

DISSERTATION

**MOLECULAR DISSECTION OF *ALTERNARIA*
BRASSICICOLA PATHOGENESIS MECHANISMS**

Submitted by

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In partial fulfillment of the requirements

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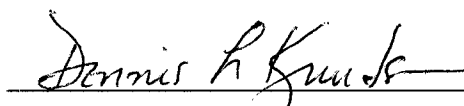
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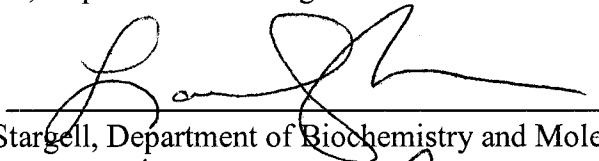
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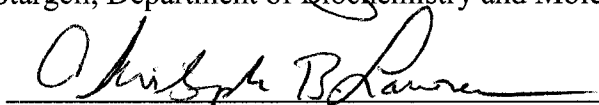
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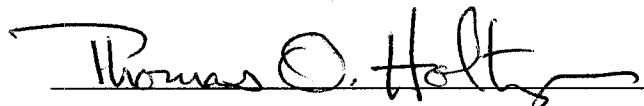
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ABSTRACT OF DISSERTATION

MOLECULAR DISSECTION OF *ALTERNARIA BRASSICICOLA* PATHOGENESIS MECHANISMS

Alternaria brassicicola is a necrotrophic fungal pathogen that causes black spot disease on cruciferous plants including economically important *Brassica* species and the model plant *Arabidopsis thaliana*. The purpose of this dissertation was to gain a better understanding of the pathogenesis mechanisms employed by *A. brassicicola* by identifying and cloning candidate pathogenicity genes. A functional genomics based approach was used to identify candidate pathogenicity genes by creating two suppression subtractive hybridization (SSH) libraries enriched for *A. brassicicola* genes during fungal pathogenesis, and one full length cDNA library of genes expressed during nitrogen starvation. The first library was created to be enriched for genes expressed during spore germination on the plant leaf surface using SSH. To create this library, RNA was 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 hours when approximately 80% of the spores had germinated either on the leaf surface or in water and used in SSH. Differentially expressed genes were further screened with RT-PCR and cDNA northern blots. Five sequences were found to be of particular interest as they were expressed on the plant leaf surface but not during spore germination in water according to cDNA northern blots. These five cDNAs were predicted to encode a cyanide hydratase, arsenic ATPase, formate dehydrogenase, major *Alternaria*

allergen, and one unknown. A second SSH library was generated from *A. brassicicola* infected green cabbage plants at 2, 4, and 6 days post-inoculation. Total RNA was pooled from these infection time points and uninfected cabbage and used to create cDNA for use in SSH. This created a library enriched for genes expressed during *A. brassicicola* pathogenesis. A total of 4224 expressed sequence tags (ESTs) were sequenced and assembled into a 3112 unisequence set using the assembly program CAP3. This unisequence set contained 608 contigs and 2504 singletons. The library had an estimated redundancy rate of 26%. BLAST algorithms were used to search publicly available databases to gain putative identities of the ESTs. BLAST searches identified 315 of the sequences as *A. brassicicola*, 1554 as cabbage, and 1243 as unknown. The unisequence set was run through the INTERPRO database and combined with the BLAST results to give Gene Ontology (GO) annotations of the ESTs. A similar analysis was conducted on a cDNA library created from *A. brassicicola* nitrogen starved mycelia. This full length cDNA library contained 1660 unisequences (253 contigs and 1407 singletons). Many ESTs from the infected cabbage and nitrogen starvation libraries had homology with known fungal pathogenicity factors. These included genes such as: ABC transporters, MAP kinases, polyketide synthases, serine proteases, and various plant cell wall degrading enzymes. One of the genes identified in all of the libraries had significant homology with the major *Alternaria* allergen *Alt a 1*. Phylogenetic analysis of *Alt a 1* homologs from other *Alternaria* species revealed that this gene is highly conserved across the genus indicating a possible important function in *Alternaria* biology. Homologous recombination was used to disrupt the cyanide hydratase gene identified in the spore germination library to assess its role in fungal pathogenesis. Cyanide hydratase mutants

were found to have increased sensitivity to varying concentrations of cyanide *in vitro*. However, cyanide hydratase mutants still caused wild-type black spot lesions on various *Brassica* species. This indicates that cyanide hydratase is likely not required for fungal pathogenesis. One fungal gene of particular interest contained in the library was a homolog of the fungal pathogenicity MAP kinase, *PMK1*. Random amplification of cDNA ends (RACE) was used to obtain a full length copy of the *Alternaria PMK1* homolog, *AMK1*. Degenerate PCR was used to clone another MAP kinase known to be involved in cell wall integrity and was named *AMK2*. Phylogenetic analysis of these 2 MAP kinases revealed that they are likely homologs of known fungal pathogenicity factors. The results of this dissertation provide the first insight into genes expressed during *A. brassicicola* infection of *Brassica* species that may be involved in fungal pathogenesis. A total of 2,002 *A. brassicicola* candidate genes were identified. Future studies will involve further characterization of important fungal genes identified in the libraries generated during the course of this research.

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takes, to be a successful primary investigator. The extra time and effort that he spent with me went a long way into helping me achieve this, and future goals.

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“This entire allegory, I said, you may now append, dear Glaucon, to the previous argument; the prison-house is the world of sight, the light of the fire is the sun, and you will not misapprehend me if you interpret the journey upwards to be the ascent of the soul into the intellectual world according to my poor belief, which, at your desire, I have expressed, whether rightly or wrongly God knows. But, whether true or false, my opinion is that in the world of knowledge the idea of good appears last of all, and is seen only with an effort; and, when seen, is also inferred to be the universal author of all things beautiful and right, parent of light and of the lord of light in this visible world, and the immediate source of reason and truth in the intellectual; and that this is the power upon which he who would act rationally either in public or private life must have his eye fixed . . . Whereas, our argument shows that the power and capacity of learning exists in the soul already; and that just as the eye was unable to turn from darkness to light without the whole body, so too the instrument of knowledge can only by the movement of the whole soul be turned from the world of becoming into that of being, and learn by degrees to endure the sight of being, and of the brightest and best of being, or in other words, of the good.”

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Chapter 1

Introduction to Fungal Plant Pathogenesis

Fungal Pathogenesis of Plants

As sessile organisms, plants are constantly exposed to a myriad of microbial invaders. To complicate matters, the inherent nature of agricultural practices leads to the development of monocultures, fields of crops with the same genotype, which sets the stage for potentially devastating disease epidemics. Because healthy plants are vital to the success of life on Earth, obtaining a greater understanding of these phytopathogenic microorganisms is important for the continued well being of mankind.

Of particular importance are diseases caused by pathogens from the Kingdom Fungi. It has been estimated that over 60% of plant diseases worldwide are caused by over 100,000 species of fungi and fungal-like organisms (Agrios 1997, Kendrick 2000). Besides their sheer abundance, the diseases caused by fungal pathogens are particularly devastating and difficult to control, and in many cases have altered the course of human history. One of the most well known examples of the significance of plant pathogens on human well being was the Irish Potato Famine caused by the fungal-like organism *Phytophthora infestans*. This devastating disease was responsible for the death of approximately 1 million people from 1845-1847, and caused the emigration of 2 million more from Ireland to the United States. Table 1.1 presents a sample of the economic losses caused by fungal diseases.

Table 1.1. Examples of economic losses caused by fungal diseases.*

Disease	Pathogen	Host	Crop Losses
Rice Blast	<i>Magnaporthe grisea</i>	Rice <i>Oryza sativa</i>	11-30% (157 million tons) 1975-1990
Rice Sheath Blight	<i>Rhizoctonia solani</i>	Rice <i>Oryza sativa</i>	10-27% in India
Wheat Rusts	<i>Puccinia</i> species	Wheat <i>Triticum aestivum</i>	100% loss in Kazakhstan 1980
Southern Corn Leaf Blight	<i>Cochliobolus heterostrophus</i>	Maize <i>Zea mays</i>	\$1 billion loss 1970 United States
Late Blight of Potato	<i>Phytophthora infestans</i>	Potato <i>Solanum tuberosum</i>	\$368.8 million loss 1986 United States

*Adapted From: Baker et al. 1997

The overall goal of this dissertation was to gain a greater understanding of the genes involved in fungal pathogenesis by the necrotrophic fungus *Alternaria brassicicola* (Schwein.) Wiltshire. *A. brassicicola* causes black spot on several economically important *Brassica* species, and there are no known sources of resistance in the cultivated germplasm (Sigareva and Earle 1999). A functional genomics based approach was used to attempt to identify genes involved in *A. brassicicola* pathogenesis. This introductory chapter provides an overview of the genes currently known to be involved in fungal pathogenesis, an overview of the biology of *Alternaria* species and specifically *A. brassicicola*, and an outline of the contents of the dissertation.

Fungal Pathogenicity

Given their economic importance and relevance to human well being, phytopathogenic fungi have been, and continue to be, the subjects of extensive research. Most fungal pathogens are found in the class Ascomycetes, while a few are also found in the Basidiomycetes. They employ diverse infection strategies including necrotrophy, biotrophy, and hemibiotrophy. Necrotrophic pathogens obtain nutrients from dead and/or dying host cells, and typically must first kill living host cells before nutrients can be obtained. This is usually accomplished through the effects of secreted toxins, which kill

host cells, and thus allows the fungus to penetrate and obtain essential nutrients to continue its life cycle. Common necrotrophic fungi are found in the genera *Botrytis*, *Sclerotinia*, and *Alternaria*. Necrotrophic fungi usually have broad host ranges, and resistance is typically mediated through the detoxification of the toxic compound produced by the fungus, or by complex actions of multiple genes.

In contrast to necrotrophs, biotrophic fungal pathogens must obtain their nutrients from living plant cells. This is usually accomplished through the use of specialized infection structures called haustoria. Haustoria mediate the uptake of nutrients from the plant cell into the fungus, but allow the plant cell to remain alive. However, if the infection is severe, the plant eventually has difficulty in producing enough nutrients for both itself and the pathogen, and succumbs to the invasion. Common biotrophs include the rusts (i.e. *Puccinia tritici*), the downy mildews (*Plasmopora viticola* and *Peronospora parasitica*), and the powdery mildews (*Erysiphe* species). Because biotrophs typically require living, intact, host cells for growth, they seldom can be cultured *in vitro* and also have very narrow host ranges. Resistance to biotrophs is usually mediated through the direct or indirect interaction of single dominant resistance genes (R genes) in the host and single dominant avirulence genes (AVR genes) in the pathogen. These interactions then lead to the induction of the hypersensitive response (HR) locally and often a systemic response called systemic acquired resistance (SAR). The HR is characterized by localized plant cell death, which inhibits the spread of the biotrophic invader.

Hemibiotrophic pathogens are unique in that they utilize both biotrophic and necrotrophic infection strategies. First, the hemibiotroph colonizes a living plant cell, keeping the cell alive long enough to acquire enough nutrients to begin its invasion of the

plant. When the pathogen is “ready,” it invades the rest of the plant killing plant cells as it progresses much like a necrotroph. One of the most well studied hemibiotrophs is the rice blast fungus *Magnaporthe grisea* and the Oomycete *Phytophthora infestans*.

A greater understanding of fungal infection strategies and mechanisms has been sought via the identification of various pathogenicity factors (genes). For the purposes of this dissertation, pathogenicity genes are defined as genes necessary for disease development, but not required for the pathogen to complete its life cycle *in vitro* (Idnurm and Howlett 2001). Though this definition does not apply to obligate pathogens which cannot be cultured *in vitro*, it is appropriate for non-obligate pathogens because a gene is termed a pathogenicity gene once its disruption shows a reduction in or complete loss of the ability to cause disease symptoms on host plants. Idnurm and Howlett (2001) recently surveyed the current literature and arbitrarily classified 79 genes found to be required for fungal pathogenesis into five categories based on their function. These categories included genes involved in infection structure development, cuticle and cell wall degradation, responses to the host environment, toxin production, signal transduction, and novel unclassified genes. It should be noted that many of the pathogenicity genes discovered to date can fit into more than one of the above categories.

Infection Structure and Signal Transduction Genes

Fungi employ diverse strategies for penetrating and invading their hosts. Some utilize wounds or natural plant openings such as stomata, while others degrade the plant cuticle and cell wall with secreted enzymes. Still others form specialized infection structures called appressoria that allow direct penetration of the plant cuticle and cell wall. Extensive analysis of appressorium formation and its importance for successful

infection has been conducted in the rice blast fungus, *Magnaporthe grisea*.

Approximately 4 hours after germination, *M. grisea* germ tubes differentiate into hemispherical infection structures called appressoria. Several genes have been identified that are required for appressorium development. A fungal hydrophobin, *MPG1*, has been proposed to mediate the contact between host surface and fungal cell wall, forming a conformational cue that triggers appressorium differentiation (Kershaw and Talbot 1998). Fungal hydrophobins are secreted into the surrounding environment and form layers, by self-assembly, between the fungal cell wall and the environment. Mutants of *MPG1* form appressoria at low frequencies and are impaired in their ability to cause disease (Beckerman and Ebbole 1996, Talbot et al. 1996). Interestingly, *mpg1* mutants can be rescued by exogenous application of cAMP, a common secondary messenger in signal transduction cascades. Thus, *MPG1* participates in the induction of a signaling pathway dependent upon cAMP. Exogenous application of cAMP can also induce appressoria formation on non-inductive, hydrophilic surfaces.

Given that cAMP is clearly involved in appressorium formation, several genes apparently involved in cAMP signal transduction pathways have been identified and shown to be required for appressorium formation. Mutations in *MAC1*, an adenylate cyclase which converts ATP to cAMP, prevent appressorium development (Choi and Dean 1997). The mutation in *MAC1* can be suppressed by exogenous cAMP application, again confirming cAMP's role as a secondary signaling molecule in appressorium formation. Cyclic AMP signaling in higher eukaryotes is typically mediated by heterotrimeric G-proteins. The first molecules involved in these signaling chains are the $G\alpha$ proteins which receive signals directly from G-protein coupled receptors. In *M.*

grisea, three Gα protein encoding genes have been cloned with one, *MAGB*, required for appressorium formation and pathogenicity (Liu and Dean 1997). Defects in *magb* mutants are similar to *mac1* mutants and are also rescued by exogenous cAMP application, suggesting that *MAGB* may activate *MAC1* resulting in elevated cAMP levels which help induce appressoria differentiation.

Mitogen activated protein (MAP) kinases (Xu and Hamer 1996, Xu et al. 1998) and cyclic AMP (cAMP) dependent kinases (Mitchell and Dean 1995) have also been shown to have important roles in fungal pathogenesis. In *M. grisea*, a MAP kinase gene, *PMK1*, was found to be essential for appressorium formation and invasive growth (Xu and Hamer 1996). *PMK1* mutants were nonpathogenic and failed to cause lesions on susceptible rice cultivars, but were not affected in their vegetative growth. In the necrotroph *Botrytis cinerea*, the gene *BMP1* was found to encode a MAP kinase homologous to *PMK1* (Zheng et al. 2000). No necrotic lesions were found on carnation and tomato plants sprayed with a *bmp1* deletion mutants. Scanning electron microscopy studies indicated that *bmp1* mutants were unable to penetrate plant cells and therefore *bmp1* mutants seemed to be defective in regulating plant cell penetration in *B. cinerea*. Other *PMK1* homologs have been shown to be important in fungal pathogenesis and include *Cochliobolus heterostrophus* (Lev et al. 1999), *Colletotrichum lagenarium* (Takano et al. 2000), *Fusarium oxysporum* (Di Pietro et al. 2001), and *Ustilago maydis* (Mayorga and Gold 1999). During the course of this dissertation, a *PMK1* homolog from *A. brassicicola* was cloned and named *AMK1* (*Alternaria* MAP Kinase 1).

Cuticle and Cell Wall Degrading Genes

Cell wall degrading (CWD) enzymes are thought to play a major role in the penetration stage of phytopathogenic fungal infection. The first barrier that a fungal spore encounters when landing on a host leaf surface is the cuticle. One of the first genes encoding a CWD to be characterized was the gene encoding the enzyme cutinase from *Fusarium solani* f.sp. *pisi* (Purdy and Kolattukudy 1975). Several fungal cutinase genes have been cloned and characterized since their initial discovery, and shown to have varying degrees of importance in pathogenesis on hosts (Sweigard et al. 1992, Yao and Koller 1994, Van Kan et al. 1997, Van der Vlugt-Bergmans et al. 1997). Cutinases are serine esterases that hydrolyze cutin into fatty acid monomers, which may serve as elicitors for the fungus to produce more cutinase and CWDs, and for the plant to initiate a defense response. Assessing the role of cutinases and other CWD enzymes in fungal pathogenesis has been problematic as many fungi possess multiple CWD enzyme isoforms and genes, and thus disruption of a single gene may or may not lead to a reduction in virulence on a particular host. To date, the role of cutinases in fungal pathogenesis is still unclear, and their importance most likely varies in each fungal-plant interaction.

The second barrier encountered by fungal pathogens is the plant cell wall which consists of a primary layer made up mostly of cellulose microfibrils embedded in a matrix of hemicelluloses and glycoproteins, and a secondary layer made up almost exclusively of cellulose, the most abundant carbon compound on Earth. Plant cells are cemented together in tissues by the middle lamella which primarily consists of pectic substances. Fungi have evolved a wide variety of enzymes that are capable of degrading the cell wall stratum. One of the most well studied are the polygalacturonases.

Endo- and exo-polygalacturonases are thought to depolymerize homogalacturan, a major component of the plant cell wall, allowing the fungus to penetrate plant cells. The oligosaccharides released by the degradation also serve as a carbon source for the invading fungus and as elicitors recognized by the plant to induce defense gene expression. The endopolygalacturonase gene *BCPG1* was found to be required for full virulence of *Botrytis cinerea* on tomato leaves (Ten Have et al. 1998). But like cutinase, endopolygalacturonases have varying degrees of importance in determining pathogenicity. For instance, an endopolygalacturonase was found to be essential for citrus black rot disease caused by *Alternaria citri*, but not essential for brown spot caused by *Alternaria alternata* (Isshiki et al. 2001). Other endopolygalacturonase genes have been cloned in important phytopathogenic fungi including *Fusarium oxysporum* (Di Pietro and Roncero 1998), *Fusarium moniliforme* (Caprari et al. 1993), *Cochliobolus carbonum* (Scott-Craig et al. 1990), *Sclerotinia sclerotiorum* (Reymond et al. 1994), and *Colletotrichum lindemuthianum* (Centis et al. 1996). However, their apparent redundancy in the genomes of fungal pathogens makes it difficult to assess their individual roles in pathogenicity.

Responses to the Host Environment

One strategy that plants employ to protect themselves from fungal invaders is to produce secondary metabolites that are toxic to fungi. These compounds have generally been classified into two classes: the phytoalexins which are produced in response to the presence of a pathogen, and the phytoanticipins which are pre-formed anti-microbial compounds (Paxton 1980, Van Etten et al. 1994). In response to these anti-fungal

compounds, pathogenic fungi have evolved various detoxification mechanisms, and in some cases, have even evolved to use these toxic compounds to their advantage.

One of the most striking and interesting examples of this interplay between fungal pathogen and plant host is found in the *Septoria lycopersici* – *Lycopersicon esculentum* (tomato) interaction. Solanaceous species such as tomato produce a family of glycosylated steroidal or steroidal alkaloid compounds that are called saponins (Roddick 1974). Saponins have anti-fungal activity, and the fungal pathogen *S. lycopersici* requires an enzyme, tomatinase, to detoxify the saponin α -tomatine (Martin-Hernandez et al. 2000). Bouarab et al. (2002) showed that not only does detoxification of the saponin assist in fungal invasion, but in fact the hydrolysis product (β -tomatine) of the reaction between tomatinase and its counterpart saponin suppresses induced defense responses by interfering with plant defense signal transduction pathways. Thus, besides detoxifying an anti-fungal compound, tomatinase also functions indirectly to suppress plant defense responses.

Another group of phytoanticipins are the cyanogenic glycosides and glucosinolates. Both compounds are not innately toxic, however, upon tissue damage, they are degraded by various enzymes (β -glucosidase and myrosinase) releasing toxic cyanide in the case of cyanogenic glycosides, and isothiocyanates, nitriles, and thiocyanates in the case of glucosinolates (Osborn 1996). Though these compounds often show anti-microbial activity, their role in plant defense against fungal pathogens is unknown. Glucosinolates, which are produced by members of *Brassicaceae*, were shown to have no affect on resistance to the necrotrophic pathogen *Leptosphaeria maculans* (Andreasson et al. 2001).

In sorghum, which produces high levels of the cyanogenic glycoside dhurrin, it was hypothesized that the sorghum fungal pathogen *Gloeocercospora sorghi* would require a cyanide detoxification system to cause disease symptoms. Wang et al. (1999) cloned and disrupted a cyanide degrading enzyme, cyanide hydratase, from *G. sorghi*, but mutants with a disrupted cyanide hydratase ORF were still capable of causing disease on sorghum. Thus, to date, the role of cyanogenic glycosides and glucosinolates in fungal-plant interactions is still unclear.

Besides direct detoxification of the anti-fungal compound, fungi have also evolved various transporter mechanisms to deal with potentially toxic metabolites produced by the plant. The role of membrane transporters in microbial pathogenesis has long been recognized in bacterial pathogens. Transporters in plant pathogens can not only secrete potential pathogenicity factors, but may also be involved in excreting toxic compounds produced by the plant's defense system, thereby protecting the fungus from potential toxic compounds (Del Sorbo et al. 2000). In the last few years, two groups of transporters have received particular attention in phytopathogenic fungi. The first, ATP binding cassette (ABC) transporters, have been shown to be important in protecting the invading fungus from plant secreted toxins. The gene *ABC1* from *M. grisea* encodes an ABC transporter that has been shown to be essential for pathogenicity. *ABC1* mutants penetrate epidermal cells with the same frequency as the wild type, but have increased sensitivity to a plant produced compound as evidenced by their subsequent death upon penetration (Urban et al. 1999). Other ABC transporter encoding genes have been identified in several phytopathogenic fungi including *Mycosphaerella graminicola* (Zwiers and De Waard 2000), *Leptosphaeria maculans* (Taylor and Condie 1999), and

Penicillium digitatum (Nakaune et al. 1998). However, to date, the specific compounds that these transporters are involved in transporting out of the fungal cell has yet to be directly determined. Vermeulen et al. (2001) demonstrated that the ABC transporter from the necrotroph *Botrytis cinerea*, *BCATRB*, appears to mediate responses to phenylpyrrole fungicides fludioxonil and fenpiclonil. *BCATRB* mutants displayed increased sensitivity to the fungicides, and mutants over expressing *BCATRB* displayed decreased sensitivity. Thus, it appears that fungal ABC transporters can and do eliminate toxic compounds from the fungal cell.

Genes Involved in Toxin Production

While ABC transporters seem to be involved in protecting the fungus from anti-fungal compounds, major facilitator superfamily (MFS) transporters appear to be involved in secreting host-specific toxins (HSTs) produced by the fungus itself. A MFS transporter gene, *TOXA*, has been identified in *Cochliobolus carbonum* that is involved in secretion of HC toxin (Pitkin et al. 1996). HC toxin acts as a histone deacetylase inhibitor, and is required for pathogenicity of *C. carbonum*. In *Cercospora kikuchii*, a MFS transporter secretes the toxin cercosporin, and has been shown to be important for pathogenicity on soybean (Callahan et al. 1999). Future studies are certain to identify more transporters of fungal toxins.

One interesting aspect of toxin production in pathogenic fungi is that genes required for toxin biosynthesis are often clustered (Walton 1996). The most well characterized example of toxin production is HC toxin from *Cochliobolus carbonum*, a fungal pathogen of maize. HC toxin biosynthesis is controlled by a single Mendelian locus called *TOX2*. *TOX2* is large gene cluster containing six known genes present in

multiple copies. These genes are lacking in non HC toxin producing strains of *C. carbonum* (Ahn, J.-H. et al. 2002). Other known toxins which appear to be synthesized from gene clusters include AK toxin from *Alternaria alternata* f.sp. *kikuchiana*, AM toxin from *Alternaria alternata* f.sp. *mali* and AAL toxin from *A. alternata* f.sp. *lycopersici* (Akamatsu et al. 1997, Johnson et al. 2000, Tanaka et al. 1999).

The fact that genes for toxin biosynthesis are clustered has led to the hypothesis that horizontal gene transfer is the source of these genes in fungal genomes (Walton 2000). Though direct evidence for this hypothesis has not been found, indirect evidence strongly suggests horizontal gene transfer as the source of toxin biosynthesis clusters in fungal pathogens. For instance, single pathotype populations of *A. alternata* do not form monophyletic groups, and no homologs of toxin biosynthesis genes have been found in strains from other *A. alternata* pathotypes or non-pathogenic isolates (Kusaba and Tsuge 1994, 1995, 1997). This significantly reduces the chance that these genes arose from mutation events such as internal rearrangements of chromosomes (Hatta et al. 2002). Indeed, Hatta et al. (2002) found that the AF toxin biosynthesis genes were clustered on a dispensable 1.05 Mb chromosome. AF toxin minus mutants, which are non-pathogenic, all lack this 1.05 Mb chromosome. It has been postulated that small dispensable chromosomes, also called supernumerary chromosomes, may have been introduced into fungal genomes by horizontal gene transfer from another species that contained the toxin gene clusters (Covert 1998).

Regardless of the pathogenicity strategy employed by pathogenic fungi, the diversity of genes shown to have potential roles in determining the pathogenic capabilities of a given fungus on its host underscores the complexity of fungal-plant

interactions. Though many of these genes show homology between diverse fungal pathogens, it seems clear that the outcome of each pathogen-host interaction is governed by many unique genes interacting in numerous ways. There exists a clear need to not only identify and clone these pathogenesis-related genes, but also to understand their function both in an autonomous and whole organism sense. Full genome sequencing of economically important fungal pathogens has been undertaken to help gain a better understanding of the evolutionary history of fungal pathogenesis. As of the writing of this dissertation, full or extensive draft genome sequences (publicly available) of 3 plant pathogenic fungi has been completed, *Magnaporthe grisea*, *Fusarium graminearum*, and *Ustilago maydis*. Full genome sequences of related non-plant pathogenic fungal species are also available and include *Neurospora crassa*, *Aspergillus nidulans*, and *Aspergillus fumigatus*. Comparative genomics studies are already underway to compare the genomes of these fungi which employ different life strategies and should yield a greater understanding of fungal pathogenesis.

***Alternaria* Diseases**

The genus *Alternaria* was established in 1817 by Nees with *A. alternata* (formerly known as *A. tenuis*) as the type isolate (Rotem 1994, Thomma 2003). *Alternaria* is part of the family Dematiaceae, order Hyphomycetes. Teleomorphs (sexual stage) of most *Alternaria* species have not been identified; however a few are anamorphs (asexual stage) of the genus *Lewia* (Simmons 1986). The important taxonomic feature that separates *Alternaria* species from other closely related fungi is the production of large, multicellular, melanized, rostrate, obclavate conidia with longitudinal and transverse

septa (Ellis 1971). *Alternaria* conidia are catenate (formed in chains) on conidiophores, and arise as protrusions from the protoplast through pores in the conidiophore cell wall.

Plant diseases caused by *Alternaria* species are typically some of the most common, affecting virtually all known economically important plants including cereals, vegetables, and fruits (Agrios 1997). All parts of the plant including stems, leaves, flowers, and fruit can be affected. *Alternaria* diseases typically appear as blights and leaf spots, but can also cause stem, tuber, and fruit rots, as well as damping-off of seedlings. Though plant pathogenic *Alternaria* species are typically necrotrophic, most species of *Alternaria* are saprophytic and opportunistic invaders (Rotem 1994).

Phytopathogenic species of *Alternaria* typically over-winter as mycelium or spores in infected plant debris. Seeds can also be infected with *Alternaria* species, and can serve as a primary source of inoculum for the following year (Agrios 1997). Germinating conidia form germ tubes which can penetrate the plant directly through the use of appressoria and cell wall degrading enzymes, or indirectly through wounds and natural plant openings such as stomata. Whether or not appressoria are formed seems to be dependent upon the particular species of *Alternaria*, and the location on the host of the penetration event (Rotem 1994). For example, the upper leaf surface of apple has relatively few stomata and *A. alternata* f.sp. *mali* (AM) penetrates the surface directly through the epidermis. However, when AM is found on the lower, stomata rich leaf surface, penetration mainly occurs indirectly through stomata (Sawamura and Yanase 1964). Following penetration, invasion of plant tissues occurs with disease symptoms typically developing 48-72 hours following infection depending on the particular interaction. New conidia are produced from infection sites and can serve as inoculum for

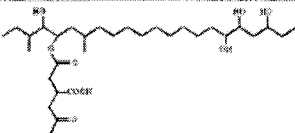
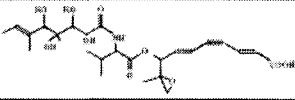
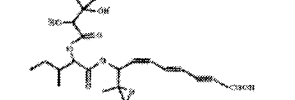
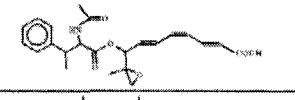
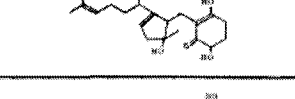
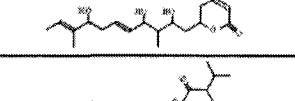
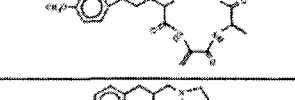
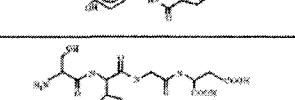
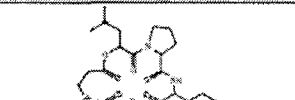
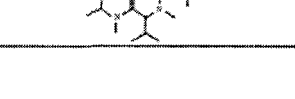
further infections during the growing season. Control of *Alternaria* diseases varies with each disease, but typically involves the use of resistant crops, disease free seed, and chemical sprays with fungicides including chlorothalonil, captafol, and mancozeb (Agrios 1997).

One of the distinguishing characteristics of *Alternaria* (and other necrotrophic fungi) diseases is the development of an un-invaded chlorotic halo surrounding the primary lesion site. This halo is thought to develop from the secretion of fungal metabolites by the invading pathogen (Tewari 1983). Specifically, *Alternaria* species are notorious for the production of host and non-host specific toxins, which are most likely the cause of the chlorotic region during infection (Thomma 2003). Phytotoxins produced by *Alternaria* species are typically low molecular weight secondary metabolites that are not required for vegetative growth or reproduction. Non-host specific toxins produced by *Alternaria* species are typically mildly toxic against a broad range of plant species, and though they contribute to disease development, are usually not required by the pathogen for pathogenesis (Ballio 1991, Thomma 2003). One of the most common non-host specific toxins produced by *Alternaria* species is tentoxin, a cyclic tetrapeptide that blocks the development of chloroplasts by binding the chloroplast coupling factor which is responsible for the synthesis of ATP from ADP and phosphate (Steele et al. 1978). Other non-host specific toxins produced by *Alternaria* species include alternaric acid, alternariol, tenuazonic acid, radiclinil, and zinniol (Rotem 1994). In contrast to non-host specific toxins, host-specific toxins (HSTs) cause extensive effects on their hosts and are absolutely required for disease development (Markham and Hille 2001). It has been hypothesized that by secreting HSTs and killing living plant cells, fungi are able to avoid

the innate defense responses of their hosts. Among *Alternaria* species, at least 12 are known to produce HSTs (Table 1.2) (Thomma, 2003).

Most of the HSTs produced by *Alternaria* species come from variants of *A. alternata*. Molecular analysis of these HST producing *Alternaria* species (rDNA and mtDNA analyses) supports the hypothesis that these variants are pathotypes of *A. alternata* (Kusaba and Tsuge, 1994, 1995, 1997). Because *A. alternata* is typically a

Table 1.2. Host-specific toxins produced by *Alternaria* species. Chemical structures of AT-toxin (*A. alternata*), ATC-toxin (*A. tenuissima*), and AB-toxin (*A. brassicicola*) are unknown and not listed. After Thomma (2003).

<i>Alternaria alternata</i> Esp. <i>lycopersici</i> (tomato pathotype)	AAL-toxin (Bottini and Gilchrist, 1981)	
<i>Alternaria alternata</i> Esp. <i>citri tangerine</i> (tangerine pathotype)	ACT-toxin (Kohmoto et al., 1993)	
<i>Alternaria alternata</i> Esp. <i>fragariae</i> (strawberry pathotype)	AF-toxin (Nakatsuka et al., 1986)	
<i>Alternaria alternata</i> Esp. <i>kikuchiana</i> (Japanese pear pathotype)	AK-toxin (Nakashima et al., 1985)	
<i>Alternaria alternata</i> Esp. <i>citri tangerine</i> (tangerine pathotype)	ACTG-toxin (Kohmoto et al., 1993)	
<i>Alternaria alternata</i> Esp. <i>citri jambhiri</i> (rough lemon pathotype)	ACR-toxin (Gardner et al., 1985)	
<i>Alternaria alternata</i> Esp. <i>mali</i> (apple pathotype)	AM-toxin (Okuno et al., 1974)	
<i>Alternaria alternata</i> (spotted knapweed pathogen)	Maculevin (Stierle et al., 1988)	
<i>Alternaria alternata</i> (sunflower pathogen)	AS-toxin (Liakopoulou-Kyriakides et al., 1997)	
<i>Alternaria brassicae</i>	Destruxin B (Bains and Tewari, 1987)	

saprophyte and opportunistic pathogen, this situation presents a unique opportunity to study the evolution of fungal pathogenesis. As discussed above in the section on toxin biosynthesis, toxin biosynthetic genes are clustered and often appear on small (<2.0 Mb), dispensable chromosomes. This has led to the hypothesis that the non-pathogenic saprophytic *A. alternata* acquired the ability to produce various HSTs from horizontal gene transfer. No direct evidence currently exists to support or refute this hypothesis.

Alternaria brassicicola

A. brassicicola is a necrotrophic fungus that causes black spot disease (also called dark leaf spot) on numerous economically important *Brassica* species (Braverman 1979, Neergaard 1945). Uncultivated weedy species are also susceptible and include the model plant *Arabidopsis thaliana*. Under appropriate conditions, *A. brassicicola* can cause a devastating disease resulting in 20-50% yield reductions (Daebeler et al. 1986). Like other diseases caused by *Alternaria* species, black spot appears on the leaves as necrotic lesions, which are often described as black and sooty with chlorotic yellow halos surrounding the lesion sites (Agrios 1997, Verma and Saharan 1994). *A. brassicicola* is not limited to infection of leaves, and can infect all parts of the plant including pods, seeds, and stems, and is of particular importance as a post-harvest disease (Rimmer and Buchwaldt 1995). The necrotrophic nature of *A. brassicicola* typically leads to extensive damage of the plant and harvest product, with seedlings seldom surviving an attack (Humpherson-Jones 1985, Neergaard 1945).

A. brassicicola is typically seed transmitted, though transmission by insects has also been reported (Dillard et al. 1998, Rotem 1994). Between 1976 and 1978 in the United Kingdom, 86% of the commercial *Brassica* seed produced was contaminated with

A. brassicicola (Maude and Humpherson-Jones 1980). Spread of the disease during the growing season is typically by rain and wind dislodged spores. Optimal conditions for sporulation and infection include a minimum wet period of 13 hours and ambient temperatures of 20-30°C (Humpherson-Jones and Phelps 1989, Rotem 1994). Consequently, black spot disease has been of particular importance in regions of the world with cool, wet weather during the growing season such as in the United Kingdom, Thailand, and the northeastern United States (King 1994, Pattanamahakul and Strange 1999).

Molecular karyotype analysis of *A. brassicicola* strain O-263 revealed the presence of 9 chromosomes and an approximate genome size of 29.6 Mb. The smallest chromosome was 1.7 Mb in size, and the largest 5.7 Mb (Akamatsu et al. 1999). Genetic variation within and between 18 isolates of *A. brassicicola* was conducted using amplified fragment length polymorphisms (AFLPs). Significant variation was seen among the 18 isolates suggesting that despite its asexual life style, *A. brassicicola* has a means for generating and maintaining genetic variation (Bock et al. 2002). The AFLPs were able to distinguish the 18 *A. brassicicola* isolates from 5 *A. alternata* isolates.

***Alternaria brassicicola* pathogenesis**

Studies on the pathogenesis mechanisms employed by *A. brassicicola* are limited. Like most *Alternaria* species, *A. brassicicola* employs a necrotrophic strategy when infecting its hosts, and analysis of culture filtrates of *A. brassicicola* has yielded a few toxic secondary metabolites. Suri and Mandahar (1984) isolated a phytotoxic fraction from culture filtrate that contained cytokinins. The authors attributed the toxicity of the filtrate to the cytokinin activity. Cooke et al. (1997) tested the toxicity of spore

germination fluid (SGF) from *A. brassicicola* and found that SGF from *A. brassicicola* produced more necrosis than SGF from the related *A. brassicae* on various *Brassica* species. Cooke et al. (1997) also noted that some non-*Brassica* species (*Nasturtium officinale* and *Iberis amara*) also displayed sensitivity to SGF from *A. brassicicola*. Cooke et al. (1997) concluded that the toxic factors produced by *A. brassicicola* were not host-specific, but host-selective (active against a broad spectrum of *Brassica* species not only against specific cultivars of *Brassica* species). An analysis of the toxic factors in the SGF was not conducted.

In another study, six fusicoccane-like diterpenoid compounds, named brassicicene A to F were isolated from culture filtrates of *A. brassicicola* (MacKinnon et al. 1999). Fusicoccane-like diterpenoids had not been previously reported to be found in the fungal genus *Alternaria*. Culture filtrates exhibited toxic activity against detached leaves of canola, but due to the small amount of Brassicicene A to F isolated, individual Brassicicenes were not tested for toxic activity, and it is unknown if these compounds are the toxic factors identified by Cooke et al. (1997) in their SGFs.

Given the results of Cooke et al. (1997), and the non-specific nature of *A. brassicicola* pathogenesis, one would not expect a host-specific toxin to be involved in the infection process. However, Otani et al. (1998) have reported the isolation of a 35 kDa host-specific toxic peptide from *A. brassicicola* SGF. SGF from these experiments did not produce any necrosis or disease symptoms on non-hosts as Cooke et al. (1997) had previously reported. However, different non-host species were used in the two studies. To date, the gene encoding the putative AB toxin has not been cloned, which brings into question the true nature of “AB toxin.”

One interesting metabolite produced by *A. brassicicola* is the histone deacetylase (HDAC) inhibitor, depudecin (Kwon et al. 1998). HDAC inhibitors are known to cause changes in gene expression, induction of apoptosis, developmental arrest, and detransformation of oncogene-transformed cells (Baidyaroy et al. 2002). HDAC inhibitors are also being explored as potential anti-cancer agents (Jung 2001). Interestingly, another HDAC inhibitor, HC-toxin, is produced by the phytopathogenic fungus *Cochliobolus carbonum* (Baidyaroy et al. 2002). HC-toxin is a cyclic tetrapeptide and is the major determinant of virulence and host specificity in the interaction between *C. carbonum* and its host, maize. This suggests that HDAC inhibitors may have a critical role in determining virulence and host specificity in plant-fungal interactions. To date, the role of depudecin in the *A. brassicicola*-*Brassica* interaction has not been examined, and no genes involved in the synthesis of depudecin have been cloned. However, given the importance of cell death in necrotrophic fungal pathogenesis, one can speculate that depudecin may be involved in stimulating apoptosis (and thus cell death) in the host.

Besides toxic components in SGFs from *A. brassicicola*, little is known about other genes employed during pathogenesis. In fact, no pathogenicity gene has been cloned, to date, from *A. brassicicola*. An examination of the role of cutinase genes in *A. brassicicola* pathogenesis was conducted by Yao and Koller (1994, 1995). Biolistic transformation resulted in disruption of the *CUTAB1* gene, and affected saprophytic growth (cutin was no longer able to be utilized as a sole carbon source). However, disruption of *CUTAB1* had no significant affect on *A. brassicicola* pathogenicity (Yao and Koller 1995). Fan and Koller (1998) found that different cutinases were sequentially induced upon landing on and penetration of cabbage leaves. After penetration of the

subcuticle layers, cutinases that were active during saprophytic growth were induced. This may suggest that there exists a specific point in time when *A. brassicicola* switches from parasitic to saprophytic growth (Thomma 2003).

Berto et al. (1997) found an extracellular lipase produced by *A. brassicicola* *in vitro*. The molecular weight of the purified lipase was determined to be 80 kDa by SDS-PAGE and estimated to be 85 kDa by gel filtration suggesting that the enzyme is a monomer. Anti-lipase antibodies were found to significantly decrease *A. brassicicola*'s ability to cause disease on cauliflower leaves. However, if the cauliflower leaves were dewaxed prior to infection with *A. brassicicola* in the presence of anti-lipase antibodies, disease progression proceeded as normal (Berto et al. 1999). This suggests that lipases may have a role early in *A. brassicicola* pathogenesis, perhaps assisting with penetration of the cuticle which is known to be composed of fatty acid monomers. To date, the gene encoding this 85 kDa lipase has not been cloned.

Plant Defense Responses to *Alternaria brassicicola*

Despite the limited understanding of *A. brassicicola* pathogenesis mechanisms, a substantial 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 *Arabidopsis* ecotypes, providing a useful situation to study resistance mechanisms (Otani et al. 2001, Kagan and Hammerschmidt 2002). Further, the enormous genomic resources that are available for *Arabidopsis* make it an ideal candidate to identify which signaling pathways are important for resistance to necrotrophic fungal pathogens such as *A. brassicicola*. For example, Peninckx et al. (1996) were able to show 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 were 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. These studies suggest that jasmonic acid (JA) signaling is required for resistance to *A. brassicicola*. Thomma et al. (1998) also found that *NahG* plants (unable to detect the presence of salicylic acid) remain resistant to *A. brassicicola* indicating that salicylic acid is not required for resistance. Camalexin, a major phytoalexin of *Arabidopsis*, also appears to have a major role in resistance, with different *Arabidopsis* ecotypes having varying levels of this important phytoalexin (Kagan and Hammerschmidt 2002). However, it was not clear from the Kagan and Hammerschmidt (2002) study what role these varying camalexin levels had on resistance to *A. brassicicola*.

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 Columbia-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 during *A. brassicicola* infection finding 35 genes with altered gene expression. Van Wees et al. (2003) have identified 645 genes induced by *A. brassicicola* infection in wild type Columbia-0 and *pad3* plants indicating that *pad3* does not have a

major effect on defense signaling. However, Narusaka et al. (2003) reported that the timing of defense related gene expression was altered in *pad3* plants subjected to *A. brassicicola* infection, suggesting a role for *PAD3* and camalexin in the defense response. Interestingly, 265 of the 645 *A. brassicicola* induced genes identified by Van Wees et al. (2003) required *COII* for full expression, perhaps confirming the role of JA signaling in resistance to *A. brassicicola*. 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.

Summary of Dissertation

Based on current knowledge in plant-microbe interactions, it is clear that genes, and the proteins and secondary metabolites that they ultimately produce, are the determinants of the interaction's outcome. Figure 1.1 demonstrates a visual model of how a pathogen and plant interact and the potential outcomes of the interaction. In this model, the pathogen has pathogenicity genes which may or may not allow infection of its host. From the host's perspective, defense genes are present which produce proteins that recognize and attempt to thwart the invading pathogen. Thus, the outcome of each plant-microbe interaction is determined by the genes employed by both the pathogen and the host. In Figure 1.1., the potential outcomes listed are disease, when the pathogen successfully overcomes the host defense response, or resistance when the pathogen is unable to breach the host's defense system. In reality, this all or nothing situation does not always exist. A gradient of potential outcomes from disease to resistance can exist

depending upon the unique genotypes of the pathogen and host involved in the interaction. In order to gain a better understanding of plant-microbe interactions, it is important to identify these genetic factors in both the pathogen and the host.

As figure 1.2 demonstrates, several strategies and techniques are available to identify genes involved in plant-microbe interactions. Before the advent of molecular biology techniques, classical genetics was often used to identify genes involved in microbial pathogenesis or plant defense responses. This required natural variation in the pathogen or host populations, and was successful in identifying many genes important in plant-microbe interactions. With the advent of insertional mutagenesis techniques, such as restriction enzyme mediated integration (REMI), forward genetic approaches became very powerful in identifying pathogenicity factors in important fungal pathogens, as non-pathogenic variants could be created by the insertion of foreign DNA into coding regions of important pathogenicity genes. By sequencing the flanking region around the insertion event, the disrupted gene could be identified and further studied to confirm its role in pathogenesis.

Though forward genetic approaches have been very successful in identifying pathogenicity genes in many plant-microbe interactions, there are several potential drawbacks with this approach. First, many phytopathogenic fungi do not have known sexual stages (teleomorphs) which makes classical genetic approaches impossible to conduct. Second, insertional mutagenesis techniques such as REMI are highly dependent on the ability to efficiently transform the pathogen in question (an often difficult proposition for phytopathogenic fungi), and even when insertional mutagenesis can be accomplished, a high frequency of untagged mutants is often obtained. To overcome

some of these limitations, reverse genetic approaches have been used to identify fungal pathogenicity genes.

Reverse genetic approaches start by identifying candidate gene sequences. Several strategies exist that can be used to identify a candidate gene sequence (Figure 1.2). These include biochemical approaches such as protein purification and amino acid sequencing, functional genomics approaches based on the transcription of genes in a given environmental condition (such as infection of a plant), and genomic approaches including full genome sequencing of the pathogen in question. Once sequence information is obtained, bioinformatic approaches are used to obtain a preliminary identity for the candidate gene sequence in question. These approaches include using the BLAST algorithms available from the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>), and protein motif databases such as the Protein Families Database of Alignments and HMMs (www.sanger.ac.uk/Software/Pfam/index.shtml). For an excellent review of these and other available bioinformatic tools for DNA and protein sequence analysis, see the review article by Rehm (2001). Finally, candidate genes chosen based on their expression profiles or sequence homology to genes of interest in other organisms, are functionally analyzed by creating mutants with disrupted or knocked out copies of the gene in question.

In order to identify candidate pathogenicity factors in *A. brassicicola*, a reverse genetics functional genomics approach was used. This approach was chosen due to the asexual lifestyle of *A. brassicicola*, and low efficiency obtained with insertional mutagenesis techniques that made a forward genetics approach impractical. The primary functional genomics based technique utilized in this dissertation to identify candidate *A.*

brassicicola pathogenesis genes was suppression subtractive hybridization (SSH). SSH was developed by Diatchenko et al. (1996) to overcome some of the difficulties in previous subtractive hybridization techniques. Subtractive hybridization techniques, such as SSH, allow the selective cloning of genes differentially expressed in two different environmental conditions. Genes that are expressed in common between the two gene populations are “subtracted” out of the final cDNA library leaving cDNA sequences that are only expressed in the environmental condition of interest. An outline of a typical SSH experiment is depicted in Figure 1.3. In this experiment, a cDNA library is created that is enriched for genes expressed by the fungus growing *in planta* (i.e. during infection). Genes expressed during plant infection by the fungus are candidate pathogenicity genes.

The following is a brief summary of the chapters contained in this dissertation. Chapter 2 details the creation of a SSH library, which contained 47 *A. brassicicola* genes upregulated during the first 24 hours of *Arabidopsis* infection. Reverse transcriptase polymerase chain reactions (RT-PCR) were used to assess expression levels during fungal pathogenesis of 5 genes including a cyanide hydratase, an ABC transporter, a formate dehydrogenase, a major *Alternaria* allergen *Alt a 1*, and an unknown clone P3F2. Of these genes, clone P3F2 was solely expressed during fungal pathogenesis, and not in nutrient rich *in vitro* conditions suggesting that P3F2 is important in fungal pathogenesis.

Chapter 3 details the creation of an SSH library enriched for genes expressed by *A. brassicicola* and the host *Brassica oleraceae* var. *capitata* (green cabbage) during fungal infection. This library contained 3112 unique expressed sequence tags (ESTs) from both *A. brassicicola* and cabbage. This library not only gave an insight into the genes expressed by *A. brassicicola* during fungal pathogenesis, but also revealed many of

the genes involved in the host's defense response. Using various bioinformatic analyses including BLAST algorithms and a gene ontology database, 315 of the 3112 sequences were able to be assigned as *A. brassicicola* sequences. Many of these genes had known homology to genes known to be involved in fungal pathogenesis and are excellent candidate pathogenicity genes. Because the majority of genes in the mixed *A. brassicicola* - cabbage SSH library were of plant origin, a full length cDNA nitrogen starvation library of *A. brassicicola* genes was also created, which contained 1660 sequences. These 1660 sequences, which by definition were all from *A. brassicicola*, were also useful in assigning genes in the mixed library as fungal genes. Combined with the SSH library created in Chapter 2, a total of 2,002 *A. brassicicola* genes were identified during the course of this dissertation.

Following the identification of these 2,002 candidate pathogenicity genes, a closer examination was taken of the gene with homology to the major *Alternaria* allergen *Alt a 1* in Chapter 4, the gene with homology to a *Leptosphaeria maculans* cyanide hydratase in Chapter 5, and the genes with homology to 2 known fungal MAP kinases involved in fungal pathogenesis in Chapter 6. Functional analysis of the cyanide hydratase in fungal pathogenesis was made possible by the generation of an *A. brassicicola* mutant with a disrupted cyanide hydratase coding sequence. Mutants with a disrupted cyanide hydratase gene did not display any clear defects in their ability to cause disease symptoms on green and red cabbage. However, mutants were significantly more susceptible to the *in vitro* presence of KCN, and KCN was able to be utilized as a sole nitrogen source by wild type *A. brassicicola*. Because *Brassica* species are not known to be cyanogenic plants, it is

unlikely that a single cyanide hydratase enzyme would be required for *A. brassicicola* pathogenesis.

Creation of mutants in the *Alt a 1* and 2 MAP kinase homologs was unsuccessful. However, phylogenetic analysis of the *Alt a 1* homologs from other *Alternaria* species strongly suggested a role in primary fungal metabolism for *Alt a 1*. Though *Alt a 1* was highly expressed during spore germination in nutrient rich environments, it was also expressed during germination and growth *in planta*, which may suggest a role in pathogenesis for the *A. brassicicola* *Alt a 1* homolog. Further, the *Alt a 1* homolog from *A. brassicicola* was suggested to have a putative glycosylation site absent in *Alt a 1* from the non-pathogenic fungus *A. alternata*. In conclusion, the results presented in this dissertation are the first reported extensive analysis of candidate pathogenicity genes in the necrotrophic fungus *A. brassicicola*, and lay the foundation for a more extensive dissection of necrotrophic fungal pathogenesis.

Figure 1.1. Plant-pathogen interactions, a model. Pathogens and their hosts interact through complex signaling pathways that are mediated by products of pathogenicity genes in the pathogen, and plant defense genes in the host. This model depicts an all or nothing result (i.e. disease or resistance), however, a gradient of outcomes is theoretically possible.

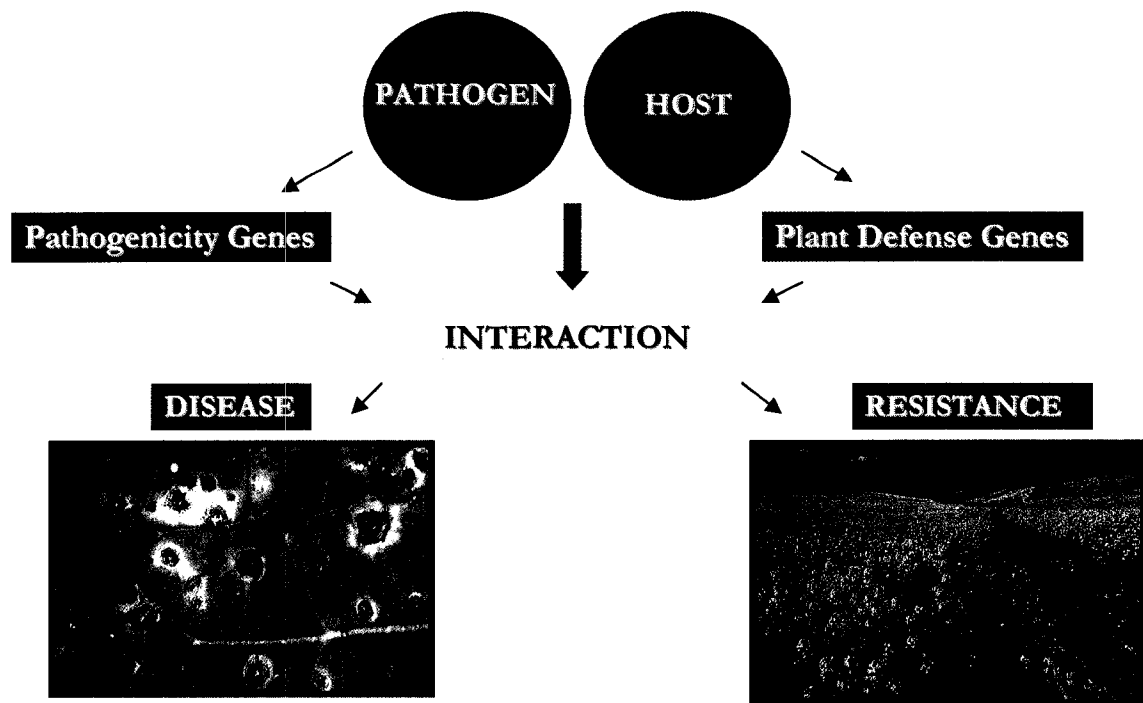
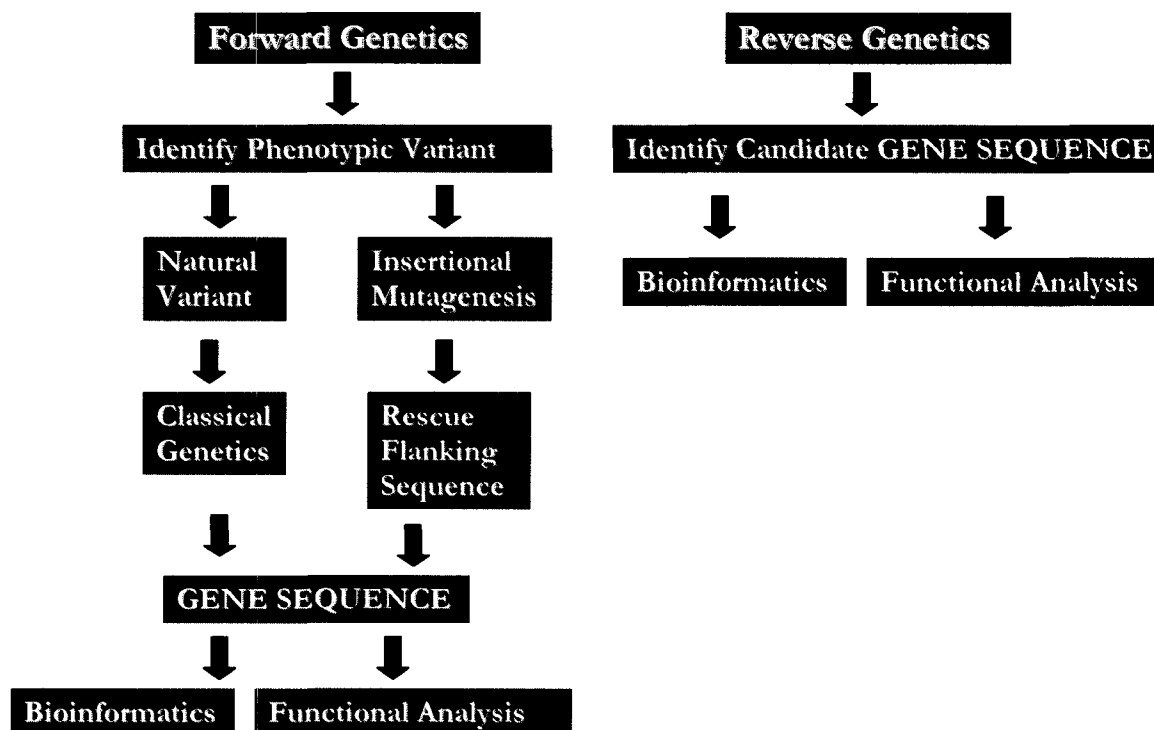


Figure 1.2. Strategies for identifying genetic factors in plant-microbe interactions. A. Comparison of forward and reverse genetic approaches. B. Comparison of different techniques for the identification of candidate gene sequences.

A.



B.

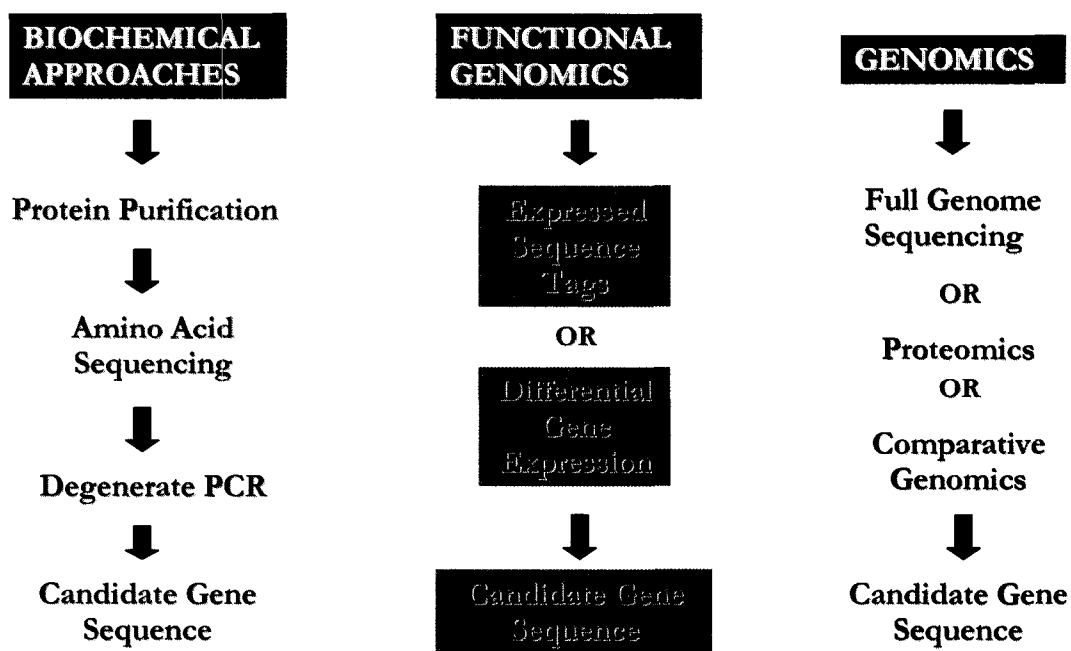
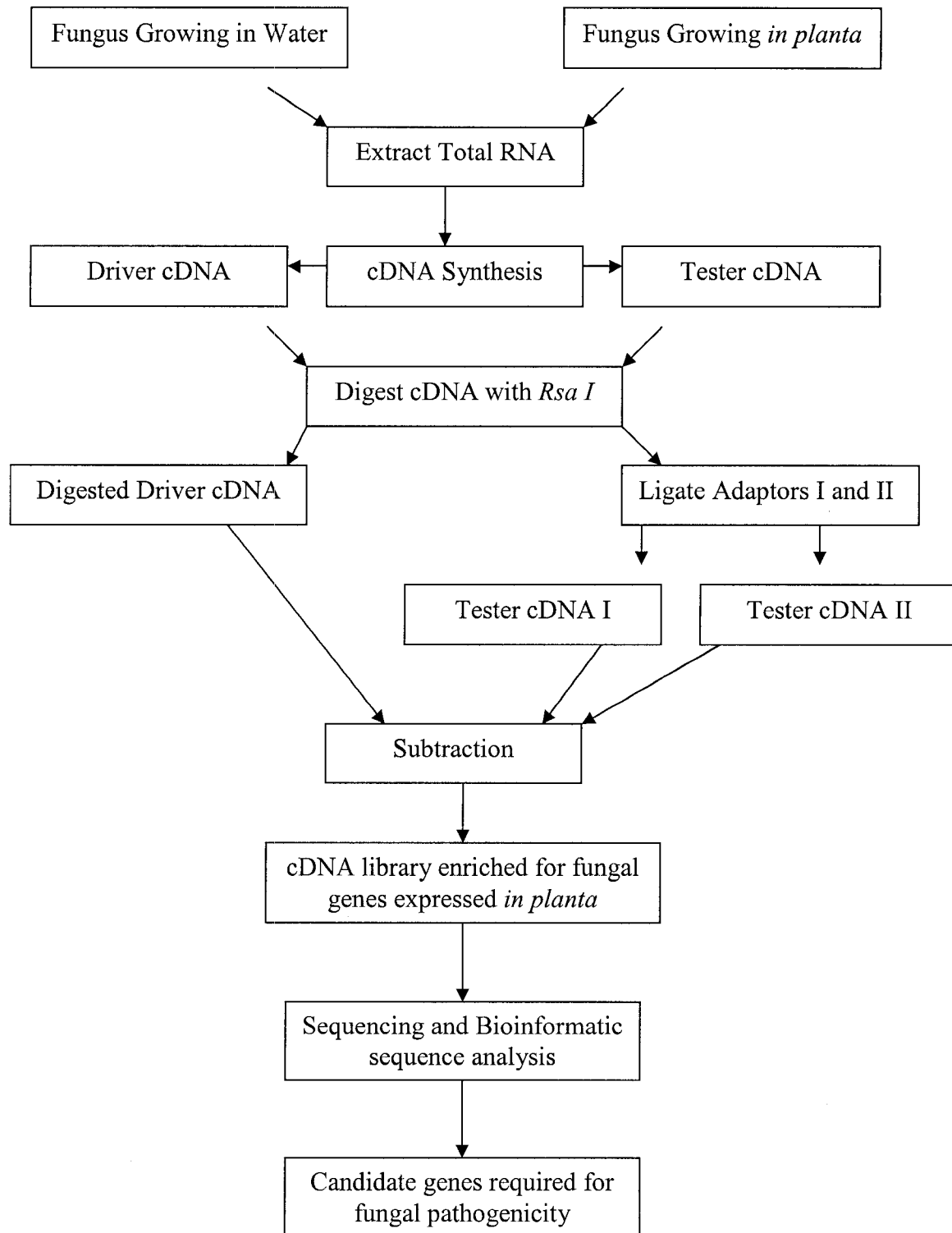


Figure 1.3. Outline of a suppression subtractive hybridization experiment to identify candidate *A. brassicicola* pathogenicity genes.



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Chapter 2

Identification of *Alternaria brassicicola* Genes Expressed *in planta* During Pathogenesis of *Arabidopsis thaliana*

Introduction

Alternaria brassicicola (Schwein.) Wiltshire 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 (Sigareva 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 *Arabidopsis* ecotypes providing a useful situation to study resistance mechanisms (Otani et al. 2001, Kagan and Hammerschmidt 2002). Penninckx 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 which include, *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. 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 Columbia-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 tissue finding 35 genes with altered gene expression. Van Wees et al. (2003) identified 645 induced genes by *A. brassicicola* in wild type Columbia-0 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 *COII* 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, little is known about the pathogen side of this interaction. In this study, suppression subtractive hybridization (SSH) was used to identify fungal genes expressed *in planta* during the first 24 hours 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 representational 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 normalizes mRNA levels between the two populations being compared. 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). Thus,

SSH was chosen to identify candidate pathogenicity genes in *A. brassicicola* given SSH's previous successes in gene discovery.

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 candidate pathogenicity genes in *A. brassicicola*.

During the course of this study, we have identified 5 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.

Materials and Methods

Experimental setup, RNA isolation, and cDNA synthesis

Spores from *Alternaria brassicicola* strain ATCC 34622 were placed either in sterile water held in a Petri-plate, or in 20 µl 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 hours, 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 $8,000 \times g$ for 5 minutes. 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).

The SMART cDNA synthesis system (BD Biosciences-Clontech, Palo Alto, CA) was used for creation of the cDNA populations due to the low amount of total RNA isolated from the germinating fungal spores. The SMART technique 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.

Suppression subtractive hybridization

Suppression subtractive hybridization (SSH) 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 units of *RsaI*. The digestion reaction was passed 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 units 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 minutes. The first hybridization was performed at 68°C for 8 hours. 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 double strand cDNAs with different adaptor sequences on each end are exponentially amplified. The conditions for the first PCR were as follows, 5 minutes at 72°C, 25 seconds at 94°C, and 27 cycles of 10 seconds at 94°C, 30 seconds at 66°C, and 1.5 minutes 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 seconds at 94°C, 30 seconds at 68°C, and 1.5 minutes at 72°C. All PCR reactions 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 *E. 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.

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 hours 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 seconds, then 23 cycles of 95°C for 10 seconds and 68° C for 3 minutes. PCR products were combined with an equal volume of 0.6 N NaOH in a 96 well plate. Two µls of this mixture, for each clone, were blotted onto a nylon membrane using a vacuum blotter (Bio-Rad, 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, 0.5% SDS) at 68° C and then a high stringency solution (0.2× SSC, 0.5% SDS) at 68° C for 30 minutes each. Membranes were then exposed to X-ray film overnight at -70° C.

cDNA Northern analysis

cDNA Northern blots (also called cDNA Southern or virtual Northern blots) 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'GCGGTAATTCCAGCTCCAATAGC-3' and 5'GATGACACGCGCTTACTAGGC-3'. Nylon membranes were then hybridized overnight 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 cDNA Northern blots were repeated a minimum of 2 times.

Clone sequencing and analysis

Selected individual cDNA clones were purified with the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). 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 characterize 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 manually 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 websites using a cutoff E-value of $1e^{-3}$ and the BLOSUM62 scoring matrix.

RT-PCR analysis

RT-PCR analysis was conducted using the Thermoscript 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 hour light/dark cycle. Leaves from 3 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 the manufacturer's protocol. One µl of first strand cDNA was used in 25 µl PCR reactions containing a 2× master mix from Promega, (Madison, WI). Primers used in the reactions are presented in Table 2.1.

Table 2.1. RT-PCR primers.

Clone/Gene	Primer Sequences
P3D7	5' CGG CAG CAG TTG CCT TCT GC 3' 5' TGC TTC CGA CTT GGT CAC GC 3'
P3G2	5' ATG CAG TTC ACC ACC ATC GC 3' 5' GTA ACG AGG GTG ACG TAG GC 3'
P2A12	5' CTC CCT CGC CAT CCA GCT TG 3' 5' TGG GCG CGG TGT CGA AGA TG 3'
P3G8	5' GCC AGG TTC GAC CAT TCT CTC CTG CTG C 3' 5' CAT TGG TGC AAA CTG GTC GGT GTG C 3'
P3F2	5' ACT CTC CGA AAT GCA TTT AGG TGC 3' 5' GCT CCA CAA TAC CTC ACG ATA CTG 3'
P2C12	5' TCA ACC AAC GCC AGT CTC CTT ATC 3' 5' ATA TCA AAG CCC GTC TTG GGT AGG 3'
18S rRNA	5' CTG TTA ATC ATT GCG GCG TCT CTA G 3' 5' GCG GTA ATT CCA GCT CCA ATA GC 3'
Beta Tubulin	5' GCC AGT CTG GTG CTG GTA ACA ACT GG 3' 5' CGA ATC CGA CCA TGA AGA AGT GGA GAC G 3'

Primer sequences for the beta-tubulin and 18S rRNA sequences were derived from *A. brassicicola* GenBank accessions Y17084 (beta tubulin) and AF229506 (18S rRNA).

PCR reactions were conducted in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following program: 94°C for 2:00 minutes followed by 35 cycles of 94°C 0:30 seconds, either 55°C or 53°C 0:30 seconds 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-PCR reactions were repeated at least 2 times with similar results.

Results

Suppression subtracted library creation

A SSH library was successfully created using the Clontech PCR-Select cDNA Subtraction kit. Figure 2.1A details the results of the double stranded cDNA synthesis.

Over-cycled cDNA is clearly visible after 18 cycles indicating that the exponential phase of amplification was exceeded. However, at 15 cycles the PCR reaction appeared to still be in the exponential phase, and 16 cycles was chosen to conduct the subtraction procedure. Following the subtraction procedure, differentially expressed cDNAs were selectively amplified by PCR. Figure 2.1B shows successful amplification of primary and secondary PCR products. Following ligation of the secondary PCR products into the appropriate vector and subsequent transformation into *E. coli*, bacteria were used as templates in a nested PCR (using nested primers 1 and 2R included with the SSH kit) (Fig. 2.1C). These primers are based on the adaptor sequences that were ligated to each cDNA in the library. Of the 288 colonies examined, 222 (77%) had inserts, with the average insert size approximately 500 base pairs. The success of the subtraction procedure was estimated by amplifying a putative house-keeping gene from the forward subtracted secondary PCR reaction and forward unsubtracted secondary PCR control. As seen in Figure 2.1D and E, a decrease in the amount of beta-tubulin transcript is apparent in the forward subtracted cDNA population indicating that at least some degree of subtraction occurred between the two cDNA populations. Because house keeping genes such as beta-tubulin can be differentially expressed, this analysis only provides an approximation of the subtraction efficiency.

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

In order to confirm the differential expression of selected cDNAs, PCR amplified inserts (e.g. Fig. 2.1C) representing 222 clones were arrayed on 3 nylon 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 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 that were subjected to this macro-array dot blot screening is presented in figures 2.2. A, B, C, D, E and F. Differences in hybridization signal intensity was apparent on the membranes probed with the forward (Fig. 2.2 A, C, E) and reverse subtracted probes (Fig. 2.2 B, D, F), which indicated the success of the SSH procedure in identifying differentially expressed sequences between the two populations being examined.

cDNA Northern analysis

After examination of the macro-array dot-blot, 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 cDNA Northern blots to further confirm their differential expression (Endege et al. 1999). Of the 47 clones examined, 35 (77%) were found to be significantly upregulated during the first 24 hours of *A. brassicicola* infection of the *Arabidopsis* ecotype Ler. Figure 2.3 presents the cDNA Northern blot results for 11 selected clones. Dramatic up-regulation on the plant leaf surface is seen in figure 2.3. 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 hours 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.

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 cDNA Northern blots. Following sequencing of the 47 selected clones, the 47 cDNA sequences were used to search the Genbank non-redundant database (Table 2.2), Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and Oomycete EST database (Table 2.3), *Magnaporthe grisea* Genome Database release 2.1 (Table 2.4), and *Neurospora crassa* Genome database 3 (Table 2.5) using the BLASTX algorithm. Thirty-six of the 47 sequences (77%) had at least 1 significant ($E \leq 1E-3$) 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 non-fungal organisms (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. Clone P3G2, had homology ($E = 1E-104$) with the major *Alternaria* allergen, *Alt a 1*, clone P2A12 had homology ($E = 4E-13$) with an arsenite translocating ATPase (ABC transporter) involved in arsenic detoxification, clone P3D7 had homology ($E = 4E-75$) with a cyanide hydratase gene from *Leptosphaeria maculans*, and clone P3G8 had homology ($E = 6E-25$) 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 Figure 2.4. 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 *Magnaporthe 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, or the *Neurospora 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 ($E = 1E-43$) 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, and may be essential for saprophytic growth.

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.

RT-PCR analysis

We used RT-PCR to examine the expression patterns of the 5 genes expressed during plant infection and not in spores germinating in water according to the cDNA Northern blots. We chose to examine the presence of transcripts from these genes for 5 days during an infection time course of the economically important *Brassica oleraceae* var. capitata (green cabbage), and at two times points during *Arabidopsis* infection (48 hours 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 hours of infection. However, visible necrosis and chlorosis were visible on the *Arabidopsis* detached leaves at 48 hours. At day 3, the cabbage leaves began to exhibit typical black spot symptoms characterized by dark brown to black lesions 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.

Figure 2.5A 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. 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.

Figure 2.5B presents expression of the 5 selected clones and clone P2C12, 2 and 7 days post-inoculation on the *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. 2.5B). The other 5 clones examined, P2A12, P3G2, P3G8, P2C12, P3D7, were all expressed in the media as well as at 7 days post-inoculation of cabbage and *Arabidopsis*.

Discussion

Despite the substantial amount of work done on resistance mechanisms to *A. brassicicola* in *Arabidopsis*, little is known about this necrotroph's pathogenicity 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 hours 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, 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 hours 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 *Magnaporthe 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 4 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. As databases become more saturated with sequence information from fungal species, identifying genus and species unique genes will become easier.

One of these unknown sequences, P3F2, was highly upregulated *in planta* according to the cDNA Northern results (Fig. 2.3). 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. 2.5B). 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 of 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 4 clones were expressed during *A. brassicicola*'s infection process on green cabbage (Fig. 2.5A, 4B) as well as *in vitro* in nutrient rich media (Fig. 2.5B). 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 hours for spores germinated in water) when the RNA was extracted. Based on the bioinformatics analyses and expression patterns of these 4 cDNA sequences, 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). However, *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 hemibiotroph *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, the *in silico* 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 mutants 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. 2.5B) and cDNA 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 up-regulated *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 cDNA 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, I 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 5 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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Table 2.2. BLASTX of Genbank Non-redundant database. Summary of 47 sequenced inserts from the SSH library enriched with *Alternaria brassicicola* genes expressed during the first 24 hours of a compatible interaction with the *Arabidopsis* ecotype Landsberg Erecta. Only scores with an E-value of $<10^{-3}$ were considered significant and reported in this table. MIPS Functional Category Numbers: 1 = Metabolism, 2 = Energy, 4 = Transcription, 5 = Protein Synthesis, 6 = Protein Destination, 7 = Transport Facilitation, 11 = Cell Rescue/Defense/Stress, 99 = Unclassified

Genbank	Clone ID	Size (bp)	Putative I.D.	Organism	E-Value	MIPS Functional Category
CA405363	P2H6	486	Acetyl-coa acetyl transferase	<i>S. 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>A. alternata</i>	1e-104	99
CA405368	P2A12	580	Aresnite translocationg ATPase	<i>S. pombe</i>	4e-13	7
CA405369	P3D7	621	Cyanide Hydratase	<i>L. 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 cis-trans 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	Botrytis cinerea 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>O. 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>H. 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>A. oryzae</i>	3e-5	7
CA405390	P3C5	500	Carbon catabolite repressor CreA	<i>C. 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

Table 2.3. TBLASTX searches of the Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and EST Database version 1.3. Putative EST ID based on the annotation according to the COGEME database. Only E-values $<10e^{-3}$ were considered significant and reported.

Genbank	Clone ID	Putative EST I.D.	Organism	E-Value	Uni-sequence ID
CA405363	P2H6	Acetyl-coa acetyl transferase	<i>M. graminicola</i>	3e-39	mg[1476]
CA405364	P3C4	Activator of Heat Shock Protein 90	<i>B. fuckeliana</i>	1e-46	BfCon[1598]
CA405365	P3A1	ADP/ATP Carrier Protein	<i>G. 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 cis-trans 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

Table 2.4. BLASTX searches of the *Magnaporthe grisea* Genome Database release 2.1. 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

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	Mitochondrial Carrier Proteins	2.1238	6e-15	MG06656
CA405366	P3E9	Mitochondrial Carrier Proteins	2.1238	9e-15	MG06656
CA405367	P3F4	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	Acetohydroxy acid isomeroreductase	2.347	1e-115	MG01808
CA405408	P1A8	Acetohydroxy acid isomeroreductase	2.347	8e-14	MG01808
CA405374	P3D8	Acetohydroxy acid isomeroreductase	2.347	1e-61	MG01808
CA405375	P3D4	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	FKBP type peptidyl prolyl cis-trans isomerase	2.1345	1e-30	MG07234
CA405379	P1D9	Hypothetical Protein/GrpE Domain	2.642	7e-07	MG03214
CA405380	P3H10	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

Table 2.5. BLASTX searches of the *Neurospora crassa* 3 Genome Database. 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.

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 cis-trans 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

Figure 2.1. Suppression subtractive hybridization. A. cDNA synthesis of tester and driver. Lane 1 = 1 kb ladder, other lanes correspond to the number of PCR cycles. Cycles 18, 21, and 24 are over cycled indicating that 15-16 cycles is the optimum number. B. PCR amplification of differentially expressed cDNAs. Lane 1 = 1 kb ladder, 2 = forward subtracted primary PCR products, 3 = forward unsubtracted control primary PCR products, 4 = reverse subtracted primary PCR products, 5 = reverse unsubtracted control primary PCR products, 6 = *Hae III* digested ϕ X174 DNA control primary PCR products, 7 = forward subtracted secondary PCR products, 8 = forward unsubtracted control secondary PCR products, 9 = reverse subtracted secondary PCR products, 10 = reverse unsubtracted control secondary PCR products, 11 = *Hae III* digested ϕ X174 DNA control secondary PCR products. C. Agarose gel showing cDNA inserts that subsequently are blotted onto nylon membranes for reverse Northern screens. Lane 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. D. Subtraction efficiency PCR, forward subtracted, Lane 1 = 100 bp ladder, other lanes correspond to number of PCR cycles. E. Subtraction efficiency PCR unsubtracted forward control, lanes as in D.

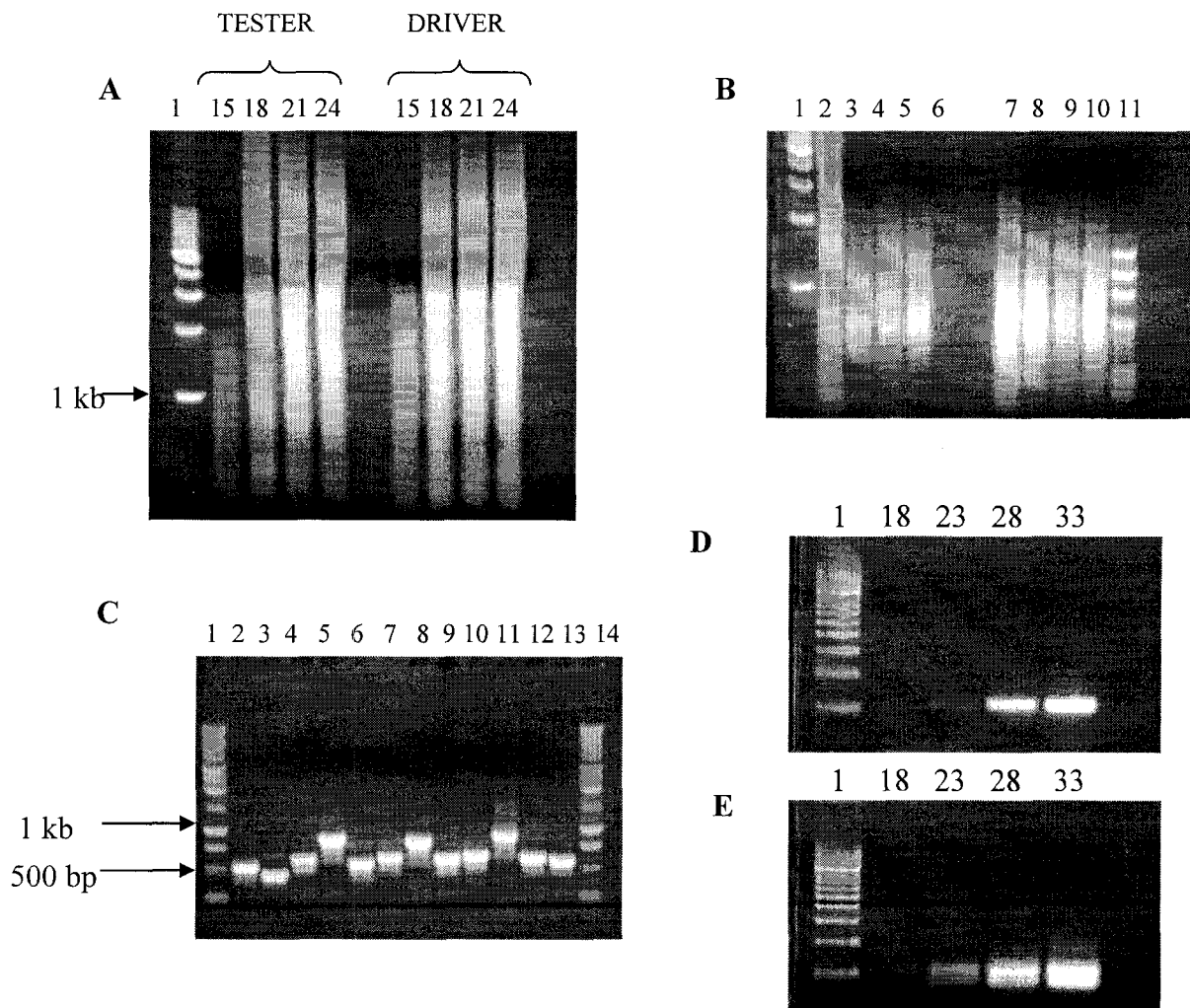


Figure 2.2. Differential gene expression macro-arrays. A, C, E. Membranes blotted with PCR amplified cDNA sequences from subtracted library and probed with the forward subtracted probe which is enriched for fungal genes expressed on the leaf surface of the plant. B, D, F. Membranes blotted with PCR amplified 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 A and B, C and D, and E and F are identical except for the probe with which they are screened. Clones sequenced in this study: blots A and B = all clones with "P1" designation in this study, i.e. G5 = clone P1G5, blots C and D = A7=P2F2 B2=P2B5 B4=P2H6 B8=P2A8 C1=P2F9 D5=P2A12 D6=P2C12 D7=P2D12. Blots E and F = clones correspond to all "P3" designated clones in this study, i.e. G2 = clone P3G2.

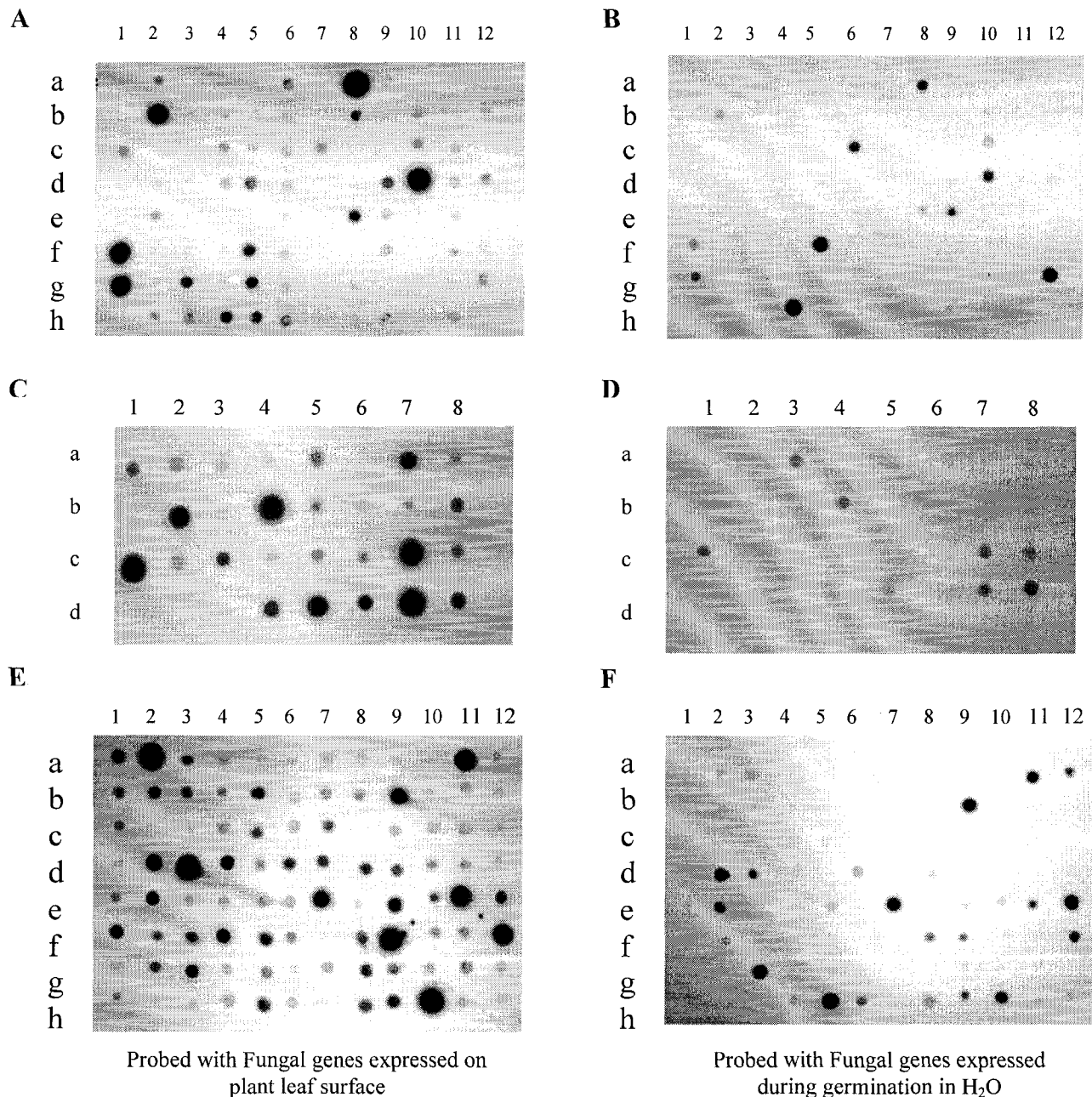


Figure 2.3. cDNA Northern blots showing differential expression of selected cDNA clones. P2C12 (unknown), P3G2 (allergen Alt a 1), P3D7 (cyanide hydratase), P3G8 (formate dehydrogenase), P2A12 (ABC Transporter), P3F2 (unknown), P3D4 (mitochondrial matrix protein), P3D8 (Ketol acid reductoisomerase), 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, lane 2 = water germinated spores 24 hours cDNA, and lane 3 = leaf surface germinated spores 24 hours 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.

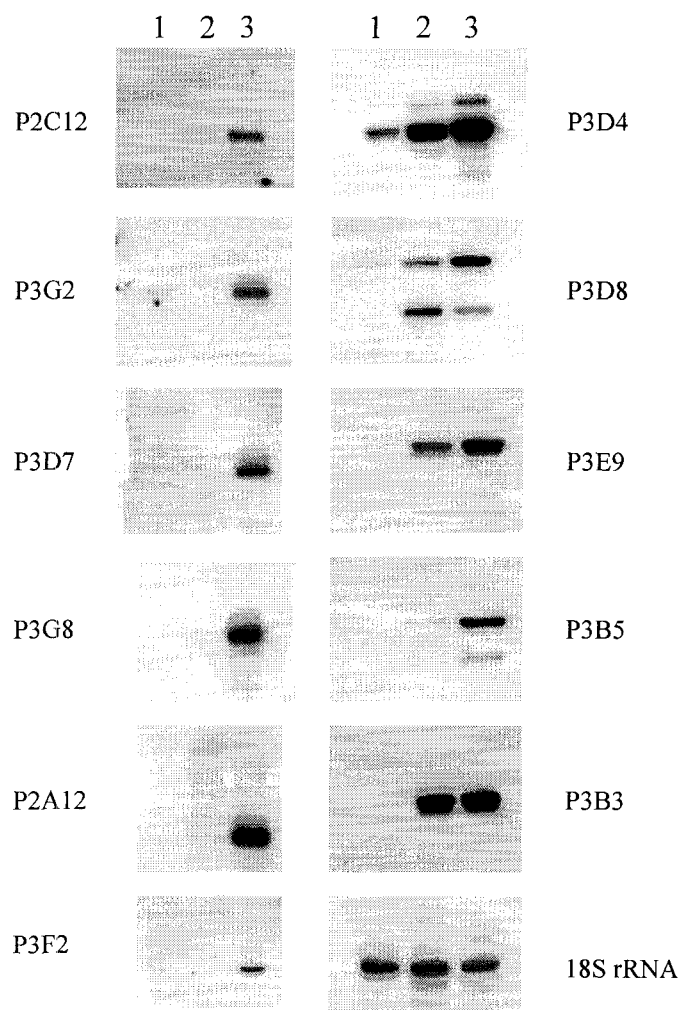


Figure 2.4. Distribution of *Alternaria brassicicola* ESTs into MIPS categories. Percentages were calculated from the total number of ESTs (47) and functional assignment was based on results from BLASTX searches of the non-redundant Genbank database.

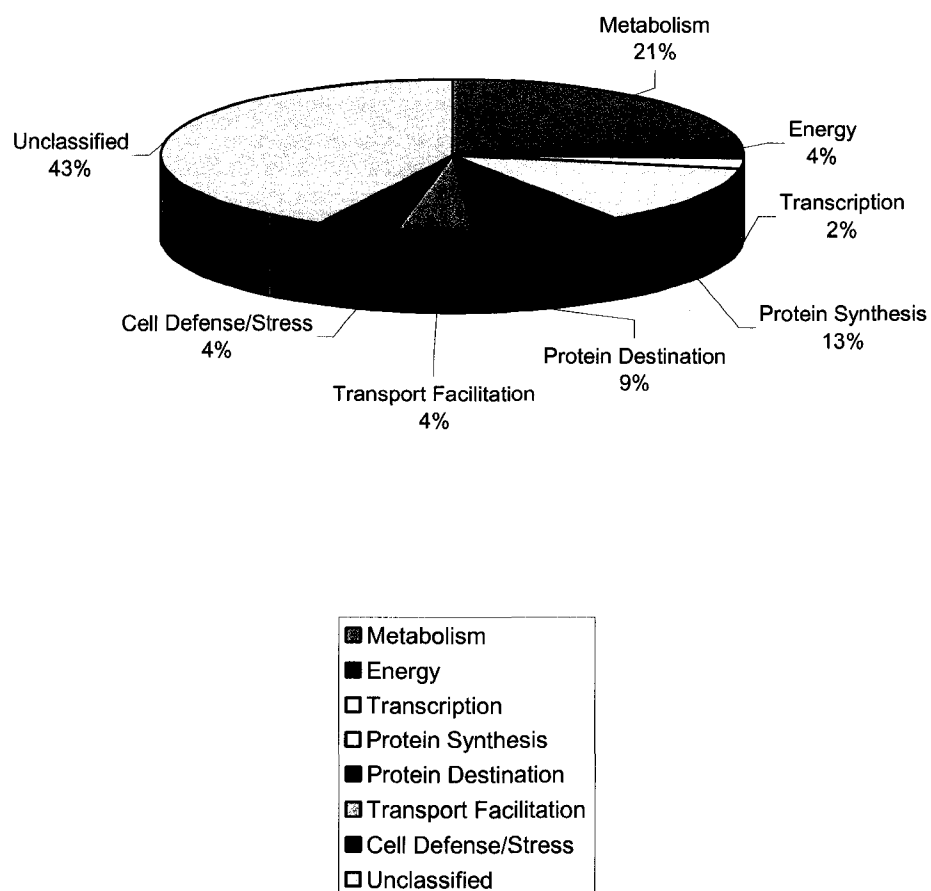
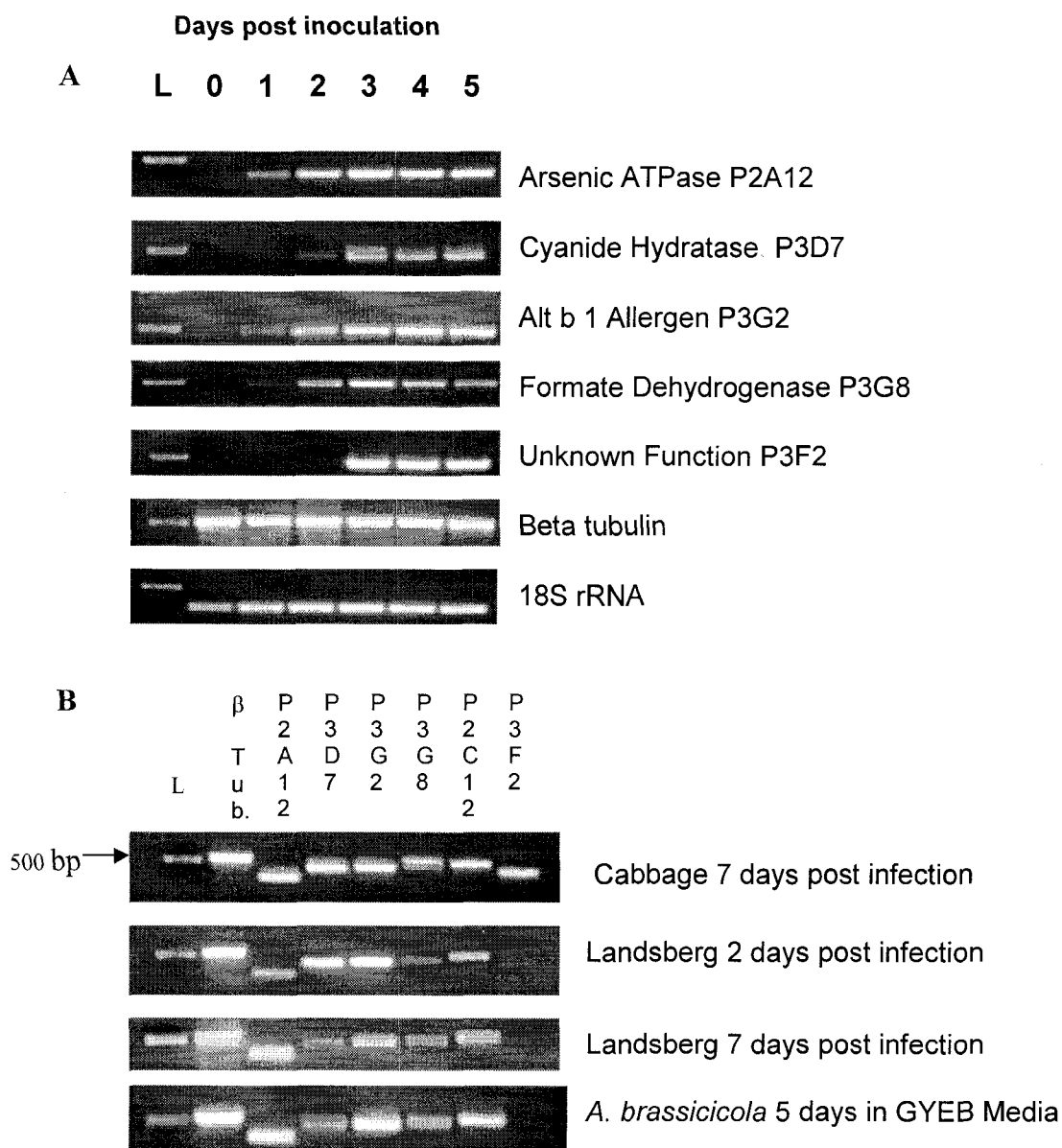


Figure 2.5. RT-PCR analysis of selected *Alternaria brassicicola* SSH clones. A. RT PCR analysis of the expression of the 5 genes upregulated *in planta* and not in spores germinating in water according to the cDNA Northern analysis. Lane L = 500 bp ladder, 0 = uninfected cabbage, 1 = cabbage infected 24 hours 2 = cabbage infected 48 hours 3 = cabbage infected 72 hours 4 = cabbage infected 96 hours and 5 = cabbage infected 120 hours. 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 a 1 allergen, P3G8 = formate dehydrogenase, P2C12 = unknown clone, and P3F2 = unknown clone.



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Chapter 3

Molecular Dissection of *Alternaria brassicicola* Pathogenesis by Expressed Sequence Tag (EST) Analyses

Introduction

Plant diseases caused by necrotrophic fungi pose serious threats to food supply quantity and quality around the world. Unfortunately, our knowledge about necrotrophic fungal pathogens and host defense responses to them is extremely limited. With the advent of the genomics era and the sequencing of whole genomes of partners in various plant-microbe interactions, the tools available to dissect fungal pathogenesis and host defense responses have greatly increased. However, full genome sequences are not available for all pathogen-host interactions, and in order to gain a greater understanding of genes employed by both partners of an interaction, expressed sequence tag (EST) sequencing and analysis has been used widely (Skinner et al. 2001). The purpose of this study was to gain a better understanding of the genes expressed during the *Alternaria brassicicola* – *Brassica oleraceae* var. *capitata* interaction.

Alternaria brassicicola (Schwein.) Wiltshire is a necrotrophic fungus that causes black spot disease (also called dark leaf spot) on numerous economically important *Brassica* species (Braverman 1979, Neergaard 1945). Uncultivated weedy species are

also susceptible and include the model plant *Arabidopsis thaliana*. Under appropriate conditions, *A. brassicicola* can be a destructive disease resulting in 20-50% yield reductions (Daebeler et al. 1986). Like other diseases caused by *Alternaria* species, black spot appears on the leaves as necrotic lesions, which are often described as black and sooty with chlorotic yellow halos surrounding the lesion sites (Agrios 1997, Verma and Saharan 1994). *A. brassicicola*, however, is not limited to infection of leaves, and can infect all parts of the plant including pods, seeds, and stems, and is of particular importance as a post-harvest disease (Rimmer and Buchwaldt 1995). The necrotrophic nature of *A. brassicicola* typically leads to extensive damage of the plant and harvest product, with seedlings seldom surviving an attack (Humpherson-Jones 1985, Neergaard 1945).

There is conflicting evidence in the literature about whether *A. brassicicola* produces a host-specific toxin (HST) like several of the *A. alternata* pathotypes. Otani et al. (1998) have reported the isolation of a 35 kDa host-specific toxic peptide (AB toxin) from *A. brassicicola* spore germination fluid (SGF). SGF from these experiments did not produce any necrosis or disease symptoms on non-hosts, but extensive necrosis on various *Brassica* species. These results are in contrast with extensive studies done by Cooke et al. (1997), which showed that SGF from *A. brassicicola* could indeed cause necrosis on non-hosts. However, different non-host species were used in the two studies.

To date, the putative gene encoding the putative AB toxin has not been cloned, which brings into question the true nature of “AB toxin.” Studies in our laboratory have recently identified two non-host specific peptide toxins from *A. brassicicola* SGF, as well

as one non-host specific peptide toxin from a culture filtrate, which seems to confirm the results presented by Cooke et al. (1997) (Pilon et al. 2004 unpublished data).

Regardless of the nature and identity of toxic compounds produced by *A. brassicicola*, little is known about its pathogenesis mechanisms, with no pathogenicity factors cloned to date. Extensive research on host defense responses to *A. brassicicola* has been conducted using the model plant *A. thaliana*. Natural differences in susceptibility and resistance to *A. brassicicola* exist in *Arabidopsis* ecotypes, which provide a useful situation to study resistance mechanisms to necrotrophic fungi (Otani et al. 2001, Kagan and Hammerschmidt 2002). Work by various authors has strongly suggested that resistance to *A. brassicicola* requires an intact jasmonic acid signaling pathway and the phytoalexin camalexin (Peninckx et al. 1996, Thomma et al. 1998, Narusaka et al. 2003, and Van Wees et al. 2003). The plethora of genomics based resources available for *Arabidopsis* make it an ideal candidate to identify which signaling pathways are important for resistance to necrotrophic fungal pathogens such as *A. brassicicola*. Part of the purpose of this study was to examine the genes expressed by an economically important agronomic crop, green cabbage, in response to *A. brassicicola* infection.

To better understand the genes involved in the interaction between *A. brassicicola* and green cabbage, gene expression from the fungus and the host was analyzed *in vivo* with ESTs during the infection. To gain a fuller appreciation of gene diversity in *A. brassicicola*, and to identify additional candidate pathogenicity genes, an *in vitro* EST library from *A. brassicicola* growth under nitrogen starvation conditions was also constructed. Fungal genes expressed during growth in a nitrogen poor environment have

previously been shown to be similar to genes expressed during fungal pathogenesis (Talbot et al. 1997). Sequence analysis of the expressed cDNAs in the two libraries yielded many potential *A. brassicicola* pathogenicity factors including several genes previously shown to be required for pathogenicity in other fungal pathogens. Genes expressed by the plant in response to *A. brassicicola* were similar to genes previously reported to be important in host defense responses. Data gained from this preliminary EST study provides the first glimpse of genes employed by *A. brassicicola* for pathogenesis, and a glimpse at genes expressed by a susceptible host during fungal infection.

Materials and Methods

Strains, culture conditions, and plant tissue inoculation

Alternaria brassicicola strain ATCC 96866 was grown on potato dextrose agar (PDA) at 25°C for 7 days. Spores for inoculation of plants were obtained from the agar cultures by rubbing spores free in sterile distilled water with a sterile pipet tip. A spore inoculum at a concentration of 1×10^6 spores/ml was used to inoculate 6 week old early green hybrid cabbage (*Brassica oleraceae* var. *capitata* cultivar ‘tendersweet F1’ (Johnny’s Selected Seeds, Albion, ME) detached leaves. Secondary leaves were removed from cabbage plants and placed in 150 mm x 15 mm sterile Petri plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ), the bottom layered with wet, and sterile paper towels. Detached leaves were sprayed with the spore inoculum until runoff occurred, Petri plate lids were sprayed with sterile water, replaced, and sealed with parafilm to maintain 100% humidity. Plates containing the detached leaves were placed in an incubator at 24°C and monitored for disease symptom development. For each time point

in the infection assayed in this experiment (0, 2, 4, and 6 days post inoculation) three sets of detached leaves from three different plants were used (a total of 6 leaves for each time point in the infection process).

For nitrogen starvation, $\sim 1 \times 10^5$ spores of *A. brassicicola* strain ATCC 96866 were inoculated into 50 ml of glucose-yeast extract broth (GYEB, 1.5% glucose, 0.3% yeast extract) and grown at 24°C, 100 rpm for 2 days. Mycelium was collected with sterile Miracloth (Calbiochem, Lajolla, CA) and rinsed 5× with sterile distilled water to remove any remaining GYEB. The mycelium was transferred to a minimal medium lacking a nitrogen source (0.5 g/L KCl, 0.5 g/L MgSO₄, 1.5 g/L KH₂PO₄, 0.1% (v/v) trace elements, and 10 g/L glucose, pH 6.5) (Talbot et al. 1997). The fungus was grown under nitrogen starvation conditions for 48 hours at 24°C, 100 rpm. Mycelium was again collected with sterile Miracloth, rinsed 5× with sterile water, and dried for RNA extraction.

cDNA library constructions

Extraction of total RNA from both plant tissue and fungal mycelium was performed with the RNeasy Plant Kit according to the manufacturer's protocol (Qiagen USA, Valencia, CA). For the mixed library (*A. brassicicola* infected cabbage), total RNA was extracted from leaves at 0, 2, 4, and 6 days post-inoculation. For the nitrogen starvation library, total RNA was extracted from the nitrogen starved mycelium. In order to obtain only infection related genes from the mixed library, we employed suppression subtractive hybridization (SSH) (Diatchenko et al., 1996).

For the SSH procedure, two cDNA populations were created using the SMART cDNA synthesis system (BD Biosciences-Clontech, Palo Alto, CA). The first cDNA

population (the driver) was generated from total RNA extracted from uninfected cabbage tissue (0 day). The second cDNA population (the tester) was generated from pooled total RNA extracted from 2, 4, and 6 days post-inoculation of *A. brassicicola* infected green cabbage leaves. Equal amounts of total RNA from each time point (2, 4, and 6 days post-inoculation) were pooled together (~2 µg) to form the second RNA population.

Approximately 1.5 µg of total RNA was used to generate the two cDNA populations for use in the subtraction procedure. Suppression subtractive hybridization (SSH) was performed between the driver (uninfected cabbage cDNA) and tester (*A. brassicicola* infected cabbage 2, 4, and 6 days post-inoculation cDNA) cDNA populations. The SSH procedure was performed with the PCR-Select cDNA Subtraction kit according to the manufacturer's directions with slight modifications as previously reported (BD-Biosciences-Clontech, Palo Alto, CA) (Cramer and Lawrence 2004, also see Chapter 2).

The resulting PCR mixture enriched for differentially expressed sequences from the tester population was non-directionally cloned using the pGEM-T vector (Promega, Madison, WI) and transformed into TOP-10 *E. coli* cells (Invitrogen, Carlsbad, CA) for blue-white selection. The subtracted library contained approximately 5,000 clones and 4,224 white colonies were randomly picked for identification and characterization.

For the nitrogen starved library, double stranded cDNA was created as above with the SMART cDNA synthesis system. Amplified double stranded cDNA was non-directionally cloned into pGEM-T (Promega, Madison, WI) and transformed by electroporation into *E. coli* DH5α cells for blue-white selection. The nitrogen starved library contained approximately 4,000 clones and 3,264 white colonies were randomly selected for identification and characterization.

DNA sequencing

DNA sequencing was conducted at the Fungal Genomics Laboratory (FGL) at North Carolina State University. Sequencing reactions were performed with standard Big Dye (Applied Biosystems, Foster City, CA) protocols for a 10 µl reaction. Cycle sequencing was performed over 35 cycles (96°C for 10 s; 50°C for 5 s; 60°C for 4 min.) in an Applied Biosystems GenAmp 9700 thermocycler. DyeEx 96-well plates (Qiagen USA, Valencia, CA) were used for dye-terminator removal prior to loading samples onto an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA). In total, 44 96-well plates were sequenced from the mixed library (4,224 sequences), and 34 96-well plates were sequenced from the nitrogen starvation library (3,264 sequences).

Sequence analysis

Raw sequence chromatogram trace files were processed by the program PHRED (version 0.990722, <http://www.phrap.org>) (Ewing et al. 1998). PHRED reads DNA sequencer trace data, calls bases, assigns quality values to the bases, and writes the base calls and quality values to output files. Sequences containing >100 bases with PHRED quality values ≥ 20 , or an average base quality ≥ 12 were retained. Cross_match was used to remove vector sequences. The output files including the base quality files were run through the assembly program CAP3 (contig assembly program) to develop a unisequence set (Huang and Madan 1999). The default parameters were used in the CAP3 assembly. The mixed library unisequence set is available for BLAST searches at <http://www.alternaria.org>.

Both the mixed and nitrogen starved library unisequence sets were run through the Genbank NR (non-redundant) database using the BLASTX algorithm with an E-value

cutoff of $E \leq 1e^{-5}$ (Altschul et al. 1990, 1997). To help confirm the identity of the sequences in the mixed library, BLASTN searches were also done against the *Magnaporthe grisea* genome genes database version 2.3 (<http://www.broad.mit.edu/annotation/fungi/magnaporthe/index.html>) and the *Arabidopsis thaliana* ATH1_cds_20030417 database (<http://www.arabidopsis.org>). All BLAST searches were conducted with stand-alone BLAST programs available from <ftp://ftp.ncbi.nih.gov/blast/executables/>. Sequences with significant homology ($E \leq 1e^{-5}$, BLASTX) were placed in a database developed with Microsoft Access (Microsoft Corporation, Redmond, WA) to allow manipulation of the BLAST reports. For example, queries could be made against the database to pull out all ESTs with significant hits to kinase-like genes. A script was developed by Dr. Dennis Knudson, Colorado State University, to separate sequences with hits ($E \leq 1e^{-5}$) in the NR database from insignificant hits. Insignificant hits from both the mixed library and nitrogen starvation library were used with the TBLASTX algorithm to search the Genbank EST_OTHERS (non-human, non-mouse ESTs) database available from <ftp://ftp.ncbi.nih.gov/blast/db/FASTA/>. The results of this analysis were also transferred to a Microsoft Access database for manipulation.

Since the databases examined are significantly more saturated with plant sequences than fungal, it was elected to utilize the sequence data from the nitrogen starved library to determine whether the remaining unknown sequences were from *A. brassicicola*. Sequence data from the nitrogen starved library (1660 unisequences) was formatted as a NCBI BLAST database using the program 'FORMATDB' available in the BLAST package at NCBI. This analysis served two purposes, first it assessed the

accuracy of the BLASTX results in assigning an origin to a given EST, and second it allowed an origin to be assigned to remaining unknown ESTs. The results of this BLASTN search were compared with previous BLASTX and TBLASTX searches to assign an EST as plant or fungal. ESTs with a BLASTN E value (against the nitrogen starvation library) less than the BLASTX or TBLASTX E value (with highest hit as plant sequence) were reassigned as fungal.

In order to identify putative functions of the EST sequences, an EST and protein sequence annotation pipeline and database currently under development at the Center for Integrated Fungal Research (<http://www.cifr.ncsu.edu>) was used. This system brings together several publicly available search tools to identify homologous proteins and protein domains, and assign descriptive terms from the Gene Ontology (GO) project (<http://www.geneontology.org>) to the EST sequences. EST sequences were translated in six frames and used to search the InterPro database (version 7, <http://www.ebi.ac.uk/interpro/>) using the iprscan search tool provided by the InterPro database project (Apweiler et al. 2001). InterPro entries that were assigned to EST sequences were translated to GO terms using a mapping file provided by InterPro. To identify putative homologous genes, a set of 32,309 manually curated protein sequences was downloaded from the Gene Ontology database and used as a BLAST database for annotating the EST sequences. A BLASTX search was performed using this database, and GO terms from the top hit that also had an E value better than $1e^{-30}$ were transferred to the EST. Gene Ontology terms from the InterPro and BLASTX searches were merged and loaded into a GO database. This database is available at

<http://www.fungalgenomics.ncsu.edu/cgi-bin/go.cgi>. Dr. Michael Thon at North Carolina State University conducted these GO annotations of the ESTs.

Results

cDNA libraries

Figure 3.1 shows the results of the subtraction procedure used to generate the mixed *Alternaria brassicicola* – *Brassica oleraceae* var. *capitata* EST library. The SSH procedure was successful, however, according to the subtraction efficiency test (Figure 3.1E) the subtraction was not 100% efficient. Because house keeping genes such as beta-tubulin can be differentially expressed, the subtraction efficiency analysis only provides an approximation of the subtraction efficiency. In this case, the results in Figure 3.1E demonstrate that at least some degree of subtraction occurred between the two cDNA populations.

Table 3.1 summarizes the results from the mixed and nitrogen starvation libraries. The rate of redundancy was similar for the mixed library (26%) and nitrogen starvation library (49%). Because the library was not directional and sequenced from only one direction, some degree of redundancy will not be detected, and more than 1 contig may correspond to the same transcript. Redundancy was calculated with the formula $[(\text{total number of sequences} - (\text{total number of singletons} + \text{total number of contigs})) / (\text{total number of sequences})]$. Both cDNA libraries were amplified prior to sequencing, and the mixed library also underwent a normalization step during the subtraction, which makes a correlation between the number of times that an EST clone appeared in the library and its expression level unreliable, so this analysis was not conducted. However, the lower redundancy rate in the mixed library may reflect the

usefulness of the normalization step in the subtraction procedure, even if it was not 100% efficient. It also reflects that more non-redundant ESTs likely exist in the mixed library.

Table 3.1. Summary of cDNA libraries created in this study.

Library	Total Sequences	Unisequences	Contigs	Singletons	Redundancy	Avg. Read Length
Mixed	4224	3112	608	2504	26%	925 bp
Nitrogen	3264	1660	253	1407	49%	920 bp

Mixed *Alternaria brassicicola* – *Brassica oleraceae* var. *capitata* library

Of the 3112 unisequences derived from the mixed library, 1584 (50.8%) exhibited significant ($E \leq 1e^{-5}$) hits with the Genbank NR database based on BLASTX search results. Of these 1584 hits, 1402 (88.5%) had their highest homology to either *Arabidopsis* (1285) or *Brassica* species (117) sequences. A summary of these hits, with genes putatively involved in plant defense responses, is presented in Table 3.2. Of the remaining 182 hits, 74 were from fungal species with the majority (44, 59%) having homology to *Neurospora crassa* sequences. Thus, 4.6% (74) of the 1584 ESTs with significant BLASTX hits are of probable fungal origin (Table 3.3).

TBLASTX searches of the EST_OTHERS database (organisms other than human and mouse) using sequences with no significant NR database BLASTX hits, yielded an additional 383 hits ($E \leq 1e^{-4}$). Of these 383 hits, 151 (39.4%) had their highest significant hit with *Arabidopsis* or *Brassicaceae* sequences. Sixty-one (15.9%) had their most significant hit with sequences from other plant species, and only 29 (7.5%) of the 383 unknown sequences had their most significant hit with a fungal EST (Table 3.4 and 3.5). The remaining 142 sequences had significant hits with organisms other than fungi or plants.

To assess whether or not the BLASTX hits were accurate in assigning a taxonomic (i.e. plant vs. fungal) identity to each EST, the BLASTN algorithm and the *Magnaporthe grisea* and *Arabidopsis thaliana* full genome sequences were utilized. Only 65 of the ESTs had significant BLASTN hits ($E \leq 1e^{-4}$) with both *M. grisea* and *A. thaliana* sequences. In only one case did the BLASTX result not concur with the BLASTN result. In this case, the BLASTN result ($E = 3.2e^{-23}$) suggested that the EST was of plant origin, while the BLASTX result ($E = 2.1e^{-59}$) suggested a fungal ribosomal protein. Because this EST (altr006xm14) is apparently a ribosomal protein, this result is not surprising and this gene was assigned as of fungal origin based on the strong BLASTX hit. Fifteen of the 65 sequences (23%) had significant BLASTN but not BLASTX or TBLASTX hits. Table 3.6 summarizes these 15 ESTs, 2 of which were assigned as fungal and 13 as plant.

BLASTN analysis of the 3112 mixed library ESTs against the *A. brassicicola* nitrogen starved library sequences yielded 408 significant hits ($E \leq 1e^{-5}$). Of these 408 hits, 134 (33%) did not previously have a significant hit in the other BLAST analyses conducted. These sequences were assigned as *A. brassicicola* sequences (Table 3.7). Of the remaining 274 sequences, 26 had significant BLASTX or TBLASTX hits of fungal origin and were already assigned as *A. brassicicola* sequences. This left 248 sequences that were originally assigned to be of plant origin based on the BLASTX or TBLASTX analysis that had significant hits with *A. brassicicola* nitrogen starvation sequences. To assign a putative origin for these sequences, BLASTN results were compared with the BLASTX and TBLASTX results. Sequences were assigned as fungal if the BLASTN E value was less than the BLASTX or TBLASTX E value. This resulted in re-assigning 75

sequences as *A. brassicicola* rather than cabbage. Table 3.8 presents the comparison of these 248 sequences.

Table 3.9 summarizes the final results of the mixed library based on the BLAST analyses. A total of 2119 sequences had significant hits in one of the analyses conducted (BLASTX of dbNR, TBLASTX of dbEST_OTHERS, BLASTN of *M. grisea* genome or *A. thaliana* genome, and BLASTN of *A. brassicicola* nitrogen starvation library). Of these 2119 sequences, 14.8% (315) are of probable fugal origin. Of the 3112 unisequences, 39.9% (1243) were unable to be assigned as either fungal or plant based on the BLAST searches conducted.

Table 3.9. Summary of mixed *Alternaria brassicicola* – *Brassica oleraceae* library based on BLAST searches of the databases utilized in this study.

<i>Alternaria brassicicola</i>	<i>Brassica oleraceae</i>	Other	Unknowns	Total
315	1554	250	993	3112

Gene ontology (GO) annotation assigned 911 of the ESTs to at least one of the 3 GO term categories (Biological Process (839, 92%), Cellular Component (786, 86%), and Molecular Function (819, 90%). The ability to assign a given EST to one of the three categories is dependent upon the amount of information returned from the BLASTX or InterPro domain analyses. For instance, it may not be possible to assign a cellular component to an EST that returns only an InterPro domain significant hit. Further, a given EST may be assigned to more than 1 functional category. Figure 3.2 summarizes the GO annotation results of the mixed library and table 3.10 summarizes the molecular functions of the ESTs assigned a GO annotation.

Table 3.10. Molecular functions of ESTs from mixed library assigned a GO annotation. An EST may be placed in more than one functional category.

Molecular Function	Number of ESTs
Enzyme Activity	532
Binding Activity	307
Transporter Activity	119
Molecular Function Unknown	56
Obsolete Function	56
Structural Molecule Activity	42
Signal Transducer Activity	27
Translation Regulator Activity	19
Transcription Regulator Activity	17
Chaperone Activity	16
Enzyme Regulator Activity	15
Motor Activity	4
Apoptosis Regulator	3
Toxin Activity	2
Antioxidant Activity	2
Defense/Immunity Protein Activity	2
Cell Adhesion Molecule Activity	1
TOTAL	1220

Nitrogen starvation library *Alternaria brassicicola*

Of the 1660 unisequences in the nitrogen starvation library, 503 (30.3%) exhibited significant ($E \leq 1e-5$) hits with the Genbank NR database based on BLASTX search results. Of these 503 hits, 314 (62.4%) had their highest significant hit with *Neurospora crassa* sequences. Only 63 of the sequences (12%) had their most significant hit with organisms other than fungi. Table 3.11 presents the non *Neurospora crassa* fungal sequences. TBLASTX searches of the EST_OTHERS database with the sequences with no significant NR database BLASTX hit, yielded an additional 285 hits ($E \leq 1e-5$). Of these 285 hits, 83 (29%) sequence had their highest significant hit with fungal ESTs while 155 (54%) had their highest significant hit with plant ESTs. These 83 sequences with highest hit to fungal ESTs are presented in table 3.12. The remaining 47 sequences

had their highest hits with organisms other than fungi or plants. A total of 788 ESTs (47.4%) had significant hits in the Genbank NR and EST_OTHERS databases. All ESTs from the nitrogen starvation library were assigned as *A. brassicicola* sequences.

For the GO annotation, 510 of the ESTs were assigned to at least one of the 3 GO term categories (Biological Process (458, 89.9%), Cellular Component (319, 63.4%), and Molecular Function (425, 83%) (Figure 3.3). Table 3.13 details the molecular function categories that 425 ESTs were assigned to. As with the mixed library, ESTs with enzyme activity and binding activity make up the two most frequent classes. The binding activity class includes a myriad of proteins that are reported to bind DNA, RNA, ATP, GTP, sugars, chitin, metal ions, actin, chromatin, and other important cell components.

Table 3.13. Molecular functions of *Alternaria brassicicola* nitrogen starvation ESTs assigned a GO annotation. An EST may be placed in more than 1 category.

Molecular Function	Number of ESTs
Enzyme Activity	147
Binding Activity	132
Transporter Activity	118
Structural Molecule Activity	79
Signal Transducer Activity	42
Molecular Function Unknown	39
Cell Adhesion Molecule Activity	17
Transcription Regulator Activity	10
Defense/Immunity Protein Activity	6
Translation Regulator Activity	6
Enzyme Regulator Activity	3
Chaperone Activity	3
Obsolete Activity	2
Toxin Activity	1
Nutrient reservoir activity	1
Anti-oxidant activity	1
TOTAL	607

Since the nitrogen starvation library did not undergo a subtraction procedure, it is likely worthwhile to examine the sequences that were assembled into the 253 contigs.

Table 3.14 below lists the contigs that consisted of 10 or more assembled sequences.

Table 3.14. Nitrogen starvation library contigs. Contigs that consisted of 10 or more sequences are listed.

Contig	Number of Sequences Assembled
Contig227	158
Contig90	147
Contig15	60
Contig116	54
Contig29	49
Contig150	42
Contig109	40
Contig172	40
Contig190	40
Contig14	36
Contig36	33
Contig236	32
Contig234	31
Contig215	30
Contig10	25
Contig16	25
Contig183	25
Contig205	22
Contig217	22
Contig178	19
Contig118	18
Contig23	17
Contig143	14
Contig166	14
Contig147	13
Contig159	13
Contig24	13
Contig50	13
Contig153	12
Contig12	11
Contig18	11
Contig59	11
Contig202	10
Contig250	10
Contig33	10
Contig63	10

Contig227, the one that consisted of the most sequences, had a significant TBLASTX ($1.00e^{-64}$) with an EST (P3D2) from the *A. brassicicola* SSH library created in Chapter 2. This sequence did not have any significant homology based on BLASTX searches of the

Genbank non-redundant database, and was not assigned a molecular function in the GO annotation, and thus its function is unknown. However, the number of sequences that correspond to this sequence may indicate that it has importance in *A. brassicicola* biology. A BLASTN search of the unisequence set from the mixed library using Contig227 revealed significant homology with Contig193 and Contig569 ($1.00e^{-12}$) indicating that this gene is also likely to be expressed during fungal pathogenesis.

Discussion

This study presents the first large scale analysis of the *A. brassicicola* – *Brassicaceae* interaction, and provides a glimpse into the biological processes occurring during necrotrophic fungal infection. One of the major hurdles to overcome when analyzing an EST library from a plant – fungal pathogen interaction is the source of each EST. Currently, there has been no defined method established for determining the origin of a given EST expressed during the interaction of a pathogen and its host. One approach has been to utilize the difference in average GC content for plant and fungal genes (Qutob et al. 2000). While *A. brassicicola* ESTs may have a higher GC content than cabbage ESTs, there were 2 problems that made this analysis impractical in this study. First, the number of full length *A. brassicicola* cDNA sequences in the Genbank database is only 3, making it impossible to determine a reasonable average GC content for *A. brassicicola* genes. Second, the infection-specific library was created by suppression subtractive hybridization. During the SSH procedure, cDNA fragments are digested with the frequent cutter *RsaI*. Thus, all the ESTs in the mixed library are partial EST sequences, and the average GC content of partial ESTs does not seem to be a reliable indicator of an EST's origin.

In order to assign a putative origin to the ESTs from the mixed library, a multifaceted sequence analysis approach was used. Because no single database contains all known sequences available for comparison, searches using BLAST algorithms were conducted against several different databases (Altschul et al. 1990, 1997). First, BLASTX searches were performed against the Genbank NR database. This was successful in assigning 74 ESTs as fungal, and 1402 as plant (Tables 3.2 and 3.3). Next, the sequences with no homology in the NR database were searched against the EST_OTHERS database with the TBLASTX algorithm (Tables 3.4 and 3.5). The accuracy of assigning an origin to an EST based on the BLASTX or TBLASTX search was estimated by using the BLASTN algorithm to search the *Magnaporthe grisea* and *Arabidopsis thaliana* full genome sequences. This analysis resulted in only 65 ESTs that had significant hits with both *M. grisea* and *A. thaliana* sequences (Table 3.6). Thus, utilizing BLASTX and TBLASTX algorithms and available sequence databases seems to be a reasonable approach for initially assigning an origin to an EST.

Because an *A. brassicicola* nitrogen starvation library was also created during the course of this study, these sequences were also utilized to help identify fungal genes in the mixed library (Table 3.7). This approach was particularly successful as 134 ESTs that did not have significant matches in the Genbank non-redundant or EST-OTHERS databases had significant matches with *A. brassicicola* sequences in the nitrogen starvation library. This result also suggests that a significant number of ESTs expressed in nitrogen starvation conditions are also expressed during fungal pathogenesis as has previously been reported (Talbot et al. 1997). This analysis was also used to check the validity of assigning an origin to an EST based on a BLASTX search of the Genbank NR

database. In this case, 248 sequences had significant hits with both the BLASTX search of the Genbank NR and BLASTN search of the *A. brassicicola* sequences. These 248 sequences all had highest significant matches with plant genes in the Genbank NR database. Because BLASTN is a direct DNA-DNA comparison, sequences with lower BLASTN E values than BLASTX E values were re-assigned as *A. brassicicola* sequences (Table 3.8). A total of 75 sequences were re-assigned as *A. brassicicola* sequences. This suggested that assigning a sequence origin based on the BLASTX result would result in roughly a 5% error rate (1476 sequences assigned either as fungal or plant based BLASTX result, $75/1476 = .05$), which seems acceptable for preliminary sequence analysis.

Combining the sequences that had significant hits with organisms other than fungi or plants and sequences with no significant hit in any of the searches performed in this study left a total of 1243 (39%) unidentified sequences (Table 3.9). It is difficult to speculate on the origin of these sequences, however, given the sheer number of plant sequences in the databases examined (including the full genome of *Arabidopsis thaliana*) one would speculate that the majority of these sequences are in all likelihood fungal in origin. Still, future studies will include confirming the origin of the sequences identified in this study with PCR and Southern blots.

GO ontology annotation of EST molecular functions

Gene ontology annotation was used to assign a putative function to the ESTs. This analysis was conducted at the Center for Integrated Fungal Research at North Carolina State University. Nine-hundred and eleven of the mixed library ESTs were able to be assigned a putative function using this annotation pipeline (Table 3.10 and Figure 3.2).

Not surprisingly, 64% of the mixed ESTs assigned a molecular function had enzyme activity. The second highest molecular function assigned (37% of the ESTs) was binding activity. Enzyme activity (34%) and binding activity (31%) were also the most frequent assignments for ESTs in the nitrogen starvation library. As expected, ESTs were also found with signal transduction, transporter, and transcription regulation activity. Genes from all of these molecular function categories have previously been shown to be important in fungal pathogenesis and host defense responses and indicate that the ESTs identified in this study represent, in part, molecular processes that occur during fungal pathogenesis.

One of the goals of this project was to identify cDNA sequences of putative toxic proteins from *A. brassicicola* that have been identified in the Lawrence laboratory. N-terminal amino acid sequencing of these toxic proteins is being conducted to obtain sequence data that will be used to BLAST against the ESTs identified in this study. Since the mode of action of these toxins is unknown, it is not possible to identify the potential toxin sequences based on the GO annotation. However, this dataset of ESTs expressed during *A. brassicicola* pathogenesis should prove to be an invaluable resource for identifying genes involved in toxin production.

Putative fungal pathogenicity genes

Virtually nothing is currently known about genes employed by *A. brassicicola* during infection of *Brassicaceae* hosts. In this study, 1975 (315 from the mixed EST library and 1660 from the nitrogen starvation library) *A. brassicicola* expressed sequences were identified. Of these, 315 were expressed during an interaction with a susceptible *Brassica oleraceae* var. *capitata* cultivar. Many of these genes were predicted

to be involved in plant cell wall degradation, secondary metabolite biosynthesis, signal transduction cascades important in fungal pathogenicity, and phytoalexin detoxification (Table 3.3). Two ESTs with homology to fungal xylanases (altr012xe13 and altr010xi20) and one with homology to an exo-alpha 1,4-polygalacturonase enzyme (contig 62) were expressed during infection of cabbage. Though it is often difficult to determine the role of a single cell wall degrading enzyme in fungal pathogenesis due to their sheer number and different isoforms, they are almost undoubtedly involved in allowing the fungus to penetrate the plant cell wall during infection (Mendgen et al. 1996).

Because necrotrophic fungi must first kill their host cells before colonization, it is expected that necrotrophs are exposed to a myriad of potentially toxic plant produced compounds. In this study, several ESTs were found to encode genes involved in detoxifying plant produced compounds by various mechanisms (Table 3.3). Indeed two fungal ABC transporters (alr016xb19 and altr013xh02) and several other genes encoding transporter like proteins were identified in the mixed library (Table 3.3). The role of membrane transporter proteins in pathogenesis has long been recognized in bacterial pathogens, and recently has gained recognition in fungal pathogenesis. Transporters in plant pathogens are not only involved in excreting toxic compounds produced by the plant's defense system, but may also secrete potential pathogenicity factors such as toxins (Del Sorbo et al. 2000). One example of the role of ABC transporters in fungal pathogenesis is *ABC1* from *Magnaporthe grisea*, which encodes an ABC transporter that has been shown to be essential for pathogenicity. *ABC1* null mutants penetrate the epidermal cells as the wild type does, but have increased sensitivity to some plant

produced compound as evidenced by their subsequent death upon penetration (Urban et al. 1999).

One of the most interesting ESTs of fungal origin in the mixed library was altr014xe01 which had significant homology (BLASTX $3.84e^{-28}$) with FUM12 from *Fusarium verticilliloides* (Table 3.3). FUM12 is part of a gene cluster that is involved in producing toxic fumonisins (Seo et al. 2001). Fumonisins are important polyketides that can cause severe symptoms in animals that ingest *Fusarium* infected plant debris (Howard et al. 1999). *Alternaria* species are not known to produce fumonisins, but this gene could be part of a gene cluster involved in producing a polyketide similar to fumonisin. Genome walking studies are currently underway to identify the genes surrounding altr014xe01.

Two ESTs of fungal origin (contig 515 and altr006xk14) in the mixed library had similarity to lipases from *Neurospora crassa* (Table 3.3). This is significant because a lipase has previously been shown to be important in the early stage of *A. brassicicola* pathogenesis (Berto et al. 1997, 1999). The molecular weight of the purified lipase was determined to be 80 kDa by SDS-PAGE and estimated to be 85 kDa by gel filtration suggesting that the enzyme is a monomer. Anti-lipase antibodies were found to significantly decrease *A. brassicicola*'s ability to cause disease on cauliflower leaves. However, the gene for this putatively important lipase has not been cloned to date, and the exact role of the lipase in fungal pathogenesis is not known. The two lipase-like ESTs identified in the mixed library that are expressed by *A. brassicicola* during pathogenesis are excellent candidates to clone and functionally analyze the role of lipases in fungal pathogenesis.

One of the signal transduction genes identified in both the mixed and nitrogen starvation libraries had homology with a *PMK1* homolog (altr012xxo05 and altr203xp18) in the necrotrophic *Brassicaceae* pathogen *Leptosphaeria maculans* (Tables 3.3 and 3.11). Though this MAP kinase has not been functionally analyzed in *L. maculans*, in *Magnaporthe grisea* *PMK1* was found to be essential for appressorium formation and invasive growth (Xu and Hamer 1996). *PMK1* mutants were nonpathogenic and failed to cause lesions on susceptible rice cultivars, but were not affected in their vegetative growth. In the necrotroph *Botrytis cinerea*, the *PMK1* homolog, *BMP1* was also found to be required for full pathogenicity (Zheng et al. 2000). No necrotic lesions were found on carnation and tomato plants sprayed with a *bmp1* deletion mutant. Scanning electron microscopy studies indicated that *bmp1* mutants were unable to penetrate plant cells and therefore *bmp1* mutants seemed to be defective in regulating plant cell penetration in *B. cinerea*. It is likely that the *A. brassicicola* *PMK1* homolog identified in this study also mediates plant penetration in some fashion.

The remaining 1660 sequences were expressed during nitrogen starvation (NS) conditions. Nitrogen starvation has previously been shown to induce expression of genes often involved in fungal pathogenesis (Talbot et al. 1997). The NS library was particularly rich in genes known to be involved in secondary metabolite production. Three polyketide synthases (PKS) were identified in the NS library (altr203xn03, contig 94, altr203xp03) (Table 3.11). Type I fungal polyketide synthases are monomodular enzymes that produce a wide variety of polyketides. Polyketides have been shown to be very important secondary metabolites in fungal-plant pathogen interactions. T-toxin, produced by *Cochliobolus heterostrophus* causal agent of Southern corn leaf blight, is a

polyketide. In order to be produced, T-toxin requires the polyketide synthase *PKS1*. Mutants with disrupted a *PKS1* gene do not produce T-toxin and are not capable of infecting maize (Yang et al. 1996). Other important polyketides in plant-fungal interactions include aflatoxin produced by *Aspergillus* species and cercosporin produced by *Cercospora* species (Diener and Davis, 1966, Mitchell et al. 2001). What metabolites these PKS genes in *A. brassicicola* produce is unknown, but they may be involved in the production of non-host or host specific toxins. Indeed, one of these PKS genes may be located in the same cluster as the FUM12 homolog identified in the mixed library.

Genes involved in plant defense responses

Resistance mechanisms to necrotrophic fungi are poorly understood. Plants recognize and often respond to microbial invaders by inducing a rapid defense response called the hypersensitive response (HR) (Baker et al. 1997). Induction of HR results in localized cell death of host cells that serves to contain the pathogen from spreading, and also induces a nonspecific resistance response known as systemic acquired resistance (SAR) throughout the plant. HR and SAR are thought to depend on the interaction of pathogen produced avirulence factors (AVR) and host produced resistance genes (R genes). AVR gene products from the pathogen have been hypothesized to serve as ligands that are recognized by corresponding receptors, the R genes, in plants (Dangl and Jones 2001). The recognition event triggers the HR, SAR, and other downstream defense responses. However, because necrotrophic pathogens actually require host cell death for successful invasion, induction of an HR response is not an effective means of plant defense, and mounting experimental evidence suggests that this would serve to promote fungal colonization.

Genes expressed by cabbage identified in this study are unique in that they represent genes expressed by a plant during a compatible (susceptible) interaction with a necrotrophic pathogen (Table 3.2). However, many of the genes expressed during *A. brassicicola* infection of cabbage are also expressed during incompatible (resistant) interactions in other pathosystems. For instance, several leucine rich repeat (LRR) receptor like kinases and disease resistance proteins of the CC-NBS-LRR and TIR-NBS-LRR class are putatively upregulated during *A. brassicicola* pathogenesis. These types of proteins are the putative R genes that recognize pathogen elicitors (such as AVR gene products) and induce a host defense response (i.e. the HR). One potential hypothesis is that necrotrophic fungal pathogens have actually evolved to take advantage of the AVR-R gene induced HR response to initiate host cell death that allows necrotrophic fungal infections. Indeed, several of the ESTs from the mixed library that are of putative plant origin are involved in cell death, senescence and apoptosis like responses. But it is not known whether these genes are induced in response to the production of a fungal toxin, which is very possible, or part of actual HR induction resulting from an AVR-R gene interaction.

What is clear, based on the types of genes expressed by the plant during infection with *A. brassicicola*, is that even though the plant is highly susceptible, it is trying to mount a defense response. Besides the disease resistance like genes, several pathogenesis related proteins (PR) including chitinase (contig 327) and other antifungal proteins are being expressed by the plant (Table 3.2). A Polygalacturonase inhibitor protein (contig 177) was also found to be expressed, which may correspond to the polygalacturonases found in the mixed library produced by *A. brassicicola*. A transcription factor from the

WRKY family (altr006xm15) was also found to be expressed during *A. brassicicola* infection. WRKY transcription factors are known to be induced by pathogens as well as salicylic acid (SA). SA is known to be a primary signaling molecule in the SAR response. Recently, it was shown that the promoter sequence of *NPR1* contains the DNA binding sequence of a WRKY transcription factor. *NPR1* is putatively involved in the signal transduction pathway that senses SA presence and ultimately leads to the coactivation of defense gene expression. Thus, WRKY transcription factors appear to act upstream of *NPR1* positively regulating the expression of plant defense response genes (Yu et al. 2001). Many other signaling molecules were also expressed in response to *A. brassicicola*. These include an ethylene response factor, phosphatidylinositol 4-phosphate 5 kinase, a MAP kinase, a serine/Threonine specific protein kinase, a calmodulin dependent protein kinase, and an adenylate kinase (Table 3.2).

An analysis of the genes expressed in an incompatible (resistant) interaction between *A. brassicicola* and the *Arabidopsis* ecotype Col-0 has been conducted using cDNA microarrays (Schenk et al. 2000). Several of the genes identified as upregulated in the resistant response to *A. brassicicola* were also identified in the susceptible response in cabbage conducted in this study. These include leucine-rich repeat proteins, putative disease resistance proteins, receptor like kinases, polygalacturonase inhibitor proteins, catalases, cytochrome P450 monooxygenases, peroxidases, and chitinases (Table 3.2). It is not known whether the *Arabidopsis* genes are homologs of the cabbage ESTs identified in this study, but it is clear that similar types of genes are being expressed by a resistant and susceptible host in response to *A. brassicicola* infection. One potential hypothesis is that the result of an interaction may be determined by the timing of the induction of

defense response pathways, which may or may not be mediated by various types of R genes. Plants susceptible to necrotrophs may be able to initiate a defense response almost identical to the one employed by a resistant plant, but susceptible plants may lack a given receptor that allows the plant to recognize the presence of the fungus before actual physical penetration has occurred. Thus, by the time the plant defense system is activated in a susceptible plant, it is too late to prevent the onslaught of the invading pathogen.

Conclusions

Interactions between pathogens and their hosts are complicated events mediated by many different signaling pathways. In this study we report the first large scale analysis of the interaction between *A. brassicicola* and *B. oleraceae* var. *capitata*. The putative identity of the genes identified in the EST library, enriched for genes expressed during fungal pathogenesis, indicates that susceptible plants do attempt to induce a defense response in response to necrotrophic fungi. Whether this defense response includes an attempted induction of an actual HR response is not known. Necrotrophic pathogens may take advantage of the induction of HR, or perhaps even induce an HR like response through the use of toxins. Further analysis of the genes identified in this study through gene replacement and disruption studies, as well as more detailed gene expression profiling experiments, will provide more details on this complex and important interaction. Fungal ESTs identified in this study will also help assist in the annotation of a potential full genome sequence of *A. brassicicola*, and will provide a starting point to begin looking for genes involved in the production of *A. brassicicola* toxic compounds which are almost certainly important in the interaction.

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Table 3.2. Subset of ESTs most likely to be of plant origin and involved in host defense based on BLASTX searches of the Genbank non-redundant database.

Query EST	Genbank Hit	E-Value	Description
Contig448	H86264	1.09E-166	Cytochrome P450 family Arabidopsis thaliana
Contig 305	AF089848_1	1.90E-130	senescence-specific cysteine protease Brassica napus
altr011xm05	NP_199923.1	6.70E-125	iron superoxide dismutase Arabidopsis thaliana
Contig309	NP_565955.2	1.30E-119	ABC transporter family protein Arabidopsis thaliana
Contig134	AAN62481.1	1.70E-117	phosphatidylinositol 3-kinase Brassica napus
Contig391	H84887	2.40E-106	probable pectinesterase Arabidopsis thaliana
altr009xg08	NP_568750.1	2.70E-103	ATP-dependent Clp protease ATP-binding subunit ClpD
altr010xe06	gi_16930430	2.17E-99	Peroxidase, Arabidopsis thaliana
altr007xe11	AAB53101.2	2.40E-96	catalase Brassica napus
Contig455	AF319169_1	7.70E-96	CBL-interacting protein kinase Brassica napus
altr016xg03	NP_176331.1	1.10E-95	receptor protein kinase (IRK1)
Contig 327	AAN23109.1	2.90E-88	chitinase Brassica rapa subsp. pekinensis
altr009xc11	AAM65906.1	3.50E-81	Nitrilase 4 (sp P46011) Arabidopsis thaliana
altr014xb21	NP_568327.1	2.00E-80	lipase-like protein Arabidopsis thaliana
Contig287	T46183	3.20E-77	zinc proteinase Arabidopsis thaliana
altr015xp18	NP_196455.1	6.80E-74	glycosyl hydrolase family 27
altr011xo21	NP_177396.1	1.50E-73	putative lipoxygenase Arabidopsis thaliana
altr014xk18	AAB23155.1	9.80E-73	COT44=cysteine proteinase homolog Brassica napus
Contig185	NP_568971.1	1.10E-72	leucine-rich repeat transmembrane protein kinase
Contig266	NP_195257.1	4.90E-72	calmodulin-domain protein kinase CPK5
altr009xk08	NP_195056.1	1.10E-71	disease resistance protein CC-NBS-LRR class
altr017xh14	NP_173546.1	4.40E-71	wall-associated kinase 5 Arabidopsis thaliana
altr006xm15	NP_181381.1	9.30E-71	WRKY family transcription factor Arabidopsis
Contig116	NP_200914.2	5.40E-67	histone deacetylase Arabidopsis thaliana
Contig 446	AAK50344.1	8.60E-63	defense-related protein Brassica carinata
altr016xa10	NP_195849.1	3.60E-62	serine/threonine-specific protein kinase NAK
Contig551	NP_197963.1	5.40E-56	disease resistance protein family Arabidopsis thaliana
altr014xn11	NP_175778.1	6.60E-56	26S proteasome AAA-ATPase subunit RPT1
altr006xa09	AAL12626.1	1.75E-53	leucine-rich repeat receptor-like kinase F21M12.36
altr013xc09	NP_172559.	8.70E-53	phosphatidylinositol-4-phosphate 5-kinase
Contig59	NP_181262.1	5.20E-52	putative adenylate kinase: At2g37250.1
Contig322	NP_172973.1	4.70E-51	ABC transporter family protein Arabidopsis thaliana
altr015xk19	NP_188548.2	1.30E-48	metalloprotease, putative Arabidopsis thaliana
Contig 177	AAM94869.2	9.40E-48	polygalacturonase inhibitor protein Brassica napus
altr007xh04	T07915	2.10E-46	probable aspartic proteinase rape seed
Contig 392	NP_188274.1	2.20E-44	putative lectin Arabidopsis thaliana
altr007xb04	NP_181250.1	8.30E-43	peroxidase, putative ATP2a Arabidopsis thaliana
altr015xc22	NP_175756.1	8.70E-40	MAP kinase ATMPK9
altr013xb17	NP_172106.1	5.60E-39	ethylene response factor, putative Arabidopsis
altr015xn15	AAN23106.1	4.5 E-30	PR4-type protein Brassica rapa subsp. pekinensis
Contig 63	Q39313	2.60E-27	Cysteine-rich antifungal protein 3 precursor (AFP3)
altr011xm15	NP_197336.1	5.90E-25	disease resistance protein TIR-NBS-LRR class
Contig406	S71591	3.70E-24	aspartic proteinase precursor, wound-induced - tomato
Contig 346	T08154	9.40E-24	pathogenesis-related protein PR1

Table 3.3. ESTs most likely to be of fungal origin based on BLASTX search of Genbank non-redundant database.

Query EST	Genbank Hit	E value	Description
altr006xm14	CAA29635	2.18E-59	ribosomal protein (AA 1-149) [<i>Neurospora crassa</i>]
altr015xf07	BAD00089	3.55E-56	1,3,8-trihydroxynaphthalene reductase [<i>Alternaria alternata</i>]
altr011xk19	XP_327208	6.84E-53	hypothetical protein [<i>Neurospora crassa</i>] similar to ER vesicle
altr004xh14	CAA23994	1.35E-48	URF 1 [<i>Emericella nidulans</i>]
altr010xf21	XP_325708	2.48E-47	hypothetical protein [<i>Neurospora crassa</i>] similar to hexose transport
altr013xh14	AL513445	9.55E-47	ribosomal protein L5 precursor, mitochondrial [<i>Neurospora crassa</i>]
Contig145	A23431	1.08E-43	NADH2 dehydrogenase (ubiquinone) [<i>Neurospora crassa</i>]
altr009xa05	AAL89551	4.91E-43	beta-glucosidase I [<i>Talaromyces emersonii</i>]
Contig518	CAA77185	1.24E-40	NADH dehydrogenase subunit 5 [<i>Trichophyton rubrum</i>]
altr015xc10	CAE76097	1.33E-40	P-type ATPase [<i>Neurospora crassa</i>]
altr010xn02	XP_329892	2.49E-40	predicted protein [<i>Neurospora crassa</i>] similar to oxidoreductase
altr017xj06	AAA82574	2.40E-34	4-hydroxyphenylpyruvate dioxygenase [<i>Coccidioides posadasii</i>]
altr006xn09	XP_330075	8.81E-32	hypothetical protein [<i>Neurospora crassa</i>] similar K+ Channel prot.
altr014xd06	XP_331977	5.63E-31	hypothetical protein [<i>Neurospora crassa</i>] similar to alcohol dehyd.
altr012xp11	XP_326182	6.27E-30	hypothetical protein [<i>Neurospora crassa</i>] similar to pisatin demeth.
altr011xm23	CAE76213	6.00E-29	GTP-binding Protein [<i>Neurospora crassa</i>]
altr015xk21	CAA30054	6.61E-29	hypothetical protein [<i>Kluyveromyces marxianus</i> var. <i>lactis</i>]
Contig348	CAB92650	8.46E-29	related to Na+/H+-exchanging protein [MIPS] [<i>Neurospora crassa</i>]
altr009xb08	XP_329201	2.23E-28	hypothetical protein [<i>Neurospora crassa</i>] similar to karyopherin
altr004xj18	XP_328751	2.63E-28	hypothetical protein [<i>Neurospora crassa</i>] similar to methyltransfer.
altr014xe01	AAN74815	3.84E-28	Fum12p [<i>Gibberella moniliformis</i>]
Contig481	XP_328439	6.58E-27	hypothetical protein [<i>Neurospora crassa</i>] similar to flavoprotein
altr006xg13	XP_327350	6.98E-26	hypothetical protein [<i>Neurospora crassa</i>] similar to racemase
altr006xc05	EAA26575	1.22E-25	amino acid permease [<i>Neurospora crassa</i>]
altr012xe13	CAB52417	5.91E-25	xylanase [<i>Helminthosporium turcicum</i>]
Contig515	XP_329934	1.76E-24	hypothetical protein [<i>Neurospora crassa</i>] similar to lipase
Contig371	EAA27941	5.88E-24	Alcohol dehydrogenase I-ADH1 [<i>Neurospora crassa</i>]
altr014xl22	P49380	8.77E-24	plasma membrane proton pump H+ ATPase [<i>Kluyveromyces lactis</i>]
altr007xp01	2107160A	2.61E-20	beta-glucosidase [<i>Candida wickerhamii</i>]
Contig62	AAC26146	1.06E-19	exo-alpha 1,4-polygalacturonase [<i>Cochliobolus carbonum</i>]
altr016xf14	XP_328439	3.16E-19	hypothetical protein [<i>Neurospora crassa</i>] similar to flavoprotein
Contig291	S54786	3.18E-19	multifunctional beta-oxidation protein [<i>Neurospora crassa</i>]
altr006xk14	XP_329338	2.42E-18	hypothetical protein [<i>Neurospora crassa</i>] similar to lipase
altr010xa05	XP_323210	4.23E-18	hypothetical protein [<i>Neurospora crassa</i>] similar to sulphoxide redu.
altr006xc18	CAA88034	4.24E-18	alcohol dehydrogenase II [<i>Emericella nidulans</i>]
altr012xo05	AAM89501	5.43E-18	mitogen-activated protein kinase [<i>Leptosphaeria maculans</i>]
altr012xo18	S09133	2.50E-17	NADH dehydrogenase subunit 5 [<i>Podospira anserina</i>]
altr016xb19	CAC42218	8.41E-17	ABC transporter protein [<i>Emericella nidulans</i>]
Contig546	AAM77705	4.44E-16	endoglucanase [<i>Nectria ipomoeae</i>]
Contig64	XP_329891	6.18E-16	hypothetical protein [<i>Neurospora crassa</i>] lysine synthesis
altr012xb19	AAB70514	8.96E-16	alpha-mannosidase [<i>Aspergillus nidulans</i>]
altr007xn23	AAC49260	1.16E-15	S-adenosylmethionine synthase [<i>Neurospora crassa</i>]
altr006xp12	EAA32047	1.69E-14	putative protein [<i>Neurospora crassa</i>] ER protein transport
altr009xl18	XP_331351	1.79E-14	hypothetical protein [<i>Neurospora crassa</i>] similar to Ypl260wp
altr009xe10	XP_328434	4.85E-14	hypothetical protein [<i>Neurospora crassa</i>] similar to AreA N respon.
Contig129	NP_862333	1.26E-13	ORF1 [<i>Trichoderma harzianum</i>]
Contig265	XP_324420	8.64E-13	hypothetical protein [<i>Neurospora crassa</i>] unknown
altr016xk22	CAB88637	9.73E-13	multicatalytic endopeptidase complex PRE1 [<i>Neurospora crassa</i>]
altr014xg18	XP_329458	1.69E-11	hypothetical protein [<i>Neurospora crassa</i>] similar to beta mannase
Contig115	CAA30000	9.55E-11	cob1 ORF [<i>Neurospora crassa</i>]
altr010xi20	CAB52417	1.37E-10	xylanase [<i>Helminthosporium turcicum</i>]
altr012xc05	XP_324159	2.45E-10	hypothetical protein [<i>Neurospora crassa</i>] similar to peroxisome prot.
altr010xf06	AAO47090	4.72E-10	ribosomal L10 protein [<i>Paracoccidioides brasiliensis</i>]

Table 3.3. Continued

Query EST	Genbank Hit	E-Value	Description
altr013xa09	XP_330355	5.33E-10	hypothetical protein [Neurospora crassa] similar to epimerase
altr014xh19	AAN77094	1.23E-09	CDC42-like protein CflB [Penicillium marneffei]
altr009xb15	CAA49629	1.61E-09	Anthranilate synthase (PRAI) [Cochliobolus heterostrophus]
Contig407	NP_862333	1.85E-09	ORF1 [Trichoderma harzianum]
altr009xf19	CAD12640	3.06E-09	putative ENA-ATPase [Pleurotus ostreatus]
altr006xg17	XP_328406	4.51E-09	elongation factor 2 (EF-2) [Neurospora crassa]
altr006xd01	XP_329959	1.09E-08	hypothetical protein [Neurospora crassa] similar to decarboxylase
altr012xk12	AAB41493	1.24E-08	fatty acid synthase, alpha subunit [Emericella nidulans]
altr004xh03	C48326	1.33E-08	cytb intron 1b protein [Podospora anserina]
altr013xh02	XP_325446	2.96E-08	hypothetical protein [Neurospora crassa] similar to ABC transport.
Contig599	BAB70707	9.72E-08	elastase-like serine protease [Metarhizium anisopliae]
altr012xe10	CAD60755	3.26E-07	unnamed protein product [Podospora anserina]
altr009xh03	CAD71059	3.41E-07	endo-1, 4-beta-xylanase B precursor [Neurospora crassa]
altr009xj06	XP_329201	1.16E-06	hypothetical protein [Neurospora crassa] similar to karyopherin
altr006xj03	CAB92681	1.69E-06	dimeric dihydrodiol dehydrogenase [Neurospora crassa]
altr012xj20	AAC69199	2.35E-06	adenosine kinase [Schizophyllum commune]
Contig283	EAA32278	3.73E-06	probable ribosomal protein S29.e.A, cytosolic [Neurospora crassa]
altr009xe07	XP_331503	6.40E-06	glucose-6-phosphate 1-dehydrogenase (G6PD) [Neurospora crassa]
Contig7	XP_323832	6.93E-06	hypothetical protein [Neurospora crassa] similar to aminopeptidase
altr007xm09	CAD70910	9.79E-06	cell division cycle 2-related protein kinase 7 [Neurospora crassa]

Table 3.4. Unknown ESTs from mixed library with highest significant hit with plant sequences based on TBLASTX searches of the Genbank EST_OTHERS database.

Query EST	Genbank Hit	E-Value	Description
Contig488	TAS007.B02	2.00E-95	Wheat cDNA Library Triticum aestivum cDNA clone
altr009xe07	WHE2970_F07_L14	2.00E-25	Wheat dormant embryo cDNA library Triticum aestivum
altr012xj07	WHE0838_G12_M24	1.00E-24	Wheat vernalized crown cDNA library Triticum aestivum
Contig345	LP254-28-d6	6.00E-19	Lotus japonicus cDNA
altr012xm18	BJ288172	7.00E-14	Wh_SL Triticum aestivum cDNA clone
altr006xf01	Gm-c1014	6.00E-13	Glycine max cDNA clone
altr012xb12	Gm-c1014	8.00E-13	Glycine max cDNA clone
altr002xj11	Ed0072D04f	1.00E-12	Gossypium arboreum 7-10 dpa fiber library
altr010xh14	BJ289230	1.00E-12	Triticum aestivum cDNA clone
altr007xc18	TaLr1137A10	1.00E-09	Triticum aestivum cDNA clone
altr017xi14	HVSMEEn0011M16f	4.00E-09	Hordeum vulgare rachis EST library
altr015xa06	HVSMEb0004E10f	2.00E-08	Hordeum vulgare seedling shoot EST library
altr016xl11	HVSMEI0022E23f	2.00E-08	Hordeum vulgare Fusarium infected spike EST library
altr016xo23	NF024C09ST	2.00E-08	Developing stem Medicago truncatula cDNA clone
altr009xc07	Gm-c1027	3.00E-08	Glycine max cDNA clone
altr014xo18	HVSMEEn0004M10f	3.00E-08	Hordeum vulgare rachis EST library
altr002xk11	Ea0010E03	4.00E-08	Gossypium arboreum 7-10 dpa fiber library
altr007xa02	Gm-c1087-5044	5.00E-08	Glycine max cDNA clone SOYBEAN
altr017xa22	HVSMEh0096C10f	6.00E-08	Hordeum vulgare 5-45 DAP spike EST library
Contig240	MTD016.F10	8.00E-08	Durum Wheat Root Library Triticum turgidum subsp. durum
Contig213	BJ298843 Y.	1.00E-07	Triticum aestivum cDNA clone
altr012xk20	Eb0038J23f	4.00E-07	Gossypium arboreum 7-10 dpa fiber library
altr016xb17	HVSMEI0002J09f	4.00E-07	Hordeum vulgare Fusarium infected spike EST library
altr012xo10	Ea0015F15r	6.00E-07	Gossypium arboreum 7-10 dpa fiber library
altr007xg14	HVSMEc0017D15f	9.00E-07	Hordeum vulgare seedling shoot EST library
altr006xg23	BJ281350 Y	1.00E-06	Triticum aestivum cDNA clone
altr012xp04	Gm-c1063	2.00E-06	Glycine max cDNA clone
altr015xm22	Ea0010M19	2.00E-06	Gossypium arboreum 7-10 dpa fiber library
altr016xn10	Gm-c1036	2.00E-06	Glycine max cDNA clone
altr016xp18	HVSMEf0004K19f	2.00E-06	Hordeum vulgare seedling root EST library
altr016xe12	HVSMEb0013B11f	3.00E-06	Hordeum vulgare seedling shoot EST library dehydration
altr014xb13	HVSMEh0096G06f	4.00E-06	Hordeum vulgare 5-45 DAP spike EST library
altr014xm18	HVSMEf0010M13f	4.00E-06	Hordeum vulgare seedling root EST library
altr015xb21	HVSMEh0096G10f	4.00E-06	Hordeum vulgare 5-45 DAP spike EST library
altr007xf06	MM04F1	5.00E-06	Mesembryanthemum crystallinum cDNA clone
altr016xk18	Ea0026H05r	5.00E-06	Gossypium arboreum 7-10 dpa fiber library
altr014xb23	HVSMEh0096G12f	7.00E-06	Hordeum vulgare 5-45 DAP spike EST library
altr014xp21	Ed0039F08f	7.00E-06	Gossypium arboreum 7-10 dpa fiber library
altr004xn05	Gm-c1069	1.00E-05	Glycine max cDNA clone
Contig65	Gm-35-10H	1.00E-05	Soybean root forward subtractive cDNA Library Glycine max
altr016xm21	HVSMEf0005M20f	1.00E-05	Hordeum vulgare seedling root EST library
altr006xc06	Ed0044G08r	2.00E-05	Gossypium arboreum 7-10 dpa fiber library
altr007xe04	HVSMEf0019K01f	2.00E-05	Hordeum vulgare seedling root EST library
altr010xo21	HVSMEEn0005F07f	2.00E-05	Hordeum vulgare rachis EST library
altr012xn17	HVSMEf0010O19f	2.00E-05	Hordeum vulgare seedling root EST library
altr014xb11	HVSMEEn0004N14f	2.00E-05	Hordeum vulgare rachis EST library
altr007xj22	Ea0009J24	3.00E-05	Gossypium arboreum 7-10 dpa fiber library

Table 3.4 Continued

Query EST	Genbank Hit	E-Value	Description
altr012xn20	HVSMEg0008P15f	3.00E-05	Hordeum vulgare pre-anthesis spike EST library
altr012xo02	HVSMEh0096K20f	3.00E-05	Hordeum vulgare 5-45 DAP spike EST library
altr017xf20	HVSMEn0013N14f	3.00E-05	HVSMEn0013N14f Hordeum vulgare rachis EST library
altr004xp16	Ed0040E07f	4.00E-05	Gossypium arboreum 7-10 dpa fiber library
altr002xo21	Ea0029A07f	5.00E-05	Gossypium arboreum 7-10 dpa fiber library
altr010xh17	MWM184c05_r	5.00E-05	Lotus japonicus young plants (two-week old) cDNA
altr014xi14	Gm-c1084	6.00E-05	Glycine max cDNA clone
altr002xa06	Ea0018C10r	7.00E-05	Gossypium arboreum 7-10 dpa fiber library
altr002xc12	HVSMEf0010K17f	7.00E-05	Hordeum vulgare seedling root EST library
altr007xl22	Ea0010I09	8.00E-05	Gossypium arboreum 7-10 dpa fiber library
altr009xk21	AJ469677	8.00E-05	Hordeum vulgare cDNA clone
altr010xe24	HVSMEf0005G24f	9.00E-05	Hordeum vulgare seedling root EST library
altr012xl21	HVSMEf0008O03f	9.00E-05	Hordeum vulgare seedling root EST library

Table 3.5. Unknown ESTs from mixed library with highest significant hit to fungal sequences based on TBLASTX searches of the Genbank EST_OTHERS database.

Query EST	Genbank Hit	E-Value	Description
Contig130	EST418639	2.00E-32	Coccidioides immitis spherule cDNA library
Contig502	NF034B01PH1F	1.00E-20	Phoma-infected Medicago truncatula cDNA
altr010xj24	NF034B01PH1F	4.00E-20	Phoma-infected Medicago truncatula cDNA
altr006xn19	hasp002xd06f	1.00E-17	Heterobasidion annosum cDNA
altr014xn09	NF034B01PH1F	3.00E-15	Phoma-infected Medicago truncatula cDNA
Contig150	EST418639	1.00E-14	Coccidioides immitis spherule cDNA library
Contig1	hasp002xd06f	9.00E-14	Heterobasidion annosum cDNA
altr006xb03	hasp002xd06f	2.00E-13	Heterobasidion annosum cDNA
altr007xe02	hasp002xd06f	1.00E-12	Heterobasidion annosum cDNA
altr012xl07	hasp002xc22f	1.00E-12	Heterobasidion annosum cDNA
altr007xk13	mgct006xc23f	2.00E-10	Magnaporthe grisea Appressorium Stage cDNA
altr004xb03	hasp002xn22f	3.00E-10	Heterobasidion annosum cDNA
altr009xj13	C00819	6.00E-10	Blumeria graminis f. sp. hordei cDNA clone
Contig283	g9g01ne.fl	4.00E-09	Neurospora crassa evening cDNA library
altr009xj07	hasp001xm02f	1.00E-08	Heterobasidion annosum cDNA
altr012xa24	mgct006xg17f	9.00E-08	Magnaporthe grisea Appressorium Stage cDNA
altr013xm23	hasp002xd06f	1.00E-07	Heterobasidion annosum cDNA
altr012xh10	mgct002xd01f	2.00E-06	Magnaporthe grisea Appressorium Stage cDNA
altr006xe12	e9e09a1.fl	3.00E-06	Aspergillus nidulans 24hr asexual developmental
altr011xa11	MgA0646f	6.00E-06	MgA Library Mycosphaerella graminicola cDNA
altr002xa02	mgct006xg17f	7.00E-06	Magnaporthe grisea Appressorium Stage cDNA
altr007xf07	hasp002xk22f	9.00E-06	Heterobasidion annosum cDNA
altr006xa13	mgct006xg17f	2.00E-05	Magnaporthe grisea Appressorium Stage cDNA
altr012xh14	D01236	2.00E-05	Blumeria graminis f. sp. hordei cDNA clone
altr017xa14	mgct001xc07f	2.00E-05	Magnaporthe grisea Appressorium Stage cDNA
altr004xl09	x3a05a1.fl	3.00E-05	Aspergillus nidulans 24hr asexual developmental
altr015xo10	mgct006xg17f	5.00E-05	Magnaporthe grisea Appressorium Stage cDNA
altr006xj01	mgct001xa08f	6.00E-05	Magnaporthe grisea Appressorium Stage cDNA
altr002xe16	mgct006xg17f	8.00E-05	Magnaporthe grisea Appressorium Stage cDNA

Table 3.6. ESTs from mixed library with both fungal (*Magnaporthe grisea*) and plant (*Arabidopsis thaliana*) BLASTN hits that did not have BLASTX hits in the Genbank non-redundant database or TBLASTX hits in the Genbank EST_OTHERS database.

Query EST	<i>M. grisea</i> BLASTN	<i>A. thaliana</i> BLASTN	Probable Origin
altr009xi02	7.3E-07	1.70E-06	Fungal
altr017xj06	2.3E-06	5.40E-06	Fungal
altr014xh06	1.3E-08	3.80E-29	Plant
altr014xi07	6.9E-04	4.00E-155	Plant
altr016xg02	6.1E-32	2.00E-117	Plant
altr016xm02	6.7E-04	6.40E-06	Plant
altr016xp15	5.20E-23	1.07E-86	Plant
altr013xl12	1.2E-11	1.00E-124	Plant
contig12	2.0E-09	0	Plant
contig171	1.9E-15	6.30E-179	Plant
contig228	7.6E-06	5.00E-163	Plant
contig308	5.30E-25	5.00E-138	Plant
contig52	3.3E-05	0	Plant
contig53	4.5E-30	2.70E-67	Plant
contig607	2.9E-07	4.00E-113	Plant
contig96	2.1E-09	3.00E-53	Plant

Table 3.7. ESTs from *Alternaria brassicicola* – *Brassica oleraceae* mixed library with significant BLASTN hits to sequences in the *Alternaria brassicicola* nitrogen starvation (NS) library, and no significant hits in the Genbank non-redundant or EST_OTHERS databases.

Query EST	NS EST Hit	E-Value
altr012xj13	altr207xj15	0
Contig106	altr211xe09	0
Contig436	Contig63	0
Contig90	altr202xj18	1.00E-167
Contig421	altr203xd12	1.00E-131
altr014xj08	altr202xk09	1.00E-128
altr015xg05	altr201xh23	5.00E-89
altr013xk05	altr207xo15	5.00E-86
altr015xa07	Contig205	3.00E-84
Contig238	Contig205	2.00E-82
altr012xm22	altr211xb07	1.00E-80
altr016xa19	altr208xl02	1.00E-58
altr009xi02	altr205xd19	5.00E-52
altr006xg08	Contig251	5.00E-49
altr016xp15	Contig206	4.00E-43
Contig523	Contig4	1.00E-40
Contig52	Contig235	6.00E-26
altr012xl20	altr211xf21	2.00E-23
altr010xk21	altr204xg08	3.00E-22
altr010xm19	altr212xk09	1.00E-21
altr010xa10	altr201xn24	7.00E-20
altr009xe13	altr213xb13	3.00E-19
altr012xg16	altr211xh15	3.00E-19
altr014xh08	altr213xb13	4.00E-19
Contig580	altr201xn24	5.00E-19
Contig386	altr204xg08	2.00E-18
Contig412	altr204xa04	8.00E-18
Contig569	Contig122	1.00E-17
altr010xc21	altr204xc22	2.00E-17
Contig339	altr213xb13	3.00E-17
Contig425	altr211xk15	4.00E-17
altr011xg13	altr213xb13	7.00E-17
altr002xp05	Contig117	8.00E-17
altr010xl16	altr213xb13	8.00E-17
altr011xo05	altr213xb13	8.00E-17
altr012xh12	altr207xf04	8.00E-17
altr014xh09	altr204xe04	8.00E-17
altr007xp02	altr201xn24	9.00E-17
Contig12	altr207xf16	2.00E-16
altr006xf10	Contig101	3.00E-16
altr007xp06	altr207xn14	3.00E-16
altr012xb06	altr213xb13	3.00E-16
altr015xj07	altr212xc20	3.00E-16
Contig522	Contig189	8.00E-16

Table 3.7 Continued

Query EST	NS EST Hit	E Value
altr010xp22	altr205xb07	1.00E-15
altr015xo19	altr205xp20	1.00E-15
Contig198	altr213xb09	1.00E-15
altr016xj19	altr201xn24	2.00E-15
Contig604	altr204xg08	2.00E-15
altr009xf14	altr204xg08	4.00E-15
altr007xf09	altr207xd12	5.00E-15
altr011xc17	altr213xb08	5.00E-15
altr012xd07	altr213xb13	5.00E-15
altr012xn10	altr213xb13	5.00E-15
altr015xl17	altr211xk15	5.00E-15
altr007xc09	altr206xj01	2.00E-14
altr007xh16	Contig3	2.00E-14
Contig71	altr203xn08	1.00E-13
Contig441	altr211xh15	2.00E-13
altr002xo21	altr205xh02	3.00E-13
altr007xp21	altr205xb07	3.00E-13
altr012xf08	altr213xb23	1.00E-12
altr016xf01	Contig164	1.00E-12
altr010xo02	altr205xp03	4.00E-12
altr015xn19	altr213xh05	4.00E-12
Contig233	altr207xf16	4.00E-12
Contig605	altr211xn15	4.00E-12
altr015xa19	Contig189	5.00E-12
Contig16	Contig41	1.00E-11
altr009xn22	altr212xm19	2.00E-11
altr014xf01	altr204xe01	2.00E-11
altr015xf12	Contig192	2.00E-11
altr017xi16	altr201xn24	3.00E-11
altr002xh21	Contig203	8.00E-11
altr007xa23	altr212xc20	3.00E-10
altr017xp18	altr212xa13	3.00E-10
altr011xe01	altr203xp22	4.00E-10
Contig277	altr211xk15	5.00E-10
altr004xl17	altr203xj20	1.00E-09
altr007xj22	Contig18	1.00E-09
altr011xh24	altr202xk13	1.00E-09
altr016xl14	altr212xc20	1.00E-09
Contig296	Contig46	1.00E-09
altr002xm02	altr202xk19	5.00E-09
altr007xn17	altr205xj24	6.00E-09
altr017xm06	altr211xh15	1.00E-08
altr017xk18	Contig124	2.00E-08
altr007xf14	altr205xj24	3.00E-08
altr015xd01	altr212xp10	7.00E-08
altr007xc16	altr204xe04	8.00E-08

Table 3.7 Continued

Query EST	NS EST Hit	E-Value
altr015xp07	altr205xp20	8.00E-08
altr016xi14	altr205xj24	8.00E-08
altr016xm19	altr212xc20	8.00E-08
altr017xb24	altr212xp10	9.00E-08
altr016xe15	altr202xe08	1.00E-07
altr016xf11	altr211xh15	1.00E-07
altr007xn16	altr201xi19	2.00E-07
altr007xg22	altr211xf09	3.00E-07
altr007xk09	altr211xf06	3.00E-07
altr012xo08	altr211xn15	3.00E-07
altr014xb05	Contig234	3.00E-07
altr015xi01	Contig205	3.00E-07
altr002xn17	altr202xm10	4.00E-07
altr007xo18	altr204xe04	4.00E-07
altr012xi12	altr206xg20	4.00E-07
altr017xf24	altr212xo07	4.00E-07
altr017xf20	altr212xk09	5.00E-07
altr007xo19	Contig189	1.00E-06
altr011xl10	altr204xg01	1.00E-06
altr012xd22	altr205xj24	1.00E-06
altr016xb09	altr206xj17	1.00E-06
Contig564	altr201xa01	2.00E-06
altr011xl06	altr211xc16	3.00E-06
altr002xd01	altr208xb10	5.00E-06
altr002xi20	altr213xc20	5.00E-06
altr004xb15	altr204xe01	5.00E-06
altr007xg09	altr202xp16	5.00E-06
altr012xe19	altr213xg08	5.00E-06
altr013xp07	Contig203	5.00E-06
altr014xl19	altr212xp10	5.00E-06
altr014xp22	altr211xf14	7.00E-06
altr004xl23	altr207xk24	2.00E-05
altr016xj05	altr211xd20	2.00E-05
altr017xn12	altr212xa21	2.00E-05
altr007xi11	altr205xj24	3.00E-05
altr017xj04	altr212xe01	5.00E-05
altr004xh10	altr212xo21	6.00E-05
altr011xl16	altr207xg18	6.00E-05
altr007xc11	altr212xd18	7.00E-05
altr010xg14	altr212xg02	7.00E-05
altr004xf16	Contig50	8.00E-05
altr007xn06	altr204xo07	8.00E-05
altr016xc08	altr201xk17	8.00E-05
altr016xl15	Contig89	8.00E-05

Table 3.8. Mixed library ESTs with significant hits ($E \leq 1 \text{ e}^{-5}$) to both the nitrogen starvation *A. brassicicola* library, and plant sequences from the Genbank non-redundant or EST_OTHERS database. If the BLASTN E value (against the nitrogen starvation sequences (NS)) was less than the BLASTX or TBLASTX E value, the sequence was assigned as fungal.

Query EST	BLASTN NS E Value	BLASTX or TBLASTX E Value	Putative Origin
altr002xe10	2.00E-11	7.57E-47	Plant
altr002xh07	1.00E-12	1.97E-018	Plant
altr002xj03	1.00E-09	2.94E-025	Plant
altr002xk07	2.00E-11	9.68E-007	Fungal
altr002xk09	4.00E-12	8.38E-071	Plant
altr002xk12	7.00E-14	7.55E-036	Plant
altr002xo09	2.00E-14	4.00E-24	Plant
altr002xp19	3.00E-19	4.32E-013	Fungal
altr004xb10	3.00E-10	7.00E-06	Fungal
altr004xb17	7.00E-14	3.16E-042	Plant
altr004xd06	2.00E-11	1.78E-051	Plant
altr004xf18	1.00E-15	2.18E-041	Plant
altr004xf24	5.00E-18	3.89E-064	Plant
altr004xh22	7.00E-11	1.00E-06	Fungal
altr004xj02	8.00E-05	1.37E-009	Plant
altr004xl13	3.00E-16	4.10E-054	Plant
altr004xl18	5.00E-12	4.41E-048	Plant
altr004xn16	3.00E-13	2.83E-006	Fungal
altr004xp20	7.00E-11	6.51E-052	Plant
altr006xc19	1.00E-15	3.80E-027	Plant
altr006xd19	2.00E-05	1.01E-059	Plant
altr006xf01	5.00E-15	6.00E-13	Fungal
altr006xk07	7.00E-45	3.62E-038	Fungal
altr006xp14	8.00E-08	2.04E-027	Plant
altr007xa04	1.00E-15	1.16E-013	Fungal
altr007xa08	8.00E-17	4.36E-010	Fungal
altr007xb10	2.00E-17	2.00E-05	Fungal
altr007xc04	2.00E-14	1.35E-007	Fungal
altr007xc15	8.00E-11	2.64E-024	Plant
altr007xd08	5.00E-18	1.23E-044	Plant
altr007xe01	3.00E-10	7.68E-014	Plant
altr007xe19	1.00E-12	5.27E-029	Plant
altr007xe20	2.00E-08	2.00E-11	Plant
altr007xf01	2.00E-17	4.96E-017	Fungal
altr007xf05	8.00E-14	3.00E-07	Fungal
altr007xf06	2.00E-17	5.00E-06	Fungal
altr007xg05	1.00E-18	6.20E-010	Fungal
altr007xi09	3.00E-10	2.03E-017	Plant
altr007xi19	7.00E-11	1.00E-64	Plant
altr007xk10	1.00E-12	2.00E-06	Fungal
altr007xk17	5.00E-12	1.02E-017	Plant
altr007xl14	1.00E-06	4.62E-086	Plant
altr007xm15	5.00E-15	1.00E-07	Fungal
altr007xo03	7.00E-08	7.00E-07	Fungal
altr007xp14	6.00E-09	4.00E-06	Fungal
altr009xb04	1.00E-21	2.29E-069	Plant
altr009xb10	7.00E-26	6.00E-09	Fungal

Table 3.8 Continued

Query EST	BLASTN NS E Value	BLASTX or TBLASTX E Value	Putative Origin
altr009xc07	2.00E-17	3.00E-08	Fungal
altr009xc23	1.00E-12	1.30E-036	Plant
altr009xd20	1.00E-08	4.73E-009	Plant
altr009xf06	7.00E-20	2.83E-025	Plant
altr009xj10	2.00E-07	2.85E-047	Plant
altr009xl04	1.00E-140	7.00E-32	Fungal
altr009xl08	2.00E-13	3.67E-074	Plant
altr009xn04	9.00E-07	2.00E-12	Plant
altr009xo06	8.00E-17	1.15E-077	Plant
altr010xa13	7.00E-23	7.19E-100	Plant
altr010xb04	5.00E-58	6.96E-030	Fungal
altr010xd24	1.00E-37	1.56E-066	Plant
altr010xi01	2.00E-17	3.00E-07	Fungal
altr010xi03	1.00E-18	5.83E-049	Plant
altr010xk23	2.00E-07	1.19E-032	Plant
altr010xm21	8.00E-17	5.43E-022	Plant
altr011xb16	3.00E-13	2.25E-011	Fungal
altr011xc07	8.00E-17	4.17E-010	Fungal
altr011xd22	8.00E-94	8.00E-05	Fungal
altr011xe19	2.00E-14	4.16E-010	Fungal
altr011xh02	9.00E-35	1.56E-038	Plant
altr011xi01	7.00E-11	2.23E-117	Plant
altr011xk15	2.00E-17	1.19E-046	Plant
altr011xk21	2.00E-17	4.77E-022	Plant
altr011xl02	1.00E-15	3.00E-35	Plant
altr011xn04	1.00E-18	6.36E-018	Fungal
altr011xn14	5.00E-12	4.18E-019	Plant
altr011xn20	5.00E-15	5.11E-021	Plant
altr011xp18	7.00E-08	2.49E-021	Plant
altr012xc08	4.00E-40	4.00E-05	Fungal
altr012xc16	4.00E-15	3.91E-059	Plant
altr012xe20	7.00E-20	7.48E-066	Plant
altr012xe22	1.00E-09	3.22E-015	Plant
altr012xf03	5.00E-18	4.57E-048	Plant
altr012xf13	1.00E-18	5.43E-026	Plant
altr012xg08	2.00E-17	1.71E-014	Fungal
altr012xh06	2.00E-08	1.00E-11	Plant
altr012xj07	5.00E-18	1.00E-24	Plant
altr012xk08	3.00E-22	2.75E-022	Plant
altr012xn12	2.00E-14	3.00E-09	Fungal
altr012xp03	4.00E-37	5.00E-18	Fungal
altr013xc09	2.00E-11	8.69E-053	Plant
altr013xd10	2.00E-08	8.88E-006	Fungal
altr013xd14	8.00E-08	3.71E-013	Plant
altr013xe11	1.00E-06	1.00E-10	Plant
altr013xl12	3.00E-10	2.81E-040	Plant
altr013xl19	5.00E-15	3.71E-091	Plant
altr013xp13	4.00E-34	2.99E-067	Plant
altr014xa04	6.00E-30	8.59E-024	Fungal
altr014xa22	2.00E-14	4.00E-10	Fungal
altr014xh06	5.00E-09	7.48E-014	Plant
altr014xi24	7.00E-05	2.08E-018	Plant

Table 3.8 Continued

Query EST	BLASTN NS E Value	BLASTX or TBLASTX E Value	Putative Origin
altr014xj02	1.00E-118	1.22E-009	Fungal
altr014xn02	1.00E-43	1.80E-053	Plant
altr014xn11	7.00E-85	6.56E-056	Fungal
altr015xa16	1.00E-06	7.93E-024	Plant
altr015xa17	4.00E-09	1.00E-08	Fungal
altr015xa23	5.00E-15	1.65E-011	Fungal
altr015xc09	5.00E-18	8.17E-018	Fungal
altr015xc12	2.00E-73	2.79E-017	Fungal
altr015xf14	5.00E-18	6.00E-05	Fungal
altr015xf22	7.00E-05	1.54E-026	Plant
altr015xg23	1.00E-06	3.00E-09	Plant
altr015xi04	1.00E-09	1.68E-011	Plant
altr015xl01	3.00E-13	2.58E-052	Plant
altr015xm24	8.00E-17	8.81E-008	Fungal
altr015xo12	1.00E-15	2.21E-011	Fungal
altr015xo20	7.00E-05	1.21E-016	Plant
altr015xp12	3.00E-07	1.37E-008	Plant
altr016xb07	5.00E-15	7.90E-047	Plant
altr016xb24	2.00E-05	2.23E-051	Plant
altr016xe12	4.00E-09	3.00E-06	Fungal
altr016xf04	4.00E-06	9.03E-030	Plant
altr016xg04	1.00E-34	8.27E-062	Plant
altr016xi19	2.00E-08	2.00E-07	Fungal
altr017xa06	1.00E-05	2.34E-082	Plant
altr017xb04	5.00E-21	8.61E-036	Plant
altr017xc02	2.00E-13	3.55E-017	Plant
altr017xc10	3.00E-22	1.13E-081	Plant
altr017xe16	7.00E-08	3.10E-032	Plant
altr017xe22	4.00E-09	1.00E-11	Plant
altr017xf06	3.00E-13	5.26E-020	Plant
altr017xf08	2.00E-14	7.27E-048	Plant
altr017xf16	1.00E-05	9.42E-072	Plant
altr017xh08	1.00E-09	1.92E-045	Plant
altr017xh16	5.00E-06	6.00E-12	Plant
altr017xi10	6.00E-14	2.06E-041	Plant
altr017xj02	1.00E-12	4.89E-038	Plant
altr017xk14	4.00E-09	1.00E-18	Plant
altr017xl24	2.00E-11	1.00E-05	Fungal
altr017xm04	2.00E-16	3.19E-020	Plant
altr017xn04	2.00E-17	2.15E-014	Fungal
altr017xn22	4.00E-18	1.08E-032	Plant
altr017xp04	4.00E-15	5.80E-081	Plant
altr017xp16	5.00E-05	1.00E-47	Plant
Contig10	2.00E-07	3.59E-042	Plant
Contig102	8.00E-07	2.02E-030	Plant
Contig103	4.00E-14	4.99E-039	Plant
Contig133	6.00E-17	5.03E-054	Plant
Contig134	6.00E-17	1.77E-117	Plant
Contig150	2.00E-23	1.00E-14	Fungal
Contig151	0	1.36E-037	Fungal
Contig155	1.00E-17	3.28E-076	Plant
Contig159	6.00E-17	8.93E-074	Plant

Table 3.8 Continued

Query EST	BLASTN NS E Value	BLASTX or TBLASTX E Value	Putative Origin
Contig160	3.00E-12	1.30E-039	Plant
Contig171	2.00E-07	8.46E-042	Plant
Contig173	5.00E-17	3.58E-047	Plant
Contig181	1.00E-11	1.17E-070	Plant
Contig187	4.00E-35	5.50E-024	Fungal
Contig193	1.00E-18	7.16E-026	Plant
Contig194	5.00E-14	2.68E-058	Plant
Contig199	2.00E-14	3.31E-108	Plant
Contig201	1.00E-05	1.54E-044	Plant
Contig205	5.00E-08	6.97E-015	Plant
Contig208	1.00E-15	2.68E-109	Plant
Contig211	2.00E-13	3.89E-071	Plant
Contig212	3.00E-21	2.03E-015	Fungal
Contig213	2.00E-25	6.66E-006	Fungal
Contig220	1.00E-14	2.65E-079	Plant
Contig224	5.00E-17	1.10E-014	Fungal
Contig234	2.00E-10	6.07E-051	Plant
Contig240	2.00E-15	8.00E-08	Fungal
Contig245	2.00E-18	3.48E-034	Plant
Contig255	2.00E-13	2.71E-054	Plant
Contig260	2.00E-13	1.75E-032	Plant
Contig278	5.00E-15	1.58E-033	Plant
Contig279	1.00E-07	1.56E-018	Plant
Contig281	4.00E-18	5.19E-059	Plant
Contig289	4.00E-17	2.00E-06	Fungal
Contig295	8.00E-14	2.34E-087	Plant
Contig3	2.00E-15	8.00E-07	Fungal
Contig308	2.00E-22	4.32E-061	Plant
Contig313	1.00E-180	9.35E-098	Fungal
Contig317	4.00E-11	2.33E-037	Plant
Contig322	2.00E-11	4.73E-051	Plant
Contig327	2.00E-13	2.93E-088	Plant
Contig328	8.00E-16	2.89E-052	Plant
Contig33	8.00E-13	3.91E-085	Plant
Contig342	5.00E-17	4.67E-021	Plant
Contig344	8.00E-13	1.14E-073	Plant
Contig346	4.00E-11	9.44E-024	Plant
Contig349	2.00E-13	2.19E-052	Plant
Contig350	4.00E-07	4.13E-121	Plant
Contig351	7.00E-10	5.71E-066	Plant
Contig352	2.00E-15	2.47E-013	Fungal
Contig354	5.00E-05	9.95E-085	Plant
Contig363	5.00E-11	4.08E-013	Plant
Contig364	8.00E-16	3.77E-062	Plant
Contig365	4.00E-18	2.02E-087	Plant
Contig38	1.00E-05	9.28E-048	Plant
Contig384	3.00E-10	6.66E-131	Plant
Contig39	2.00E-16	2.85E-023	Plant
Contig393	1.00E-12	1.38E-039	Plant
Contig394	4.00E-11	4.15E-022	Plant

Table 3.8 Continued

Query EST	BLASTN NS E Value	BLASTX or TBLASTX E Value	Putative Origin
Contig402	4.00E-11	5.34E-041	Plant
Contig405	3.00E-25	5.79E-064	Plant
Contig406	2.00E-10	3.67E-024	Plant
Contig424	2.00E-25	3.00E-08	Fungal
Contig429	1.00E-100	1.07E-026	Fungal
Contig432	6.00E-12	3.14E-042	Plant
Contig435	1.00E-06	1.01E-050	Plant
Contig44	5.00E-05	1.04E-019	Plant
Contig450	5.00E-14	1.20E-009	Fungal
Contig459	1.00E-12	9.00E-07	Fungal
Contig46	1.00E-13	7.00E-09	Fungal
Contig464	4.00E-06	9.07E-064	Plant
Contig470	3.00E-09	3.56E-063	Plant
Contig477	2.00E-80	8.49E-039	Fungal
Contig48	1.00E-10	1.84E-013	Plant
Contig482	1.00E-129	1.76E-016	Fungal
Contig484	5.00E-13	3.37E-161	Plant
Contig485	4.00E-14	2.26E-054	Plant
Contig488	0	4.72E-006	Fungal
Contig492	1.00E-176	8.00E-05	Fungal
Contig494	2.00E-17	3.97E-007	Fungal
Contig50	1.00E-08	2.40E-094	Plant
Contig516	4.00E-11	8.30E-044	Plant
Contig521	1.00E-06	3.40E-092	Plant
Contig527	4.00E-15	1.23E-056	Plant
Contig529	1.00E-130	1.00E-31	Fungal
Contig535	2.00E-09	1.00E-13	Plant
Contig537	7.00E-10	8.16E-014	Plant
Contig547	2.00E-10	2.94E-024	Plant
Contig551	1.00E-08	5.47E-056	Plant
Contig557	9.00E-08	3.26E-031	Plant
Contig576	1.00E-19	6.88E-013	Fungal
Contig583	4.00E-07	1.22E-021	Plant
Contig584	5.00E-12	5.40E-021	Plant
Contig590	1.00E-14	3.79E-065	Plant
Contig591	1.00E-11	2.01E-087	Plant
Contig595	3.00E-12	1.80E-046	Plant
Contig596	1.00E-55	2.85E-049	Fungal
Contig597	1.00E-12	1.39E-111	Plant
Contig603	3.00E-06	1.22E-022	Plant
Contig63	2.00E-13	2.59E-027	Plant
Contig65	1.00E-09	1.00E-05	Fungal
Contig67	6.00E-11	6.39E-055	Plant
Contig70	6.00E-14	9.62E-060	Plant
Contig89	4.00E-16	6.98E-043	Plant
Contig9	8.00E-18	5.95E-016	Fungal
Contig92	5.00E-17	2.39E-024	Plant

Table 3.11. ESTs from *Alternaria brassicicola* nitrogen starvation library with significant BLASTX hits ($E \leq 1e^{-5}$) to fungal sequences (other than *Neurospora crassa*) in the Genbank non-redundant database.

Query EST	Hit	E Value	Description
Contig14	P28844	1E-179	glyceraldehyd-3-phosphate dehydrogenase [Cochliobolus lunatus]
Contig209	BAA36503	1E-133	1,3,8-trihydroxynaphthalene reductase [Alternaria alternata]
Contig4	AAK19308	1E-114	polyubiquitin [Tuber borchii]
altr212xh02	AAM89498	1E-110	isocitrate lyase [Leptosphaeria maculans]
altr212xe24	AAM81358	1E-106	aspartyl proteinase [Leptosphaeria maculans]
altr211xc11	AAG32629	1E-104	mannose phospho-dolichol synthase [Hypocrea jecorina]
altr202xn23	AAN28666	2E-95	putative mannitol dehydrogenase [Alternaria alternata]
Contig222	P04828	3E-95	triosephosphate isomerase [Emericella nidulans]
altr213xm12	AAN28666	2E-93	putative mannitol dehydrogenase [Alternaria alternata]
altr208xh14	AAL26313	1E-91	formaldehyde dehydrogenase [Pichia angusta]
altr213xo24	AAF66992	4E-83	beta-tubulin [Leptosphaeria maculans]
altr211xk04	CAD80253	1E-76	phosphoglucose isomerase [Aspergillus niger]
altr203xn03	AAN59953	1E-76	polyketide synthase 1 [Glarea lozoyensis]
altr212xp05	AAO64256	5E-75	putative ribosomal protein S28 [Aspergillus fumigatus]
altr203xp18	AAM89501	2E-74	mitogen-activated protein kinase [Leptosphaeria maculans]
altr203xn04	NP_011768	2E-73	acetylate nucleosomal histones [Saccharomyces cerevisiae]
Contig48	CAA59939	8E-72	delta-9 fatty acid desaturase [Ajellomyces capsulatus]
altr211xk03	P41752	1E-68	translation elongation factor 1 alpha [Eremothecium gossypii]
Contig13	CAD80254	3E-66	6-phosphogluconate dehydrogenase [Aspergillus niger]
Contig55	P24590	3E-66	phosphoglycerate kinase (EC 2.7.2.3) - fungus (Trichoderma viride)
altr205xd13	NP_596350	2E-64	40s ribosomal protein S4A/S4.1 [Schizosaccharomyces pombe]
Contig146	AAC13691	7E-63	poly-ubiquitin [Magnaporthe grisea]
altr213xd15	AAF40140	4E-62	beta (1-3) glucanosyltransferase Gel3p [Aspergillus fumigatus]
Contig206	NP_593923	5E-62	ubiquitin fusion protein [Schizosaccharomyces pombe]
altr211xe23	CAD60755	4E-61	unnamed protein product [Podospora anserina]
altr205xb19	AAM18717	9E-61	major allergen-like protein precursor [Alternaria brassicicola]
Contig168	AAO38980	2E-60	40S ribosomal S12 protein [Paracoccidioides brasiliensis]
altr211xp02	CAA59939	2E-59	delta-9 fatty acid desaturase [Ajellomyces capsulatus]
altr213xb09	AAD54383	1E-58	ribosomal protein L16a [Emericella nidulans]
Contig128	AAM44826	1E-56	stearic acid desaturase [Emericella nidulans]
altr201xl02	P27800	2E-56	[Sporidiobolus salmonicolor] NADPH-dependent aldehyde reductase
altr201xj02	CAC85639	3E-56	serine protease 2 [Pyrenopeziza brassicae]
altr206xj23	P34054	4E-56	INDA1 [Trichoderma harzianum]
Contig12	AAO24266	4E-56	histone [Alternaria arborescens]
altr202xe19	P56525	9E-56	ATP synthase delta chain, [Magnaporthe grisea]
altr203xd14	AAC09045	2E-54	GATA transcription factor [Penicillium chrysogenum]
Contig91	AAO24266	1E-53	histone [Alternaria arborescens]
Contig94	AAM93545	3E-53	putative polyketide synthase [Xylaria sp. BCC 1067]
altr213xp12	P23753	2E-52	histone H3 [Penicillium funiculosum]
Contig68	CAD28443	6E-50	possible swi2/snf2-like protein [Aspergillus fumigatus]
altr212xa05	P52810	2E-49	cytoplasmic ribosomal protein S7 [Podospora anserina]
Contig233	AAM18717	3E-48	major allergen-like protein precursor [Alternaria brassicicola]
altr211xh07	P29497	1E-47	glyceraldehyde 3-phosphate dehydro [Cochliobolus heterostrophus]
altr203xf24	O74238	8E-46	Protein SNODPROT1 [Phaeosphaeria nodorum]
altr201xn12	S59950	1E-45	het-c2 protein [Podospora anserina]
altr211xn21	P42058	1E-45	Minor allergen Alt a 7 (Alt a VII) [Alternaria alternata]
altr212xg09	P14862	1E-45	mitochondrion ATPase subunit 6 [Cochliobolus heterostrophus]
altr207xh08	O94083	5E-45	Eukaryotic translation initiation factor 5A eIF-5A [Candida albicans]
altr203xh24	O74196	7E-44	Ubiquitin carrier protein (Colletotrichum hard-surface- 1)
altr205xf14	NP_595126	1E-43	similar to yeast cdc50 [Schizosaccharomyces pombe]

Table 3.11 Continued

Query EST	Genbank	E Value	Description
altr202xj23	NP_595071	1E-42	adenosine deaminase [Schizosaccharomyces pombe]
Contig249	Q00251	2E-42	translation elongation factor eEF-1 [Aureobasidium pullulans]
altr207xm21	CAD21517	2E-41	putative iron transferase [Claviceps purpurea]
altr211xn03	S51497	8E-41	peptidylprolyl isomerase (EC 5.2.1.8) A - cyclosporin fungus
altr201xl08	AAG40862	2E-38	ribonucleotide reductase [Emericella nidulans]
Contig181	P23751	3E-38	histone H4.2 [Emericella nidulans]
altr202xf21	O93866	1E-37	heat shock protein 70 [Trichophyton rubrum]
altr212xa13	P55059	4E-37	protein disulfide-isomerase precursor [Humicola insolens]
altr213xl10	NP_074923	9E-37	GIY Cytb i2 grp ID protein [Podospora anserina]
altr202xj11	AAF82115	2E-36	cobalamin-independent methionine synthase [Aspergillus nidulans]
altr211xf06	AAL76065	4E-36	Gap1 protein [Candida albicans]
altr203xl07	NP_595126	5E-36	similar to yeast cdc50 [Schizosaccharomyces pombe]
altr213xn21	P29497	1E-35	glyceraldehyde 3-phosphate dehydrogenase [Cochliobolus heterostrophus]
altr212xf01	CAD60618	2E-33	unnamed protein product [Podospora anserina]
altr203xd19	NP_011990	8E-32	Yhr122wp [Saccharomyces cerevisiae]
altr206xl15	S59950	2E-30	het-c2 protein [Podospora anserine]
altr201xh23	NP_012628	3E-30	YJR094w-a [Saccharomyces cerevisiae]
altr212xo07	Q9Y8A7	4E-30	small zinc finger-like protein [Emericella nidulans]
altr204xm17	CAD80253	3E-29	phosphoglucose isomerase [Aspergillus niger]
altr211xe09	P14862	2E-28	ATPase subunit 6 (AA 1-257) [Cochliobolus heterostrophus]
altr213xn16	S59950	1E-27	het-c2 protein [Podospora anserine]
altr212xj04	CAD80253	5E-27	phosphoglucose isomerase [Aspergillus niger]
altr207xj15	AAF89327	1E-26	ORF1 [Trichoderma harzianum]
altr203xd12	AAO14099	2E-26	NADH-ubiquinone oxidoreductase subunit 5 [Cryphonectria parasitica]
altr202xf09	CAB65007	9E-26	transmembrane protein [Erysiphe pisi]
altr213xm22	AAM48764	1E-25	cytochrome P450 [Fusarium cerealis]
altr208xl02	AAK69534	8E-25	CAP20-like protein [Blumeria graminis]
altr203xh09	CAD43407	1E-24	tetraspanin [Colletotrichum lindemuthianum]
altr211xn10	CAD60692	3E-24	unnamed protein product [Podospora anserina]
altr206xf07	Q12731	1E-22	TATA-box binding protein [Emericella nidulans]
altr207xd16	BAC57614	1E-22	translation elongation factor 1-alpha [Piptopezalis freseniana]
altr207xk08	AAK31199	1E-22	general amidase-C [Emericella nidulans]
altr211xe03	P40918	1E-22	Heat shock 70 kDa protein (Allergen Cla h 4) [Cladosporium herbarum]
altr206xe05	CAA75566	3E-22	cytochrome P450 monooxygenase [Gibberella fujikuroi]
altr201xp01	CAA10666	3E-21	ATP-citrat-lyase [Gibberella pulicaris]
altr202xm13	P29429	1E-20	thioredoxin [Aspergillus nidulans, Peptide, 109 aa]
altr206xi10	AAK60140	3E-20	ribosomal protein L39 [Candida albicans]
altr213xj22	NP_015182	8E-20	YPL143w [Saccharomyces cerevisiae]
altr205xj02	NP_594214	9E-20	YGL010W [Schizosaccharomyces pombe]
altr201xd10	NP_594017	4E-19	dolichol-phosphate mannosyltransferase [Schizosaccharomyces pombe]
altr207xa15	BAA36216	6E-19	endoglucanase II [Trichoderma viride]
altr202xf16	CAC17834	1E-18	secretion related GTPase (SrgC) [Aspergillus niger]
altr207xo17	AAK11296	2E-18	scytalone dehydratase [Ophiostoma floccosum]
altr202xi19	T30568	5E-18	acetyl-CoA carboxylase [Emericella nidulans]
altr211xe15	Q9P4C8	4E-16	GTP-binding protein [Pichia pastoris]
altr204xg20	P02382	5E-16	L-rRNA intron frame (AA 1-410) [Emericella nidulans]
altr203xp03	S60224	3E-15	polyketide synthase-1 [Colletotrichum lagenarium]
altr206xi24	CAD32176	1E-14	multidrug resistant protein [Acremonium chrysogenum]
altr201xb13	NP_587891	2E-14	60s ribosomal protein 110a. [Schizosaccharomyces pombe]
altr205xd03	NP_588270	3E-13	putative short chain dehydrogenase [Schizosaccharomyces pombe]
altr207xh11	AAO38980	2E-12	40S ribosomal S12 protein [Paracoccidioides brasiliensis]
altr206xa03	NP_587948	1E-11	hypothetical protein [Schizosaccharomyces pombe]

Table 3.11 Continued

Query EST	Genbank	E Value	Description
altr211xj23	AAB41493	2.00E-11	fatty acid synthase, alpha subunit [Emericella nidulans]
Contig240	P04828	2.00E-11	triosephosphate isomerase [Emericella nidulans]
altr205xd16	BAB79692	1.00E-10	chitin binding protein [Magnaporthe grisea]
altr204xo22	CAD60610	5.00E-10	unnamed protein product [Podospira anserina]
Contig230	P14862	6.00E-10	ATPase subunit 6 (AA 1-257) [Cochliobolus heterostrophus]
altr212xa14	AAN11327	9.00E-10	ADP-ATP translocase [Gaeumannomyces graminis var. tritici]
altr203xp19	AAL27457	1.00E-09	manganese-superoxide dismutase [Glomerella graminicola]
altr207xd21	Q9Y7B6	2.00E-09	ketopantoate hydroxymethyltransferase [Emericella nidulans]
altr211xf13	pdb 1K3I A	3.00E-09	Chain A, Crystal Structure Of The Precursor Of Galactose Oxidase
altr211xf02	NP_012955	6.00E-09	gea1-6 membrane-associated suppressor [Saccharomyces cerevisiae]
altr212xk14	NP_588218	6.00E-09	putative amino-acid permease. [Schizosaccharomyces pombe]
altr212xm19	Q00746	8.00E-09	Aspergillus nidulans antigen 1 [Emericella nidulans]
altr203xd09	AAK84438	9.00E-09	chitin deacetylase [Blumeria graminis]
altr213xj07	pdb 1N8P A	1.00E-08	Chain D, Crystal Structure Of Cystathionine Gamma-Lyase From
altr202xo15	BAC65217	2.00E-07	trichothecene 3-O-acetyltransferase [Fusarium sp. IFO 7772]
altr201xn11	NP_011445	4.00E-07	RNA polymerase II subunit RPB9 [Saccharomyces cerevisiae]
altr201xn16	Q9HFR7	5.00E-07	60S ribosomal protein [Trichoderma hamatum]
altr213xc20	NP_014398	1.00E-06	mitochondrial citrate synthase [Saccharomyces cerevisiae]
altr203xf03	P06835	3.00E-06	beta-glucosidase precursor [Pichia anomala]
altr207xb21	AAA98076	4.00E-06	nuclear protein
altr206xo05	JC4564	5.00E-06	antifungal protein precursor [Penicillium chrysogenum]
altr211xo11	O60039	5.00E-06	Anucleate primary sterigmata protein apsB [Emericella nidulans]
altr207xm09	NP_013897	6.00E-06	DNA damage inducibleDdr48p [Saccharomyces cerevisiae]
altr212xh21	NP_593235	8.00E-06	DNA-RNA polymerase III C11 subunit [Schizosaccharomyces pombe]

Table 3.12. TBLASTX hits from *Alternaria brassicicola* nitrogen starvation library with highest significant hit to fungal ESTs in Genbank EST_OTHERS database.

Query EST	Genbank Hit	E-value	Description
Contig189	BI416947	2.00E-70	Heterobasidion annosum SSH cDNA
Contig8	CB331858	3.00E-70	Aspergillus fumigatus cDNA
altr205xj06	BU635792	7.00E-69	Infected Arabidopsis Leaf Arabidopsis thaliana
Contig227	CA405393	1.00E-64	A. brassicicola SSH cDNA clone P3D2
Contig69	AW792429	9.00E-64	Blumeria graminis f. sp. hordei cDNA clone
altr204xc09	BQ750908	2.00E-61	Colletotrichum trifolii cDNA clone
altr213xb02	CA405389	3.00E-56	A. brassicicola cDNA clone P3H1
Contig154	CA405393	9.00E-56	A. brassicicola SSH cDNA clone P3D2
altr207xf07	CA405393	3.00E-55	A. brassicicola SSH cDNA clone P3D2
altr205xh20	BG370084	5.00E-53	V8 mycelial library Leptosphaeria maculans cDNA
Contig132	CA580379	6.00E-48	Paracoccidioides brasiliensis cDNA
altr211xf11	CA405393	1.00E-47	A. brassicicola SSH cDNA clone P3D2
altr202xm19	CA405393	2.00E-42	A. brassicicola SSH cDNA clone P3D2
altr205xb04	BF253295	3.00E-39	Coccidioides immitis spherule cDNA library
altr202xh01	CA405393	3.00E-38	A. brassicicola SSH cDNA clone P3D2
Contig169	BG370066	3.00E-38	V8 mycelial library Leptosphaeria maculans
altr207xj21	CA405393	5.00E-38	A. brassicicola SSH cDNA clone P3D2
altr207xf08	CA405393	1.00E-37	A. brassicicola SSH cDNA clone P3D2
altr212xe13	CA405393	7.00E-36	A. brassicicola SSH cDNA clone P3D2
altr207xh04	CA405395	3.00E-35	A. brassicicola SSH cDNA clone P2B11
altr207xd04	CA405404	2.00E-33	A. brassicicola SSH cDNA clone P3B2
Contig152	CA405393	8.00E-32	A. brassicicola SSH cDNA clone P3D2
altr202xc13	CF943837	1.00E-29	Hypocrea jecorina cDNA clone
altr205xd16	BM872642	5.00E-29	Magnaporthe grisea cDNA clone
altr201xo07	BF253295	5.00E-29	Coccidioides immitis spherule cDNA library
altr206xe01	CB688528	3.00E-28	Cryphonectria parasitica cDNA clone
altr207xd14	BF253295	3.00E-26	Coccidioides immitis spherule cDNA library
Contig36	BF251125	5.00E-26	Coccidioides immitis spherule cDNA library
altr212xn18	AI210086	3.00E-25	Aspergillus nidulans 24hr asexual developmental
altr213xl14	BF253295	1.00E-24	Coccidioides immitis spherule cDNA library
altr212xn17	CA405393	3.00E-23	A. brassicicola SSH cDNA clone P3D2
altr204xc04	BQ139997	3.00E-21	Phoma-infected Medicago truncatula cDNA clone
Contig202	CB900321	3.00E-21	T.reesei mycelial culture, Version 3
Contig179	BG370130	7.00E-21	V8 mycelial library Leptosphaeria maculans cDNA
Contig124	BG370130	4.00E-20	V8 mycelial library Leptosphaeria maculans cDNA
altr212xe23	BM864436	7.00E-20	Magnaporthe grisea cDNA clone
altr207xg19	CA405393	1.00E-19	A. brassicicola SSH cDNA clone P3D2
altr206xh19	CA405376	2.00E-19	A. brassicicola SSH clone P3E1 NADH dehydroge.
Contig236	BG370064	2.00E-18	V8 mycelial library Leptosphaeria maculans cDNA
altr207xi17	CF943837	3.00E-18	Hypocrea jecorina cDNA clone
altr204xe01	BG370064	5.00E-18	V8 mycelial library Leptosphaeria maculans cDNA
Contig32	BF251125	1.00E-17	Coccidioides immitis spherule cDNA library
Contig166	BG370064	2.00E-17	V8 mycelial library Leptosphaeria maculans cDNA
altr201xn06	BG370064	2.00E-16	V8 mycelial library Leptosphaeria maculans cDNA
altr213xn22	BF253295	3.00E-16	Coccidioides immitis spherule cDNA library
altr213xg04	CA405374	5.00E-16	A. brassicicola SSH cDNA clone P3D8
altr207xo10	CB331858	5.00E-16	Aspergillus fumigatus cDNA Library
altr205xl15	BP099243	1.00E-15	Chaetomium globosum cDNA clone
Contig162	BF251275	1.00E-15	Coccidioides immitis spherule cDNA library
altr201xc24	CA405393	2.00E-15	A. brassicicola SSH cDNA clone P3D2

Table 3.12 Continued

Query EST	Genbank Hit	E-Value	Description
altr202xb20	BE759358	7.00E-15	<i>Aspergillus niger</i> cDNA clone
altr207xb02	BF251275	8.00E-15	<i>Coccidioides immitis</i> spherule cDNA library
altr201xn13	BQ495604	2.00E-14	<i>Paracoccidioides brasiliensis</i> cDNA
altr206xk17	AW790255	3.00E-14	<i>Blumeria graminis</i> f. sp. <i>hordei</i> cDNA clone
altr206xd21	BG370131	3.00E-14	V8 mycelial library <i>Leptosphaeria maculans</i> cDNA
altr206xh11	CA405393	2.00E-13	<i>A. brassicicola</i> SSH cDNA clone P3D2
Contig127	BF251125	3.00E-13	<i>Coccidioides immitis</i> spherule cDNA library
altr204xg14	BG370026	1.00E-12	V8 mycelial library <i>Leptosphaeria maculans</i> cDNA
Contig174	BG370066	2.00E-12	V8 mycelial library <i>Leptosphaeria maculans</i> cDNA
altr212xb13	BF253295	2.00E-11	<i>Coccidioides immitis</i> spherule cDNA library
altr207xm08	CA405393	3.00E-11	<i>A. brassicicola</i> SSH cDNA clone P3D2
altr211xf21	BI416947	3.00E-11	<i>Heterobasidion annosum</i> infection stage SSH cDNA
altr213xh20	CA405393	3.00E-11	<i>A. brassicicola</i> SSH cDNA clone P3D2
altr211xo12	BE758769	4.00E-11	<i>Aspergillus niger</i> cDNA clone
altr213xe06	CA405393	4.00E-11	<i>A. brassicicola</i> SSH cDNA clone P3D2
Contig89	BG370094	1.00E-10	V8 mycelial library <i>Leptosphaeria maculans</i> cDNA
Contig172	BG370094	1.00E-10	V8 mycelial library <i>Leptosphaeria maculans</i> cDNA
altr212xi11	BP099767	4.00E-10	<i>Chaetomium globosum</i> cDNA clone
altr213xl20	BQ504111	9.00E-10	<i>Paracoccidioides brasiliensis</i> cDNA
altr207xj09	CA405393	2.00E-09	<i>A. brassicicola</i> SSH cDNA clone P3D2
altr212xd23	CD036985	5.00E-09	<i>Magnaporthe grisea</i> subtracted mycelial library
altr211xl11	CF874342	7.00E-09	<i>T.reesei</i> mycelial culture, Version 6
altr205xl11	BQ751427	7.00E-09	<i>Colletotrichum trifolii</i> cDNA
altr201xh19	BQ751215	3.00E-08	<i>Colletotrichum trifolii</i> cDNA
altr212xk07	BQ498600	4.00E-08	<i>Paracoccidioides brasiliensis</i> cDNA
altr207xe07	BQ752241	4.00E-08	<i>Colletotrichum trifolii</i> cDNA
altr213xd08	BU642803	1.00E-07	<i>Magnaporthe grisea</i> cDNA
altr213xb23	BG810400	1.00E-07	<i>Magnaporthe grisea</i> Appressorium Stage cDNA
altr204xo03	BQ110342	2.00E-07	<i>Verticillium dahliae</i> cDNA
altr202xg21	CF877117	6.00E-07	<i>T.reesei</i> mycelial culture, Version 6
altr212xl10	CF873255	2.00E-06	<i>T.reesei</i> mycelial culture, Version 6
altr205xb02	CA405374	3.00E-06	<i>A. brassicicola</i> SSH P3D8 <i>Ketol Acid Reductoiso.</i>
altr213xb05	CF874342	3.00E-06	<i>T.reesei</i> mycelial culture, Version 6

Figure 3.1. Suppression subtractive hybridization of mixed *Alternaria brassicicola* – *Brassica oleracea* var. *capitata*. A. Double stranded cDNA synthesis of the driver (Lane 2) and tester (Lane 3) cDNA, 17 cycles. Ladder in lane 1 is 1 kb. B. *RsaI* digestion of double stranded cDNA. Lane 1 = undigested tester cDNA, 2 = digested tester cDNA, 3 = undigested driver cDNA, 4 = digested driver cDNA. C. Primary PCR of subtracted products. Lane 1 = 500 bp ladder, 2 = forward subtracted products, 3 = unsubtracted tester control, 4 = reverse subtracted products, 5 = unsubtracted reverse control. D. Secondary PCR of subtracted products. Lane 1 = 500 bp ladder, 2 = forward subtracted products, 3 = unsubtracted tester control, 4 = reverse subtracted products, 5 = unsubtracted reverse control. E. Subtraction efficiency PCR. Beta tubulin was used to assess the subtraction efficiency. Lanes 1-4 are the forward subtraction, 18, 23, 28, and 33 cycles respectively. Lanes 5-8 are the unsubtracted tester control, 18, 23, 28, and 33 cycles respectively.

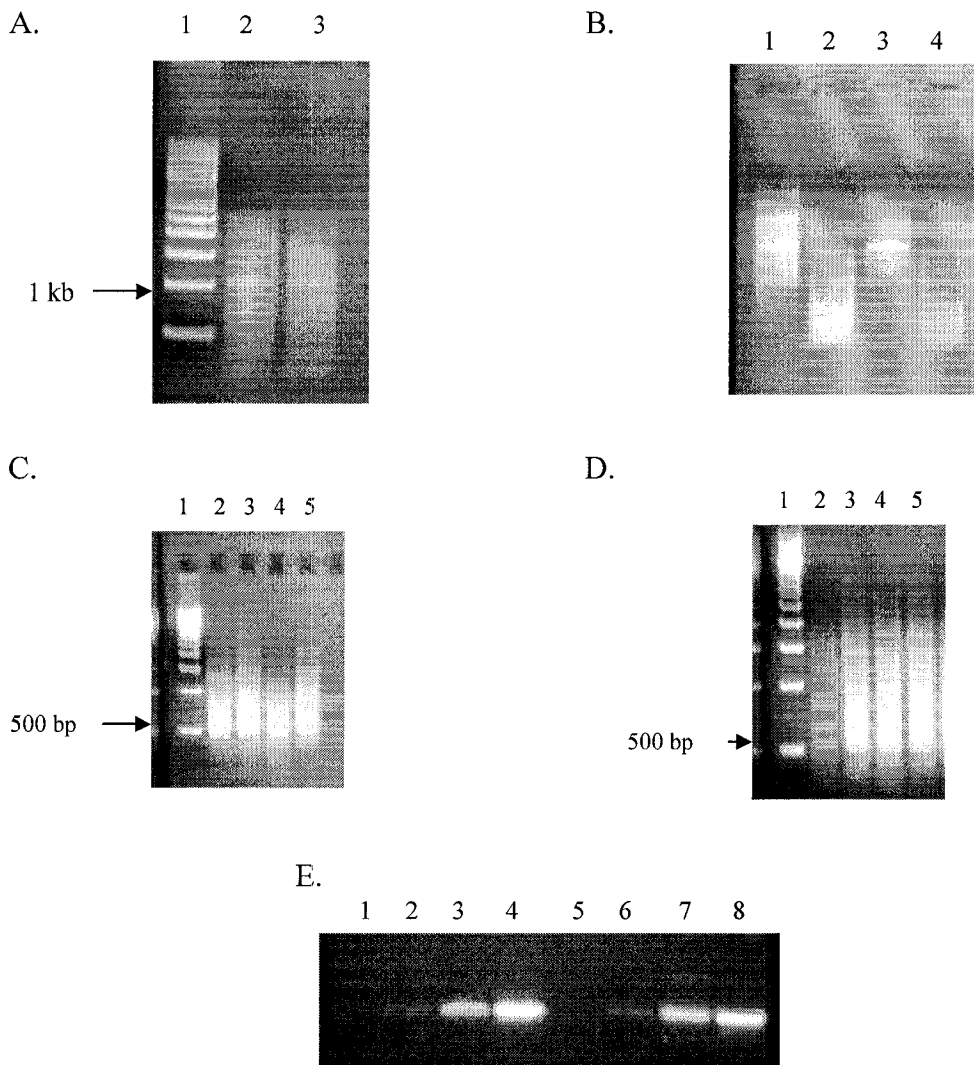


Figure 3.2. GO annotation of ESTs in *Alternaria brassicicola* – *Brassica oleraceae* var. *capitata* mixed library.

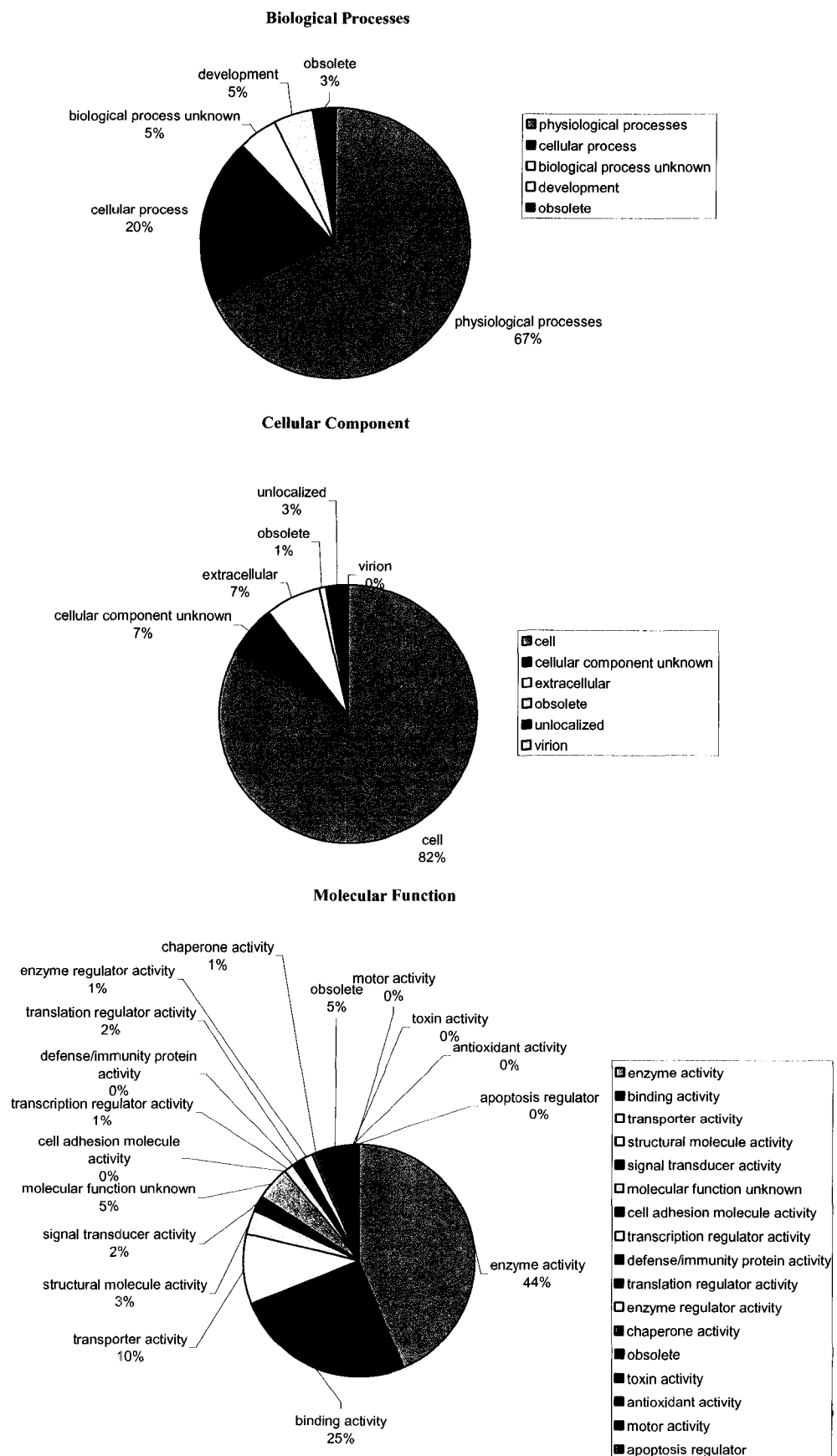
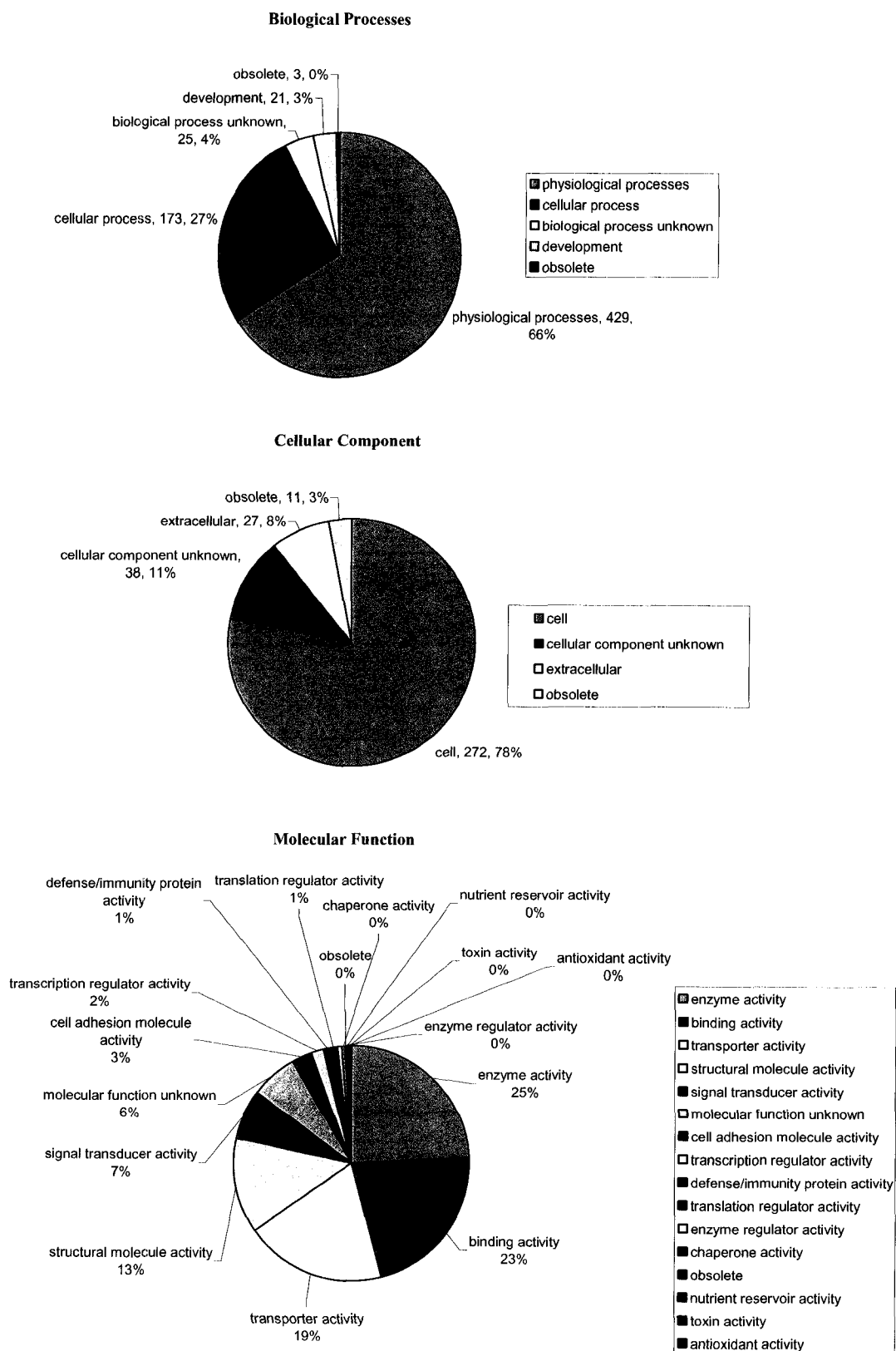


Figure 3.3. GO annotation of ESTs from *Alternaria brassicicola* nitrogen starvation library.



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Chapter 4

Cloning of a Gene Encoding an *Alt a 1* Isoallergen Differentially Expressed by the Necrotrophic Fungus *Alternaria brassicicola* during *Arabidopsis* Infection

Introduction

The genus *Alternaria* is considered one of the most important producers of fungal allergens in the world (Al-Doory et al. 1984). *Alternaria* allergens have been shown to be associated with childhood asthma, and recently, sensitivity to fungal allergens was shown to be a risk factor for life threatening asthma (Halonen et al. 1997; Black et al. 2000). Furthermore, the genus *Alternaria* is responsible for some of the world's most devastating plant diseases (Rotem 1994). Given its direct relevance to human well being, a need exists to gain a better understanding of the biology of *Alternaria* species, and in particular, to identify genes responsible for its pathogenic and allergenic capabilities.

The most well known of these allergens is *Alt a 1*, produced by *A. alternata*. To date, the N-terminal amino acid sequence of this protein has been obtained and subunits of *Alt a 1* have been cloned (Barnes et al. 1996; DeVouge et al. 1996; Zhang et al. 1995). The major *Alt a 1* protein is a dimer of about 29 kDa that when exposed to reducing agent dissociates into 14.5 kDa and 16 kDa subunits (DeVouge et al. 1996; Zhang et al. 1995). A common epitope exists on both *Alt a 1* subunits that can serve as a binding site for a

novel 2-site monoclonal antibody (Aden et al.1999). No known biological function has been assigned to *Alt a 1* to date, and surprisingly, no known homologs in other *Alternaria* species have been identified.

Recently, germination of *A. alternata* spores was shown to increase allergen release (Mitakakis et al. 2001). Allergen release was measured by immunostaining and showed a marked increase in allergen discharge from germinating spores compared with ungerminated spores. The authors hypothesized that *Alt a 1* is secreted or released from growing germ tubes as has been reported in *Aspergillus* (Sporik et al. 1993). Thus, environmental conditions that trigger *Alternaria* spore germination may also trigger allergen production, and *Alt a 1* may have some function during the initial stages of spore germination. Identifying when and where *Alternaria* allergens are produced may lead to a further understanding of how patients are sensitized to *Alternaria* allergens, and to the development of novel control strategies for their mitigation. As *Alternaria* spores are too large to reach the alveoli of the lung, a high probability exists that sensitization to *Alternaria* is due to inhalation of dried mycelia or spores, which then germinate and release various allergenic proteins such as *Alt a 1*.

In this study, we report the identification of an *Alt a 1* homolog in the necrotrophic fungus *A. brassicicola* (Schwein.) Wiltshire, the causal agent of black spot disease of many cruciferous plant species. This is the first report of an *Alt a 1* homolog in another species of *Alternaria*. This homolog, which we have designated *Alt b 1*, is preferentially expressed in spores germinating on the leaf surface of *Arabidopsis*, and not in spores germinating in water. These results may indicate that *Alt b 1* expression in *A. brassicicola* is controlled by specific environmental factors, and may play a role in

necrotrophic fungal pathogenesis of plants. Other *Alt a 1* homologs were also identified in other species of *Alternaria*, and phylogenetic analysis suggests that *Alt a 1* is conserved throughout the genus *Alternaria*. This conservation may suggest that *Alt a 1* and *Alt b 1* are involved in some basal, conserved process in *Alternaria* species. Understanding the regulation of gene expression and the biological function of this major fungal allergen may lead to novel control strategies and a further understanding of the major *Alternaria* allergen, *Alt a 1*.

Materials and Methods

Identification and cloning of *Alt a 1* homolog, *Alt b 1*, from *A. brassicicola*

A homolog of the major *Alternaria* allergen *Alt a 1* was identified in a suppression subtractive library (SSH) (Cramer and Lawrence 2004). The cDNA fragment, clone P3G2, was 464 base pairs in length and was from the 5' end of the gene. Details of the subtraction procedure are available in Chapter 2 of this dissertation. Briefly, *A. brassicicola* strain 34622 was obtained from the American Type Culture Collection (ATCC) and grown on potato dextrose agar (Sigma Chemical CO, St Louis, MO.) for 14 days. Twenty µl aliquots of the spore suspension were placed on detached leaves of the susceptible *Arabidopsis* ecotype Landsberg and were incubated at 24°C in 100% humidity for 24 hours. The remaining spores were allowed to germinate in water for 24 hours under similar conditions. A third population, ungerminated spores, was also collected for comparison purposes in expression studies, but not used in the subtraction.

Germinating spores were collected and used to extract total RNA. RNA extraction was done according to the manufacturer's instructions using the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Following RNA extraction, approximately 100 ng of total

RNA was used to create cDNA from each of the 3 RNA samples described above using the SMART PCR cDNA Synthesis kit (Clontech, Palo Alto, CA). RNA extracted from the water only germinated spores was used to create the driver cDNA, while RNA extracted from leaf germinated spores was used to create the tester cDNA. Double stranded cDNA from the tester and driver populations generated from the SMART PCR cDNA synthesis kit was used in suppression subtractive hybridization (SSH) (Diatchenko et al. 1996). SSH was performed with the PCR Select cDNA Subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions.

Random amplification of cDNA ends (RACE) was used to clone the full length *Alt b 1* cDNA sequence. The GeneRacer kit (Invitrogen, Carlsbad, CA) was used to obtain the full-length 3' end of the *Alt b 1* cDNA. Mycelia plugs from a 7 day old culture of *A. brassicicola* grown on potato dextrose agar (PDA) were transferred to 50 ml of glucose yeast extract broth (GYEB). The culture was incubated at 24°C, 100 rpm, for 5 days. Mycelia was collected by filtering the culture through 4 layers of sterile cheesecloth, dried, and total RNA was extracted with the RNeasy Plant Kit according to the manufacturer's protocol (Qiagen USA, Valencia, CA). One µg of total RNA was used to create RACE ready cDNA following the manufacturer's instructions (Invitrogen, Carlsbad, CA).

RACE reactions were performed with 45 µl of Platinum PCR Supermix (Invitrogen, Carlsbad, CA), 3 µl of GeneRacer 3' primer (10 µM stock), 1 µl of 3' *Alt b 1* 3' RACE primer (10 µM stock), and 1 µl of RACE ready cDNA. Table 4.1 gives a list of the primers used in this study. PCR reactions were carried out in an Applied Biosystems

Table 4.1. Primers used in this study.

Primer	Primer Sequence
<i>Alt b 1</i> 3' RACE	5' TCT GCT CGG GCC GAC AAG CTT GAG GAC C 3'
5' <i>Alt b 1</i>	5' ATG CAG TTC ACC ACC ATC GC 3'
3' <i>Alt b1</i> degen	5' ACG AGG GTG AYG TAG GCG TC 3'
5' <i>Alt b 1</i> RTPCR	5' ATG CAG TTC ACC ACC ATC GC 3'
3' <i>Alt b 1</i> RTPCR	5' GTA ACG AGG GTG ACG TAG GC 3'
5' 18s rRNA	5' GCG GTA ATT CCA GCT CCA ATA GC 3'
3' 18 s rRNA	5' GAT GAC ACG CGC TTA CTA GGC 3'

GeneAmp 2400 thermocycler (Applied Biosystems, Foster City, CA) with the following conditions: 94°C for 2 minutes, 5 cycles of 94°C for 30 seconds and 72°C for 1 minute, 5 cycles of 94°C for 30 seconds and 70°C for 1 minute, and 25 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute, followed by an extension for 10 minutes at 72°C. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized with ultra-violet light. Gel fragments containing DNA were excised with a sterile scalpel, transferred to 1.5 ml microcentrifuge tubes, and DNA extracted with the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen USA, Valencia, CA). Four µls of the eluted DNA was cloned into the TOPO TA cloning vector pCR4-TOPO (Invitrogen, Carlsbad, CA) and transformed into DH5α *E. coli* cells.

Selected individual RACE cDNA clones were obtained with the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). 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.

Cloning and identification of *Alt a 1* homologs from other *Alternaria* species

PCR primers, based on the sequences of *Alt a 1* and *Alt b 1*, were designed to amplify full length homologs from other *Alternaria* species (Table 4.1, 5' *Alt b 1* and 3' *Alt b 1* degen.). These species included 5 strains of *A. brassicicola* (21-71-01, 21-71-04, 21-71-05, 21-71-06, and ATCC 34622), one strain of *A. brassicae* (21-61-002), one strain of *A. alternata* (21-41-054), one strain of *A. japonica* (21-72-002), *A. alternata* f.sp. *lycopersici* (AS273), and 1 strain of *A. porri* (AP256). Strains with the prefix 21- were obtained from Dr. Barry Pryor at the University of Arizona, Tucson, AZ. AS273 and AP256 were obtained from Dr. Howard Schwartz at Colorado State University, Fort Collins, CO.

PCR reactions consisted of 12.5 µl of 2X PCR master mix (Promega, Madison, WI.), 10.5 µl of water, 1 µl of genomic DNA (50 ng), and 0.5 µl of each primer (10 µM stock). PCR reactions were conducted in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following conditions, 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. PCR products were subsequently treated as above for the RACE products.

Sequence analysis

The BLAST algorithms at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) were used to initially characterize sequences in the non-redundant GenBank database (Altschul et al., 1997). Multiple alignments were

conducted with ClustalX 1.81 and the BLOSUM series protein weight matrix (Thompson et al., 1997). A phylogenetic tree was calculated from the multiple alignment using the BOOTSTRAP N-J TREE option in ClustalX 1.81. This method derives confidence values for the groupings in a tree. For this analysis, 1000 bootstrap replicates were used. Primer sequences were removed from the sequences prior to alignment and tree calculations. The tree was rooted with the outgroup *A. brassicae* isolate 21.61.002.

cDNA Northern Blots

cDNA Northern blots (also called virtual northern blots) were used to initially characterize the expression of *Alt b 1* (Endege et al., 1999). Blots for cDNA Northern analysis were created using 2 µg each of double stranded cDNA from the tester, driver, and ungerminated spore populations. The cDNAs were electrophoresed in a 1% agarose gel and blotted onto positively charged nylon membranes (Roche Diagnostics, Mannheim, Germany). The original *Alt b 1* cDNA clone was used as a probe and labeled using the PCR DIG Probe Synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). Filters were hybridized with the PCR amplified Alt b1 DIG labeled probe at a probe concentration of 25 ng/ml. Filters were washed in a final solution of 0.1X SSC and 0.1% SDS at 68° C.

RT-PCR

RT-PCR reactions were performed to assess transcript accumulation of *Alt b 1* under different environmental conditions. Two µls of first strand cDNA created using the SMART PCR cDNA Synthesis kit (Clontech, Palo Alto, CA) were used in a 25 µl PCR reaction. Primers used in the amplification to detect the presence of *Alt b 1* transcript were 5' Alt b1 RTPCR and 3' Alt b1 RTPCR (Table 4.1). Transcript levels of *Alt b 1*

were compared with 18s rRNA using primers 5' 18s rRNA and 3' 18s rRNA (Table 4.1). PCR amplification conditions were 95°C for 2 minutes followed by 40 cycles of 95°C for 30 seconds, 53°C for 45 seconds, and 72°C for 1 minute. A final 5 minute extension was done at 72° C.

For Southern blot analysis, *A. brassicicola* genomic DNA was extracted using the Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Solana Beach, CA). Restriction digests were performed by digesting 3 µg aliquots of genomic DNA with 6 individual restriction enzymes (*Bam*HI, *Hind*III, *Pst*I, *Eco*RI, *Eco*RV, and *Xba*I). Restriction digests were separated on a 1% agarose gel and blotted onto nylon membranes as for cDNA Northern blots. DNA probe for *Alt b 1* was labeled as the cDNA Northern probe above. Concentration of the probe in hybridization solution was 50 ng/ml and hybridization was carried out at 50°C. Membranes were washed in a final solution of 0.1X SSC and 0.1% SDS at 68°C.

Results

Cloning of *Alt b 1* cDNA

Sequence analysis of the 464 base pair cDNA SSH clone using BLASTN and BLASTX revealed significant homology ($1e^{-104}$ BLASTN, $2e^{-34}$ BLASTX) with the major *Alternaria* allergen *Alt a 1* (GenBank accession number AAU86752).

Subsequently, we named this gene *Alt b 1*. 3' random amplification of cDNA ends (RACE) was successful in obtaining the 3' end of the *Alt b 1* cDNA (Figure 4.1A). Figure 4.1B shows the full length cDNA sequence of *Alt b 1* (GenBank accession number AF499002). The cDNA sequence is 815 base pairs in length, including a 205 base pair 5' untranslated region and poly A tail. The initiation codon (ATG) starts at position 206 and

the termination codon (TAA) ends at position 680. The 205 base pair 5' untranslated region also appears in the *Alt a 1* sequence (data not shown). The cDNA sequence of *Alt b 1* was 92% similar to *Alt a 1* and thus can be considered an isoallergen (Hoffman et al. 1994). Southern blot analysis of *Alt b 1* (Fig. 4.2) was conducted and revealed the apparent presence of one copy of *Alt b 1* in the *A. brassicicola* genome. The enzymes *Bam*HI and *Eco*RI gave two large fragments, while the enzyme *Hind*III gave one fragment of about 1500 bp that hybridized with the *Alt b 1* cDNA probe. The two large fragments produced by *Bam*HI and *Eco*RI can be explained by the presence of restriction sites for these two enzymes in the middle of the *Alt b 1* cDNA clone used as a probe in the Southern blot.

The amino acid sequence of *Alt b 1* was 90% similar to the previously identified *Alt a 1* (Figure 4.3). The 18 amino acid cleaved signal peptide located at the N-terminus that directs *Alt a 1* for export from the cell also appears to be present in *Alt b 1*. The amino acid sequences of *Alt a 1* and *Alt b 1* were used to scan the PROSITE database, release 18.17, (<http://us.expasy.org/tools/scanprosite/>) for the occurrence of patterns and profiles. These searches revealed several patterns with high probabilities of occurrence, but no unique patterns that could suggest a biological role for these allergens (Table 4.2). However, several differences between the amino acid sequences of *Alt a 1* and *Alt b 1* are readily apparent. Interestingly, *Alt b 1* contains a putative N-glycosylation site which is lacking in *Alt a 1*. A search of the protein families database of alignments and HMMs (Pfam, <http://www.sanger.ac.uk/Software/Pfam/index.shtml>) revealed no significant hits for either *Alt a 1* or *Alt b 1*.

Table 4.2 PROSITE patterns of occurrence for *Alt a 1* and *Alt b 1* amino acid sequences

PROSITE Pattern	<i>Alt a 1</i> – residue number	<i>Alt b 1</i> – residue number
Protein kinase C Phosphorylation site	101-103 Sdr	33-35 Stk 75-77 Sar 101-103 Sdr
Casein kinase II phosphorylation site	23-26 SrqD 34-37 TegD 88-91 ScgE 93-96 SfmD	23-26 TrqD 34-37 TkgD 88-91 ScgE 93-96 SfmD
Tyrosine kinase phosphorylation site	48-55 Rkp.EgtyY 80-87 Kle.DhkwY 111-118 KvsdDit.Y	48-55 Rkp.EgsyY 80-87 Kle.DhkwY 111-118 KvddDtt.y
N-myristoylation site	14-19 GLaaAA 52-57 GTyyNS 142-147 GVadAY	14-19 GLaaAA 52-57 GSyyNN 131-136 GGngAN 132-137 GNgaND 142-147 GVsdAY
Amidation site	46-49 yGRK	46-49 yGRK
N glycosylation site	NA	27-30 NASC

Cloning and identification of *Alt a 1* homologs from other *Alternaria* species

Amplification of *Alt a 1* homologs was successful from all *Alternaria* isolates examined, including: 5 isolates of *A. brassicicola*, 1 *A. japonica*, 1 *A. brassicae*, 1 *A. porri*, 1 *A. alternata*, and 1 *A. alternata* f.sp. *lycopersici*. This suggests that *Alt a 1* is a conserved gene throughout the genus *Alternaria*. A multiple alignment of the genomic nucleotide sequences revealed relatively little variation among the different isolates (Figure 4.4). An intron near the 3' end of the sequence occurs approximately 352 base pairs from the ATG site in all the isolates. Significant variation occurs among the isolates over the sequence of this intron. In the *A. brassicicola*, *A. alternata*, *A. alternata* f.sp. *lycopersici*, and *A. porri* isolates the intron is 69 base pairs in length, and 71 nucleotides in *A. japonica* and *A. brassicae*.

Neighbor-joining analysis of the *Alt a 1* genomic sequences placed the *A. brassicicola* isolates in a clade separate from the other *Alternaria* species examined

(Figure 4.5). This clade was supported by a bootstrap value of 100%. The *A. japonica* isolate was placed in the *A. brassicicola* clade (bootstrap value 97.6%). The second clade distinct from the *A. brassicicola* clade consisted of *A. alternata* and *A. alternata* f.sp. *lycopersici* (bootstrap value 98.8%), and the *A. porri* isolate (bootstrap value 98.3%). The outgroup, *A. brassicae*, formed a third clade distinct from the other isolates in the analysis.

Expression analysis of *Alt b 1*

cDNA Northern analysis using *Alt b 1* cDNA as a probe revealed drastic up-regulation on the leaf surface of *Alt b 1* precursor when compared with ungerminated spores and spores germinated only in water based on relative mRNA transcript abundance (Fig. 4.6A). *Alt b 1* was highly expressed *in vitro* in three different media, minimal medium (MM), potato dextrose broth (PDB), and glucose-yeast extract broth (GYEB) (Figure 4.6B). RT-PCR experiments (Fig. 4.6D) confirmed that *Alt b 1* is highly expressed in *A. brassicicola* cultures growing in various liquid media (glucose-yeast extract broth, minimal media, and potato dextrose broth). RT-PCR experiments also demonstrated that *Alt b 1* is expressed by spores germinating on the leaf surface of a resistant *Arabidopsis* ecotype, Columbia.

Discussion

This is the first study to identify an *Alt a 1* homolog in *Alternaria* species other than *A. alternata*. Presence of *Alt b 1* in *A. brassicicola* and other *Alternaria* species presents another source of *Alternaria* allergen, particularly because many of these *Alternaria* species infect economically important plants grown for human consumption. Of potential interest to both allergen researchers and plant pathologists, *Alt b 1* from *A.*

brassicicola appears to be drastically up-regulated during the initial stages of infection on *Arabidopsis*. This may indicate that *Alt b 1* has a role in fungal pathogenesis, though further studies are required to elucidate its requirement in infection. To date it is not known whether allergenic proteins play a role in plant pathogenesis and it is difficult to speculate on this concept due to the diverse structures and biological functions of known fungal allergens.

Phylogenetic analysis of *Alt a 1* homologs in other *Alternaria* species revealed that this protein is highly conserved across the genus. Considerable variation does occur within a small intron in the *Alt a 1* homologs' nucleotide sequences, but little variation is seen across the coding regions. The phylogeny of *Alt a 1* closely resembles the phylogeny of *Alternaria* species based on nuclear ITS and SSU rDNA sequences (Pryor and Gilbertson 2000). This is a significant finding for elucidating the classification of *Alternaria* species, and suggests that the phylogenetic relationships seen with nuclear ITS and mt SSU rDNA sequences are correct. If indeed the phylogenetic relationships between *Alt a 1* and the ITS and mt SSU rDNA sequences are correct, the *A. porri* isolate utilized in this study may be mis-identified. The conserved nature of *Alt a 1* may suggest that it has a role in primary metabolism rather than secondary metabolism (i.e. toxin production). Secondary metabolite production tends to differ among and between species and isolates of *Alternaria*, and one would not expect a gene involved in secondary metabolism to be conserved across a wide range of species and isolates.

Analysis of the amino acid sequence of *Alt a 1* from *A. alternata* and *Alt b 1* from *A. brassicicola* suggests that *Alt b 1* is N-glycosylated, while *Alt a 1* is not. What effect this putative difference in glycosylation status between *Alt a 1* and *Alt b 1* has on

biological function is unknown. The role of N-glycosylation in determining the biological function of a given protein seems to vary with different types of proteins. Introduction of an N-glycosylation site has been shown to dramatically increase the secretion of heterologous proteins in *Saccharomyces cerevisiae* (Sagt et al. 2000). Perhaps N-glycosylation of *Alt b 1* increases its secretion and this may be important for the biological function of Alt b1.

Recently, it has been reported that germinating spores of *Alternaria* release greater amounts of allergen than non-germinating spores (Mitakakis et al. 2001). The results presented here, based on cDNA Northern analysis, seem to confirm these results to a degree. If one assumes that transcript abundance corresponds to allergen release, then clearly more allergen is being released from *A. brassicicola* spores germinating in nutrient rich environments than non germinating spores. Interestingly, in this study spores that were germinated in water alone did not have high levels of *Alt b 1* transcript. This may be due to the nutrient deficient environment of the sterile distilled water used to germinate the spores (though the water was not tested for presence of sugars). Based on these results, simple spore germination does not seem to increase *Alt b 1* transcription. Also, it is important to note that the study by Mitakakis et al. (2001) used spores that were harvested from vegetable juice media which contains plant products. The results presented in this study, along with Mitakakis et al. (2001), suggest that a signal(s) present on the plant leaf surface may trigger an increase in *Alt b 1* gene expression. RT-PCR experiments demonstrate that this signal is also present in nutrient rich media, and thus a nutrient rich environment (such as the nasal passage) conducive to fungal growth may be the only requirement for *Alt b 1* production. Identification of the specific environmental

conditions that trigger *Alt b 1* production and its release should help elucidate mechanisms that regulate its production and provide clues to its potential biological function.

In this study, the identification of an *Alt a 1* isoallergen from the phytopathogenic fungus, *A. brassicicola*, is reported. The degree of sequence homology between *Alt a 1* and *Alt b 1* (92% at the cDNA level) indicates that *Alt b 1* may be considered an isoallergen (Hoffman et al. 1994, and www.allergen.org). Further, *Alt b 1* appears to have the 18 amino acid signal peptide described for *Alt a 1* that is required for secretion (DeVouge et al. 1996). Genomic homologs of *Alt a 1* were also identified in: 5 isolates of *A. brassicicola*, 1 isolate of *A. japonica*, *A. brassicae*, *A. alternata* f.sp. *lycopersici*, and *A. porri*. Phylogenetic relationships based on the *Alt a 1* genomic sequences were consistent with those seen with nuclear ITS and mt SSU rDNA sequences.

Though fungi have long been known to be sources of allergens, recent studies have demonstrated their scope and impact on human health (Black et al. 2000; Shelton et al. 2002). Successful control of allergies caused by fungal allergens will require knowledge of the potential sources of allergens, as well as an understanding of the biological functions of allergenic proteins. Future studies on *Alt b 1* precursor will include examination of its role in the pathogenesis process on crucifer species by gene disruption as well as defining its allergenic properties. The role of N-glycosylation in determining *Alt b 1* function should also be examined. Identification of a biological function for *Alt b 1* and its homolog *Alt a 1* will certainly provide new ideas for potential control of this important fungal allergen, and may help answer the question of how patients are sensitized to *Alternaria* allergens.

Acknowledgements

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Another portion of this chapter, along with additional, more extensive phylogenetic analyses on *Alt a 1* homologs from other *Alternaria* species was in review at the time of the writing of this dissertation as: **Hong, S.G., Cramer, R.A., Lawrence, C.B., and Pryor, B.A.** 2004. Use of *Alt a 1* gene sequence in phylogenetic study of *Alternaria* and related genera. Fungal Genet. Biol. Manuscript in review as of March 2004.

Figure 4.1. *Alt b 1* 3' RACE result and nucleotide cDNA sequence of *Alt b 1*. A. 3' RACE result, *Alt b 1*. B. Nucleotide cDNA sequence *Alt b 1*. Initiation (ATG) and stop codons (TAA) are underlined and the sequence encoding the 18 amino acid signal peptide is in bold. The open reading from position 206 to 680 is italicized. Genbank accession number AF499002.

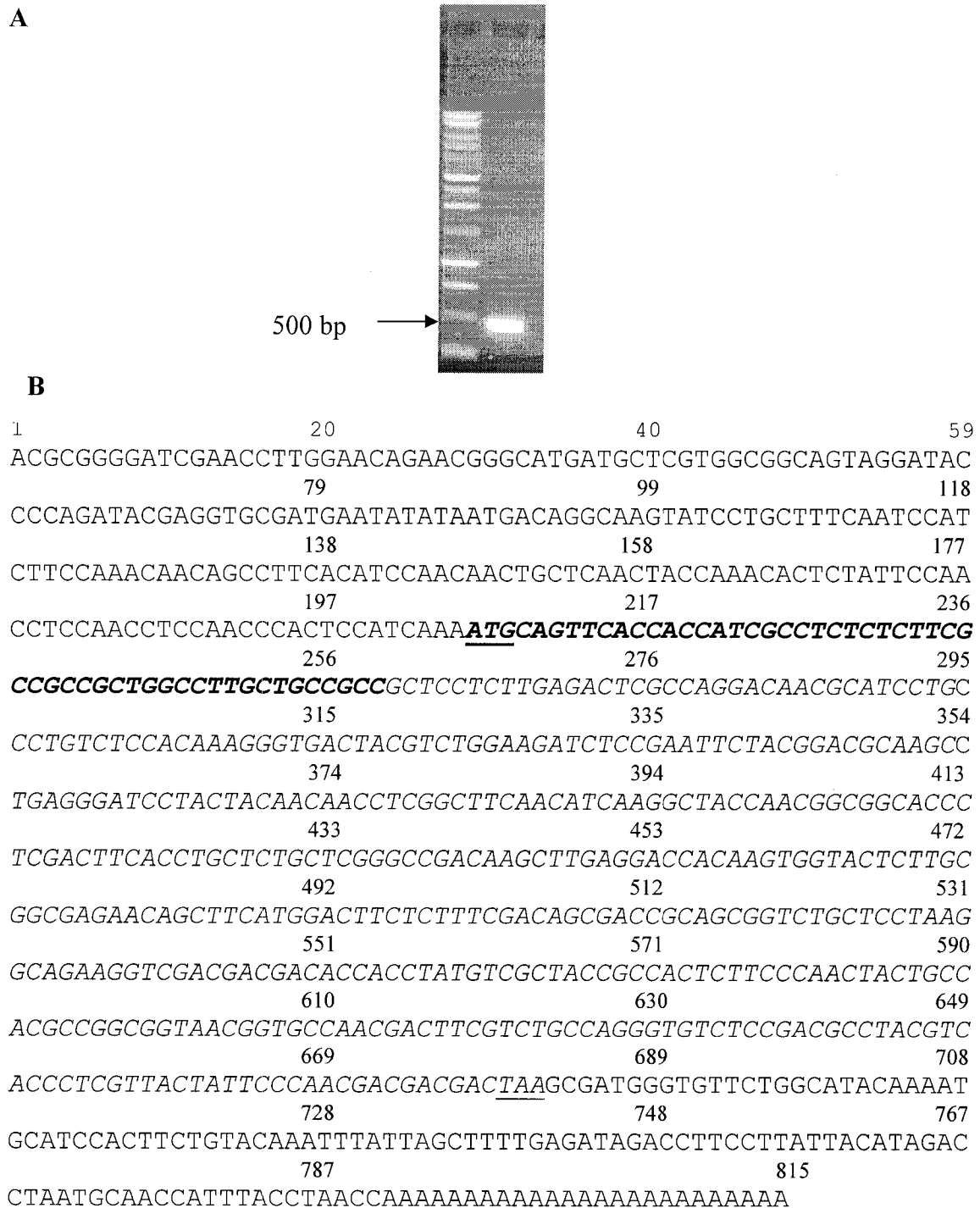


Figure 4.2. Southern analysis of *Alt b 1*. Lane 1 = DIG ladder, lanes 2-7 *A. brassicicola* genomic DNA digested with *Bam*HI, *Hind*III, *Pst*I, *Eco*RI, *Eco*RV, and *Xba*I I respectively. *Alt b 1* appears to be a single copy gene.

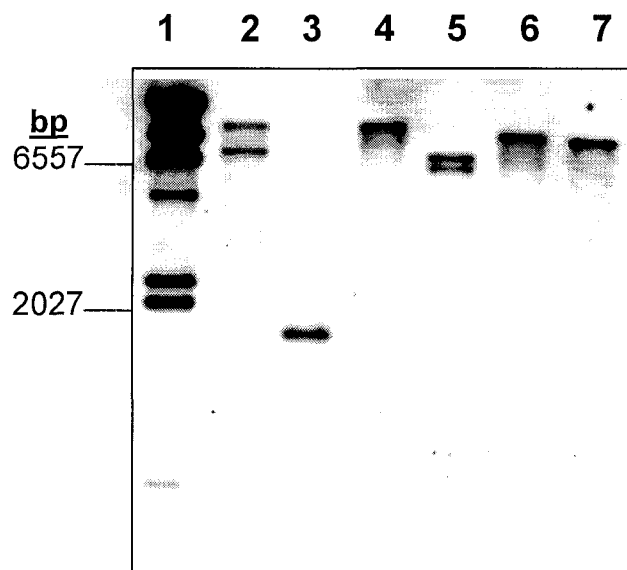


Figure 4.4. Multiple alignment of *Alt a 1* genomic DNA sequences from *Alternaria* species. Multiple alignment was created with ClustalX 1.81 and the BLOSUM protein weight matrix. 5' and 3' primer sequences were removed prior to conducting the alignment. AB = *A. brassicicola*, AJ = *A. japonica*, AP = *A. porri*, AS = *A. alternata* f.sp. *lycopersici*, AA = *A. alternata*. * denotes the intron sequence.

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21.71.006AB CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGACTCGCCAGGACAACGCATCCTGC
34622AB CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGACTCGCCAGGACAACGCATCCTGC
21.71.005AB CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGACTCGCCAGGACAACGCATCCTGC
21.71.001AB CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGACTCGCCAGGACAACGCATCCTGC
21.71.004AB CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGACTCGCCAGGACAACGCATCCTGC
21.72.002AJ CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGTCCGCCAGACAACGCATCCTGC
21.41.054AA CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGTCTCGCCAGGACAACGCATCCTGC
AS273 CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGTCTCGCCAGGACAACGCATCCTGC
AP256 CTCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAGTCTCGCCAGGACAACGCATCCTGC
21.61.002ABS CACTCTCTTCGCCGCCGCCGCCCTCGCTGCCGCCGCTCCTCTTGAGTCTCGCCAGGACAACGCATCCTGC
consensus CtCTCTCTTCGCCGCCGCTGGCCTTGCTGCCGCCGCTCCTCTTGAG CtCGCCAGGACAACGCATCCTGC

21.71.006AB CCTGTCTCCACAAAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCTGAGGGATCCT
34622AB CCTGTCTCCACAAAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCTGAGGGATCCT
21.71.005AB CCTGTCTCCACAAAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCTGAGGGATCCT
21.71.001AB CCTGTCTCCACAAAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCTGAGGGATCCT
21.71.004AB CCTGTCTCCACAAAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCTGAGGGATCCT
21.72.002AJ CCACTCTCCACCAAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCGAGGGAACTT
21.41.054AA CCTGTCAACCACTAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCGAGGGAACTT
AS273 CCTGTCAACCACTAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCGAGGGAACTT
AP256 CCTGTCAACCACTAGGGTGACTACGTCTGGAAGATCTCCGAATTCTACGGACGCAAGCCGAGGGAACTT
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21.71.006AB ACTACAACAACCTCGGCTTCAACATCAAGGCTACCAACGGCGGCACCCTCGACTTCACCTGCTCTGCTCG
34622AB ACTACAACAACCTCGGCTTCAACATCAAGGCTACCAACGGCGGCACCCTCGACTTCACCTGCTCTGCTCG
21.71.005AB ACTACAACAACCTCGGCTTCAACATCAAGGCTACCAACGGCGGCACCCTCGACTTCACCTGCTCTGCTCG
21.71.001AB ACTACAACAACCTCGGCTTCAACATCAAGGCTACCAACGGCGGCACCCTCGACTTCACCTGCTCTGCTCG
21.71.004AB ACTACAACAACCTCGGCTTCAACATCAAGGCTACCAACGGCGGCACCCTCGACTTCACCTGCTCTGCTCG
21.72.002AJ ACTACAACAACCTCGGCTTCAACATCAAGGCTACCAACGGCTGGCACTTCGACTTCACCTGCTCTGCTCA
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AP256 ACTACAACAACCTCGGCTTCAACATCAAGGCTACCAACGGAGGAACCTCGACTTCACCTGCTCTCACTC
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consensus ACTACAACAACCTCGGCTTCAACATCAAGGCTACCAACGGCGGCACCCTCGACTTCACCTGCTCTGCTcg

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21.72.002AJ GGCCGACAAGCTTGAGGACCACAAGTGGTACTCTTGCGGCGAGAACAGCTTCATGGACTTCTCTTCGAC
21.41.054AA GGCCGACAAGCTTGAGGACCACAAGTGGTACTCTTGCGGCGAGAACAGCTTCATGGACTTCTCTTCGAC
AS273 GGCCGACAAGCTTGAGGACCACAAGTGGTACTCTTGCGGCGAGAACAGCTTCATGGACTTCTCTTCGAC
AP256 GGCCGACAAGCTTGAGGACCACAAGTGGTACTCTTGCGGCGAGAACAGCTTCATGGACTTCTCTTCGAC
21.61.002ABS GGCCTCCAGACTCGAGGACCACAAGTGGTACTCTTGCGGCGTGAACACTTCATGAGTTTGCTTTCAG
consensus gGCcgacAagCTTGAGGACCacAagTGGTACTCTTGcGGcGaGAACAgCTTCATggacTTctCTTTCgAc

21.71.006AB AGCGACCGCAGCGGTCTGCTCCTTAGGCAGAAGGTCGACGACGAGTAAGTC--CCCTACACCGGCGAGTG
34622AB AGCGACCGCAGCGGTCTGCTCCTTAGGCAGAAGGTCGACGACGAGTAAGTC--CCCTACACCGGCGAGTG
21.71.005AB AGCGACCGCAGCGGTCTGCTCCTTAGGCAGAAGGTCGACGACGAGTAAGTC--CCCTACACCGGCGAGTG
21.71.001AB AGCGACCGCAGCGGTCTGCTCCTTAGGCAGAAGGTCGACGACGAGTAAGTC--CCCTACACCGGCGAGTG
21.71.004AB AGCGACCGCAGCGGTCTGCTCCTTAGGCAGAAGGTCGACGACGAGTAAGTC--CCCTACACCGGCGAGTG
21.72.002AJ AGCGACCGCAGCGGTCTGCTCCTTAGGCAGAAGGTCGACGAGTAAGTTTTCCTATATCTCAGATA
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AP256 AGCGACCGCAGCGGTCTGCTCCTTAGGCAGAAGGTCGACGAGTAAGTTACCTTTTACCTTTCATT
21.61.002ABS AGCGACCGCAGCGGTCTGCTCCTTAGGCAGAAGGTCGACGAGTAAGTTACCTTTTACCTTTCATT
consensus AGCGACCGCAGCGGTCTGCTCCTaAgGCAGAagGTcgaCGACGAGTAAGtc CCcta AcC gcgagtg

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Figure 4.4. Continued

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21.71.006AB CTTCTCATCTTTTACACATACTAACATCT-CTTCAGCACCACCTATGTCGCTACCGCCACTCTTCCCAAC
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21.71.005AB CTTCTCATCTTTTACATATACTAACATCT-CTTCAGCACCACCTATGTCGCTACCGCCACTCTTCCCAAC
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AP256 CTTGGCAGATTTC-AGATATACTAACATGT-TTCCAGCATCACCTATGTCGCTACCGCCACTCTTCCCAAC
21.61.002ABS CTTCCACTTACC-AGATATACTAACATTTTTCAGTATCACCAACGTCGGTACCGTCACTCTTCCCAAC
consensus CTTctCatcttttA AtATACTAACATcT cttCAGcAcCACctAtGTCGcTACcgCACTCTTCCCAAC

21.71.006AB 418 TACTGCCACGCCGGCGGTAACGGTGCCAACGACTTCGTCTGCCAGGGTGTCTCC
34622AB 418 TACTGCCACGCCGGCGGTAACGGTGCCAACGACTTCGTCTGCCAGGGTGTCTCC
21.71.005AB 418 TACTGCCACGCCGGCGGTAACGGTGCCAACGACTTCGTCTGCCAGGGTGTCTCC
21.71.001AB 418 TACTGCCACGCCGGCGGTAACGGTGCCAACGACTTCGTCTGCCAGGGTGTCTCC
21.71.004AB 418 TACTGCCACGCCGGCGGTAACGGTGCCAACGACTTCGTCTGCCAGGGTGTCTCC
21.72.002AJ 420 TACTGCCACGCCGGTGGTAACGGCCCAACGACTTCGTCTGCCAGGGTGTCTCC
21.41.054AA 419 TACTGCCCGGTGGCGGTAACGGCCCTAAGGACTTTGTCTGCCAGGGTGTCTCC
AS273 419 TACTGCCCGGTGGCGGTAACGGCCCAAGGACTTTGTCTGCCAGGGTGTCTCC
AP256 419 TACTGCCCGGTGGCGGTAACGGCCCTAAGGACTTTGTCTGCCAGGGTGTCTCC
21.61.002ABS 420 AACTGCCCGGTGGCGGCAACGGTCTCAGGACTTTGTCTGCACTGGTGTCTCC
consensus 421 tACTGCCaCGCCGGcGgtAACGGt CcaAcGACTTcGTCTGccagGGTGTctCC

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Figure 4.5. Bootstrap Neighbor-Joining phylogram of *Alt b 1* genomic DNA homologs. Multiple alignments were conducted with ClustalX 1.81 (Thompson et al., 1997). A phylogenetic tree was calculated from the multiple alignment using the BOOSTRAP N-J TREE option in ClustalX 1.81. For this analysis, 1000 bootstrap replicates were used. Primer sequences were removed from the sequences prior to alignment and tree calculations. Scale relative to branch = 10 base substitutions(0.1) per 100 positions.

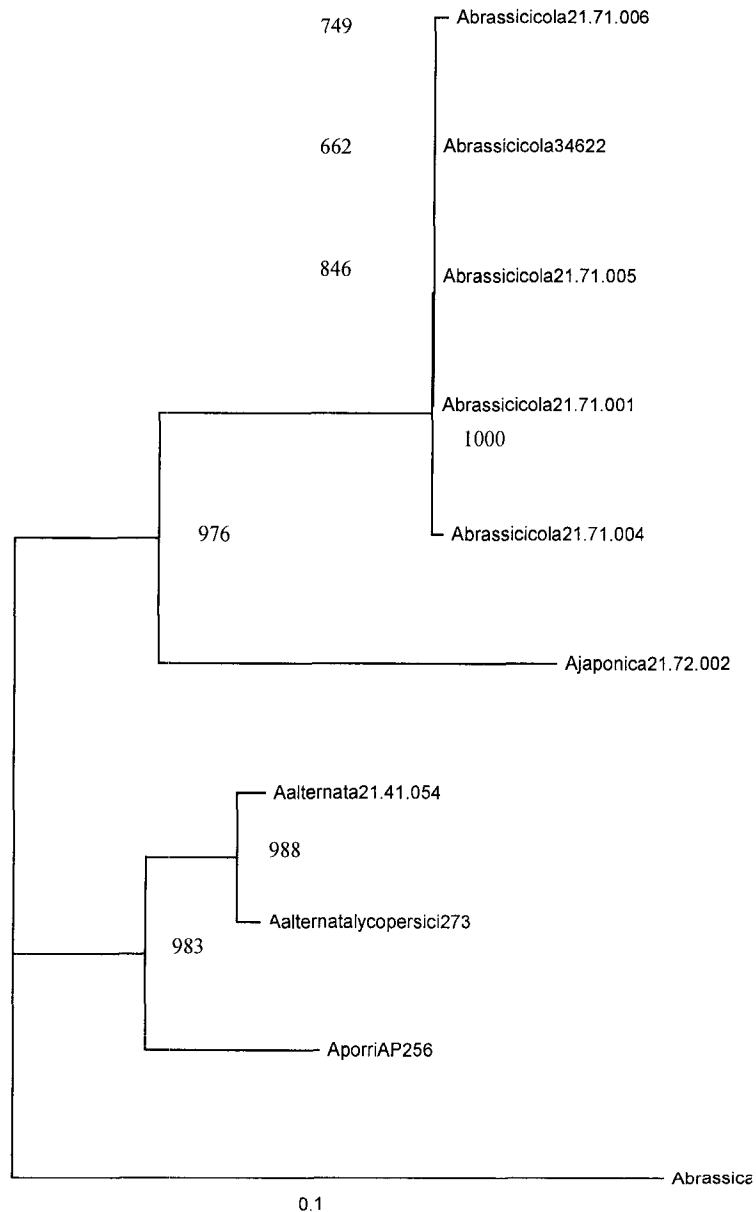
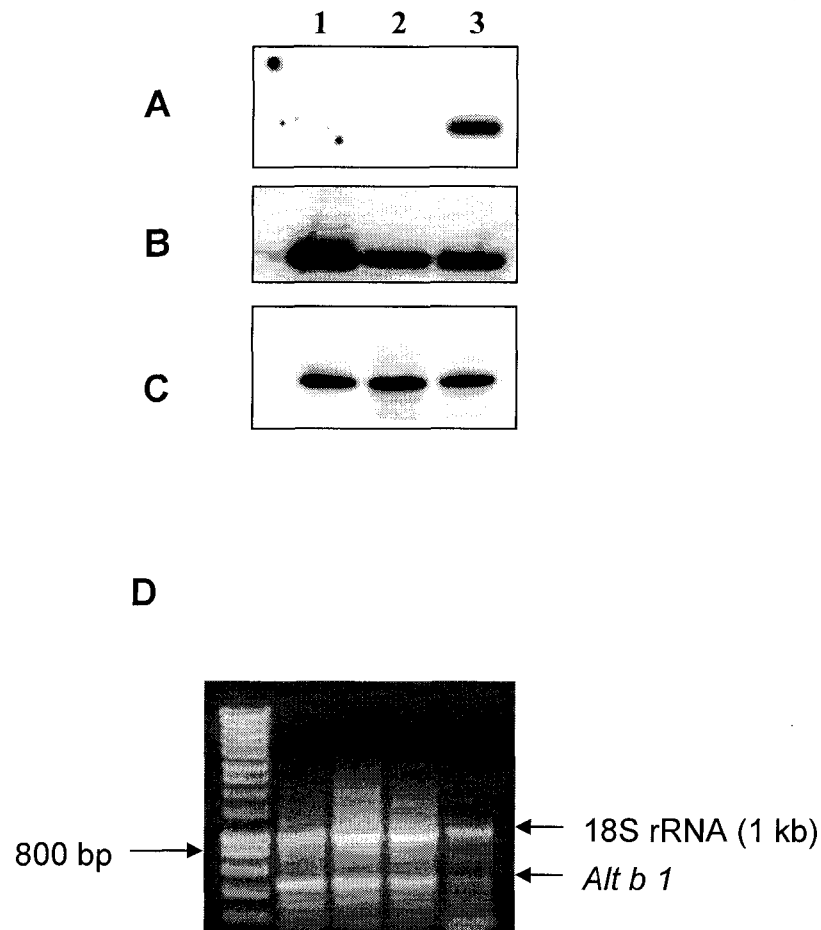


Figure 4.6. Expression analysis of *Alt b 1*. A-C. cDNA Northern analysis of *Alt b 1* expression. A. Lane 1 = cDNA from ungerminated *A. brassicicola* spores, 2 = water germinated spores, 3 = plant leaf surface 24 hours germinated spores. B. Lane 1 = minimal medium germinated spores, 2 = potato dextrose broth germinated spores, 3 = glucose-yeast extract broth germinated spores. C. 18S rRNA with lanes corresponding to cDNA as in A. D. RT-PCR analysis of *Alt b 1* expression. Lane 1 = 1 kb ladder, lane 2 = *A. brassicicola* grown in glucose yeast extract broth (GYEB) for 6 days, lane 3 = *A. brassicicola* grown in minimal medium (MM) for 6 days, lane 4 = *A. brassicicola* grown in potato dextrose broth (PDB) for 6 days, and lane 5 = *A. brassicicola* spores grown on the leaf surface of *Arabidopsis* for 24 hours. The upper band corresponds to 18S rRNA which allows comparison of *Alt b 1* expression across samples. The bottom band corresponds to *Alt b 1* transcript.



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Chapter 5

Cloning and Characterization of a Cyanide Hydratase from the Necrotrophic Fungal Pathogen *Alternaria brassicicola*

Introduction

Plants produce a wide variety of compounds thought to play a role in the defense against microbial invaders. These compounds have been broadly classified into two categories corresponding to how and when they are produced. Phytoalexins are the most well known, and most studied, of the two types of compounds. These anti-microbial secondary metabolites are produced by the plant in a specific response to a pathogen. For example, camalexin is a phytoalexin produced by *Arabidopsis thaliana* and other wild crucifers, and has been shown to be involved in resistance to the necrotrophic fungus *Alternaria brassicicola* (Schw.) Wiltshire (Thomma et al. 1998). Numerous other phytoalexins have been identified and shown to be important in various plant-pathogen interactions.

Recently, a second group of compounds, the phytoanticipins, have gained prominence. Unlike their phytoalexin counterparts, which are produced in response to the presence of a pathogen, phytoanticipins are constitutively expressed at low levels throughout the plant. These compounds are often stored as inactive precursors and become biologically active when exposed to particular plant enzymes (Van Etten et al.

1994). One of the most well studied groups of phytoanticipins are the glucosinolates, sulfur containing glucosides found in the *Brassicaceae* including the model plant *Arabidopsis thaliana*.

Glucosinolates themselves show little biological activity, but when they are hydrolyzed by the enzyme myrosinase (β -thioglucosidase glucohydrolase) toxic compounds including nitriles, isothiocyanates, and thiocyanates are formed (reviewed by Bones and Rossiter 1996). One mechanism by which glucosinolates and the corresponding myrosinase enzyme come in contact to produce bioactive antimicrobial compounds is by tissue damage. The role of glucosinolate breakdown products has been well established in plant-insect interactions where they can act as herbivore deterrents or feeding stimuli, but is still unclear in plant-fungal interactions (Andreasson et al. 2001, Ratzka et al. 2002). Like some insects which have evolved mechanisms to evade the glucosinolate – myrosinase system, the *Brassica* fungal pathogen *Leptosphaeria maculans* also has been reported to be insensitive to glucosinolate hydrolysis products (Giamoustaris and Mithen 1997, Andreasson et al. 2001).

The *Brassica* pathogens *Alternaria brassicae* and *Alternaria brassicicola* also do not appear to be sensitive to increased concentrations of glucosinolates (Giamoustaris and Mithen 1997). Tierens et al. (2001) explored the role of the glucosinolate breakdown product 4-methylsulphinylbutyl isothiocyanate (ITC) against various microbial pathogens including *A. brassicicola*. Since *A. brassicicola* was not inhibited *in vitro* by ITC, their results, along with those of Giamoustaris and Mithen (1997) suggest that *A. brassicicola* has evolved a mechanism(s) to deal with these toxic compounds.

One potential detoxification mechanism of glucosinolate hydrolysis products may occur by enzymes that are capable of breaking carbon-nitrogen bonds such as nitrilases and cyanide degrading enzymes (Sexton and Howlett 2000). In this study, the cloning and characterization of a cyanide hydratase enzyme from *A. brassicicola*, the causal agent of black spot disease on many economically important *Brassica* species is reported. Cyanide hydratase enzymes (EC 4.2.1.6.6) are capable of converting HCN to formamide which is non-toxic to fungi. Several other groups of enzymes also have carbon-nitrogen hydrolase domains including the nitrilases (EC 3.5.5.1), aliphatic amidases (EC 3.5.1.4), beta alanine synthase (EC 3.5.16), biotinidase (EC 3.5.1.12), and pantetheinase (EC 3.5.1). Mutants with a disrupted cyanide hydratase open reading frame (ORF) displayed increased sensitivity to KCN *in vitro*, normal vegetative growth and conidiation, and no defects in pathogenicity. It is apparent that *A. brassicicola* and other *Alternaria* species have developed powerful cyanide detoxification mechanisms which do not solely depend on the production of a cyanide hydratase enzyme. Thus, it is still not clear what exact role cyanide hydratase enzymes play in fungal metabolism, though they may be involved in nitrogen metabolism as well as the detoxification of cyanide.

Materials and Methods

Cloning of full length cyanide hydratase cDNA

A 621 base pair cDNA clone (P3D7) with homology to a *Leptosphaeria maculans* cyanide hydratase ($E = 4e^{-75}$, BLASTX) was identified in a suppression subtractive library (SSH) enriched for *A. brassicicola* genes expressed during infection of *Arabidopsis thaliana* (Cramer and Lawrence 2004). The cDNA clone contained the 3' end of the putative cyanide hydratase, and was named *CYHAB* (cyanide hydratase

Alternaria brassicicola). Details of the subtraction procedure are available in Chapter 2 of this dissertation and Cramer and Lawrence (2004).

Random amplification of cDNA ends (RACE) was used to clone the full length *CYHAB* cDNA sequence. The GeneRacer kit (Invitrogen, Carlsbad, CA) was used to obtain the full-length 5' end of the *CYHAB* cDNA. Mycelia plugs from a 7 day old culture of *A. brassicicola* grown on potato dextrose agar (PDA) were transferred to 50 ml of glucose yeast extract broth (GYEB). The culture was incubated at 24°C, 100 rpm, for 5 days. After 5 days, a freshly prepared solution of potassium cyanide (KCN) was added to the culture at a final concentration of 10 mM to induce *CYHAB* expression. After a 4 hour induction, mycelia was collected by filtering the culture through 4 layers of sterile cheesecloth, dried, and total RNA extracted with the RNeasy Plant Kit according to the manufacturer's protocol (Qiagen USA, Valencia, CA). Five micrograms of total RNA was used to create RACE ready cDNA following the manufacturer's instructions (Invitrogen, Carlsbad, CA).

RACE reactions were performed with 45 µl of Platinum PCR Supermix (Invitrogen, Carlsbad, CA), 3 µl of GeneRacer 5' primer (10 µM stock), 1 µl of 5' *CYHAB* 5' RACE primer (10 µM stock) (5' GGG AGC CAG GGT CCA CGT GCC AGT 3'), and 1 µl of the KCN induced RACE ready cDNA. PCR reactions were carried out in a GeneAmp 2400 thermocycler (Applied Biosystems, Foster City, CA) with the following conditions: 94°C for 2 minutes, 5 cycles of 94°C for 30 seconds and 72°C for 1 minute, 5 cycles of 94°C for 30 seconds and 70°C for 1 minute, and 25 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute, followed by an extension for 10 minutes at 72°C.

PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized with ultra-violet light. Gel fragments containing DNA were excised with a sterile scalpel, transferred to 1.5 ml microcentrifuge tubes, and DNA extracted with the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen USA, Valencia, CA). Four micro-liters of the eluted DNA was cloned into the TOPO TA cloning vector pCR4-TOPO (Invitrogen, Carlsbad, CA) and transformed into *DH5α E. coli* cells by heat shock.

Selected individual RACE cDNA clones were obtained with the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). 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.

Southern analysis

For Southern analysis, *A. brassicicola* genomic DNA was extracted using the Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Solana Beach, CA). Restriction digests were performed by digesting 3 µg aliquots of genomic DNA with 5 individual restriction enzymes (*Bam*HI, *Hind*III, *Pst* I, *Eco*RI, and *Eco*RV). Restriction digests were separated on a 1% agarose gel and blotted onto nylon membranes with a vacuum blotter (Bio-Rad, Hercules, CA). The DNA probe for *CYHAB* was labeled using DIG with the PCR DIG Probe Synthesis Kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, a 1:100 dilution of the original P3D7 clone (621 base pairs) in pCR4-TOPO was made, and 1 µl was used as template in

the DIG PCR labeling reaction. Concentration of the probe in hybridization solution was 100 ng/ml and hybridization was carried out at 50°C overnight with constant agitation. Membranes were washed in a final solution of 0.1× SSC and 0.1% SDS at 68°C. Chemiluminescent detection was used to develop the blots with CDP-Star and the DIG Block and Wash Buffer set according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

Cloning of *CYHAB* homologs in other *Alternaria* species

PCR primers based on the full length *CYHAB* cDNA sequence were developed to amplify potential homologs in other *Alternaria* species. These species included 7 isolates of *A. brassicicola* (21-71-01, 21-71-02, 21-71-04, 21-71-05, 21-71-06, 21-71-07, and ATCC 34622), one isolate of *A. brassicae* (21-61-002), one isolate of *A. alternata* (21-41-054, ATCC 11680), one isolate of *A. japonica* (21-72-002), *A. alternata* f.sp. *lycopersici* (AS273), 1 isolate of *A. porri* (AP256), and 1 isolate of *A. ethzedia* (21-16-001). Isolates with the prefix 21- were obtained from Dr. Barry Pryor at the University of Arizona, Tucson, AZ. AS273 and AP256 were obtained from Dr. Howard Schwartz at Colorado State University, Fort Collins, CO.

PCR reactions consisted of 12.5 µl of 2× PCR master mix (Promega, Madison, WI.), 10.5 µl of water, 1 µl of genomic DNA (50 ng), and 0.5 µl of each primer (10 µM stock). Primer sequences were 5' TGA GCC ATG CTG GTT CGA CCT G 3' and 5' TCG CAA GGT CTA CAT GAC AGA GC 3'. PCR reactions were conducted in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following program: 94°C for 2:00 minutes followed by 35 cycles of 94°C 0:30 seconds, 55°C 0:30 seconds, 72°C 1:30, 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 PCR reactions were repeated 2 times with identical results. To confirm the PCR results a Southern blot was conducted. Five μ gs of genomic DNA from the above *Alternaria* strains was digested with *Hind*III overnight at 37°C. The Southern blot was treated and processed as described above. Under these stringent hybridization conditions (T_m -4 of cyanide hydratase DNA probe and final wash at 68°C, 0.1 $\times$ SSC), the cyanide hydratase homologs in other *Alternaria* species would have to be 95.6% homologous to *A. brassicicola* *CYHAB* in order to see a clear hybridization signal. This was calculated by first calculating the effective T_m of the probe with the formula $81.5 + 16.6[\log [Na^+]] + 0.41(GC\% \text{ probe})$. The concentration of Na^+ for a 0.1 $\times$ SSC solution is known to be 0.0165. The percent homology was then calculated with the formula $100 - [(Effective\ T_m - Final\ Wash\ Temperature)/1.4]$ using the fact that a 1% mismatch of two DNA molecules lowers the T_m by 1.4°C.

Expression analysis of *CYHAB*

Expression analysis of *CYHAB* during *A. brassicicola* infection of cabbage was previously described (Chapter 2 this dissertation, Cramer and Lawrence 2004). RT-PCR was also used to assess whether KCN could induce *CYHAB* transcription *in vitro*. RT-PCR reactions were conducted using the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA). Agar plugs (2 cm in diameter) were taken from 7 day old *A. brassicicola* cultures grown on potato dextrose agar (PDA) and inoculated into various media. Media used were glucose-yeast extract broth (GYEB, 1.5% glucose and 0.3% yeast extract), minimal medium (0.5 g/L KCl, 0.5 g/L MgSO₄, 1.5 g/L KH₂PO₄, 0.1% (v/v) trace elements, 6 g/L NaNO₃, and 10 g/L glucose, pH 6.5) (Talbot et al. 1997), V8 media (20%

V8 juice, 3 g/L CaCO₃), and potato dextrose broth (PDB) produced by Difco (Becton Dickson and Company, Sparks, MD). Cultures were incubated at 24°C, 100 rpm for 5 days. At day 5, KCN was added to a culture in GYEB to a final concentration of 10 mM and incubated for an additional 4 hours.

RNA was extracted with the RNeasy Plant Mini-Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. 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 the manufacturer's protocol. One µl of first strand cDNA was used in 25 µl PCR reactions containing a 2× master mix from Promega, (Madison, WI). PCR reactions were conducted in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following program: 94°C for 2:00 minutes followed by 30 cycles of 94°C 0:30 seconds, either 55°C or 53°C 0:30 seconds depending on each primer set, 72°C 0:30 seconds, and a final extension for 5:00 at 72°C. Primers used to amplify *CYHAB* were 5' CGG CAG CAG TTG CCT TCT GC 3' and 5' TGC TTC CGA CTT GGT CAC GC 3'. Primers used to amplify *A. brassicicola* 18S rRNA were 5' CTG TTA ATC ATT GCG GCG TCT CTA G 3' and 5' GCG GTA ATT CCA GCT CCA ATA GC 3' obtained from Genbank accession number AF229506 (18S rRNA). PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized with ultra-violet light. All RT-PCR reactions were repeated 2 times with similar results.

***CYHAB* gene disruption**

The *CYHAB* disruption vector was created by cloning a 429 base pair fragment of the *CYHAB* cDNA into the *Hind*III and *Eco*RI site of the fungal transformation vector

pCB1636, which contains the bacterial hygromycin B phosphotransferase gene for resistance to hygromycin B (Sweigard et al. 1997). The *Hind*III and *Eco*RI sites were engineered into the cDNA fragment by adding their cut sequences onto the primers used for PCR amplification. Primer sequences were *CyHyEco*RI 5' GCG TGA ATT CTC ACG AGT GGT GTA AGT AGC 3' and *CyHyHind*III 5' GCG CAA GCT TCA AGG AGA CTC TCA AGT ACC 3' (engineered sequence underlined). The resulting disruption vector was named pCB1636-CyHy.

Protoplasts for transformation were prepared by the methods of (Akamatsu et al. 1997 and Tsuge et al. 1990) with modifications. Spores of *A. brassicicola* strain ATCC 34622 were grown in 50 ml of glucose-yeast-extract broth (GYEB) in the dark at 25°C, 100 rpm for 48 hours. Mycelia were collected by pouring off the GYEB and rinsing 1× with sterile water and 1× with sterile 0.7 M NaCl buffer. Mycelia were mixed with 5 ml of sterile enzyme solution containing 10 mg/ml Kitalase (Wako Pure Chemical Industries USA, Richmond, VA) in 0.7 M NaCl buffer. This mixture was incubated at 25°C, 100 rpm in the dark for 3-5 hours. The digestion was checked every hour with light microscopy for protoplast development. When the digestion was complete, this mixture was filtered through one layer of sterile Miracloth (Calbiochem, La Jolla, CA) to collect the protoplasts but remove remaining mycelia. A 0.7 M NaCl (5-10 ml) solution was added to the protoplast solution which was centrifuged at 3,000 rpm (Eppendorf 5804R centrifuge) for 10 minutes at 4°C. Following this centrifugation, protoplasts formed a white pellet at the bottom of the centrifuge tube and this tube was immediately placed on ice. The digestion solution was decanted, and the protoplasts were gently washed with 14 ml of 0.7 M NaCl by centrifuging at 3,000 rpm for 10 minutes. Following this

centrifugation, the 0.7 M NaCl solution was decanted and gently replaced with 500 µl of STC buffer (1 M sorbitol, 50 mM CaCl₂, 50 mM Tris-HCL pH 8.0). After the protoplast pellet was gently resuspended in 500 µl of STC, an additional 13 ml of STC was added. The protoplast solution in STC was centrifuged at 3,000 rpm for 10 minutes and 4°C. Following this centrifugation, STC was decanted and the protoplasts resuspended in 200 µl of STC and immediately placed on ice. The final concentration of protoplasts was measured with a hemacytometer and was approximately 1×10^8 protoplasts/ml.

Transformation was conducted by mixing 80 µl of the protoplast suspension with 20 µl of 40% PEG-3000 (polyethylene glycol) and 10 µl (3 µg DNA) of the disruption vector pCB1636-CyHy. The transformation mixture contained in a 1.5 ml microcentrifuge tube was placed on ice for 30 minutes. Following the 30 minute incubation, 900 µl of the 40% PEG solution was added. This mixture was placed at room temperature (23°C) for 20 minutes. The transformation mixture was transferred to 5 ml of liquid regeneration media (0.5 M sucrose, 0.1% yeast extract, 0.1% casamino acids) and incubated overnight in the dark at 25°C. The next day, the liquid regeneration transformation mixture was plated onto regeneration agar plates containing 1 M sucrose, 0.1% yeast extract, 0.1% casamino acids, 1.5% agar, and 50 µg/ml of hygromycin B to select transformants. Plates were sealed with parafilm and incubated at 25°C in the dark for 5-7 days when transformants appeared.

Analysis of transformants

For Southern analysis, *A. brassicicola* genomic DNA was extracted using the Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Solana Beach, CA). Restriction digests were performed by digesting 3 µg aliquots of genomic DNA with *EcoRI* and

*Hind*III. Restriction digests were separated on a 1% agarose gel and blotted onto a positively charged nylon membrane using a vacuum blotter (Bio-Rad, Hercules, CA). The 429 base pair cDNA probe for *CYHAB* was labeled using the PCR DIG Probe Synthesis kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Concentration of the probe in the DIG Easy Hyb hybridization solution was 50 ng/ml, and hybridization was carried out at 50°C overnight. Membranes were washed in a final solution of 0.1× SSC and 0.1% SDS at 68°C.

Transformants were also assessed by using PCR. Long distance PCR was performed with REDAccuTaq LA DNA Polymerase according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). A total of 100 ng of *A. brassicicola* genomic DNA, wild type and mutants, was used in each PCR reaction. Primers used to amplify the entire *CYHAB* ORF were 5' TGA GCC ATG CTG GTT CGA CCT G 3' and 5' TCG CAA GGT CTA CAT GAC AGA GC 3'. LD-PCR reactions were conducted in a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA) with the following program: 94°C for 3:00 minutes followed by 25 cycles of 94°C 30 seconds, 55°C 30 seconds, 68°C 6:00 minutes, and a final extension for 5:00 at 68°C.

PCR was also conducted with a non-LD Taq polymerase and short extension time capable of amplifying the wild type *CYHAB* ORF, but not the disrupted locus. These PCR reactions consisted of 12.5 µl of 2X PCR master mix (Promega, Madison, WI.), 10.5 µl of water, 1 µl of genomic DNA (50 ng), and 0.5 µl of each full length *CYHAB* primer (10 µM stock). PCR reactions were conducted in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following conditions, 94°C for 3 minutes, followed

by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes.

Finally, PCR was also conducted using the 5' *CYHAB* full length primer and a 3' Hygromycin B resistance gene primer (5' CGC GTC TGC TGC TCC ATA CAA G 3'). The PCR reaction conditions and thermocycler conditions were as above for the full length PCR with short extension time. All PCR reactions were repeated at least 2 times, and all PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized with ultra-violet light.

Cyanide sensitivity assays

A fresh KCN stock solution at a concentration of 50 mg/ml was added in varying concentrations to molten potato dextrose agar (PDA) prior to pouring in Petri plates (100 mm x 15 mm). For controls, PDA without KCN was used. Plates were inoculated with 2 mm agar plugs from 5-7 day old *A. brassicicola* cultures grown on PDA. For plates inoculated with cyanide hydratase mutants, these agar plugs came from plates of the mutant supplemented with 10 µg/ml of hygromycin B (to select for transformants). However, the mutant PDA plates in the KCN assays did not contain hygromycin B. All plates were sealed with parafilm and incubated at 25°C in a dark growth chamber. Diameters of colonies on plates with and without KCN were measured after 10 days to determine the growth inhibition by KCN. Experiments were repeated at least two times with similar results.

Pathogenicity assays

Red and green cabbage plants, 5-7 weeks old, were grown under greenhouse conditions at Colorado State University. Typical greenhouse conditions were 16/32°C

night/day temperatures with supplemental lighting provided 12 hours per day. Plants were watered on a daily basis as needed, and fertilized once per week with Miracle-Gro fertilizer (The Scotts Company, Marysville, OH). However, it should be noted that these greenhouse conditions were not monitored on a daily basis, and slight variations in the temperature and lighting were possible. Seeds were obtained from Johnny's Selected Seeds (Albion, ME) and the red cabbage was cultivar 'Super Red 80 F1' while the green cabbage cultivar was 'Tendersweet F1.' The second set of full true leaves was used in all pathogenicity assays with red and green cabbage. Petri plates (150 x 15 mm) were prepared by lining the bottom with wet paper towels. Detached leaves, with cuticle wax removed, were placed on the wet paper towels. Cuticle waxes were removed with a brief wash in 70% ethanol to allow the spore suspensions to stick to the cabbage leaf surface. Spore suspensions were created using 7-14 day old *A. brassicicola* cultures grown on potato dextrose agar (PDA). Spores were collected by flooding the plates with sterile distilled water, and gently rubbing the cultures with sterile pipet tips. A hemacytometer was used to calculate spore concentrations. Spore suspensions were placed on the leaf surfaces in 20 µl aliquots. Petri plate lids were sprayed with sterile water and sealed with parafilm to maintain 100% humidity required for *A. brassicicola* infection. Plates were incubated in growth chambers at 24°C with 12 hour day/night cycles for 5 days. After the 5 day incubation period the diameters of any lesions were measured.

Sequence analysis

The BLAST algorithms at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) were used to initially characterize sequences in the non-redundant GenBank database (Altschul et al. 1997). Multiple alignments were

conducted with ClustalX 1.81 and the BLOSUM series protein weight matrix (Thompson et al. 1997). A phylogenetic tree was calculated from the multiple alignment using the BOOTSTRAP N-J TREE option in ClustalX 1.81. This method derives confidence values for the groupings in a tree. For this analysis, 1000 bootstrap replicates were used.

Results

Cloning and sequence analysis of *CYHAB*

5' random amplification of cDNA ends (RACE) was successful in obtaining the 5' end of the *CYHAB* cDNA (Figure 5.1A). Figure 5.1B shows the full length cDNA sequence of *CYHAB* (GenBank accession number AY325811). The cDNA sequence is 1228 base pairs in length, including a 25 base pair 5' untranslated region and a 111 base pair 3' untranslated region including a 30 base pair poly-A tail, which encodes a predicted protein of 363 amino acids. The initiation codon (ATG) started at position 26 and the termination codon (TAG) ended at position 1117. A signal peptide was not predicted by the SignalP serverII (<http://www.cbs.dtu.dk/services/SignalP-2.0/>), and thus *CYHAB* appears to be a non-secretory protein. (Nielson et al. 1997).

Southern analysis of *CYHAB* (Fig. 5.2) was conducted and revealed the apparent presence of one copy of *CYHAB* in the *A. brassicicola* genome. *Bam*HI yielded a single fragment of approximately 2300 base pairs, *Hind*III a single 4300 base pair fragment, *Pst*I a single 4000 base pair fragment, *Eco*RI a single 9400 base pair fragment, and *Eco*RV a single 6500 base pair fragment.

The amino acid sequence of *CYHAB* was used to scan the PROSITE database, release 18.17, (<http://us.expasy.org/tools/scanprosite/>) for the occurrence of patterns and profiles. These searches revealed two patterns and one profile with unique patterns of

occurrence that suggested a biological role for *CYHAB*. The first pattern, nitrilase/cyanide hydratase signature1 (amino acids 38-53 GckLIaFpEvwIpGYP) and the second pattern, nitrilase/cyanide hydratase signature 2 (amino acids 159-172 GQlnCWENmnPFIK) suggest that *CYHAB* is a cyanide hydratase and perhaps may even have nitrilase activity. The profile (PDOC50263), carbon-hydrolase domain (amino acids 6-303) confirms the signature pattern results. A search of the protein families database of alignments and HMMs (Pfam, <http://www.sanger.ac.uk/Software/Pfam/index.shtml>) also returned a carbon-nitrogen hydrolase domain ($E=1.2e^{-72}$).

***CYHAB* homologs in other *Alternaria* species**

Amplification of *CYHAB* homologs was successful for all *A. brassicicola* strains examined, but failed for all other species of *Alternaria* (Figure 5.3A). To confirm that the PCR results were not due to sequence differences at the primer sites among the species examined, a Southern blot probed with a conserved region of cyanide hydratase enzymes was conducted. Three species, *A. brassicae*, *A. japonica*, and *A. ethzedia* apparently have *CYHAB* like genes as indicated by the Southern blot, however, their genomic location and nucleotide sequences are apparently divergent from the *CYHAB* homologs in *A.*

brassicicola. *A. brassicicola* isolates had a *Hind*III fragment approximately 4300 base pairs in size, while *A. brassicae* had a ~9400 base pair fragment, *A. japonica* a ~7000 base pair fragment, and *A. ethzedia* a ~8500 base pair fragment. It is not known whether these genomic sequences actually encode transcriptionally active genes. No Southern blot signal or PCR amplification product was detected for *A. alternata* f.sp. *lycopersici*, *A. alternata*, or *A. porri*. However, it should be noted that the hybridization conditions utilized in this study were extremely stringent and would require sequence homology of

at least 95% for a hybridization signal to occur. Thus, these results do not conclusively rule out the presence of a cyanide hydratase homolog in *A. alternata* f.sp. *lycopersici*, *A. alternata*, or *A. porri*.

Phylogenetic analysis of cyanide hydratase and nitrilase homologs

Multiple alignment of predicated amino acid sequences of known fungal cyanide hydratase and nitrilase enzymes revealed 2 conserved sequences (Figure 5.4). The first, amino acids 41-53 in *CYHAB*, has been predicted to form a hydrophobic β -strand (Bork and Koonin 1994). The second conserved sequence, amino acids 159-172, is predicted to contain the putative active site of the protein. The cysteine at position 163 has been shown to be required for cyanide hydratase activity (Brown et al. 1995). Several other residues are conserved among the enzymes examined in this study, however their functional role is not known. These include a lysine at position 26 (all positions based on the amino acid sequence of *CYHAB*), a glutamic acid at positions 33, 103, and 135, a leucine-tyrosine at positions 109-110, a proline at position 118, an arginine-lysine at positions 127-128, glycines at positions 140, 142, and 144, a valine at position 150, a threonine at position 153, and an alanine at position 185.

The phylogenetic tree calculated from the multiple alignment revealed a trichotomous tree. The first clade, supported by a bootstrap value of 1000, contained all the fungal cyanide hydratases examined including *CYHAB* which was most closely related to the cyanide hydratase from the *Brassica* pathogen *Leptosphaeria maculans*. The second clade contained the 2 nitrilase enzymes and was supported by a bootstrap value of 1000 (out of 1000 possible replicates). The outgroup was the cyanide degrading enzyme from the bacteria *Pseudomonas stutzeri* and this was the third clade. These

results indicate that cyanide hydratase enzymes from different fungal pathogens are highly conserved.

***CYHAB* expression analysis**

CYHAB was previously shown to be expressed, by RT-PCR, throughout the 5 day *A. brassicicola* infection of green cabbage (Chapter 2, this dissertation). Figure 5.6 demonstrates that *CYHAB* is highly upregulated when KCN is present in the environment. Though low levels of expression of *CYHAB* are found in media not containing cyanide (see Chapter 2, Figure 2.5), it is evident that *CYHAB* is upregulated in response to the presence of cyanide, further strengthening the conclusion that *CYHAB* is a cyanide hydratase.

***CYHAB* gene disruption**

To disrupt the *CYHAB* locus, a plasmid containing a 429 base pair cDNA fragment of *CYHAB* was used to transform protoplasts of *A. brassicicola* (Figure 5.7). A total of 20 transformants (~6 transformants per 1 µg of DNA) were collected from the *CYHAB* disruption transformation. These 20 transformants were single spored, taking 2 single spore isolates from each transformant for a total of 40 transformants. Two transformants named M1-S1 (mutant 1 single spore 1) and M2-S1 (mutant 2 single spore 1) had a disrupted *CYHAB* locus according to PCR and Southern blot analyses, which indicated that homologous recombination had occurred between the introduced plasmid and the *CYHAB* locus (Figure 5.8). The Southern blot was digested with *EcoRI* and *HindIII* and probed with the 429 base pair cDNA insert used to drive homologous recombination. A 4.2 kb fragment was indicative of the wild type intact *CYHAB* locus, and was also seen in mutants M3-S1 and M4-S1. Mutants M1-S1 and M2-S1 both did not

have this 4.2 kb fragment, but rather had 2 fragments corresponding to approximate 2500 and 1900 base pair fragments indicative of homologous recombination at the *CYHAB* locus. Along with the wild type 4.2 kb fragment, a 4.4 kb fragment is apparent in mutants M1-S1, M3-S1, and M4-S1, which is indicative of the introduced plasmid. Also, mutants M1-S1, M3-S1 and M4-S1 all have a 429 base pair fragment that demonstrates that homologous recombination did not occur between the introduced plasmid and the *CYHAB* locus. Because mutant M1-S1 has both this 429 base pair fragment, and the 2 fragments indicative of homologous recombination at the *CYHAB* locus, it apparently is a double insertion mutant with one of the these insertions occurring ectopically.

PCR analysis of the integration event in mutant M2-S1 also confirmed that the plasmid integrated into the *CYHAB* locus. Long distance PCR was used to amplify the entire *CYHAB* ORF, which yielded a 6 kb product in mutant M2-S1 consistent with integration of the plasmid into the *CYHAB* locus, and a 1100 base pair fragment in a wild type isolate (Figure 5.8 B-I). PCR with a short extension time and normal Taq polymerase was unable to amplify the *CYHAB* locus in the mutant M2-S1 as expected, but did amplify the 1100 base pair ORF in the wild type (Figure 5.8 B-II). Finally, PCR using a primer which anneals to the hygromycin B resistance gene and a primer which anneals to the 5' end of the *CYHAB* cDNA yielded a 1 kb fragment as expected for a homologous recombination event (Figure 5.8 B-III). Taken together, these results clearly demonstrate that the *CYHAB* locus is disrupted in mutant M2-S1.

CYHAB mutants displayed normal growth *in vitro* (Figure 5.9), as well as normal pigmentation and conidiation. *CYHAB* mutants did exhibit increased sensitivity to KCN *in vitro*, but were not completely inhibited even at high concentrations of KCN (Figure

5.10). These results confirm that *A. brassicicola* has evolved multiple detoxification mechanisms to deal with cyanide, and is not solely relying on a cyanide hydratase enzyme for cyanide detoxification. Table 5.1 shows typical results from the cyanide sensitivity assays.

Table 5.1. KCN sensitivity assays. Colony diameter measurements (cm) were conducted 10 days following inoculation of the agar plates. Results presented are the mean of 3 plates for each isolate with standard deviation. KCN concentrations examined were 0.9 mm, 1.1 mm, 1.3 mm, 1.6 mm, and 1.8 mm.

Genotype	0.9 mm	1.1 mm	1.3 mm	1.6 mm	1.8 mm
WT	5.60 ± 0.14	4.75 ± 0.07	4.50 ± 0.28	4.00 ± 0.00	3.85 ± 0.07
M2-S1	4.45 ± 0.07	3.95 ± 0.07	3.45 ± 0.07	3.15 ± 0.21	3.00 ± 0.14

***CYHAB* pathogenicity screens**

Pathogenicity screens with mutants M1-S1 and M2-S2 were inconclusive in determining a role for *CYHAB* in fungal pathogenesis, though they suggested that *CYHAB* is not required for *A. brassicicola* pathogenesis. Results of three pathogenicity screenings on red cabbage detached leaves are presented in Table 5.2. Results from replication 1 indicated that mutant M2-S1 may have a statistically significant ($p < 0.05$ student's T test) reduction in virulence as indicated by a 33% reduction in lesion size. However, no statistically significant ($p > 0.05$) reduction in virulence was seen with M2-S1 in replication 2 and 3. Similar results were obtained for M1-S1. In replication 2, M1-S1 exhibited a marked 58% decrease in lesion size ($p < 0.05$) compared with the wild type, but in replications 1 and 3, no difference was seen.

Interestingly, M1-S1 and M2-S1 had almost doubled the number of lesions that did not take compared with the wild type. Figure 5.11 demonstrates the definition of a no take lesion, as well as typical results obtained with red cabbage pathogenicity assays. However, given the apparent variability in the 3 experiments conducted, these results

needs to be examined further. Varying greenhouse conditions may be responsible for the variability in these pathogenicity assays.

Table 5.2 Pathogenicity assay results. Data is reported as the mean lesion diameter (cm) and standard deviation for each replication. Lesions that did not develop, non takes, were not included in mean lesion diameter analysis. Lesions measured do not include non-takes.

Isolate	Rep 1	Rep 2	Rep 3	Pooled Reps	Non Takes	Lesions Measured
Wild Type	1.56 ± 0.27	2.29 ± 0.42	1.70 ± 0.48	1.86 ± 0.51	23	83
M1-S1	1.61 ± 0.21	0.97 ± 0.59	1.55 ± 0.54	1.32 ± 0.54	41	65
M2-S1	1.05 ± 0.43	2.23 ± 0.42	1.58 ± 0.39	1.66 ± 0.70	42	62

Pathogenicity assays with green cabbage also did not reveal any apparent differences between the *CYHAB* mutants and wild type. Only 1 experiment was actually measured due to the extensive necrosis caused by *CYHAB* mutants on green cabbage (Table 5.3 and Figure 5.12).

Table 5.3. Pathogenicity assay results with green cabbage. Results reported as mean lesion diameter (cm).

<i>Isolate</i>	<i>Experiment Result</i>
Wild Type	1.20 ± 0.32
M1-S1	1.19 ± 0.19
M2-S1	1.35 ± 0.25

Taken together, these results indicate that *A. brassicicola* pathogenesis is not likely dependent on a functional cyanide hydratase enzyme.

Discussion

In this study, the cloning and characterization of a cyanide hydratase from the cruciferous necrotrophic pathogen *Alternaria brassicicola* is reported. Previous reports have suggested a correlation between the ability of a fungus to produce cyanide hydratase

and its ability to parasitize cyanogenic plants (Cluness et al. 1993, Fry and Evans 1977, and Wang et al. 1992). Wang et al. (1999) were able to clone and disrupt a cyanide hydratase from the sorghum pathogen *Gloeocercospora sorghi*, but mutants with a disrupted cyanide hydratase locus were not affected in their pathogenic capabilities. This was surprising because sorghum is a known cyanogenic plant, and previous studies by Fry and Meyers (1981) had suggested a role for cyanide hydratase in *G. sorghi* pathogenesis. *Brassica* species, and the model plant *Arabidopsis*, are not considered cyanogenic plants. However, all plants are capable of producing cyanide during ethylene biosynthesis and senescence (Goudey et al. 1989, Yip and Yang 1988).

This results from one molecule of cyanide being produced from the oxidation of ACC (1-aminocyclopropane-1-carboxylic acid) by ACC oxidase in the presence of iron and oxygen (Yang and Hoffman 1984). This may have direct relevance to the presence of cyanide hydratase enzymes in necrotrophic fungal pathogens. While senescence and apoptosis are two distinct processes, they both result in plant cell death which can cause the release of cyanide. Since necrotrophs appear to induce plant cell death before colonization of the host, they must have evolved some mechanism to deal with the cyanide produced as the result of the cell death. Thus, a cyanide hydratase enzyme appeared to be a good candidate gene to be required for *A. brassicicola* pathogenesis. The amount of cyanide that *A. brassicicola* would be exposed to during necrotrophic fungal pathogenesis was not examined in this study.

However, in comparison with *G. sorghi*, *A. brassicicola*'s ability to tolerate KCN *in vitro* was quite remarkable, and apparently is not solely dependent on cyanide hydratase activity. *G. sorghi* mutants with a disrupted cyanide hydratase were 91%

reduced in their ability to grow in the presence of 1 mM KCN, and wild type strains could be completely inhibited at a KCN concentration of 2 mM. In contrast, *A. brassicicola* cyanide hydratase mutants showed only a 20% reduction in growth at a KCN concentration of 0.9 mM. At a KCN concentration of 1.8 mM, cyanide hydratase mutants showed a 42% reduction in growth compared with *G. sorghi* wild type which was completely inhibited at 2 mM. Wild type *A. brassicicola* could be completely inhibited by KCN at a concentration of approximately 3.5 mM KCN. These results indicate that *A. brassicicola* has developed powerful cyanide detoxification mechanisms that do not solely rely on cyanide hydratase activity. Another potential explanation for these results is that the disrupted mutant was still capable of producing a truncated, but functional, copy of cyanide hydratase. Future studies should focus on creating a gene replacement mutant of *CYHAB* to completely rule out this possibility.

Since *A. brassicicola* apparently is not dependent upon a single cyanide hydratase enzyme for cyanide detoxification, it was not surprising that pathogenicity assays with the *cyhab* mutants did not reveal an apparent change in virulence. Further, even with a disrupted cyanide hydratase enzyme, *A. brassicicola* is clearly still producing its host-selective and non-host selective toxins. While the plant cell death caused by the action of *A. brassicicola*'s toxins may release HCN, it is not known what these levels are. Thus, at the spore concentrations used to assay pathogenicity with the *cyhab* mutants, the effect of the loss of *CYHAB* may not be evident due to the amount of toxins produced by *A. brassicicola*.

However, it should be noted that there was a significant amount of variation evident in the pathogenicity assays. For instance, the difference in the number of lesions

that developed between the mutants and the wild type may suggest a role for *CYHAB* in fungal pathogenesis. This variability was likely due to the growth conditions of the cabbage plants used in the study. Or, because *A. brassicicola* requires conditions with 100% humidity for pathogenesis, this condition may have not been met with the experimental set up used for the pathogenicity assays in this study. Future pathogenicity assays with the *cyhab* mutants need to be conducted in environmentally controlled growth chambers with an inoculum dilution series to better elucidate *CYHAB*'s role in fungal pathogenesis. Another explanation is that cyanide production, released from the dying plant cells, varies considerably from lesion to lesion and leaf to leaf. This may possibly explain the difference in the number of “no take lesions” between the wild type and *cyhab* mutants. Future studies should explore this possibility in more detail.

Phylogenetic and bioinformatics analyses have discovered cyanide hydratase enzymes in a wide variety of fungi with different life styles including necrotrophs, hemibiotrophs and even non-pathogenic saprophytes. This evidence alone may suggest that cyanide hydratase enzymes have not evolved from selection pressures encountered during plant pathogenesis, and thus are not required for fungal pathogenesis of plants. But under what selection pressures did the need for a cyanide hydratase enzymes arise during the course of fungal evolution? One potential explanation is that saprophytic fungi that normally inhabit the soil and feed on decaying material are often exposed to high levels of cyanide from cyanogenic bacteria which are also present in the soil (Handelsman and Stabb 1996). This may potentially explain why some saprophytic fungi like the *Alternaria* species examined in this study, and the model filamentous fungus *Neurospora crassa* contain cyanide hydratase enzymes. The fact that cyanide hydratase

enzymes are found in fungal pathogens that do not infect cyanogenic plants, i.e. the rice blast fungus *Magnaporthe grisea*, and fungi that are saprophytes, also seems to suggest that this gene is not required solely for fungal pathogenesis, and perhaps is more important for another aspect of fungal biology that is conserved throughout the Kingdom Fungi.

Interestingly, Sexton and Howlett (2000) found that the promoter of the *Leptosphaeria maculans* cyanide hydratase gene contained 4 putative target sites for GATA transcription factors which are known to regulate genes involved in nitrogen metabolism. Thus, it may be possible that cyanide hydratase enzymes are involved in providing a nitrogen source to fungi when exposed to cyanogenic compounds, which may be frequently encountered during saprophytic growth. Figure 5.13 is a potential model whereby cyanide hydratase could serve to provide a nitrogen source for fungal growth. This model depends on the presence of an enzyme that is capable of metabolizing formamide produced from the hydrolysis of HCN by cyanide hydratase. *Aspergillus nidulans* is known to produce such an enzyme, *FMDS*, that releases formate and ammonia from formamide, and thus some fungi do have formamidase enzymes (Fraser 1997). This model would also result in the production of NADH, through the action of a formate dehydrogenase, which could be used in electron transport chains. A formate dehydrogenase from *A. brassicicola* was identified in the suppression subtractive library created in this dissertation (Chapter 2).

Still, it does appear that *A. brassicicola* has evolved other mechanisms not solely dependent on a cyanide hydratase enzyme to deal with cyanide. In animals, cyanide is detoxified into the less toxic thiocyanate (SCN) by rhodanese (EC 2.8.1.1) and 3-

mercaptopyruvate sulphur transferase (EC 2.8.1.2) (Mintel and Westly 1966, Qarawi et al. 2001). Using the rhodanese cDNA sequence from *Homo sapiens*, a BLASTX search of fungal sequences in the Genbank non-redundant database (NR) revealed several putative rhodanese homologs in fungi such as *N. crassa* ($E = 1e^{-41}$) and *A. nidulans* ($E = 1e^{-45}$). Because fungi are more closely related to humans than plants, rhodanese enzymes might be responsible for the ability of some fungi to tolerate more cyanide than others. *G. sorghi* was reported not to have rhodanese activity, which may explain its increased sensitivity to cyanide (Myers and Fry 1978).

Recently, a cyanide hydratase from *Fusarium lateritium* was shown to have nitrilase activity (Nolan et al. 2003). Cyanide hydratases and nitrilases have been shown to catalyze distinct reactions, with cyanide hydratase catalyzing the conversion of HCN to formamide with the addition of one water molecule, while nitrilases hydrolyze RCN substrates into the corresponding carboxylic acid. As the phylogenetic analysis of amino acid sequences of fungal cyanide hydratases and various nitrilases show, there is a high degree of conservation between these two enzymes. Thus, if cyanide hydratase enzymes are capable of using nitriles as substrates, it may be possible that nitrilases are capable of utilizing cyanide as a substrate. Future studies should examine the ability of nitrilases and cyanide hydratases to utilize various substrates.

Possible other substrates for cyanide hydratase and nitrilase activity include the glucosinolates, which are broken down into isothiocyanates, thiocyanates, and nitriles. It has been previously hypothesized, but not proven, that a cyanide hydratase from the *Brassica* pathogen *L. maculans* is involved in detoxifying glucosinolate breakdown products (Sexton and Howlett 2000). Sexton and Howlett (2000) found that propionitrile

could induce cyanide hydratase expression and also found that *L. maculans* could utilize 2-propenyl glucosinolate as a sole nitrogen source. Though the effects of these glucosinolate breakdown products on *cyhab* mutants were not directly examined in this study, the pathogenicity assays suggest that the *A. brassicicola* cyanide hydratase is not directly involved in detoxifying glucosinolate breakdown products. Or, if it is, there are other enzymes that can compensate for its loss. Thus, *A. brassicicola*'s apparent ability to detoxify glucosinolate breakdown products is still not known (Giamoustaris and Mithen 1997, Tierens et al. 2001).

In conclusion, the results of this study indicate that the necrotrophic fungus *A. brassicicola* possesses a cyanide hydratase enzyme (*CYHAB*) that does not appear to be required for pathogenesis on *Brassica* species. *CYHAB* was also not solely required for detoxification of cyanide *in vitro* as mutants with a disrupted *CYHAB* locus displayed increased sensitivity, but not a complete loss of the ability to grow, in the presence of varying concentrations of KCN. This result suggests that *A. brassicicola* has evolved other mechanisms to detoxify cyanide. Since other fungi that are typically saprophytic and not plant pathogens also contain cyanide hydratase enzymes, it is likely that the presence of cyanide hydratase enzymes in fungi was not the result of selection pressures brought about by pathogenesis on cyanogenic plants. Future research should focus on the role of cyanide hydratase enzymes in nitrogen metabolism and dealing with cyanide produced by other soil inhabiting microorganisms.

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Figure 5.1. Random amplification of *CYHAB* 5' cDNA end and full length *CYHAB* cDNA. A. 5' RACE product for *CYHAB*. Lane L = 1 KB ladder, 1 = HeLA RNA control, 2 = *CYHAB* 5' race product. B. Full length cDNA sequence of *CYHAB*. Genbank accession AY325811, start codon (ATG) and termination codon (TAG) bold and underlined.

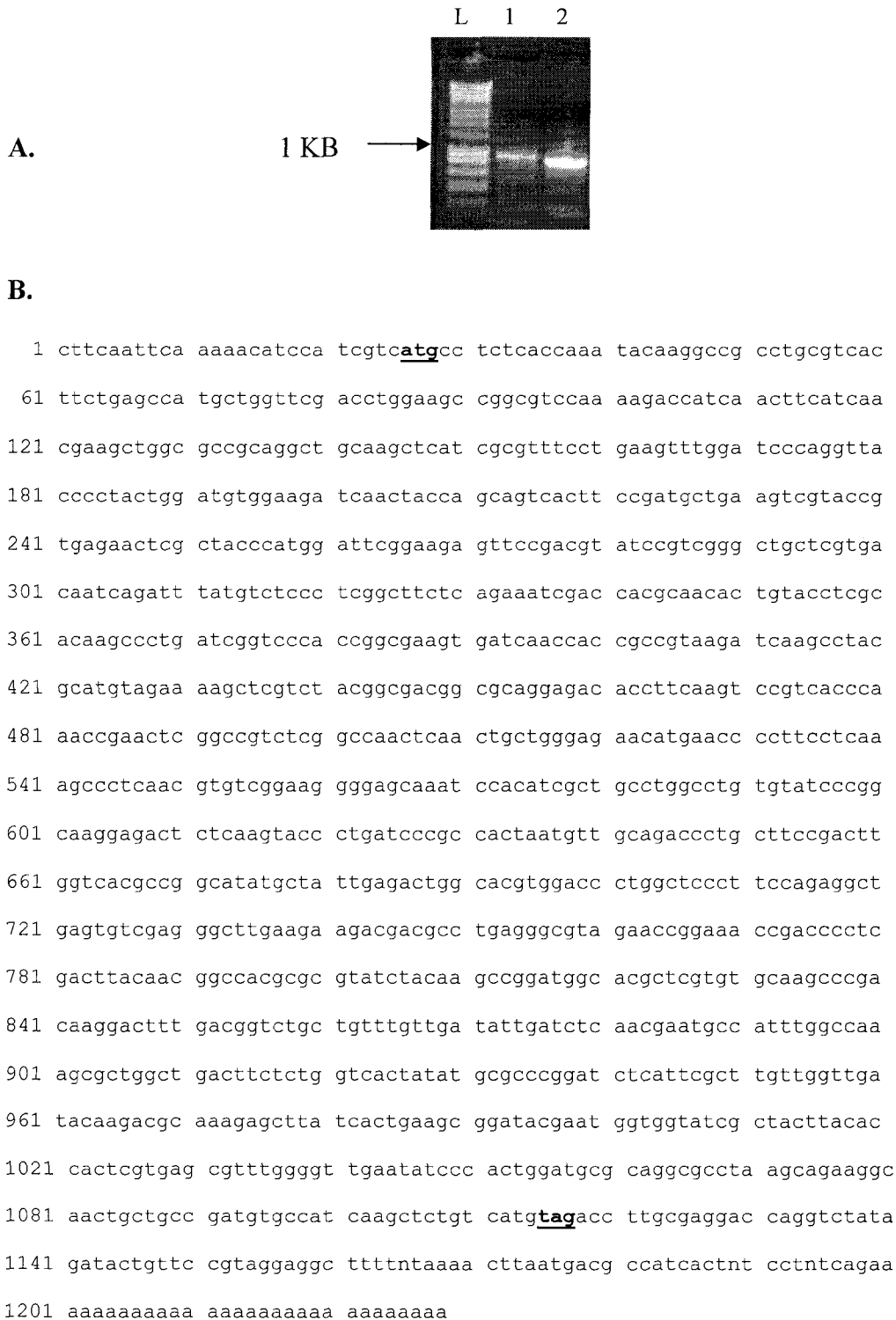


Figure 5.2. Southern blot of *CYHAB Alternaria brassicicola* strain ATCC 34622. Lane 1 = *Bam*HI, 2 = *Hind*III, 3 = *Pst*I, 4 = *Eco*RI, 5 = *Eco*RV.

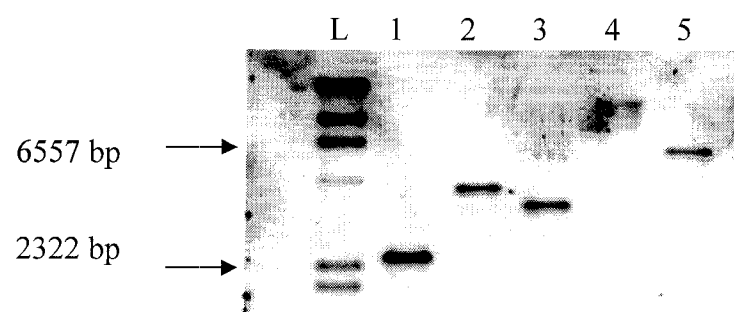
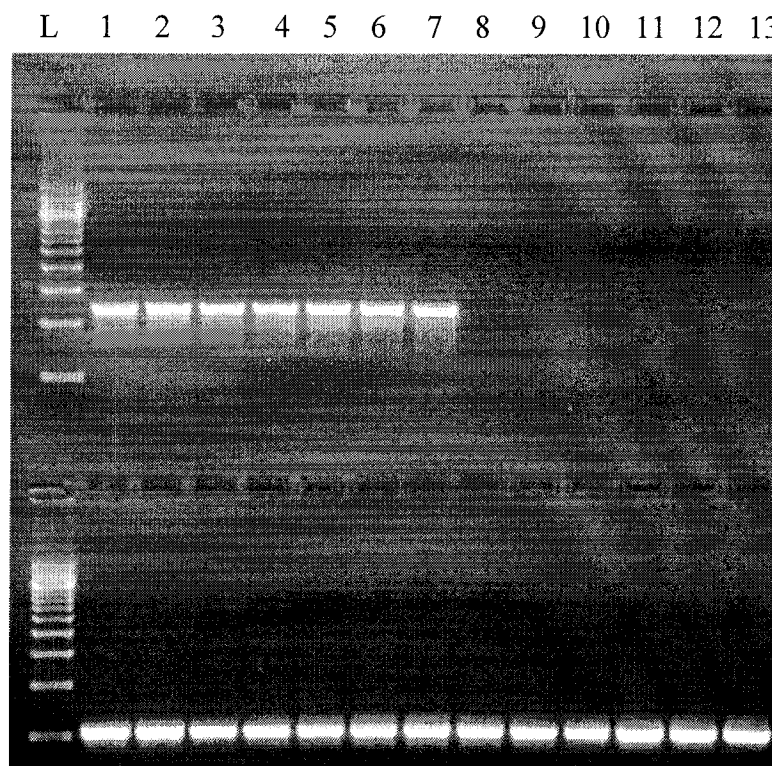


Figure 5.3. PCR amplification and Southern blot analysis of *CYHAB* homologs from other *Alternaria* species. A. PCR, Top = *CYHAB* amplification, bottom = beta-tubulin amplification to check template integrity. Lanes L = 1 kb ladder, 1 = *A. brassicicola* (AB) ATCC 34622, 2 = AB 21-71-001, 3 = AB 21-71-002, 4 = AB 21-71-004, 5 = AB 21-71-005, 6 = AB 21-71-006, 7 = AB 21-71-007, 8 = *A. brassicae* 21-61-002, 9 = *A. japonica* 21-72-002, 10 = *A. alternata* f.sp. *lycopersici* AS273, 11 = *A. porri* O256, 12 = *A. ethzedia* 21-16-001, 13 = *A. alternata* ATCC 11680. B. Southern blot digested with *Hind*III. Lanes L = DIG marker, 1-9 as above in A. Lane 10 = *A. ethzedia* 21-16-001, 11 = *A. porri* O256, 12 = *A. alternata* f.sp. *lycopersici* AS273, 13 = *A. alternata* ATCC 11680

A.



B.

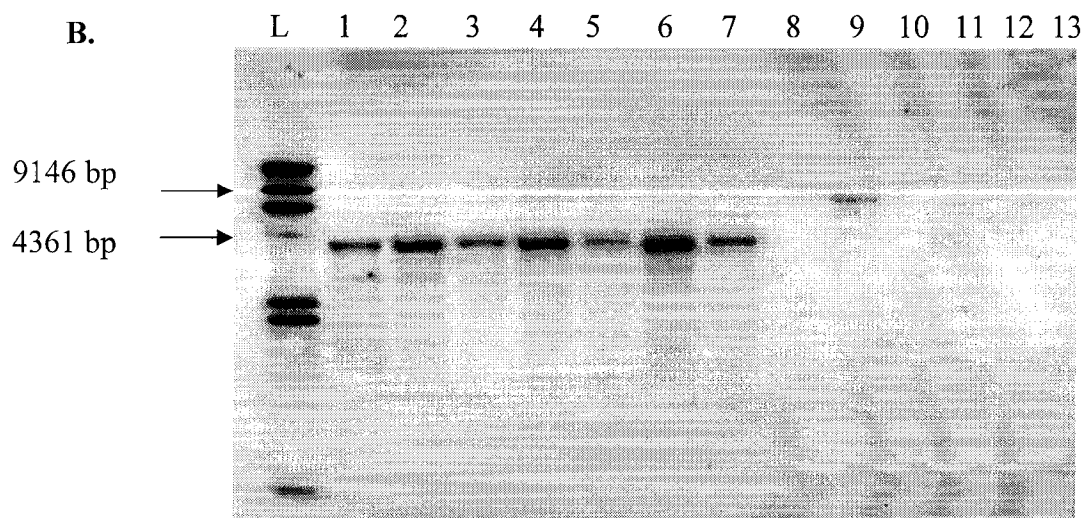


Figure 5.4. Multiple alignment of cyanide hydratase and nitrilase homologs. Multiple alignment performed with Clustal X v. 1.81. Genbank accessions:

L_maculans_AF192405, A_brassicicola_AAP88966, N_haematococca_CAC69666, M_grisea_MG10714, G_sorghii_JQ1613, P_stutzeri_BAA11653, A_thaliana_CAB88999, NP_012102 S_cerevisiae

```

L_maculans_CYHY      1  -----MPLTKYKAAAVTSEPAWFLLEAGVOKTIDFINEAGAGCKL
A_brassicicola_CYHY  1  -----MPLTKYKAAAVTSEPCWFDLEAGVOKTINFINEAGAGCKL
N_haematococca_CYHY  1  -----MPLTKYKAAAVTSEPCWFDLEAGVOKTIDFINEAGAGCKL
M_grisea_CYHY        1  -----MSITKYKAAAVTSEPCWFDLEAGVOKTINFINEAGAGCKL
G_sorghii_CYHY       1  -----MPENKYKAAAVTSEPVWENLEGGVVKTIETFINEAGAGCKL
P_stutzeri_cyanideenz 1  -----MAHYPLKAAAVQDAVWLLDPAVENSVKLLEASNGAKL
A_thaliana_nitrilase1 1  MSSTKDMSTVQNATPFNGVAPSTTVVVTIVQSTVINDTPATDPAEKYIVEASKGAEL
S_cerevisiae_nitrilase 1  -----MAKHIVAAQIGSCPGSTKDTKRIISSEKEIKESGAKL
consensus            1  mpltkykaaavtsep wfdleagv kti finEag ag kL

L_maculans_CYHY      42  VAFPEVWIPGYP----YWMWKVNYQOSLPMLKKYRENSAVDS---DEERRIRRAARD
A_brassicicola_CYHY  42  VAFPEVWIPGYP----YWMWKVNYQOSLPMLKSYRENSEPMDS---DEERRIRRAARD
N_haematococca_CYHY  42  VAFPEVWIPGYP----YWMWKVNYLQSLPMLKKYRENSITVDS---DEERRIRRAARD
M_grisea_CYHY        42  VAFPEVWIPGYP----YWMWKVNYLQSLPMLKAYRENSKFKVDS---DEERRIRRAARD
G_sorghii_CYHY       42  VAFPEVWIPGYP----YWMWKVNYLQSLPMLKAYRENSITVDS---DEERRIRRAARD
P_stutzeri_cyanideenz 43  VAFPEAIPGYP-----TAALGHPEYRRFYLTLYLNAEAPS---EAVQISAAARK
A_thaliana_nitrilase1 61  VLFPEGHICGYPRGRFRGLAVGVHNEGRDEFKYHASTHVPG---PEVARADVARK
S_cerevisiae_nitrilase 40  VVIPFATIGGYPKGSNFGVYLGRLQGREYAKYLAELENGGEKYPELSQCALSEA
consensus            61  vafPEvwipGYP      ywmkwkyny qslpmlkkyrensm vds      demrrirraard

L_maculans_CYHY      93  NQIYVSLGFSEIDHATLYLAQALISPTGEVFNHRRKIKPTHVEKLVYGDGAGDFTSVVP
A_brassicicola_CYHY  93  NQIYVSLGFSEIDHATLYLAQALIGPTGEVFNHRRKIKPTHVEKLVYGDGAGDFTKSVTQ
N_haematococca_CYHY  93  NQIYVSLGFSEIDHATLYLAQVLIGPDGSVFNHRRKIKPTHVEKLVYGDGPGDFTSVSVL
M_grisea_CYHY        93  NQIYVSLGFSEIDHATLYLAQVLIGPDGSVFNHRRKIKPTHVEKLVYGDGSGDSFPPVITQ
G_sorghii_CYHY       93  NQIYVSLGFSEIDHATLYLQVLISPLGIVFNHRRKIKPTHVEKLVYGDGSGDSFPPVITQ
P_stutzeri_cyanideenz 94  NKLYVCSQSEKDGSLYLAQWENPEGLTGKRMFYVAEFLCAGDGNCSMPVPT
A_thaliana_nitrilase1 117  NHLYVVCAILKKGTYLCTVFFSPQOFLGKARKMPTSLERCTNGQCDG-STIPVYV
S_cerevisiae_nitrilase 100  TDASLCYGCIERDGTLLCTMVYDEKDGYYGKRMPTAGELNGQCDG-STIPVYV
consensus            121  nqiYvslgfsEidhatLYlaqvli P geVlnhrRKikpthvEklyvGdG GdsfmpVte

L_maculans_CYHY      153  TEIGRLGQLNCWENMNPFLKALNVSAEQIHAAPVPYPGKETLKYDPDPATNVADPASDL
A_brassicicola_CYHY  153  TEIGRLGQLNCWENMNPFLKALNVSEGEQIHAAPVPYPGKETLKYDPDPATNVADPASDL
N_haematococca_CYHY  153  TEIGRLGQLNCWENMNPFLKALNVSCGEQIHAAPVPYPGRRERQVAPDPATNYADPASDL
M_grisea_CYHY        153  TEIGRLGQLNCWENMNPFLKSLVSSGVQHHAAPVPYPGVQQVAPDPATNYADTASDL
G_sorghii_CYHY       153  TEIGRLGQLNCWENMNPFLKSLAVERGEQIHAAPVPYHDLKGVHPDPATNYADPASDL
P_stutzeri_cyanideenz 153  TEIGNLGGIMCWEHNVPLDIAAMNSQNECHHAAPVPGSFDDDET-----
A_thaliana_nitrilase1 176  TPICRLGAAICWENRMPLYETALYKGIETLYCAPTADGSKWEQS-----SMLH
S_cerevisiae_nitrilase 159  TAACKLGGAICWENMNPFLHYAMYKKGVEIWCAPTVDARPI-----
consensus            181  TeiGrlGqlnCwEnmnpflkal vs geqihiaawpvpyg e      pdpatn adpasdl

L_maculans_CYHY      213  VTPAYAIETGWTLPAPFQRLSAEGLKMNTPGVEPETDPTTYNGHARIYRPDGSILVVR-P
A_brassicicola_CYHY  213  VTPAYAIETGWTLPAPFQRLSVEGLKKTTPGVEPETDPTTYNGHAXXYTPDCILVCK-P
N_haematococca_CYHY  213  VTPAYAIETGAWTLAPFQRLSVEGLKKNTPGVEPETDPTTYNGHARIYRPDGSILVVR-P
M_grisea_CYHY        213  VTPAYAIETGWTLPAPFQRLSVEGLKKTTPAGVEPETDPTPYKHARIYRPDGSILVVKP
G_sorghii_CYHY       213  VTPAYAIETGWTLPAPFQRLSVEGLKKTTPGVEPETDPTPYKHARIYRPDGSILVYK-P
P_stutzeri_cyanideenz 196  ASHYAICNQAFLMTSSITYSEMKDMLCETQERQYFNIFKSGHTRIYGPDGEPISDLV
A_thaliana_nitrilase1 225  ATEGCFVLSACQFCQRKFFPDHPDYLFIDWYDKKHLSIVSGGGSVITSLQLQVAG-P
S_cerevisiae_nitrilase 225  -----
consensus            241  vtpayaietgtw lapfqrslsveglkkt tp gvepetdpt ynghariyrpdgslv k p

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Figure 5.4. Continued.

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L_maculans_CYHY      272 DKDFDGLFVVDIDLNECHLTKALADFSGHYMR-----PDLIRLLVDTRR
A_brassicicola_CYHY  272 DKDFDGLFVVDIDLNECHLTKALADXXGHYMR-----PDLIRLLVDTRR
N_haematococca_CYHY  272 DKDFDGLFVVDIDLNECHLTKVLADEFAGHYMR-----PDLIRLLVDTRR
M_grisea_CYHY        273 DKDFDGLFVVDIDLNECHLTKVLADEFAGHYMRPLLDANILQSIRNQLRSDLIRLLVDTRR
G_sorghii_CYHY       272 AVDFDGLFVVDIDLNECHLTKALADFAHYMR-----PDLIRLLVDTRR
P_stutzeri_cyanideen  256 PATEGTAFAIDIEKIIDEKYYIDPVGHYSN-----QSLSSNFNQSP
A_thaliana_nitrilas1 284 FFSGLTADIDLGAIARAKLYFDSVGHYSF-----FDMLHITVNEEP
S_cerevisiae_nitrilas
consensus            301 dkdfdgllfvdidlne hltk ladf ghymr                pdlirllvdtrr

L_maculans_CYHY      316 KELVTEAAG--RDCIKAYSTRERLGLNLPLDGSKEDKVPVRL-----
A_brassicicola_CYHY  316 KELVTEAAT--GGIATYSTRERLGLNIPDAQAPKQKATADVPSSSVM---
N_haematococca_CYHY  316 KELVTEAP--VGTATYSTRRLGLDKPLDGEKKEKEATKRDSEAEEL---
M_grisea_CYHY        333 KELVTEAP--GGIKSYSTRRLGLGKALDDVEEDTERTV-----
G_sorghii_CYHY       316 KELVTEVGGGDNGGIQSYETMARLGLDRPLDEEDYRQGTDAETEKAASSNGHA
P_stutzeri_cyanideenzym1 299 NPVVRKIGE---RDSVFTYDDNLISVSDDEPVRSLRK-----
A_thaliana_nitrilas1 328 RKSVEFVTKVEKEEDDNK-----
S_cerevisiae_nitrilaselp
consensus            361 kelvteae  nggi tystr rlg1  pld                a

```

Figure 5.5. Phylogenetic analysis of cyanide hydratase and nitrilase homologs. Tree created from multiple alignment made with Clustal X v. 1.81 and the nearest neighbor joining method. 1000 bootstrap replicates were used. The tree was rooted with the cyanide degrading enzyme from *Pseudomonas stutzeri*.

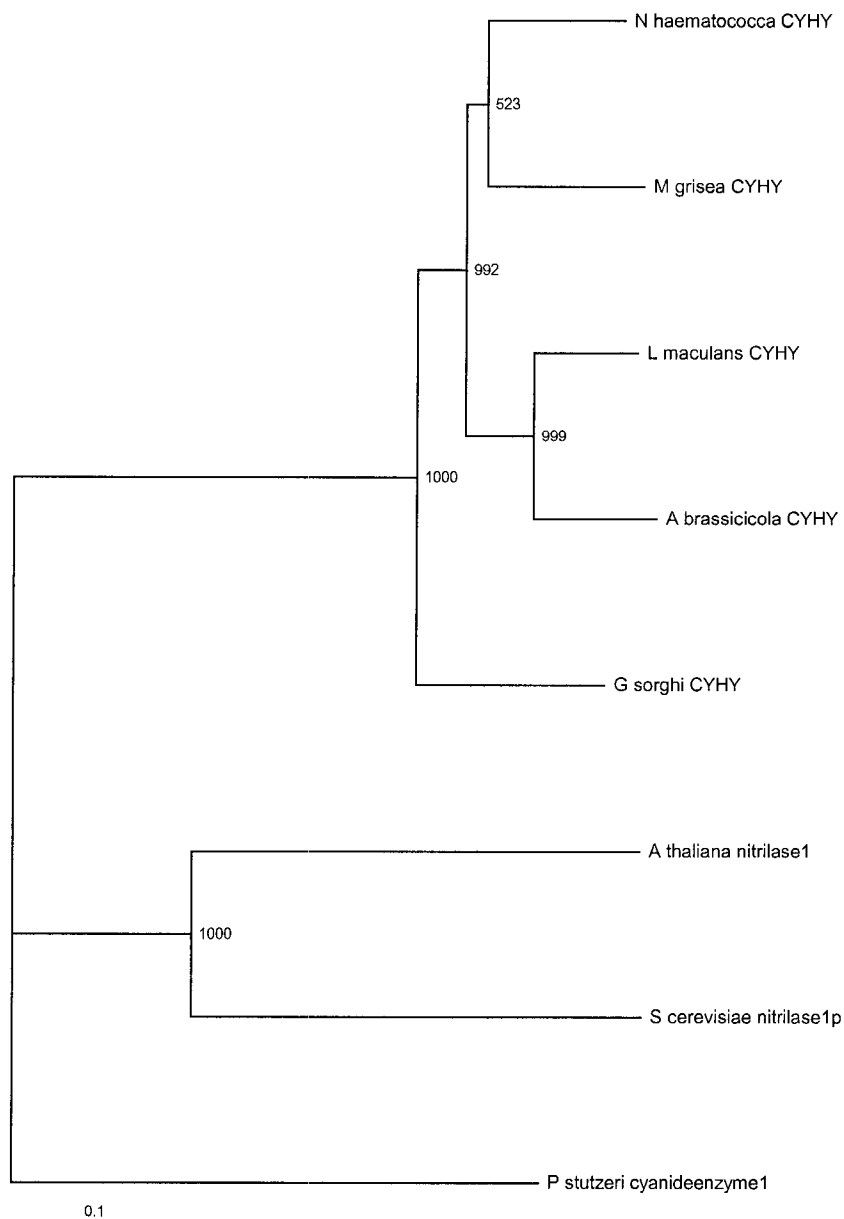


Figure 5.6. Expression of *CYHAB* examined with RT-PCR. Lanes L = 1 KB ladder, 1 = blank lane water control, 2 = *Arabidopsis* ecotype Landsberg Erecta, 3 = *A. brassicicola* grown in V8 broth for 5 days, 4 = *A. brassicicola* grown in minimal medium broth for 5 days, 5 = *A. brassicicola* grown in potato dextrose broth for 5 days, 6 = *A. brassicicola* grown in glucose yeast extract broth (GYEB) for 5 days, 7 = *A. brassicicola* grown in GYEB for 5 days, then 10 mM KCN was added and *A. brassicicola* was grown an additional 4 hours.

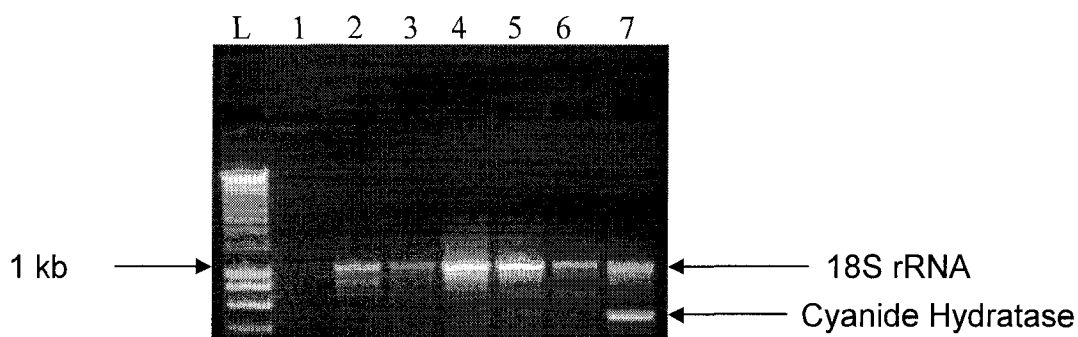


Figure 5.7. Disruption strategy for *CYHAB*.

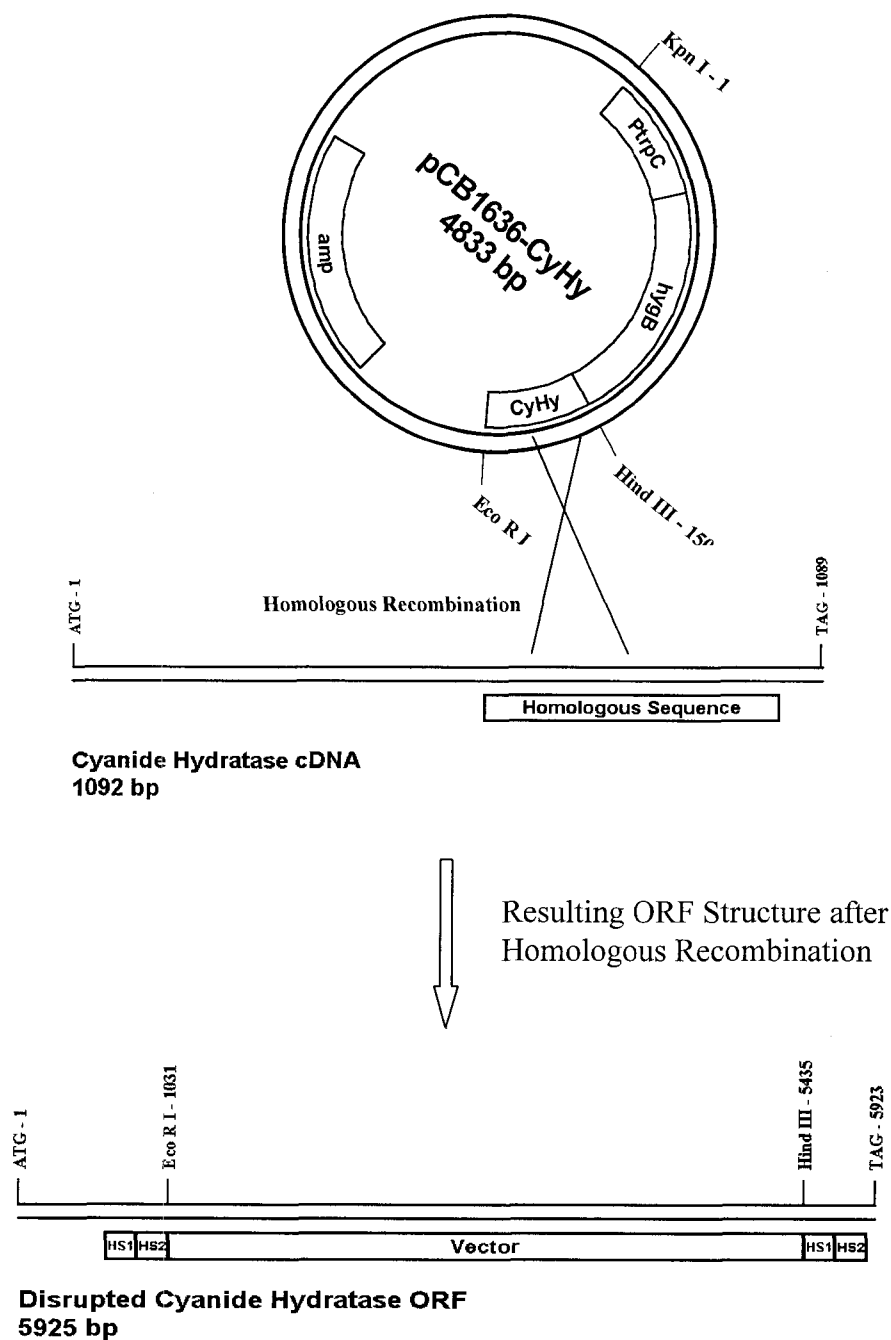


Figure 5.8. Confirmation of *CYHAB* gene disruption. A. Southern blot, Lanes = L = DIG marker, 1 = wild type *A. brassicicola* ATCC 34622, 2 = *CYAHAB* mutant M1, 3 = *CYAHAB* mutant M2, 4 = *CYAHAB* mutant M3, 5 = *CYAHAB* mutant M4. Genomic DNA digested with *EcoRI* and *HindIII*. B. PCR confirmation of *CYHAB* disruption. I = long distance PCR with full length *CYHAB* primers Lanes = L = 1 KB ladder, 1 = wild type *A. brassicicola*, 2 = mutant M1 3 = mutant M2, II = full length *CYHAB* primers short extension time Lanes = L = 1 KB ladder, 1 = wild type, 2 = mutant M2 III = *CYHAB* 5' primer and 3' Hygromycin B primer Lanes = L = 1 KB ladder, 1 = wild type, 2 = mutant M2, 3 = mutant M1, 4 = wild type beta tubulin, 5 = mutant M2 beta tubulin.

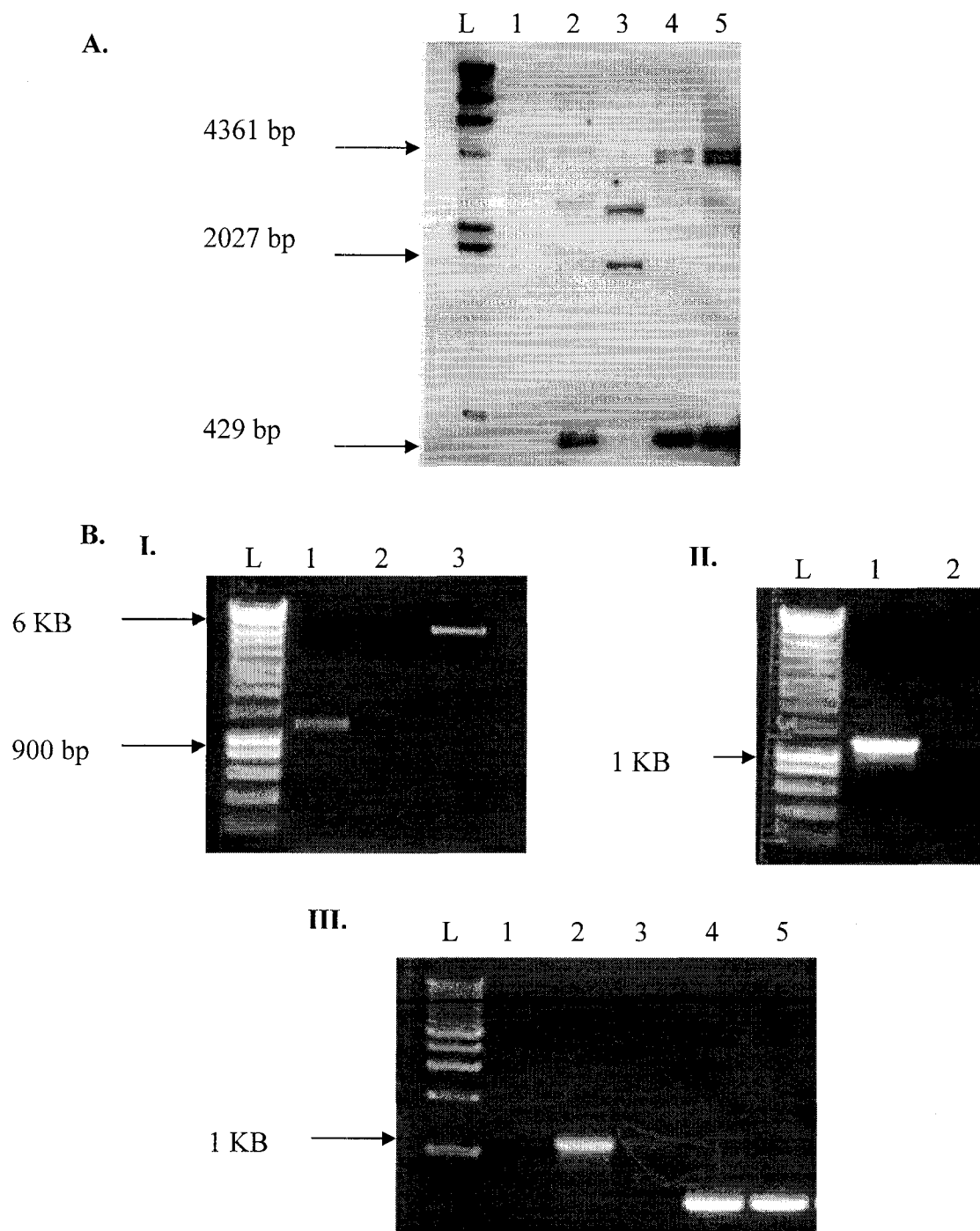


Figure 5.9. In vitro growth of cyanide hydratase mutants. Mutants with a disrupted cyanide hydratase locus show normal vegetative growth *in vitro*.

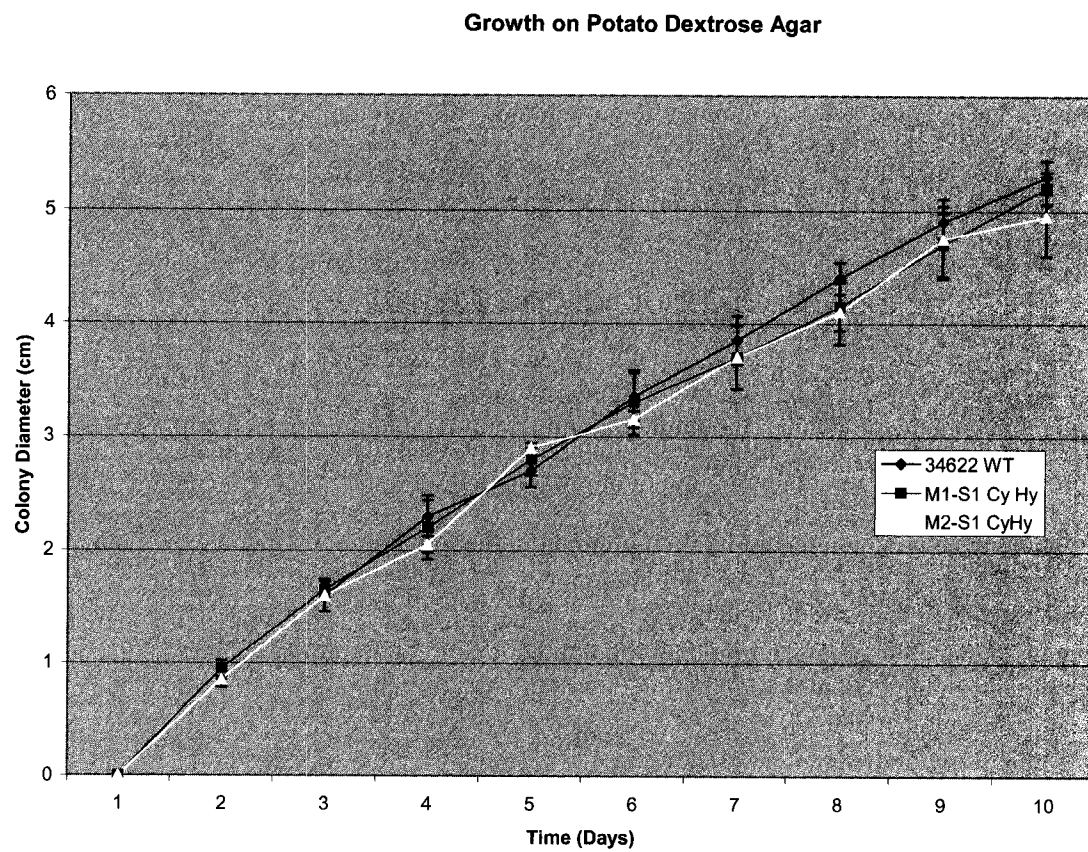


Figure 5.10. Sensitivity to KCN *in vitro*. Wild type *Alternaria brassicicola* isolate ATCC 34622 and cyanide hydratase mutant M2-S1 were grown on PDA media containing varying amounts of KCN. Results presented are 10 days following inoculation of the plates.

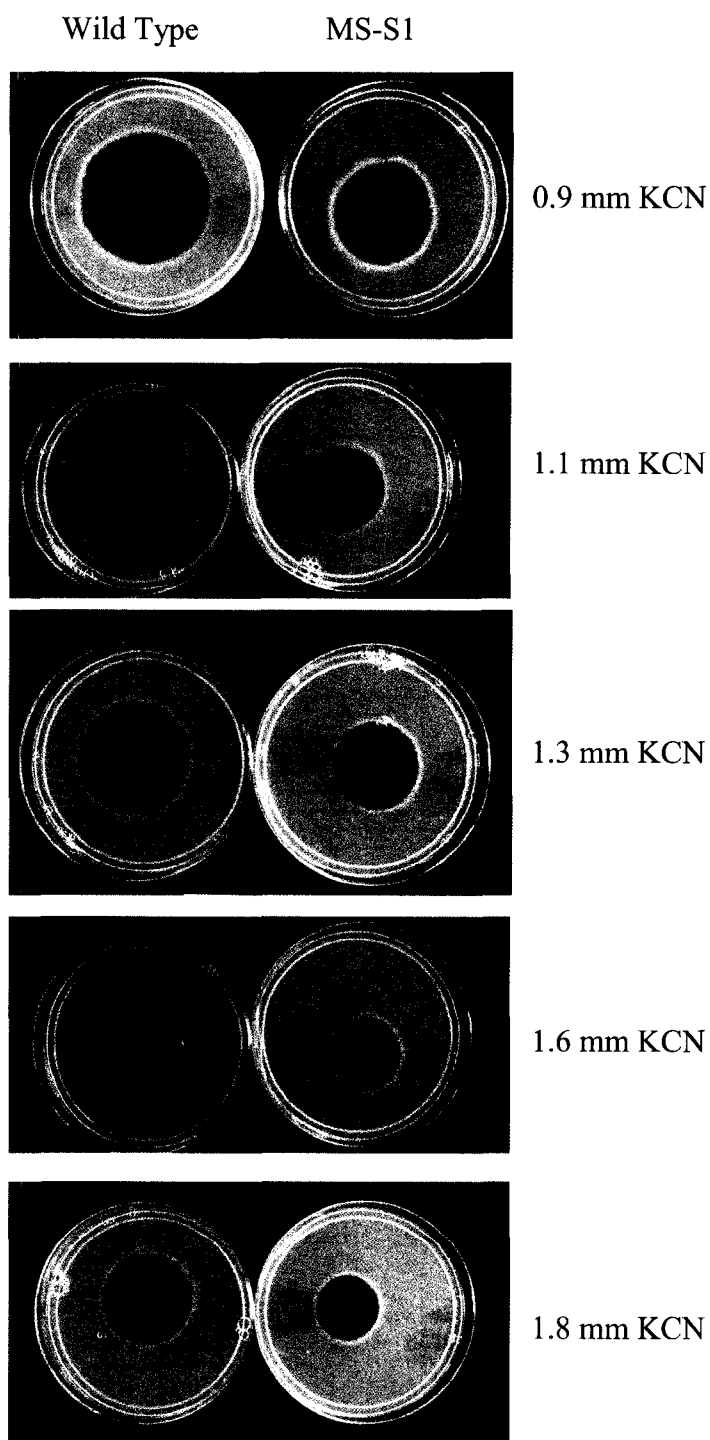
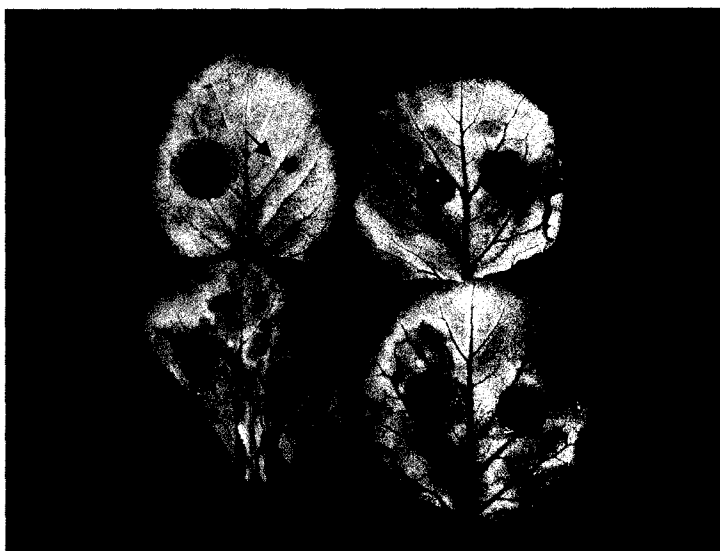


Figure 5.11. Red cabbage pathogenicity assay. Typical pathogenicity assay result with red cabbage, wild type *Alternaria brassicicola* isolate ATCC 34622, and cyanide hydratase mutant M2-S1. Arrows point to lesions classified as no-takes.

Wild Type



M2-S1

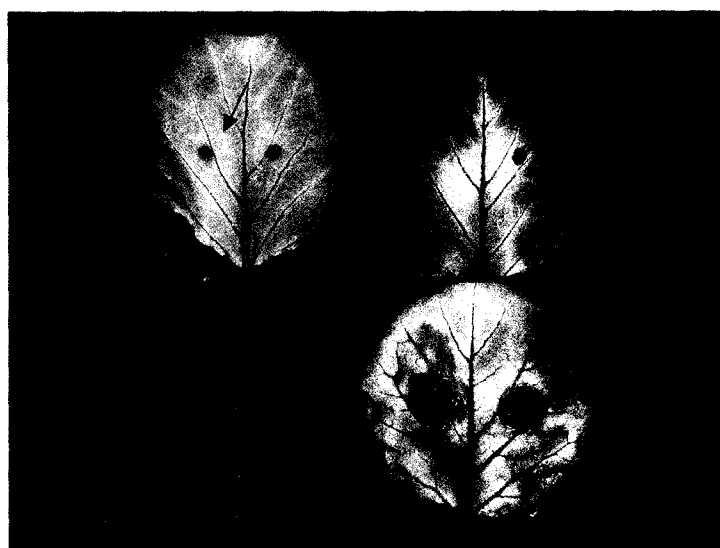


Figure 5.12. Green cabbage pathogenicity assay. Typical pathogenicity assay result with green cabbage, wild type *Alternaria brassicicola* isolate ATCC 34622, and cyanide hydratase mutant M2-S1.

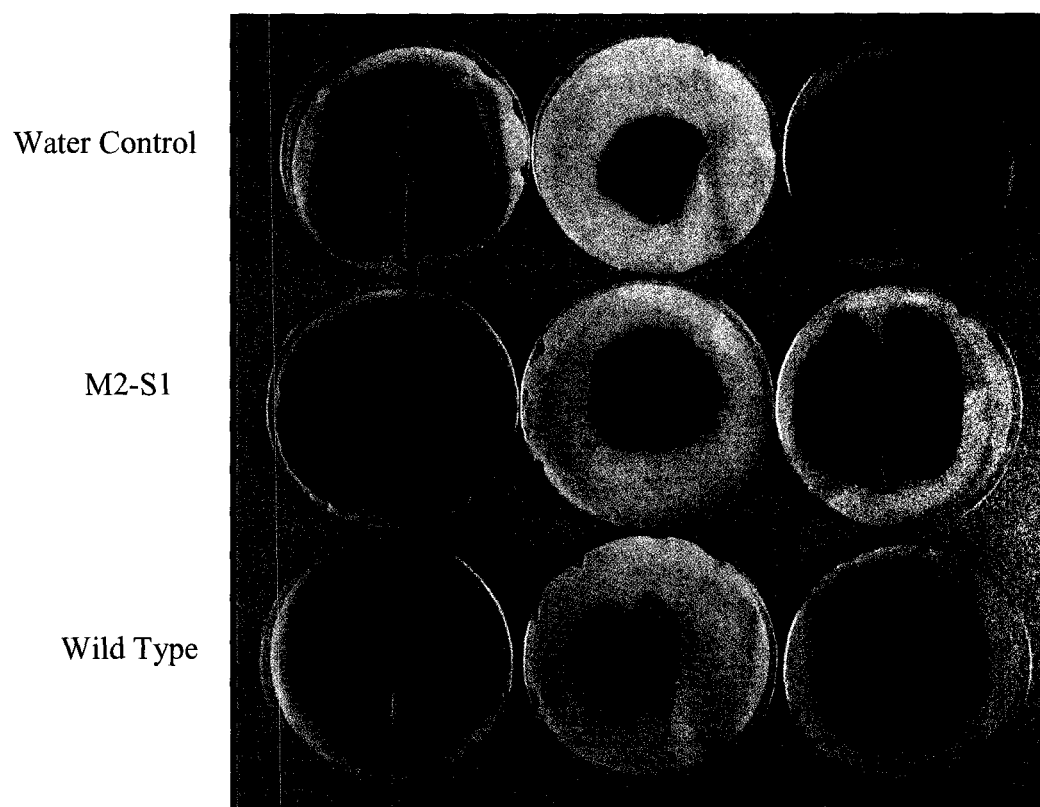
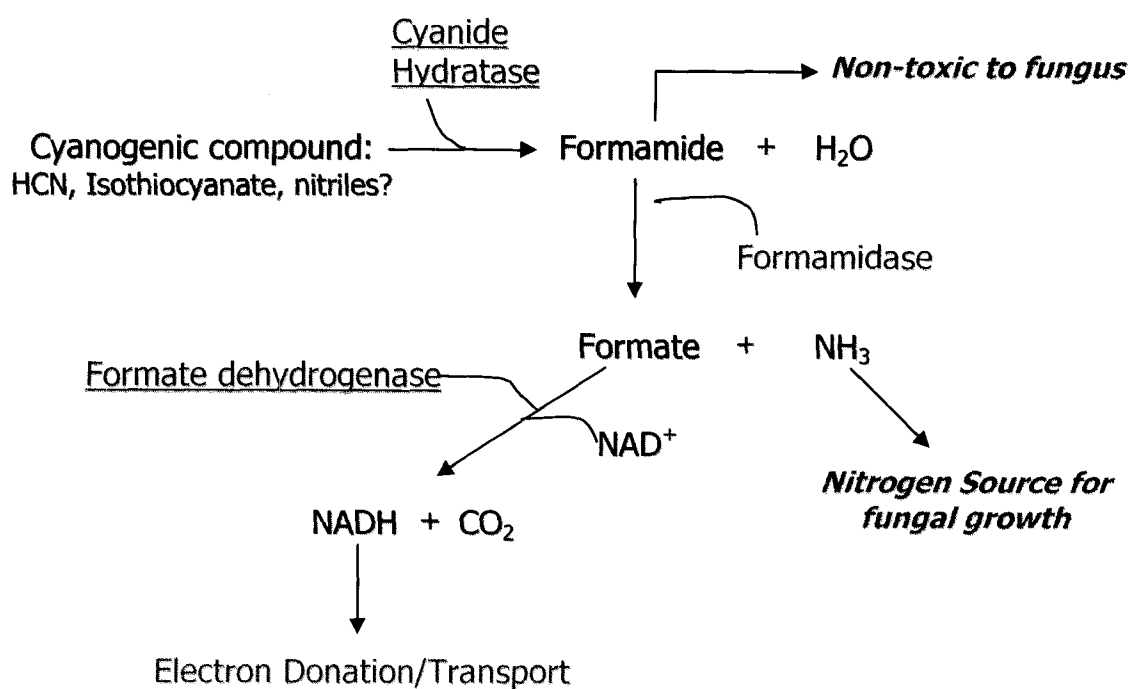


Figure 5.13. A model for the role of cyanide hydratase in fungal biology. Underlined enzymes are genes that have been cloned from *Alternaria brassicicola*.



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Chapter 6

Cloning and Expression Analysis of the *Alternaria brassicicola* MAP

Kinases *AMK1* and *AMK2*

Introduction

Mitogen-activated protein kinase (MAPK) cascades are widely conserved in eukaryotes and regulate a diverse set of cellular processes including programmed cell death, mitosis, growth, and reproduction. Like other protein kinases, MAPKs are enzymes that covalently attach phosphate to the side chain of either serine or threonine of specific proteins. Phosphorylation of proteins can control many aspects of their function including interactions with other proteins, cellular location, and enzymatic activities. MAPKs, like their substrates, are regulated by phosphorylation and are part of a phospho-relay system that consists of three sequentially activated kinases (Cobb and Goldsmith 1995; Johnson and Lapadat 2002). When a MAPK cascade is activated, a MAP kinase kinase kinase (MAPKKK) phosphorylates a MAP kinase kinase (MAPKK), which in turn phosphorylates the MAPK (Cooper 1994).

Baker's yeast, *Saccharomyces cerevisiae*, has become the model organism for the study of MAP kinase cascades. Sequencing of the yeast genome along with genetic and

biochemical studies have revealed the presence of 5 MAPKs that are part of 5 functionally distinct signal transduction cascades (Gustin et al. 1998; Hunter and Plowman 1997). Perhaps the most well studied of these pathways is the mating-pheromone response pathway which involves the MAPK *FUS3*. Secreted pheromones (α -factor and α -factor) stimulate cell cycle arrest, morphological changes, and transcriptional induction of mating related genes. These responses are mediated through the *FUS3* MAPK pathway. Initially, the pheromones bind to and activate a 7 transmembrane domain receptor (*STE2/STE3*), which induces the dissociation of a heterotrimeric G protein. The G β -G γ (*STE4/STE18*) complex then activates downstream proteins *STE5* (a scaffold protein) and *STE20* (a protein kinase), which in turn activate the *STE11* (MAPKKK), *STE7* (MAPKK), *FUS3* (MAPK) cascade. *FUS3* phosphorylates several downstream targets including *FAR1*, *DIG1*, *DIG2* and *STE12* that regulate various responses required for successful mating (Errede et al. 1993; Leberer et al. 1993; Marcus et al. 1994; Ramer and Davis 1993).

The identification of a *FUS3/KSS1* homolog, *PMK1*, in the hemibiotroph *Magnaporthe grisea*, was a major breakthrough in the study of fungal pathogenesis (Xu and Hamer 1996). *M. grisea* germ tubes produced from germinating conidia differentiate into specialized infection structures called appressoria on hydrophobic surfaces such as the plant leaf surface. Turgor pressure is generated in the appressoria by accumulation of a high concentration of glycerol (Howard and Valent 1996; Howard et al. 1991; Thines et al. 2000). Once enough turgor pressure is accumulated, the plant surface is penetrated and infectious hyphae continue the infection process. *PMK1* disruption mutants are significantly reduced in their ability to form appressoria, and consequently, are non-

pathogenic on compatible rice cultivars. Further, direct infection of rice leaves by inoculation with *pmk1* mutants through wound sites fails to produce rice blast lesions indicating that *pmk1* mutants are also deficient in invasive growth (Xu and Hamer 1996). *PMK1* gene replacement mutants produce normal mycelia and conidia, and vegetative growth *in vitro* is unaffected. *PMK1* driven by the GAL1 promoter can complement the mating defect of a *fus3/kss1* double mutant, indicating that *PMK1* is a functional homolog of *FUS3/KSS1* (Xu and Hamer 1996). Further studies with *pmk1* mutants revealed that *PMK1* is required for the mobilization of conidial glycogen and triacylglycerol to the appressorium during its formation (Thines et al. 2000). Glycogen and triacylglycerol can then be used in the synthesis of glycerol which is required for turgor pressure generation.

Several other *PMK1* homologs have been identified in phytopathogenic fungi. In *Colletotrichum lagenarium*, *cmk1* mutants exhibited reduced conidiation on rich media and germination in water. Like *pmk1* mutants, *cmk1* mutants are non-pathogenic and fail to form appressoria (Takano et al. 1999). The southern corn leaf blight fungus, *Cochliobolus heterostrophus*, also requires a *PMK1* homolog for pathogenicity. *CHK1* is essential for appressorium formation and *chk1* mutants have poorly developed aerial hyphae which fail to produce conidia (Lev et al. 1999). In *Ustilago maydis*, casual agent of corn smut, the *PMK1* homolog *UBC3/KPP2* is required for mating, virulence, and dikaryon formation (Mayorga and Gold 1999; Muller et al. 1999). Thus, *FUS3/KSS1* homologs clearly play an important role in fungal pathogenesis even though different infection strategies exist for various phytopathogenic fungi. The exact role of *FUS3/KSS1* homologs in fungal pathogenesis has yet to be elucidated.

Another important MAPK that has been shown to be required for maintaining cell wall integrity and plant penetration is *MPS1* (Xu et al. 1998). *MPS1* is a homolog of the *S. cerevisiae* MAPK *SLT2*. In *S. cerevisiae*, cell wall growth in response to membrane stress is controlled through a protein kinase C pathway that ultimately activates *SLT2*. In the rice blast fungus *M. grisea*, *MPS1* is required for sporulation, fertility, and appressoria penetration (Xu et al. 1998). *SLT2* homologs are also required for virulence in the non-appressoria producing *Fusarium graminearum* and human pathogen *Candida albicans* (*MKC1*) (Diez-Orejas et al. 1997; Hou et al. 2002).

The purpose of this study was to clone and characterize the expression patterns of the *FUS3/KSS1* and *SLT2* MAPK homologs in the *Brassicaceae* necrotrophic pathogen *Alternaria brassicicola* (Schw.) Wilts. Under appropriate conditions, *A. brassicicola* can cause a devastating disease resulting in 20-50% yield reductions (Daebeler et al. 1986). The necrotrophic nature of *A. brassicicola* pathogenesis typically leads to extensive damage of the plant and harvest product, with seedlings seldom surviving an attack (Humpherson-Jones, 1985, Neergaard, 1945). Despite its economic importance, studies on *A. brassicicola* pathogenesis mechanisms have been limited, and to date no known pathogenicity factor has been cloned. Various phytotoxic components have been identified in *A. brassicicola* spore germination fluids, but none of the genes for these toxins have been cloned (Cooke et al. 1997, MacKinnon et al. 1999, Otani et al. 1998). In this study, we have cloned and characterized the expression patterns of two MAPKs from *A. brassicicola*, named *AMK1* (*Alternaria* MAP Kinase 1) and *AMK2* (*Alternaria* MAP Kinase 2). *AMK1* is a *FUS3/KSS1* homolog, while *AMK2* is a *SLT2* homolog. Given that homologs of *FUS3/KSS1* and *SLT2* have previously been shown to be required for

pathogenicity in other pathogenic fungi, *AMK1* and *AMK2* are excellent candidates for potential *A. brassicicola* pathogenicity genes.

Materials and Methods

Cloning of *AMK1* and *AMK2* cDNA sequences

A 385 base-pair cDNA fragment with homology to the *Botrytis cinerea* *FUS3/KSS1* homolog *BMP1* (2e-16, BLASTX) was identified from an expressed sequenced tag library created from total RNA extracted from *A. brassicicola* infected *Brassica oleraceae* var. *capitata* (green cabbage) (Chapter 3 this dissertation). Random amplification of cDNA ends (RACE) was used to clone the full length *AMK1* cDNA sequence. The GeneRacer kit (Invitrogen, Carlsbad, CA) was used in all RACE reactions in this study. Three µg of total RNA extracted from *A. brassicicola* infected cabbage tissue (2, 4, and 6 days post-inoculation) was used to generate the RACE ready cDNA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RACE reactions were performed with 45 µl of Platinum PCR Supermix (Invitrogen, Carlsbad, CA), 3 µl of GeneRacer 5' or 3' primer (10 µM stocks), 1 µl of 5' RACE *AMK1* or 3' RACE *AMK1* primers (10 µM stocks) (Table 6.1), and 1 µl of RACE ready cDNA.

PCR reactions were carried out in an Applied Biosystems GeneAmp 2400 thermocycler (Applied Biosystems, Foster City, CA) with the following conditions: 94°C for 2 minutes, 5 cycles of 94°C for 30 seconds and 72°C for 1 minute, 5 cycles of 94°C for 30 seconds and 70°C for 1 minute, and 25 cycles of 94°C for 30 seconds, 63°C for 30 seconds (3' RACE) or 60°C for 30 seconds (5' RACE), and 72°C for 1 minute, followed by an extension for 10 minutes at 72°C. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized with ultra-violet light. Gel

fragments containing DNA were excised with a sterile scalpel, transferred to 1.5 ml microcentrifuge tubes, and DNA extracted with the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen USA, Valencia, CA). Four μ ls of the eluted DNA was cloned into the TOPO TA cloning vector pCR4-TOPO (Invitrogen, Carlsbad, CA) and transformed into *DH5 α* *E. coli* cells.

Table 6.1. Primers used in this study.

Primer	Primer Sequence
5' RACE AMK1	5' GCG CGC GGA GGG TCT GGT AGA TGA AG 3'
3' RACE AMK1	5' CTA CCA GAC CCT CCG CGC GCT GAA GG 3'
5' AMK1 FL ORF	5' ATG CCA CCA GCG GCG AGC GGT AGC 3'
3' AMK1 FL ORF	5' TTA TCG CAT AAT CTC CTG GTA GAT GAG G 3'
M3K1	5' GGN GCN TAY GGN ATH GTN TG 3'
M3K2	5' ATY TCN GGN GCN CKR TAC CA 3'
5' RACE AMK2	5' TGC GGT GGC CTC GGA AGT GCT GGA GC 3'
3' RACE AMK2	5' GCG ACC TGG CAG CCA TCA TCC GCT CTG GC 3'
5' RACE AMK2	5' TAA GCG GCT GGC CAG AGC GGA TGA TGG C 3'
3' RACE AMK2	5' GCG ACC TGG CAG CCA TCA TCC GCT CTG GC 3'
5' AMK1 RT PCR	5' AGC TGC TTC GGT ACT TTA ATC ACG 3'
3' AMK1 RT PCR	5' TCA TGA AAC CTG AGT TGT CCT CG 3'
5' AMK2 RT PCR	5' CGA TGC TCA CTT CCA GTC CTT CAT 3'
3' AMK2 RT PCR	5' CGA GAG TGT CTC TTC GTT TGG TGT 3'
5' Beta tubulin	5' GCC AGT CTG GTG CTG GTA ACA ACT GG 3'
3' Beta tubulin	5' CGA ATC CGA CCA TGA AGA AGT GGA GAC G 3'

Selected individual RACE cDNA clones were obtained with the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). 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.

Degenerate primers based on conserved amino acid residues from *SLT2* (*S. cerevisiae*), *MPS1* (*M. grisea*), and *SPM1* (*S. pombe*) were used to amplify *AMK2* from *A. brassicicola*. These degenerate primers were originally designed by Hou et al. (2002)

and designated M3K1 and M3K2 (sequences available in Table 6.1). PCR reactions consisted of 12.5 µl of 2X PCR master mix (Promega, Madison, WI.), 10.5 µl of water, 1 µl of first strand cDNA generated from *A. brassicicola* infected cabbage tissue, and 0.5 µl of each primer (10 µM stock). PCR reactions were conducted in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following conditions, 94°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 48°C for 1 minute, and 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. PCR products were subsequently treated as above for the *AMK1* RACE products.

RACE was then used as detailed previously with *AMK1*, to obtain the full length *AMK2* cDNA. Primers 5' RACE AMK2 and 3' RACE AMK2 (Table 6.1) were used in RACE reactions according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The annealing temperature used for 5' RACE was 64°C and 63°C for the 3' RACE reaction. RACE products were treated the same as *AMK1* products described above.

Sequence analysis

The BLAST algorithms at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) were initially used to search for homologs of sequences identified via PCR and RACE reactions (Altschul et al. 1997). The non-redundant GenBank database was used in all searches. Multiple alignments were conducted with ClustalX 1.81 and the BLOSUM series protein weight matrix (Thompson et al. 1997). Phylogenetic trees were calculated from the respective multiple alignment using the BOOSTRAP N-J TREE option in ClustalX 1.81. This method derives confidence values for the groupings in a tree. For the trees in this study, 1000 bootstrap replicates were used.

Expression analysis

RT-PCR was used to monitor the expression of *AMK1* and *AMK2* during the infection process, as well as *in vitro* in various environments. RT-PCR analysis was conducted using the Thermoscript 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 ensure that a high humidity was maintained as is required for *A. brassicicola* infection. Plates were incubated at 24° C with a 12 hour light/dark cycle. Leaves from 3 separate plants were used and pooled together at 0 (uninfected), 1, 2, 3, 4, and 5 days post-inoculation. For *in vitro* analysis, liquid cultures with defined media were inoculated with 4 or 5 2 cm agar plugs from *A. brassicicola* cultures grown on PDA and incubated at 24°C, 100 RPM. The first medium was a glucose-yeast extract broth (GYEB), which contained 1.5% glucose and 0.3% yeast extract. The second medium was a potato dextrose broth (PDB) produced by Difco (Becton Dickson and Company, Sparks, MD). The third growth condition was oxidative stress. To create an oxidative stress environment, H₂O₂ (final concentration 100 mM), and NaCl (final concentration 1.2 M) was added to a 7 day old culture of *A. brassicicola* mycelium growing in GYEB. The fungus was incubated under these conditions for 1 hour to induce gene expression related to oxidative stress. The final *in vitro* growth condition was nitrogen starvation. For nitrogen starvation, $\sim 1 \times 10^5$ spores of *A. brassicicola* strain ATCC 96866 were inoculated into 50 ml of GYEB and grown at 24°C and 100 RPM for 2 days. The mycelium was collected with sterile Miracloth

(Calbiochem, La Jolla, CA) and rinsed 5X with sterile distilled water to remove any remaining GYEB. The mycelium was transferred to a minimal medium lacking a nitrogen source (0.5 g/L KCl, 0.5 g/L MgSO₄, 1.5 g/L KH₂PO₄, 0.1% (v/v) trace elements, and 10 g/L glucose, pH 6.5) (Talbot et al., 1997). The fungus was grown under nitrogen starvation conditions for 48 hours at 24°C, 100 rpm. Mycelium was again collected with sterile Miracloth, rinsed 5X with sterile water, and dried for RNA extraction.

All RNA was extracted with the RNeasy Plant Mini-Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). 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 µl of first strand cDNA was used in 25 µl PCR reactions containing a 2× master mix from Promega (Madison, WI). Primers used in the reactions are presented in Table 6.1 (5' and 3' AMK1 RT PCR for *AMK1* and 5' and 3' AMK2 RT PCR for *AMK2*). Beta tubulin primer sequences were derived from *A. brassicicola* GenBank accession Y17084. PCR reactions were conducted in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following program: 94° C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, either 55° C or 53°C for 30 seconds depending on each primer set, 72°C for 30 seconds, and a final extension for 5 minutes at 72° C. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized with ultra-violet light. All RT-PCR reactions were repeated at least 2 times with similar results.

Results

Cloning of *AMK1* and *AMK2* cDNA sequences

The 5' RACE reaction for *AMK1* yielded a 727 base pair cDNA fragment with 429 base pairs of this fragment from a 5' untranslated region (Figure 6.1A). The 3' RACE reaction yielded a 775 base pair cDNA fragment with 89 base pairs of 3' untranslated sequence, including a poly A tail (Figure 6.1B). The full-length ORF product was 1059 base pairs and the sequence of this product was deposited in Genbank (accession AY515257) (Figure 6.1C). The full length *AMK1* cDNA was 89% similar at the nucleotide level to the *PTK1* MAP kinase from *Pyrenophora teres*, a *FUS3/KSS1* homolog.

The amino acid sequence of *AMK1* was used to scan the PROSITE database, release 18.17 (<http://us.expasy.org/tools/scanprosite/>) for the occurrence of patterns and profiles. These searches revealed three patterns and one profile with unique patterns of occurrence that suggested a biological role for *AMK1*. The first pattern, amino acids 26-50, was a protein kinase ATP binding region signature (PDOC00100). The second pattern, amino acids 140-152, was a serine/threonine protein kinase active site signature (PDOC00100), and the third pattern, amino acids 54-156, was a MAP kinase signature pattern (PDOC01049). The profile, amino acids 20-308, was a protein kinase domain profile. A search of the protein families database of alignments and HMMs (Pfam, <http://www.sanger.ac.uk/Software/Pfam/index.shtml>) also returned a protein kinase domain ($E = 2.7e^{-95}$). These results suggest that *AMK1* is a mitogen-activated protein kinase.

For cloning *AMK2*, degenerate PCR with Primers M3K1 and M3K2 yielded a degenerate PCR product of 504 base pairs that had significant homology (5e-92 BLASTX) with *MGV1* from *Fusarium graminearum* (Figure 6.2A). RACE primers were designed based on the sequence obtained from the degenerate PCR. 5' RACE yielded a 340 base pair product homologous to *MGV1* ($4e^{-36}$ BLASTX) (Figure 6.2B) and 3' RACE yielded an 824 base pair product also homologous to *MGV1* ($1e^{-129}$ BLASTX) (Figure 6.2B). Put together, the cDNA sequence was 1149 base pairs in length. However, according to the BLAST analysis, this is not the full length cDNA of *AMK2*, and the 3' end appears to be missing approximately 99 nucleotides (based on comparisons with other SLT2 homologs). Attempts to acquire the 3' end of *AMK2* were unsuccessful at the writing of this dissertation.

The 3' truncated amino acid sequence of *AMK2* was used to scan the PROSITE database, release 18.17 (<http://us.expasy.org/tools/scanprosite/>) for the occurrence of patterns and profiles. This search revealed three patterns and one profile with unique patterns of occurrence that suggests a biological role for *AMK2* as a MAP kinase. The first pattern, amino acids 29-53, was a protein kinase ATP binding region signature (PDOC00100). The second pattern, amino acids 145-157, was a serine/threonine protein kinase active site signature (PDOC00100), and the third pattern, amino acids 58-161, was a MAP kinase signature pattern (PDOC01049). The profile, amino acids 23-314, was a protein kinase domain profile. A search of the protein families database of alignments and HMMs (Pfam, <http://www.sanger.ac.uk/Software/Pfam/index.shtml>) also returned a protein kinase domain ($E = 1.1e^{-87}$). These results suggest that *AMK2* is a mitogen-activated protein kinase.

Phylogenetic analysis of *AMK1*

In order to compare the amino acid sequence of *AMK1* with other *FUS3/KSS1* homologs, nearest-neighbor-joining-phylogenetic analysis was conducted from the multiple alignment of *FUS3/KSS1* MAP kinase homologs (Figure 6.3 and 6.4) (Saitou and Nei 1987). According to this phylogeny, *AMK1* is most closely related to the MAP kinase from *Pyrenophora teres* (*PTK1*) and is in a clade with other necrotrophic fungi which produce toxins (*Leptosphaeria maculans* and *Cochliobolus heterostrophus*) (Figure 6.4). The first clade contains two phytopathogenic fungi known to produce well defined appressoria, *Magnaporthe grisea* and *Colletotrichum lagenarium*, as well as the root pathogen *Fusarium oxysporum* f.sp. *lycopersici* and the ubiquitous *Botrytis cinerea*. The third clade contains the outgroup, *FUS3*, from *Saccharomyces cerevisiae*.

Phylogenetic Analysis of *AMK2*

As with the *AMK1* phylogeny, a tree with the structure of a trichotomy was produced from the multiple alignment of *AMK2* truncated amino acid sequences (Figure 6.5 and 6.6). Unlike the *FUS3* homologs, *SLT2* homologs have significant degrees of variation in the C terminus of the protein. *SLT2* from *S. cerevisiae* was used as the outgroup for this analysis and the tree was rooted with *SLT2*. *AMK2* was most closely related to *MPKA* from *Aspergillus nidulans*, typically a saprophyte. This is relatively surprising as the other *SLT2* homologs from phytopathogenic fungi appear in a clade together separate from the clade containing *AMK2* and *MPKA*. However, the bootstrap values are relatively low for this analysis which may indicate that not enough phylogenetic information was available at the variation sites. This is likely due to the fact that C terminus truncated amino acid sequences were used in the analysis.

When compared, *AMK1* and *AMK2* are only 50% identical at the amino acid level, which is a reflection of similar general biological functions as MAP kinases. Yet, they are divergent enough that they likely mediate different signaling events.

Expression analysis

The presence of transcripts from *AMK1* and *AMK2* was examined using RT-PCR during a 5 day infection of the economically important *Brassica oleraceae* var. *capitata* (green cabbage). No visible symptoms could be seen on infected cabbage leaves for the first 48 hours of infection. However, expression of both *AMK1* and *AMK2* is apparent according to the RT-PCR results (Figure 6.7). At day 3, the cabbage leaves began to exhibit typical black spot symptoms characterized by dark brown to black lesions with a chlorotic halo indicative of toxin production. By days 4 and 5, black spot lesions were present on the majority of the cabbage leaf and accompanied by extensive chlorosis. *AMK1* and *AMK2* were both expressed throughout the entire infection time course. Neither *AMK* nor *AMK2* amplified transcripts from the uninfected cabbage as expected. The beta-tubulin control, which was derived from an *Alternaria* sequence, did amplify the plant homolog making it impossible to determine relative amounts of fungal biomass present at each infection time point in this experiment.

Though RT-PCR is not quantitative as conducted in this study, the use of house keeping genes such as 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. Based on examining the relative intensity of the bands during the course of the infection, it appears that both *AMK1* and *AMK2* are expressed at their highest points during the third day of the

infection process, when symptoms first appear. Expression of these two genes appears to decrease following day 3 of the infection.

Expression of *AMK1* and *AMK2* was also examined *in vitro* in various environmental conditions. Both *AMK1* and *AMK2* are readily expressed in nutrient rich environments such as glucose-yeast extract and potato dextrose broths (Figure 6.7B). Both are also expressed during nitrogen starvation conditions, a condition which has previously been shown to induce pathogenicity related gene expression (Talbot et al. 1997). Interestingly, it does not appear that *AMK2* is expressed during oxidative stress conditions, however, it should be noted that apparently the RT reaction was inefficient for this environmental condition as seen by the low intensity of the β -tubulin control (Figure 6.7B). However, *AMK1* transcript was able to be detected in this environmental condition and from the same RNA sample.

Discussion

In this study the cloning and expression analysis of two *Alternaria brassicicola* MAPKs, *AMK1* and *AMK2* is reported. Phylogenetic analysis of the amino acid sequences of *AMK1* and *AMK2* revealed that *AMK1* is most closely related to the *FUS3/KSS1* MAPKs which are known to regulate the pheromone response pathway in yeast. *AMK2* was most closely related to the cellular integrity *SLT2*-like MAPKs. Both of these types of MAPKs have previously been shown to be required for fungal pathogenicity in phytopathogenic fungi (Xu 2000).

Homologs of *FUS3/KSS1* appear to be required for appressorium function in pathogenic fungi. *PMK1* in *Magnaporthe grisea* and *CHK1* in *Cochliobolus heterostrophus* are both required for normal appressorium development (Xu and Hamer

1996, Lev et al. 1999). Plant penetration was also affected in an *fmk1* mutant in the soil-borne pathogen *Fusarium oxysporum* f.sp. *lycopersici*. *FMK1* was required for the development of penetration hyphae and invasive growth (Di Pietro et al. 2001). In the necrotroph *Botrytis cinerea*, *BMP1* was also required for the penetration of carnation flowers and tomato leaves and *bmp1* deletion mutants could not grow invasively through wounds (Zheng et al 2000). Taken together, these results suggest that *FUS3/KSS1* homologs mediate plant penetration and invasive growth during fungal pathogenesis.

The mechanism of how the *FUS3/KSS1* homolog in *M. grisea*, *PMK1*, controls appressorium function was explored by Thines et al. (2000). They found that *PMK1* was required for the transfer of carbohydrate (glycogen) and lipid (triacylglycerol) reserves from the germinated conidia into the developing appressoria. This transfer appears to be required for the development of turgor pressure inside the appressoria, which is required for penetration of the plant cuticle. Though *A. brassicicola* does not produce well defined appressoria like *M. grisea*, it does produce small appressoria-like structures. What role these putative appressoria have in *A. brassicicola* pathogenesis is currently unknown. Generation of an *AMK1* replacement mutant will provide the necessary tool to examine the role of *AMK1* in *A. brassicicola* pathogenesis, and perhaps confirm or refute the hypothesis that these small appressoria-like structures are required for penetration of the plant cuticle and cell wall. Because *FUS3/KSS1* homologs in plant pathogenic fungi also appear to be required for invasive growth, *AMK1* could simply be required for this process as well. The movement of carbohydrates and lipid reserves throughout the *in planta* developing mycelium must be of vital importance to the invading fungus. The inability of *FUS3/KSS1* homolog mutants to grow invasively may reflect their inability to

move these important nutrient and energy sources throughout the developing mycelium. RT-PCR analysis of *AMK1* expression appeared to reveal that transcript levels of *AMK1* decreased after the third day of the infection process. This may imply that *AMK1* is required for plant penetration, but not necessarily invasive growth once the fungus has established itself and can obtain nutrients from the plant. A more robust expression analysis technique, such as Real Time PCR, capable of quantifying the actual amount of transcript levels is needed to confirm the results obtained here with standard RT-PCR.

Like *FUS3/KSS1* homologs, *SLT2* homologs also appear to be required for fungal pathogenesis. *M. grisea* requires an intact and functional *SLT2* homolog, *MPS1*, for pathogenesis of rice. *MPS1* null mutants are incapable of causing disease and apparently are deficient in the ability to penetrate the plant cuticle (Xu et al. 1998). *MPS1* mutants also have cell walls that are more sensitive to cell wall degrading enzymes, reduced sporulation, and fertility defects. Unlike *pmk1* mutants, *mps1* mutants can form appressoria, however, these appressoria are non-functional and do not penetrate the plant cuticle. In the cucumber anthracnose fungus, *Colletotrichum lagenarium*, the *SLT2* homolog, *MAF1*, is required for appressoria formation as well as conidiation (Kojima et al. 2002). In the biotrophic rye pathogen *Claviceps purpurea*, which does not produce appressoria, the *SLT2* homolog, *CPMK2*, is still required for fungal pathogenesis (Mey et al. 2002). Interestingly, northern blot analyses showed that the pentahydrophobin gene, *CCPH1*, was down regulated in *cpmk2* mutants suggesting that the *CPMK2* dependent signaling pathway may regulate hydrophobin production. Hydrophobins have previously been shown to be required for fungal pathogenesis, though their exact role in fungal pathogenesis is currently not known (Whiteford and Spanu 2002).

In this study an *SLT2* homolog from *A. brassicicola*, *AMK2*, was cloned and expression analysis revealed that it is expressed throughout the 5 day infection of green cabbage. With only expression data, it is not possible to speculate on the role of *AMK2* in *A. brassicicola* pathogenesis. It seems that *SLT2* homologs, like *FUS3* homologs, play an important role in plant penetration. However, the result by Mey et al. (2002) suggesting that hydrophobin production may be regulated by *SLT2* homologs is noteworthy. Indeed, to date, none of the downstream effector proteins regulated by *FUS3* or *SLT2* homologs in phytopathogenic fungi have been identified. Two genes with unknown function, *GAS1* and *GAS2*, were identified in a subtracted library enriched for genes regulated by *PMK1* in *M. grisea* (Xue et al. 2002). Null mutants of these 2 genes did have reduced virulence on rice, but were still able to form appressoria, and thus had mutant phenotypes different than *pmk1* mutants which are unable to form appressoria.

Still, it has become clear that at least two signaling pathways mediated by *FUS3* and *SLT2* MAPKs are conserved and required for fungal pathogenicity of plants. In this study we report the cloning and expression analysis of the *Alternaria brassicicola* *FUS3* homolog *AMK1* and *SLT2* homolog *AMK2*. Given the high degree of conservation of these MAPKs in other phytopathogenic fungi, *AMK1* and *AMK2* are excellent candidate *A. brassicicola* pathogenicity genes. A gene disruption protocol is currently being developed for *A. brassicicola*. Preliminary attempts at disrupting *AMK1* using gene disruption via PEG mediated protoplast transformation and homologous recombination has been unsuccessful. However, it is anticipated that as the transformation protocol becomes optimized, that null mutants of both *AMK1* and *AMK2* will become available. This will allow extensive analysis of the roles of *AMK1* and *AMK2* in *A. brassicicola*

pathogenesis, and further confirm the role of the signal transduction pathways regulated by these two important MAPKs in fungal pathogenesis.

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Figure 6.1. RACE reactions for cloning of full length *AMK1* cDNA. A. 5' RACE Lane L = 500 base pair ladder, 1 = 5' RACE *AMK1* product B. 3' RACE Lane L = 500 base pair ladder, 1 = 3' RACE *AMK1* product, lower band is the correct band C. Full length *AMK1* cDNA sequence Genbank accession AY515257. Initiation codon ATG and stop codon TAA in bold.

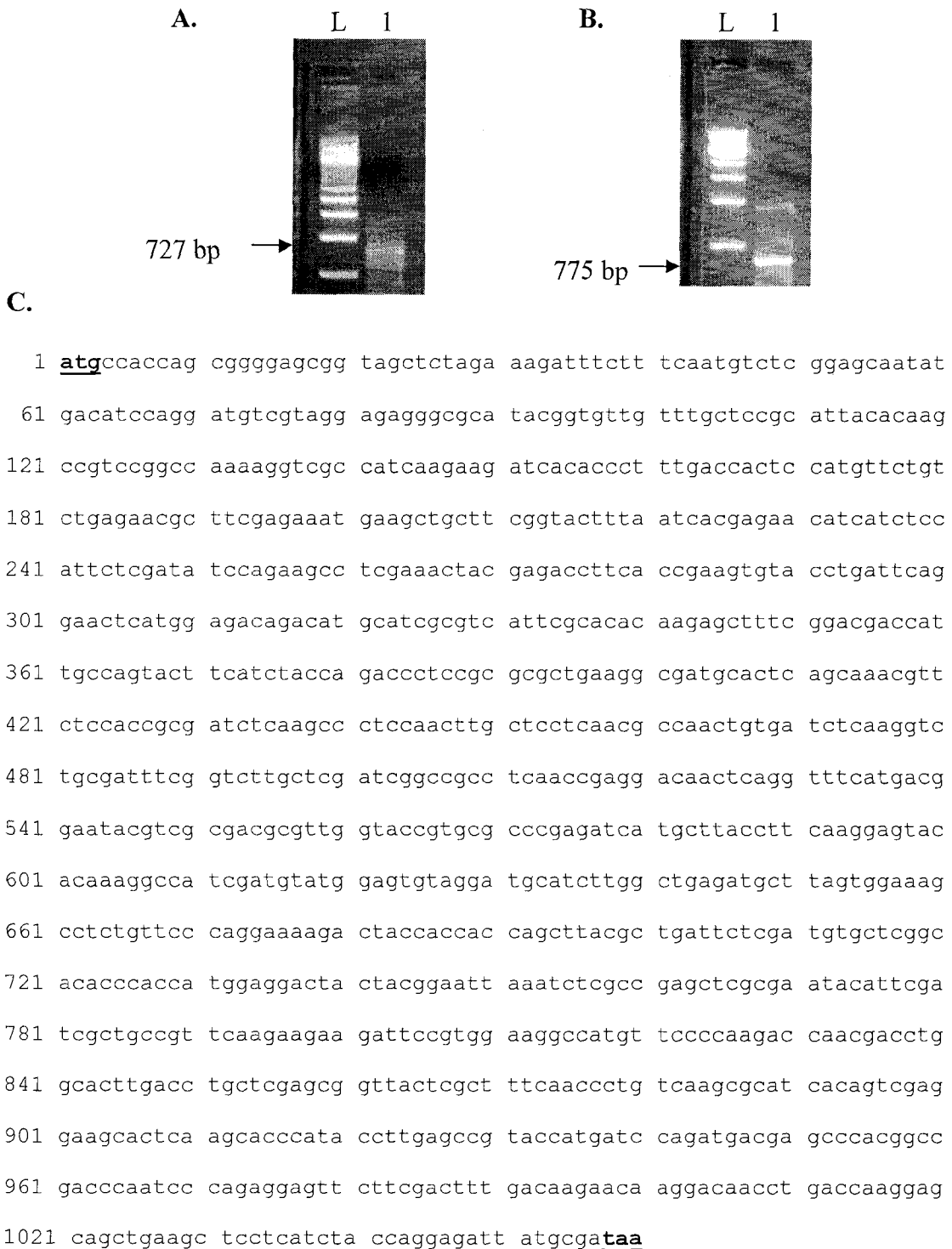


Figure 6.2. Cloning of *AMK2* cDNA sequence. A. Degenerate PCR result. Lane 1 = 500 base paid ladder, 2 = blank lane, 3 = degenerate *AMK2* PCR product. B. RACE reactions, Lane 1 = 1 kb ladder, 2 = 5' RACE *AMK2* product, 3 = blank lane, 4 = 3' RACE product 3 day *A. brassicicola* infected cabbage template, 5 = 3' RACE product *A. brassicicola* grown in GYEB medium template, 6 = 3' RACE product *A. brassicicola* grown in GYEB + 10 mM KCN template. C. *AMK2* cDNA sequence. Initiation codon ATG in bold.

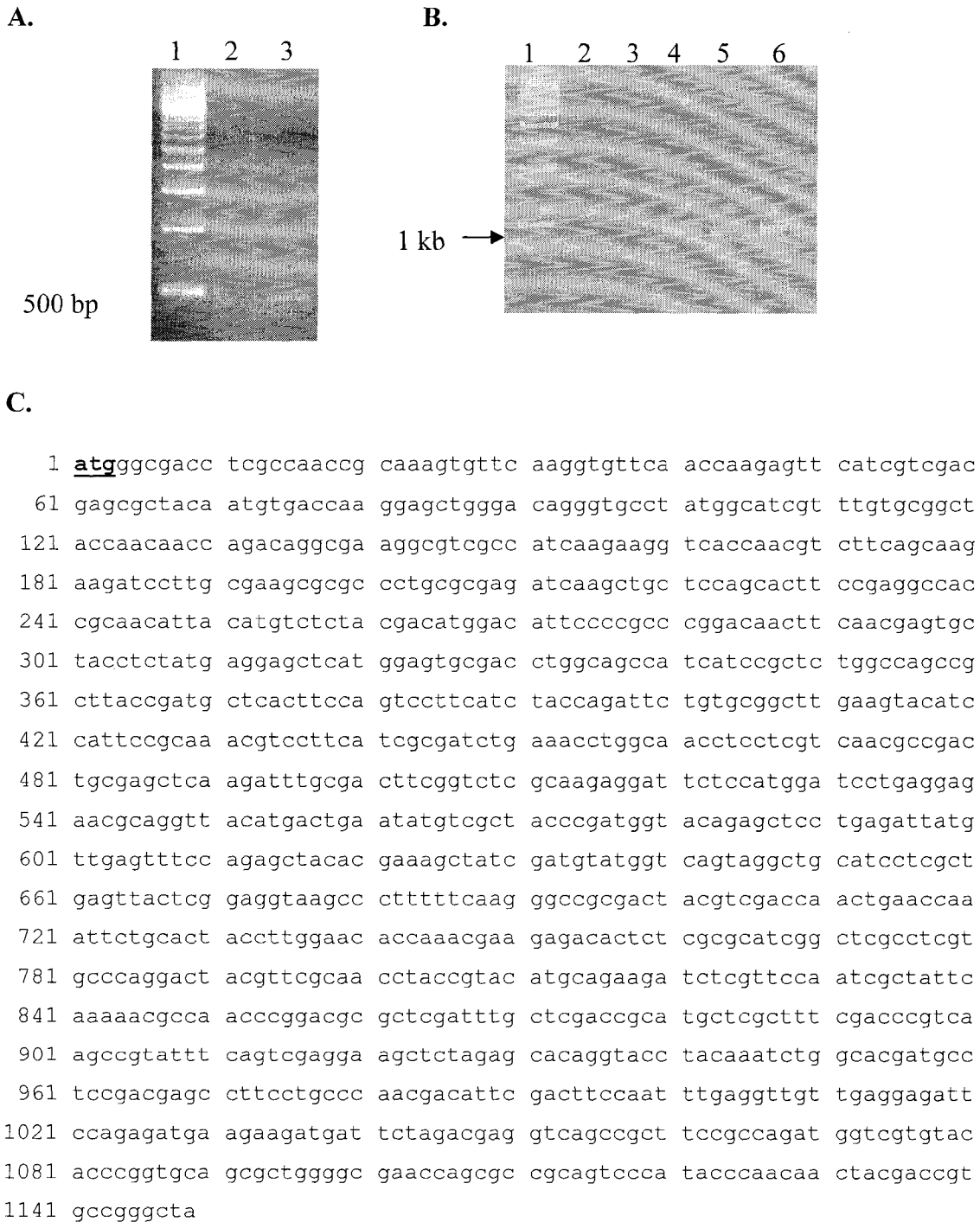


Figure 6.3. Multiple alignment of *FUS3/KSS1* MAP kinase homologs.

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FMK1 1 MSRSNPP-NPSSSRKISFNVSSEQYDIQDVVGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
CMK1 1 MSRANAP-NPSGSRKISFNVSSEQYDIQDVVGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
PMK1 1 MSRANPPSNSSGSRKISFNVSSEQYDIQDVVGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
BMP1 1 -MTARAPNPSSGSRKISFNVSSEQYDIQDVVGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
PTK1 1 ----MPPAGSSSRKISFNVSSEQYDIQDVVGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
AMK1 1 ----MPPAGSSSRKISFNVSSEQYDIQDVVGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
CHK1 1 ----MPPAGSSSRKISFNVSSEQYDIQDVVGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
LMK1 1 ----MPPTGSSSRKISFNVSSEQYDIQDVVGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
FUS3 1 -----MPPPTIVYNSSDQKSGEGAYGVVCSAHHKPSGQKVAIKKITPFDDHSMFCLRTLRE
consen. 1 pp sa srkIsfNvSeqydiqdvvgEGAYGVVCSAHHKPsGqkVAIKKITPFDDHsmFcLRTLRE

FMK1 70 MKLLRYFNHENIISILDIQKPRNYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
CMK1 70 MKLLRYFNHENIISILDIQKPRSYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
PMK1 71 MKLLRYFNHENIISILDIQKPRSYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
BMP1 70 MKLLRYFNHENIISILDIQKPRNYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
PTK1 67 MKLLRYFNHENIISILDIQKPRNYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
AMK1 67 MKLLRYFNHENIISILDIQKPRNYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
CHK1 67 MKLLRYFNHENIISILDIQKPRNYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
LMK1 67 MKLLRYFNHENIISILDIQKPRNYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
FUS3 60 MKLLRYFNHENIISILDIQKPRNYETFEVYLIQELMETDMHRVIRTQLSDDHCQYFIYQTLRALKAMH
consen. 71 mKLLryFnHENIIsIldIQkPrnyEtFtEVYLIQELMeTdmHRVIRtQdLSDDHcQYFIYQTLRALKamH

FMK1 140 SANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
CMK1 140 SANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
PMK1 141 SANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
BMP1 140 SANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
PTK1 137 SANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
AMK1 137 SANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
CHK1 137 SANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
LMK1 137 SANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
FUS3 130 GNVVHRDLKPSNLLLNANCDLKVCDFGLARSAASQEDNSG-----FMTEYVATRWYRAPEIMLTfKE
consen141 saNVlHRDLKPSNLLlNaNCdLKVCDFGLARsaasteDnsg fMTEYVATRWYRAPEiMLTfke

FMK1 203 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
CMK1 203 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
PMK1 204 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
BMP1 203 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
PTK1 200 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
AMK1 200 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
CHK1 200 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
LMK1 200 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
FUS3 200 YTKAIDVWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTF-TMEDYYGIKSRRAREYIRSLPFKKKV
consen211 YtkAiDVWSvGCILAEmlsgkPlfPFGkDYhHQLtLiLdvlGTF tmeDyygIkSrRAREYIRsLPfkkkv

FMK1 272 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
CMK1 272 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
PMK1 273 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
BMP1 272 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
PTK1 269 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
AMK1 269 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
CHK1 269 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
LMK1 269 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
FUS3 270 PFTLPFKTSDALDLELLAFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLt
consen281 Pfk mFPktndlalDLerllaFnPvKRitveeAlKHPYLePYHDPdDEPTadPIPeEFFdFdknKdnLt

FMK1 342 KEQLKQLIYQEIMR
CMK1 342 KEQLKQLIYQEIMR
PMK1 343 KEQLKQLIYQEIMR
BMP1 342 KEQLKQLIYQEIMR
PTK1 339 KEQLKQLIYQEIMR
AMK1 339 KEQLKQLIYQEIMR
CHK1 339 KEQLKQLIYQEIMR
LMK1 339 KEQLKQLIYQEIMR
FUS3 340 TKDLKQLIYQEIMR
consen351 keqLK lIyqEImr

```

Figure 6.4. Phylogentic analysis of *FUS3/KSS1* MAP kinase homologs. Genbank accession numbers = *CMK1* = AAD50496, *PMK1* = AAC49521, *FMK1* = AAG01162, *BMP1* = AAG23132, *PTK1* = AAK52840, *AMK1* = AY515257, *CHK1* = AAF05913, *LMK1* = AAM89501, *FUS3* = P16892. Phylogram created with Clustal X v. 1.81. The tree was rooted with *FUS3*.

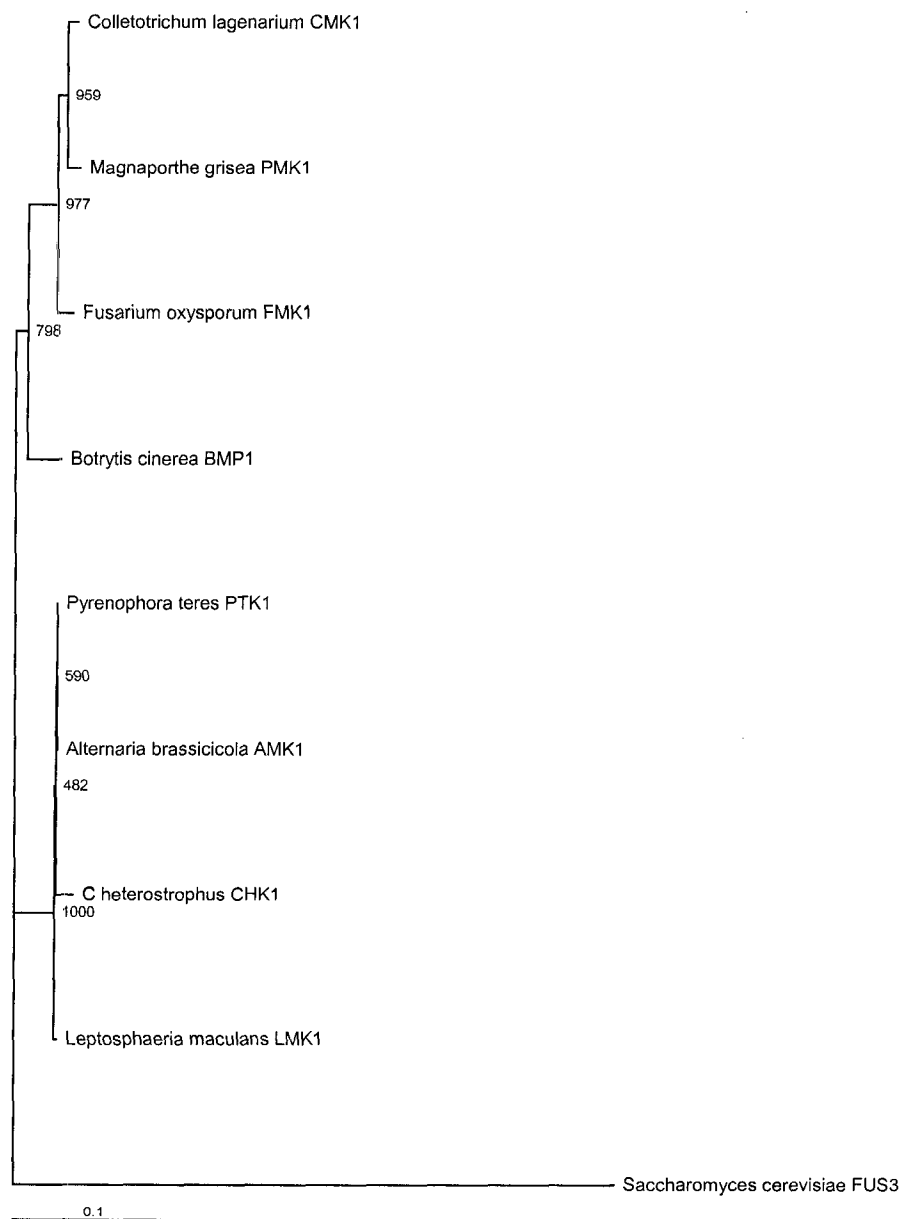


Figure 6.5. Multiple alignment of *SLT2/MPS1* homologs

MAF1	1	--MCDLQGRKVFVKFNQDFIVDERYTVTKELGQGAYGIVCAAVNNQTQEG--VAIKKVTNVFSKKILAKR
Mps1	1	--MSDLQGRKVFVKFNQDFIVDERYTVTKELGQGAYGIVCAAVNNQTSEG--VAIKKVTNVFSKKILAKR
MGV1	1	--MCDLQGRKVFVKFNQDFIVDERYTVTKELGQGAYGIVCAAVNNQTNEG--VAIKKVTNVFSKKILAKR
CPMK2	1	--MTDTPGRFAFCFNOEFIVDERYTVTKELGQGAYGIVCAAVNNQTES--VAIKKVTNVFSKKILAKR
MPKII	1	--MSDMQGRKVFVKFNQDFIVDERYTVTKELGQGAYGIVCAATNISTQEG--VAIKKVTNVFSKKILAKR
MPKA	1	MSDLQGRKVFVKFNQDFIVDERYTVTKELGQGAYGIVCAATNVQTGEG--VAIKKVTNVFSKKILAKR
AMK2	1	--MCDLANRKVFVKFNQDFIVDERYTVTKELGQGAYGIVCAATNNQTGEG--VAIKKVTNVFSKKILAKR
SLT2	1	--MDKIERATFKVFNQDFSVKPKRQSLKEGCGAYGIVCAARFAAANDTTVAIKKVTNVFSKKILAKR
consensus	1	mgdlqgRkvFkvFNQdFiVDeRytvtKELGqGAYGIVCaAvnnqt Eg VAIKKVTNVFSKKiLaKR

MAF1	67	ALREIKLLQHFRGHRNITCLYDMDIPRPD--NFNETYLYEELMECDLAAIIRSGQPLTDAHFSQFIYQIL
Mps1	67	ALREIKLLQHFRGHRNITCLYDMDIPRPD--NFNETYLYEELMECDLAAIIRSGQPLTDAHFSQFIYQIL
MGV1	67	ALREIKLLQHFRGHRNITCLYDMDIPRPD--NFNETYLYEELMECDLAAIIRSGQPLTDAHFSQFIYQIL
CPMK2	67	ALREIKLLQHFRGHRNITCLYDMDIPRPD--VFNETYLYEELMECDLAAIIRSGQPLTDAHFSQFIYQIL
MPKII	67	ALREIKLLQHFRGHRNITCLYDMDIPRPD--NFNETYLYEELMECDLAAIIRSGQPLTDAHFSQFIYQIL
MPKA	69	ALREIKLLQHFRGHRNITCLYDMDIPRPD--NFNETYLYEELMECDLAAIIRSGQPLTDAHFSQFIYQIL
AMK2	67	ALREIKLLQHFRGHRNITCLYDMDIPRPD--NFNECYLYEELMECDLAAIIRSGQPLTDAHFSQFIYQIL
SLT2	69	SLREIKLLRHFRGHNITCLYDMDIVFYPDGSINGLYEELMECDLAAIIRSGQPLTDAHFSQFIYQIL
consensus	71	aLREiKLLqHFRGHRnITCLYDMDIprpd nfNetYLYEELMECDlaaIIRSGQPLTDAHfSQfiYQIL

MAF1	135	CGLKYIHSANVLHRDLKPGNLLVNADCELCICDFGLARGFSVDPEENAGYMTYVATRWYRAPEIMLSFQ
Mps1	135	CGLKYIHSANVLHRDLKPGNLLVNADCELCICDFGLARGFSVDPEENAGYMTYVATRWYRAPEIMLSFQ
MGV1	135	CGLKYIHSANVLHRDLKPGNLLVNADCELCICDFGLARGFSVDPEENAGYMTYVATRWYRAPEIMLSFQ
CPMK2	135	CGLKYIHSANVLHRDLKPGNLLVNADCELCICDFGLARGFSVDPEENAGYMTYVATRWYRAPEIMLSFQ
MPKII	135	CGLKYIHSANVLHRDLKPGNLLVNADCELCICDFGLARGFSVDPEENAGYMTYVATRWYRAPEIMLSFQ
MPKA	137	CGLKYIHSANVLHRDLKPGNLLVNADCELCICDFGLARGFSVDPEENAGYMTYVATRWYRAPEIMLSFQ
AMK2	135	CGLKYIHSANVLHRDLKPGNLLVNADCELCICDFGLARGFSVDPEENAGYMTYVATRWYRAPEIMLSFQ
SLT2	139	CGLKYIHSANVLHRDLKPGNLLVNADCELCICDFGLARGFSVDPEENAGYMTYVATRWYRAPEIMLSFQ
consensus	141	cGLKYiHSAnVLHRDLKPGNLLVNADCeLCICDFGLARGfSvdPeeNagymTEYVATRWYRAPEIMLSfQ

MAF1	205	SYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQLNQILHLGTPNEETLSRIGSPRAQYEVNRLPFMPKK
Mps1	205	SYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQLNQILHLGTPNEETLSRIGSPRAQYEVNRLPFMPKK
MGV1	205	SYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQLNQILHLGTPNEETLSRIGSPRAQYEVNRLPFMPKK
CPMK2	205	SYTKAIDVWSVGCILAEILGSRPFFFKGRDYVDQLNQILHLGTPNEETLSRIGSPRAQYEVNRLPFMPKK
MPKII	205	SYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQLNQILHLGTPNEETLSRIGSPRAQYEVNRLPFMPKK
MPKA	207	SYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQLNQILHLGTPNEETLSRIGSPRAQYEVNRLPFMPKV
AMK2	205	SYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQLNQILHLGTPNEETLSRIGSPRAQYEVNRLPFMPKH
SLT2	209	GYTKAIDVWSAGCILAEFLGGRPFIFKGRDYVDQLNQILHLGTPPETIRRIGSKNVLDYINQILGTPHNV
consensus	211	syTKAIDVWSvGCILAEiLGgrPfFFKGRDYvDQLNQILhiLGTpNeETLSrIGSpRaQeYvnrLpfmpKk

MAF1	275	PEPQLEFANPDALDLDRLMAFDPSRSISVEALEHPYLHIWHDASDEPDCPTTFNFDFEVVDVGEMR
Mps1	275	PEPTLFPNANPDALDLDRLMAFDPSRSISVEALEHPYLHIWHDASDEPDCPTTFNFDFEVVDVGEMR
MGV1	275	PEPSLEFANPDALDLDRLMAFDPSRSISVEALEHPYLQIWHHDASDEPDCPTTFNFDFEVVDVGEMR
CPMK2	275	NEATLFPANPDALDLDRLMAFDPSRSISVEALEHPYLQIWHHDASDEPDCPTTFNFDFEVVDVGEMR
MPKII	275	PFAQLFPNANPDALDLDRLMAFDPSRSISVEALEHPYLQIWHHDASDEPDCPTTFNFDFEVVDVGEMR
MPKA	277	PEQRLFPNANPDALDLDRLMAFDPSRSISVEALEHPYLAIWHDASDEPSCPTTFNFDFEVVDVGEMR
AMK2	275	SFQQLFKKNANPDALDLDRLMAFDPSRSISVEALEHRYLQIWHHDASDEPSCPTTFNFDFEVVDVGEMR
SLT2	279	PFVNLFPNANSQALDLLEQLMAFDPOKRIEVEALEHPYLSIWHHDASDEPVSEKFEESFESVNDMEELK
consensus	281	pF tlfpnAnpdALDLDrlMAFDpssRiSvE ALeHpyLqiWHDasDEP CpttFnFdFEVvdvgemr

MAF1	345	KMILDEVYRFRQLVRTVPGSTQAGAAAGQAGAGQVPIP
Mps1	345	KMILDEVYRFRQLVRTAPGAGGHEAPHAPQVPIPAGGQ
MGV1	345	GMILDEVQRFQNVRTVPGQSGGELGGGVPLPQANG
CPMK2	345	KMILDEVIRFRQNVRTASSAEPTTGCACTAAAGQVMPMPQ
MPKII	345	KMILDEVARFRHHVRVQPMQSPSNDPSVPTLQTSVAVWG
MPKA	347	GMIVDEVVRFRQSVRMPEQAQSLAQCCIGNIPIPEH--
AMK2	345	KMILDEVSRFRQNVYTRCSAGANQRRSPIPNNYDRGL
SLT2	349	QMDEVQDFELFVQPLLEEQRQLLQQQQQQQ-----
consensus	351	kmildEV rFRqlVrt pg g q q a

Figure 6.6. Phylogenetic analysis of *SLT2/MPS1* homologs.

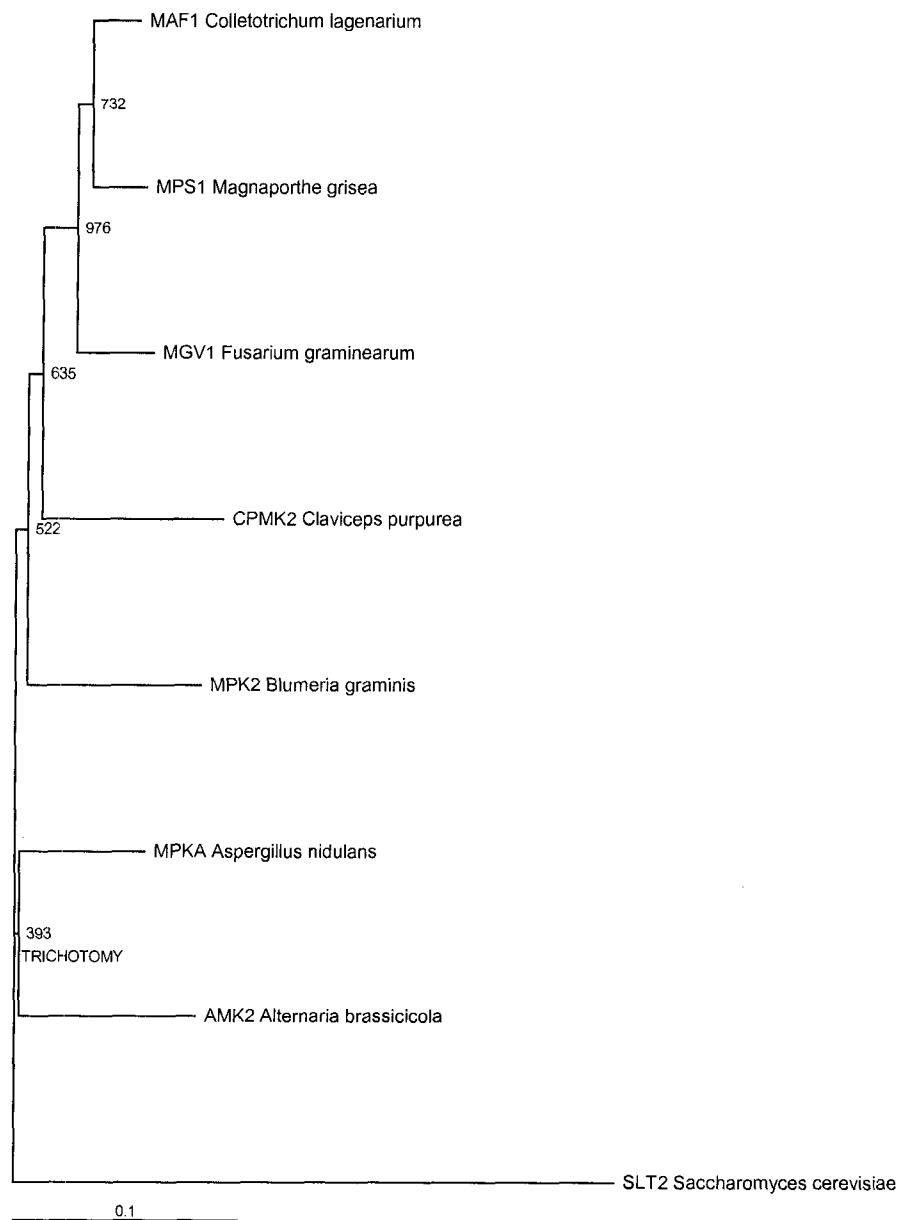
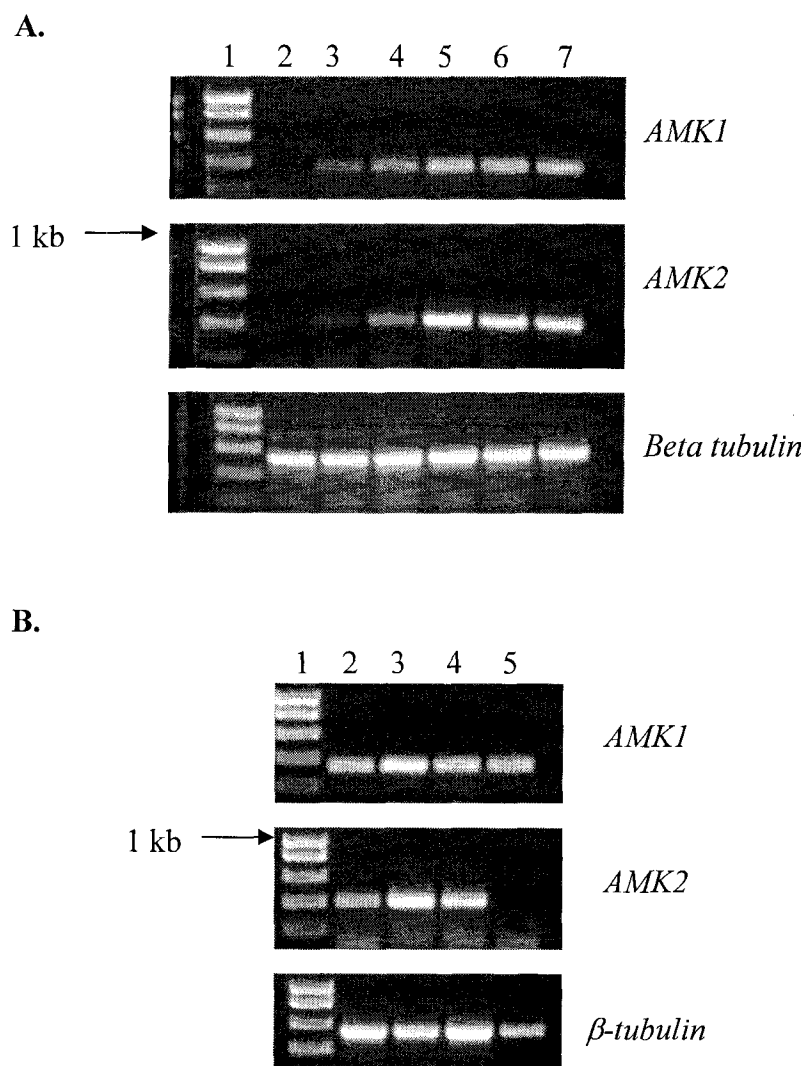


Figure 6.7. Expression analysis of *AMK1* and *AMK2* using RT-PCR. A. Lanes 1 = 1 KB ladder, 2 = 0 day uninfected cabbage, 3 = 1 day infected cabbage, 4 = 2 days infected cabbage, 5 = 3 day infected cabbage, 6 = 4 day infected cabbage, 7 = 5 day infected cabbage. B. Lanes 1 = 1 KB ladder, 2 = glucose-yeast extract broth, 3 = potato dextrose broth, 4 = nitrogen starvation, 5 = oxidative stress conditions.



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Chapter 7

Dissertation Summary and Future Directions

The outcome of plant-pathogen interactions is primarily determined by genetic factors possessed by the invading pathogen and defending host (Chapter 1, Figure 1.1). Thus, there exists a clear need to identify and clone pathogenesis-related genes from the pathogen and host, and to understand the function of these genes in an autonomous and whole organism sense. A better understanding of the genes and gene products involved in fungal-plant interactions will lead to the development of effective control strategies for these socially and economically important pathogens.

The overall goal of this dissertation was to identify candidate *Alternaria brassicicola* pathogenicity genes and establish a foundation for future studies on the *Alternaria brassicicola* – *Brassicaceae* interaction. A functional genomics based approach was used to accomplish this goal, and was successful in identifying 2,002 *A. brassicicola* genes. The identification of these genes represents the first large scale analysis of genetic factors involved in *A. brassicicola* pathogenesis. Many of these genes show significant homology with genes in other fungal pathogens known to be required for pathogenesis. Gene expression analyses demonstrated that many of these genes are upregulated during *A. brassicicola* infection of cabbage and *Arabidopsis*. Thus, these genes are excellent candidates to be involved in *A. brassicicola* pathogenesis.

The next step is to gain a better understanding of how genes identified in this study function during *A. brassicicola*'s interaction with its host plant. Approximately 1554 plant genes expressed during the interaction with *A. brassicicola* were also identified during the course of this dissertation. An analysis of the genes expressed by the pathogen and host while they are interacting provides a glimpse into the genetic factors involved in the complex interaction that leads to disease or resistance. Once genes are identified that play significant roles in the infection process (i.e. pathogenicity genes), novel and effective control strategies may be developed using these pathogenicity genes as targets.

Based on the genes identified in this study, a picture of the complex interplay between *A. brassicicola* and its hosts has emerged. Since *A. brassicicola* is a necrotroph, the production of toxic compounds is likely to be important and required for successful invasion of the host. Several genes were identified in the EST libraries that may be candidates in toxin production. Perhaps the most noteworthy was the identification of 3 polyketide synthases (PKS). PKSs are well known to be involved in the production of phytotoxic proteins in fungi. The most well known PKS synthesized fungal toxin is T-toxin from *Cochliobolus heterostrophus*. Functional analysis of PKS sequences may reveal polyketide toxins produced by *A. brassicicola* that are important for pathogenesis. Since PKS genes are typically located in gene clusters, it would be worthwhile to examine the genomic regions upstream and downstream of the PKS sequences. This may identify other genes in the putative toxin biosynthesis gene cluster.

A serine protease (*PSP1*) homolog from the *Brassica* pathogen *Pyrenopeziza brassicae* was also identified. This serine protease with an estimated molecular weight of

34 kDa appears to be required for *P. brassicae* pathogenesis of oilseed rape (Batish et al. 2003). UV mutants of *P. brassicae* that no longer produced extracellular protease were previously found to be deficient in pathogenicity (Ball et al. 1991). A 35 kDa protein (AB-toxin) of similar size to *PSP1* has been reported previously to be produced by *A. brassicicola* (Otani et al. 1998). The gene encoding this putative host-specific toxin has not been cloned. The similarity in size and host specificity between *PSP1* and AB-toxin may indicate that these proteins are closely related. These results indicate that the *PSP1* homolog from *A. brassicicola* is worth further investigation. Future studies may include cloning the full length *PSP1* homolog and developing gene replacement mutants to examine its role in *A. brassicicola* pathogenesis.

Another protein found to be upregulated during plant infection was a homolog (*Alt b 1*) of the major *Alternaria* allergen *Alt a 1*. No known function has been reported for this major allergenic protein. *Alt a 1* is a secreted protein and likely has an important role in fungal biology. One potential function may be in forming an extra cellular matrix (ECM) that is often secreted by germinating fungal spores. The ECM allows the fungal spore to attach and adhere to the substrate. ECMs are known to contain glycoproteins, though an extensive analysis of fungal ECMs has not been conducted. *Alt b 1* expression dramatically increases with spore germination and is highly conserved across the genus. Slight differences between *Alt a 1* and *Alt b 1* exist. The main difference is that *Alt b 1* may be a glycosylated protein based on bioinformatics analyses (see Chapter 4). What effect glycosylation has on *Alt b 1* function is not known. Preliminary results obtained from injection of pure *Alt b 1* protein into plant leaves indicate that *Alt b 1* may be toxic

to plant cells. This would be an extremely exciting and significant discovery with regards to *A. brassicicola* pathogenesis and the function of this major human allergenic protein.

One of the most biologically interesting secondary metabolites produced by *A. brassicicola* is the histone deacetylase inhibitor, depudecin. Research on depudecin has focused on its ability to induce morphological reversion of oncogenic NIH 3T3 cells (Kwon et al. 1998). Transcriptionally active regions of the genome typically contain hyperacetylated chromatin, while silent regions are associated with hypoacetylated histones. Thus, depudecin likely modulates gene expression by altering the acetylation state of histones by inhibiting histone deacetylases. Depudecin's role in *A. brassicicola* plant pathogenesis, however, has not been examined in detail. One of the plant genes upregulated during *A. brassicicola* infection of green cabbage was a histone deacetylase. Depudecin may prevent gene expression of regulators that positively regulate negative regulators of plant defense responses. If these positive regulators could not be transcribed and translated into their functional protein products to alleviate the negative regulation of important plant defense response pathways, the fungus would have an easier time of overcoming the weakened host defense response. Or, the inhibition of the histone deacetylase may lead to increased programmed cell death (PCD) by a similar mechanism of positive and negative regulation of genes required for PCD. Necrotrophic fungi are known to require host cell death in order to successfully colonize and acquire nutrients from host cells.

One of the first barriers encountered by all fungi is the leaf cuticle and underlying plant cell wall. Fungi have evolved diverse mechanisms for penetrating the plant cuticle and cell wall as discussed in Chapter 1. The exact mechanism that *A. brassicicola* utilizes

to invade *Brassica* leaves is unknown. Genes identified in this study indicate that *A. brassicicola* may utilize cuticle and cell wall degrading enzymes. Several well known cell wall degrading enzymes were identified in the *A. brassicicola* EST libraries including polygalacturonases, xylanases, β -glucosidases, and endoglucanases. Two clones identified in the EST libraries with homology to lipases may prove to encode a putative lipase shown previously to be involved in the early stages of *A. brassicicola* pathogenesis. The gene for this lipase has not been previously cloned. Lipases may serve similar roles as cell wall degrading enzymes by helping to degrade the plant cuticle. Release of fatty acid monomers from cutin in the cuticle may serve as an elicitor for plant defense responses, or as an elicitor for the fungus to begin toxin production.

Plant genes known to be induced in response to the presence of fungal cuticle and cell wall degrading enzymes were identified in the EST libraries. These include putative chitinase and polygalacturonase inhibiting proteins (PGIP). PGIPs are known to inhibit, but not completely abolish, fungal endo-polygalacturonase (EG) activity. By allowing continued EG activity at low levels, PGIPs allow the continued production of oligogalacturonides which serve as elicitors of plant defense responses. Genes such as chitinases and pathogenesis related proteins may be induced by oligogalacturonides. Further, phytoalexin production is known to be triggered by EG activity.

Some fungi also produce infection structures called appressoria that allow direct physical penetration of the plant cuticle and cell wall. MAP kinase cascades have been previously shown to be important for appressorium morphogenesis and function (Xu et al. 2000). One of the most important genes in appressorium development appears to be a MAP kinase with homolog to yeast *FUS3/KSS1*. MAP kinases with homology to

FUS3/KSSI have been identified in a number of fungal pathogens, and are all required for fungal pathogenesis. A MAP kinase with significant homology to *FUS3/KSSI* like MAP kinases was identified in the EST libraries in this study. The full length cDNA sequence of this putative MAP kinase was cloned and named *AMK1* (*Alternaria* MAP Kinase 1). Thus, it may be possible that *A. brassicicola* is capable of forming appressoria to directly penetrate the plant cuticle and cell wall. This finding indicates that an examination of whether *A. brassicicola* produces appressoria during infection is warranted.

Once *A. brassicicola* has penetrated the plant's cuticle and cell wall, it likely is exposed to a myriad of plant produced antifungal compounds. Presumably, detoxification of these compounds would be essential for a successful invasion. One of the genes identified in the EST libraries had significant homology to a fungal cyanide hydratase. Since this gene appeared to be a good candidate to be involved in the detoxification of an antifungal compound such as cyanide, the full length cDNA sequence was cloned and a gene disruption mutant was created. However, cyanide hydratase mutants did not show any defects in pathogenicity on green and red cabbage. As the mutants were still capable of growing in the presence of cyanide at various concentrations, *A. brassicicola* likely contains other cyanide detoxification mechanisms. Thus, the role of cyanide hydratase in fungal pathogenesis remains unclear. Phylogenetic analyses revealed that cyanide hydratase enzymes are also found in non-plant pathogenic fungi. These results suggest that cyanide hydratase enzymes likely are not required for fungal pathogenicity, and serve some other role in fungal metabolism. A role for cyanide and cyanide detoxification

in plant-fungal interactions can not be ruled out, however, since cyanide is known to be released from senescent plant cells.

In conclusion, the results presented in this dissertation provide the first insight into the genes and gene products involved in the complex interaction between the necrotrophic fungus *A. brassicicola* and its *Brassica* hosts. The outcomes of plant-fungal interactions are mediated by the complex interplay between pathogen and host derived compounds. By identifying genes that are expressed during the interaction between the pathogen and its host, a better understanding of the factors that lead to the outcome can be obtained. This greater understanding can be used to design novel control strategies to prevent devastating disease epidemics from occurring. The findings in this dissertation clearly indicate that necrotrophic fungal pathogenesis is a complex process mediated by many genes and gene products. The ultimate question is how these gene products interact with each other, and gene products from the host, to determine the outcome of plant-fungal interactions.

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