPase	<i>B. fuckeliana</i>	6e – 24	BfCon[0917]
CA405369	P3D7	Cyanide Hydratase	<i>M. grisea</i>	7e – 73	MagCon[5383]
CA405370	P2F12	Unknown Protein	<i>M. graminicola</i>	2e – 18	Mga1033f
CA405371	P3F6	S-adenosyl methionine:sterol methyl transferase	<i>M. grisea</i>	5e – 52	Mag14180412
CA405372	P3G8	No Significant Match	NA	NA	NA
CA405373	P3F5	Ketol Acid Reductoisomerase	<i>G. zeae</i>	1e – 135	GzCon[0603]
CA405408	P1A8	Ketol Acid Reductoisomerase	<i>F. sporotrichoides</i>	7e – 17	FsCon[-134]
CA405374	P3D8	Ketol Acid Reductoisomerase	<i>G. zeae</i>	4e – 65	GzCon[0603]
CA405375	P3D4	ADP/ATP Carrier Protein	<i>B. fuckeliana</i>	1e – 34	BfCon[1221]
CA405376	P3E1	NADH–ubiquinone oxidoreductase subunit 5	<i>B. fuckeliana</i>	6e – 12	BfCon[1930]
CA405377	P3C1	35 kDa nucleotide binding protein	<i>M. grisea</i>	2e – 20	MagCon[1254]
CA405378	P3C6	Peptidyl Prolyl <i>cis</i> – <i>trans</i> isomerase FK506	<i>M. grisea</i>	4e – 32	MagCon[0603]
CA405379	P1D9	Heat Shock Protein involved in protein import into mitochondria	<i>M. grisea</i>	3e – 09	MagCon[6538]
CA405380	P3H10	Prolidase	<i>B. fuckeliana</i>	2e – 28	BfCon[0773]
CA405381	P1D10	Ribosomal Protein L19	<i>B. fuckeliana</i>	2e – 57	BfCon[1265]
CA405382	P3F12	Unknown Protein	<i>B. graminis</i>	1e – 11	Bga03g03f
CA405383	P3B3	60s ribosomal protein L12	<i>M. graminicola</i>	6e – 83	mg[1404]
CA405384	P3G9	60s ribosomal protein L21	<i>G. zeae</i>	2e – 32	GzCon[0198]
CA405385	P1G1	60s ribosomal protein L30	<i>M. graminicola</i>	1e – 11	mg[1171]
CA405386	P1C7	60s ribosomal protein L7	<i>G. zeae</i>	2e – 52	GzCon[0793]
CA405387	P3F1	Unknown Protein	<i>B. graminis</i>	1e – 11	Bga03g03f
CA405388	P3B5	Eukaryotic translation initiation factor 4A	<i>M. graminicola</i>	9e – 67	mg[1180]
CA405389	P3H1	Vacuolar membrane ATPase	<i>G. zeae</i>	5e – 08	GzCon[0138]
CA405390	P3C5	No Significant Matches	NA	NA	NA
CA405391	P3B7	No Significant Matches	NA	NA	NA
CA405392	P3D6	No Significant Matches	NA	NA	NA
CA405393	P3D2	No Significant Matches	NA	NA	NA
CA405394	P2F2	Cytochrome C	<i>M. graminicola</i>	2e – 13	mg[1026]
CA405395	P2B11	No Significant Matches	NA	NA	NA
CA405396	P1G5	No Significant Matches	NA	NA	NA
CA405397	P2B5	No Significant Matches	NA	NA	NA
CA405398	P1F1	No Significant Matches	NA	NA	NA
CA405399	P2F9	No Significant Matches	NA	NA	NA
CA405400	P3A11	Unknown Protein	<i>B. graminis</i>	4e – 06	Bga03g03f
CA405401	P3D3	Unknown Protein	<i>B. graminis</i>	9e – 06	Bga03g03f
CA405402	P2A8	No Significant Matches	NA	NA	NA
CA405403	P2C12	No Significant Matches	NA	NA	NA
CA405404	P3B2	60s Ribosomal Protein L36	<i>V. dahlia</i>	7e – 08	VD109B12
CA405405	P3F2	No Significant Matches	NA	NA	NA
CA405406	P3D9	No Significant Matches	NA	NA	NA
CA405407	P2D12	No Significant Matches	NA	NA	NA

Putative EST ID based on the annotation according to the COGEME database. Only E-values < 10e – 3 were considered significant and reported.

with a chlorotic halo indicative of toxin production. By day 4, black spot lesions were present on the majority of the cabbage leaf accompanied by extensive chlorosis. By day 7 leaves were completely necrotized and chlorotic.

Fig. 4A presents the RT-PCR results of this experiment and shows that none of the genes have transcripts in the uninfected cabbage, further strengthening our conclusion that these genes are of fungal origin.

Table 3
Blastx searches of the *Magnaporthe grisea* Genome Database release 2.1

GenBank	Clone ID	Gene name/protein family	Sequence Contig	E-value	Locus ID
CA405363	P2H6	Hypothetical Protein/Thiolase Domain	2.331	3e–22	MG01755
CA405364	P3C4	Hypothetical Protein/Unknown	2.146	9e–40	MG00814
CA405365	P3A1	Hypothetical Protein/Mitochondrial Carrier Proteins	2.1238	6e–15	MG06656
CA405366	P3E9	Hypothetical Protein/Mitochondrial Carrier Proteins	2.1238	9e–15	MG06656
CA405367	P3F4	Hypothetical Protein/Mitochondrial Carrier Proteins	2.1238	8e–15	MG06656
AF499002	P3G2	No Significant Match	NA	NA	NA
CA405368	P2A12	Hypothetical Protein/Anion-transporting ATPase	2.1821	8e–19	MG09535
CA405369	P3D7	Hypothetical Protein/Carbon–nitrogen hydrolase	2.2064	1e–62	MG10714
CA405370	P2F12	Predicted Protein	2.1391	5e–19	MG07482
CA405371	P3F6	Hypothetical Protein	2.2030	2e–61	MG10568
CA405372	P3G8	No Significant Match	NA	NA	NA
CA405373	P3F5	Hypothetical Protein/Acetohydroxy acid isomeroreductase	2.347	1e–115	MG01808
CA405408	P1A8	Hypothetical Protein/Acetohydroxy acid isomeroreductase	2.347	8e–14	MG01808
CA405374	P3D8	Hypothetical Protein/Acetohydroxy acid isomeroreductase	2.347	1e–61	MG01808
CA405375	P3D4	Hypothetical Protein/Mitochondrial Carrier Proteins	2.1238	6e–28	MG06656
CA405376	P3E1	No Significant Match	NA	NA	NA
CA405377	P3C1	Hypothetical Protein	2.1146	8e–17	MG06151
CA405378	P3C6	Predicted Protein/FKBP type peptidyl prolyl <i>cis</i> – <i>trans</i> isomerase	2.1345	1e–30	MG07234
CA405379	P1D9	Hypothetical Protein/GrpE Domain	2.642	7e–07	MG03214
CA405380	P3H10	Hypothetical Protein/Metallopeptidase Family M24	2.1053	2e–25	MG05684
CA405381	P1D10	Hypothetical Protein/Ribosomal L19e Domain	2.875	3e–42	MG04681
CA405382	P3F12	Unknown Protein	NA	NA	NA
CA405383	P3B3	Hypothetical Protein/Ribosomal L11 Domain	2.1280	2e–66	MG06895
CA405384	P3G9	Hypothetical Protein/Unknown	2.596	9e–22	MG02953
CA405385	P1G1	Hypothetical Protein/Ribosomal L7Ae Domain	2.625	3e–08	MG03135
CA405386	P1C7	Hypothetical Protein/Ribosomal L7Ae Domain	2.868	1e–47	MG04612
CA405387	P3F1	No Significant Matches	NA	NA	NA
CA405388	P3B5	Hypothetical Protein/DEAD DEAH box helicase	2.828	1e–55	MG04400
CA405389	P3H1	Hypothetical Protein/ATP Synthase subunit C	2.505	7e–06	MG02466
CA405390	P3C5	No Significant Matches	NA	NA	NA
CA405391	P3B7	No Significant Matches	NA	NA	NA
CA405392	P3D6	Predicted Protein	2.1032	1e–28	MG05566
CA405393	P3D2	No Significant Matches	NA	NA	NA
CA405394	P2F2	No Significant Matches	NA	NA	NA
CA405395	P2B11	No Significant Matches	NA	NA	NA
CA405396	P1G5	No Significant Matches	NA	NA	NA
CA405397	P2B5	No Significant Matches	NA	NA	NA
CA405398	P1F1	No Significant Matches	NA	NA	NA
CA405399	P2F9	No Significant Matches	NA	NA	NA
CA405400	P3A11	No Significant Matches	NA	NA	NA
CA405401	P3D3	No Significant Matches	NA	NA	NA
CA405402	P2A8	No Significant Matches	NA	NA	NA
CA405403	P2C12	Predicted Protein	2.922	2e–05	MG04929
CA405404	P3B2	Predicted Protein/Ribosomal Protein L36e	2.692	5e–04	MG03554
CA405405	P3F2	No Significant Matches	NA	NA	NA
CA405406	P3D9	No Significant Matches	NA	NA	NA
CA405407	P2D12	No Significant Matches	NA	NA	NA

Locus ID does not necessarily imply location in the genome, but rather is an ID number according to the *M. grisea* database. Only E-values <10e–3 were considered significant and reported in this table. Reported matches are the hits with highest significance for each clone.

Beta-tubulin and 18s rRNA controls, which were derived from *Alternaria* sequences, did amplify the plant homologs making it impossible to determine relative amounts of fungal biomass present at each infection time point in this experiment. Clone P2A12, the arsenic ATPase, clone P3G2, the *Alternaria* allergen, and clone P3G8, the formate dehydrogenase, appear to be expressed throughout the 5 day infection process. Clones P3D7, the cyanide hydratase, and the unknown clone P3F2, appear to be expressed at higher levels later in the

infection process. Clone P3F2 in particular appears to be highly expressed during the later stages of infection. Though RT-PCR is not quantitative, the use of house keeping genes such as 18s rRNA and beta-tubulin allow a relative comparison of transcript abundance between the different time points of the infection, but as stated above, cannot be used in this experiment to determine approximate fungal biomass levels.

Fig. 4B presents expression of the 5 selected clones and clone P2C12 2 and 7 days post-inoculation on the

Table 4
Blastx searches of the *Neurospora crassa* 3 Genome Database

GenBank	Clone ID	Gene name/protein family	Sequence Contig	E-value	Locus ID
CA405363	P2H6	Hypothetical Protein/Thiolase Domain	3.133	1e–42	NCU02571
CA405364	P3C4	Hypothetical Protein	3.215	2e–36	NCU04087
CA405365	P3A1	ADP/ATP Carrier Protein/Mitochondrial Carrier Proteins	3.606	4e–15	NCU09477
CA405366	P3E9	ADP/ATP Carrier Protein/Mitochondrial Carrier Proteins	3.606	4e–15	NCU09477
CA405367	P3F4	ADP/ATP Carrier Protein/Mitochondrial Carrier Proteins	3.606	3e–15	NCU09477
AF499002	P3G2	No Significant Match	NA	NA	NA
CA405368	P2A12	Hypothetical Protein/Anion-transporting ATPase	3.388	2e–18	NCU06717
CA405369	P3D7	Hypothetical Protein/Carbon–Nitrogen hydrolase	3.257	3e–76	NCU04697
CA405370	P2F12	Hypothetical Protein	3.200	9e–17	NCU03660
CA405371	P3F6	Delta (24)-Sterol C-Methyltransferase	3.153	4e–60	NCU03006
CA405372	P3G8	Hypothetical Protein	3.26	1e–43	NCU00758
CA405373	P3F5	Ketol Acid Reductoisomerase Precursor	3.199	1e–116	NCU03608
CA405408	P1A8	Ketol Acid Reductoisomerase Precursor	3.199	6e–15	NCU03608
CA405374	P3D8	Ketol Acid Reductoisomerase Precursor	3.199	1e–63	NCU03608
CA405375	P3D4	Mitochondrial Processing Peptidase Alpha Subunit	3.364	2e–36	NCU06270
CA405376	P3E1	No Significant Matches	NA	NA	NA
CA405377	P3C1	Hypothetical Protein	3.548	3e–16	NCU08825
CA405378	P3C6	Hypothetical Protein/Peptidyl Prolyl <i>cis</i> – <i>trans</i> isomerase FKBP	3.217	1e–27	NCU04140
CA405379	P1D9	Probable heat shock protein MGE1 precursor	3.64	3e–07	NCU01516
CA405380	P3H10	Hypothetical Protein/Metallopeptidase Family M24	3.544	1e–28	NCU08782
CA405381	P1D10	Hypothetical Protein/Ribosomal Protein L19e	3.331	3e–42	NCU05804
CA405382	P3F12	No Significant Matches	NA	NA	NA
CA405383	P3B3	Hypothetical Protein/Ribosomal L11	3.51	4e–66	NCU01317
CA405384	P3G9	Hypothetical Protein/Ribosomal protein L21e	3.86	2e–23	NCU01948
CA405385	P1G1	Hypothetical Protein/Ribosomal protein L7Ae	3.561	7e–09	NCU08963
CA405386	P1C7	Hypothetical Protein/Ribosomal protein L7Ae	3.262	2e–48	NCU04779
CA405387	P3F1	No Significant Matches	NA	NA	NA
CA405388	P3B5	Eukaryotic initiation factor 4A/DEAD DEAH box	3.433	2e–55	NCU07420
CA405389	P3H1	Vacuolar ATP Synthase 16 kDa Proteolipid Subunit	3.51	7e–06	NCU01332
CA405390	P3C5	No Significant Matches	NA	NA	NA
CA405391	P3B7	No Significant Matches	NA	NA	NA
CA405392	P3D6	No Significant Matches	NA	NA	NA
CA405393	P3D2	No Significant Matches	NA	NA	NA
CA405394	P2F2	No Significant Matches	NA	NA	NA
CA405395	P2B11	No Significant Matches	NA	NA	NA
CA405396	P1G5	No Significant Matches	NA	NA	NA
CA405397	P2B5	No Significant Matches	NA	NA	NA
CA405398	P1F1	No Significant Matches	NA	NA	NA
CA405399	P2F9	No Significant Matches	NA	NA	NA
CA405400	P3A11	No Significant Matches	NA	NA	NA
CA405401	P3D3	No Significant Matches	NA	NA	NA
CA405402	P2A8	No Significant Matches	NA	NA	NA
CA405403	P2C12	No Significant Matches	NA	NA	NA
CA405404	P3B2	Hypothetical Protein/Ribosomal Protein L36e	3.172	2e–04	NCU03302
CA405405	P3F2	No Significant Matches	NA	NA	NA
CA405406	P3D9	No Significant Matches	NA	NA	NA
CA405407	P2D12	No Significant Matches	NA	NA	NA

Locus ID does not necessarily imply location in the genome, but rather is an ID number assigned by the *N. crassa* genome database. Only E-values <10e–3 were considered significant and reported in this table. Reported matches are the hits with highest significance for each clone.

Arabidopsis ecotype Ler, and from a 5 day old culture of *A. brassicicola* grown in glucose yeast extract broth (GYEB). Interestingly, clone P3F2 was not expressed at 7 days post-inoculation on *Arabidopsis* or in the nutrient rich media. We then performed RT-PCR on 7 day infected cabbage which revealed that clone P3F2 is expressed at 7 days post-inoculation on cabbage (Fig. 4B). The other 5 clones examined, P2A12, P3G2, P3G8, P2C12, and P3D7, were all expressed in the media as

well as at 7 days post-inoculation of cabbage and *Arabidopsis*.

4. Discussion

Despite the substantial amount of work done on resistance mechanisms to *A. brassicicola* in *Arabidopsis*, little is known about this necrotroph's pathogenicity

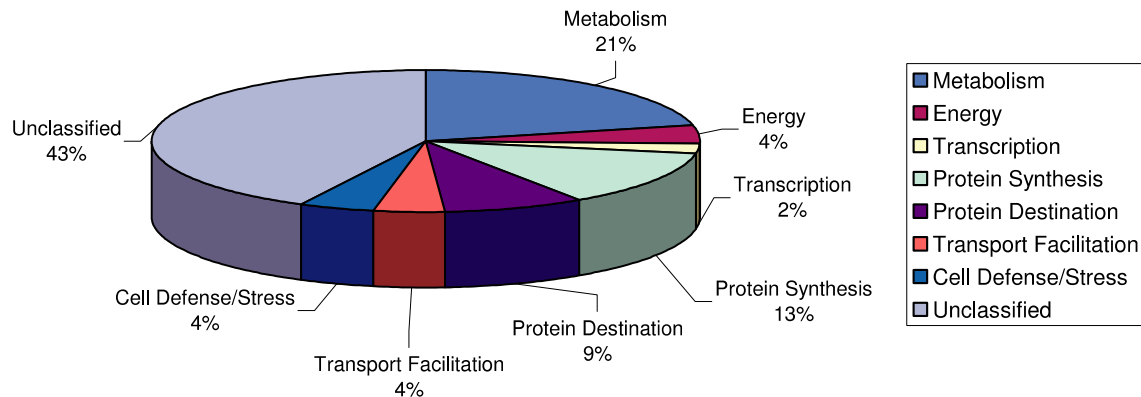


Fig. 3. Distribution of *A. brassicicola* ESTs into MIPS categories. Percentages were calculated from the total number of ESTs (47) and functional assignment was based on results from Blastx searches of the non-redundant GenBank database.

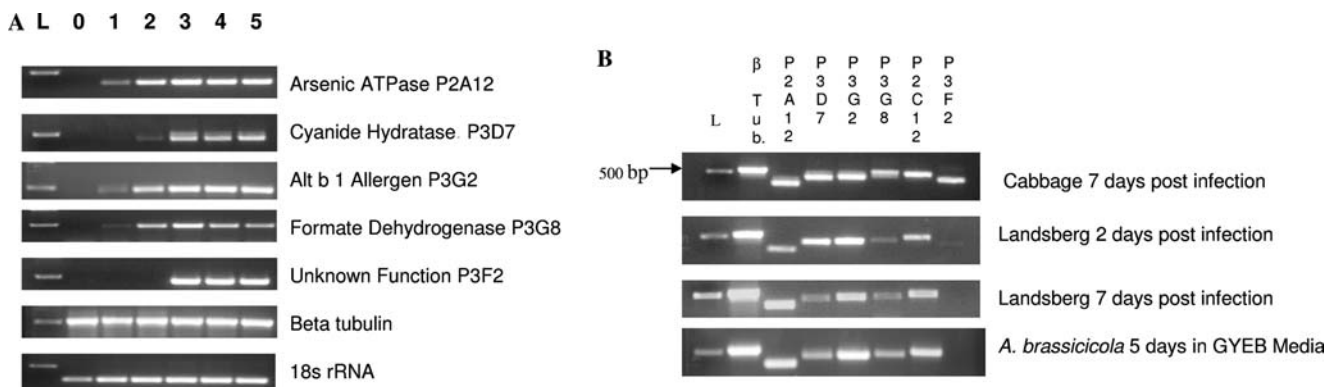


Fig. 4. RT-PCR analysis of selected *A. brassicicola* clones. (A) RT-PCR analysis of the expression of the 5 genes upregulated in planta and not in spores germinating in water according to the virtual Northern analysis. Lane L, 500 bp ladder; 0, uninfected cabbage; 1, cabbage infected 24 h; 2, cabbage infected 48 h; 3, cabbage infected 72 h; 4, cabbage infected 96 h; and 5, cabbage infected 120 h. (B) RT-PCR analysis 2 and 7 days post-inoculation on *Arabidopsis* ecotype Landsberg Erecta, 7 days post-inoculation on green cabbage, and 5 days post-inoculation in Glucose–yeast extract broth (GYEB). Lanes: L, 500 bp, ladder beta tubulin control; P2A12, ATPase arsenic transporter; P3D7, cyanide hydratase; P3G2, Alt b 1 allergen; P3G8, formate dehydrogenase; P2C12, unknown clone; and P3F2, unknown clone.

mechanisms. The purpose of this study was to identify *A. brassicicola* genes upregulated during pathogenesis of the *Arabidopsis* ecotype Ler, with the hypothesis that genes expressed in planta may be important for necrotrophic pathogenesis. A time point of 24 h post-inoculation was chosen to extract total RNA for use in SSH because at this time point in the infection process, the putative AB toxin has been shown to be present in high levels in spore germination fluid (Otani et al., 1998). Thus, we felt that this initial period of infection was an excellent starting point to potentially identify genes involved in toxin production and host specificity. Unfortunately, however, studies on the infection process of *A. brassicicola* on *Arabidopsis* are lacking. At 24 h post-inoculation, the majority of spores have germinated on both cabbage and *Arabidopsis* ($\geq 80\%$), and germ tubes have begun to swell into structures resembling appressoria. These appressoria-like structures are not well-rounded appressoria as seen in *M. grisea* (data not shown). Whether these swellings are true appressoria is

unknown, but none of the cDNAs found in this study encoded genes reported to be involved in appressoria formation in other fungi. Additional microscopic studies are currently being performed to further characterize the infection process in this patho-system.

Bioinformatic searches of four available databases, including the non-redundant GenBank database, COGEME Phytopathogenic Fungi and Oomycete EST database, *M. grisea* Genome database and *N. crassa* Genome database, revealed that 77% (36) of the sequences had at least one significant hit in the databases examined. Further, all clones with significant hits had the highest significant hit in at least one database that was of fungal origin. This supports our experimental strategy of trying to obtain only fungal sequences during the infection process. Not surprisingly, given the relative lack of fungal sequences available, 43% (20) of the sequences identified are unclassified proteins. Eleven clones had no significant matches in the databases perhaps indicating that these sequences are specific to

Alternaria species or Loculoascomycetes in general. As databases become more saturated with sequence information from fungal species, identifying species specific genes will become easier.

One of these unknown sequences, P3F2, was highly upregulated in planta according to the virtual Northern results (Fig. 2). RT-PCR analysis with this clone revealed that it was preferentially expressed during the later stages of infection on cabbage but not *Arabidopsis*. Further, P3F2 does not appear to be expressed in vitro in a nutrient rich broth of 5 day old mycelia (Fig. 4B). It is difficult to speculate on the difference between P3F2 expression on cabbage and *Arabidopsis* without having a putative function. Perhaps the difference seen at 7 days post-inoculation is due to the fact that the infection process is in all likelihood over at 7 days post-inoculation on *Arabidopsis* (indicated by complete tissue maceration). Given that P3F2 expression was not seen in mycelia in a 5 day old culture but was seen in 5 day old mycelia during infection, we do not believe P3F2 expression is simply related to the age of the mycelia. Rather, based on our expression data, we believe that the difference in P3F2 expression between *Arabidopsis* and cabbage is likely due to an intrinsic property of P3F2's function during the infection process (which is of course related to the inherent differences between *Arabidopsis* and cabbage). Attempts to clone the full length cDNA and disrupt this putatively important pathogenesis gene are underway.

Four of the clones that were expressed in planta and not in the spores germinating in water had significant hits in one or more of the databases examined. These included an arsenite translocating ATPase (ABC transporter), cyanide hydratase, major *Alternaria* allergen *Alt a 1*, and a formate dehydrogenase. Further examination of these clones with RT-PCR revealed that all four clones are expressed during *A. brassicicola*'s infection process on green cabbage (Figs. 4A and B) as well as in vitro in nutrient rich media (Fig. 4B). The fact that these genes were expressed in GYEB media and not water may suggest that their expression is induced by a nutrient rich environment, or perhaps the expression in the nutrient rich media is a reflection of the difference in age of the mycelium (5 days old vs 24 h for spores germinated in water) when the RNA was extracted. Based on the bioinformatics analyses and expression patterns of these four cDNA sequences, we suggest that these genes are candidates to be involved in *A. brassicicola* pathogenesis during fungal invasion of host tissues.

Two of these sequences, the arsenic translocating ATPase (P2A12) and the cyanide hydratase (P3D7), may be involved in detoxifying plant produced toxic compounds. By macerating host plant tissue, necrotrophic fungi expose themselves to a myriad of potentially toxic plant compounds. The arsenic ATPase is a part of the ABC superfamily and is involved in arsenic detoxification in *Saccharomyces cerevisiae* (Ghosh et al.,

1999). To our knowledge, *Arabidopsis* does not contain or sequester arsenic compounds, and thus the role of this putative ABC transporter in *A. brassicicola* pathogenesis is unclear. However, previous studies have demonstrated that ABC transporters may serve as novel pathogenicity factors in phytopathogenic fungi. In the hemi-biotroph *M. grisea*, an ABC transporter (*ABC1*) was shown to be required for fungal growth early in pathogenesis (Urban et al., 1999), and recently a copper transporting ATPase was cloned and found to be involved in the infection process of *Colletotrichum lindemuthianum* (Parisot et al., 2002). Thus, transporters involved in metallic ion transport appear to have roles in fungal pathogenicity.

Though *Arabidopsis* plants do not sequester arsenic, they do contain high levels of glucosinolates (Hansen et al., 2001; Tierens et al., 2001). Glucosinolates are broken down by myrosinases when tissue damage occurs into a wide variety of toxic compounds such as nitriles, isothiocyanates and thiocyanates. In this study, a cyanide hydratase homolog (P3D7) was identified that was expressed on the host leaf surface. Enzymes, such as cyanide hydratase, that degrade cyanide and act on carbon–nitrogen bonds may be candidates for glucosinolate by-product degradation (Sexton and Howlett, 2000). The role of cyanide hydratases in pathogenicity is still not clear, and does not seem to be required for *Gloeocercospora sorghi* infection of the cyanogenic plant sorghum (Wang et al., 1999). Interestingly, our bioinformatics analysis revealed that *M. grisea* and the saprophyte *N. crassa* both have cyanide hydratase homologs in their genomes. This may indicate that cyanide hydratases are common enzymes in filamentous fungi and could potentially explain why cyanide hydratase disruptants have so far been shown to maintain virulence. What functional role cyanide hydratases have in fungal metabolism is still not clear.

Several other genes identified in this study may have potential roles in fungal pathogenesis despite the fact that they are also expressed during spore germination in water. Clone P3B5 encoding a translation initiation factor, was also found to be upregulated in a highly virulent *Candida albicans* isolate (Mirbod et al., 1996). Though this may be a reflection of increased gene expression in a challenging environment, the fact that this translation initiation factor was upregulated in the pathogenic *C. albicans* isolate, and not a weakly virulent isolate, may indicate a general role in fungal pathogenesis for this translation initiation factor. Two other clones of particular interest are the unknown clones P2C12 and P3D6, which only had significant matches in the *M. grisea* genome database. Because these 2 clones were not found in the saprophyte *N. crassa* genome, they may be genes specifically involved in fungal pathogenesis. Clone P2C12 was shown to be expressed during pathogenesis of *Arabidopsis* and green cabbage (Fig. 4B) and virtual Northern blots

demonstrated that this clone is significantly upregulated in planta compared to spores germinated in water. We are in the process of using random amplification of cDNA ends (RACE) to obtain the full length cDNAs of these two clones. Further sequence information may reveal potential homologs and putative functions for these interesting genes.

In conclusion, we have successfully used SSH to identify 47 genes that are upregulated in planta during *A. brassicicola* pathogenesis. Five genes that were expressed during *Arabidopsis* infection, but not in spores germinated in water, were further examined with RT-PCR and found to be also expressed during infection of green cabbage and in vitro in a nutrient rich broth (except clone P3F2). This is the first study to report on the types of genes that are expressed during *A. brassicicola* pathogenesis. Of the 47 genes identified, 77% (35) were confirmed to be highly upregulated in planta according to virtual northern blots. All of these genes had at least one significant hit of fungal origin in the 4 databases examined indicating the success of our experimental technique to obtain only fungal genes. Thus, we feel that SSH was successful in identifying a set of 47 candidate genes that may be involved in *A. brassicicola* pathogenesis. Future studies will initially focus on the five genes shown to be expressed in planta on *Arabidopsis* and during the course of infection on green cabbage. These studies will include cloning full length cDNA sequences for these genes and using homologous recombination as a gene disruption technique.

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