SCREENING GENES ENCODING TRANSCRIPTION FACTORS ASSOCIATED WITH PATHOGENESIS IN *Alternaria brassicicola*

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Krushitonaasti durbhiksham

"Dearth of Agriculture is Famine" (In Sanskrit)

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Akhil Srivastava
Honolulu, Hawai'i, USA.

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ABSTRACT

The necrotrophic fungus *Alternaria brassicicola* causes black spot disease of brassicaceous plants, including green cabbage (*Brassica oleracea*) and the oil-producing *B. napus*. Pathogenesis is a multistep process that includes germination, penetration and colonization of host tissues, and survival structure formation. The *de novo* synthesis of various catabolic enzymes and secondary metabolites needed during each step in pathogenesis are under the regulation of transcription factors. Zinc finger DNA-binding domains containing transcription factor forms the largest family of transcription factors in eukaryotes. Based on the zinc binding motifs transcription factors are of three classes: Cys_2-His_2, Cys_2-Cys_2, and the fungal specific, binuclear $Zn(II)_2Cys_6His_6$. In the present study we made knock out mutants of all 184 C2H2 Zinc finger transcription factor encoding genes by targeted gene disruption and 35 $Zn(II)_2Cys_6His_6$ motif containing transcription factors through targeted gene deletion/replacement in *Alternaria brassicicola.* Our bioassays on detached leaves of green cabbage have identified twelve genes associated with early and late stages of plant infection. Among these twelve genes, two genes are pathogenicity factors as their mutants were nonpathogenic, while one showed increase in pathogenicity by almost 100%. Remaining nine genes were strong virulence factors whose mutants showed 50-90% reduction in disease symptoms compared to the wild type. We also discovered a unique gene whose mutants showed complete loss of conidia yet no effect on pathogenesis. We further report a gene encoding transcription factor associated with detoxification of phytoalexins, which is important for early plant colonization. All the discovered pathogenesis associated genes were novel pathogenicity or virulence factors and only one gene (*PacC*) was previously identified as a pathogenicity factor

in other fungal species. The molecular mechanisms of pathogenesis and their regulation in necrotrophic fungi are in an early stage of research. This study sets the platform for discovery of downstream genes associated with pathogenesis and the characterization of their functions. Currently available data from this study indicate the importance of transcription factors as regulators of pathogenesis and as future targets for disease management.

CHAPTER 1

Introduction

1.1 Plant pathogenic fungi:

The kingdom Fungi consists of simplest living eukaryotic, single to multicellular organisms that are characterized by lack of photosynthetic pigments. The cell wall of the fungus is structurally unique and differs from cellulose-based plant cell wall and mainly composed of glycoprotein and polysaccharide (Bowman and Free, 2006). There are approximately 1.5 million fungal species out of which only around 100,000 have been described so far (Hawksworth, 1991). Most fungi are nonpathogenic saprophytes (surviving on dead and decaying organic matter), and about 15000 species are parasitic on plants and animals (Gonzalez-Fernandez *et al.*, 2010). The role of fungi as plant pathogen was realized as early as 1729 by Micheli. In 1807, Prevost showed conclusively that bunt on wheat is caused by a fungus (Agrios, 2004). Fungal phytopathogens are responsible for huge losses of cultivated crops, forest trees, ornamental plants and urban landscapes. In the United States alone, over $600 million is spent annually on fungicides (Arora, 2003). Pathogenic fungi can be designated as biotrophs, necrotrophs or hemibiotrophs based on their life cycle and mode of parasitism to obtain nutrition. Biotrophs depend on living host cells and tissues and utilize the metabolic machinery of its host for survival. Biotrophs are obligate parasites in nature. They solely depend on the host plants for their survival and cannot live freely or on an axenic medium. Necrotrophs on the other hand invade, kill, and colonize the host plants. They secrete hydrolytic enzymes, extracellular toxins and other secondary metabolites that allow the necrotrophs to colonize the host. Hemi-biotrophs

are characterized by a lifestyle involving a biotrophic phase, before switching to necrotrophic growth and killing their host (Voegele, 2010).

In favorable environment fungi grow by expanding their hypha or mycelia. The diameter of colony can be from a few millimeters to many meters. The major function of a mycelium is to absorb nutrition from the substrate. Spores are another important morphological feature of fungi. Based on their spore bearing structure fungi are classified as: Ascomycetes, Basidiomycetes, Zygomycetes and Chytridiomycetes. Members of class ascomycetes form the largest group of phytopathogens and therefore among fungal pathogens they are the most studied ones. Though huge numbers of publications are available describing various aspects of fungal pathogenesis still there is a large knowledge gap in understanding of the patho-physiology of phytopathogenic fungi.

1.2 Genus *Alternaria*:

Alternaria speciesare one of the most diverse and ubiquitous forms of fungi (Hyde and Williams, 1949), commonly found as saprophytes in soil or decaying plant tissues. Alternaria belongs to phylum Ascomycota and class dothediomycetes. This genus was first described in 1816 by Nees and *Alternaria tenius* (presently known as *Alternaria alternata*) was the sole species known (Esenbeck, 1816). The taxonomy of *Alternaria spp*. is largely based on morphological characteristics and structure of conidia (Pattanamahakul and Strange, 1999). Meena *et al*. (Meena *et al.*, 2010) have compared the structural features of three important

species of *Alternaria* which are pathogenic on brassica. These species of *Alternaria* are often taken as reference in identification and differentiating from each other (table 1.1)

Table1.1: Morphological comparison of three important Alternaria spp. pathogens on brassica. (table modified from (Meena *et al.*, 2010)

Fungal Structures	***A. brassicae***	***A. brassicicola***	***A. raphani***
Mycelium	Septate, brownish gray	Septate, olive gray to grayish black	Cottony whitish to greenish gray or dark olive
Conidiophore	dark, septate, arise in fascicles, 14-74µ x 4-8µ	Olivaceous, septate branched, 35-45µ x 5-8µ	Septate, olive brown, single or branched, 29-160µ x 4-8µ
Conidia	Brownish black obclavate, muriform, produced singly or in chain, 2-3 in number	Dark, cylindrical, oblong, muriform, produce in chains of 8-10 spores.	Olive- brown to dark, obclavate, muriform, more or less pointed at each end, appear singly or in chains up to 6 spores.
Spore body (µ)	96-114 x 17-24	45-55 x 11-16	45-58 x 13-21
Spore beak length (µ)	45-65	None	1-25
Rate of growth and sporulation on media	Rudimentary slow growth	Black sooty colony with distinct zonation, fast growing with abundant sporulation	Cottony mycelial colony with less abundant sporulation.

Alternaria spp have emerged as plant pathogens that collectively cause disease on various agro-economically important crops including, cereals, ornamentals, oil crops, vegetables, fruits as well as post-harvest pathogens (Thomma, 2003). Many *Alternaria* spp. synthesize secondary metabolites such as mycotoxins that are considered as potential carcinogens in animals and humans. In immune-compromised patients *A.alternata* causes allergies and inflammations of respiratory tract (Lawrence *et al.*, 2008).

1.3 Alternaria brassicicola

Alternaria brassicicola (Schwein) Wiltshire (1947) is a necrotroph and that causes black spot disease in almost all cultivated Brassica species (Cramer and Lawrence, 2003). *A.brassicicola* is a seed-borne pathogen (Pattanamahakul and Strange, 1999). Host plants include broccoli and cauliflower *(Brassica oleracea L.),* field mustard or turnip mustard *B. rapa L.* (synonym: *B. campestris L.),* leaf or Chinese mustard *(B. juncea),* Chinese or celery cabbage *(B. pekinensis),* cabbage *(B. oleracea* var. *capitata),* rape *(B. campestris),* and radish *(Raphanus sativus)* (Meena *et al.*, 2010). In canola it causes premature pod and seed loss resulting in reduction of crop yield (Hodgkins, 1986). The destruction to crops due to *A.brassicicola* has been reported from many countries throughout the world. In UK between 1976 and 1978 about 86% of commercial brassica seed production has been found contaminated with *A.brassiciciola* (Maude and Humpherson-Jones, 1980). In Germany 20-50% of loss in yield of rape seed was due to *A.brassicicola* (MacKinnon *et al.*, 1999). Similar losses due to black spot disease have also been reported from Australia (Sivapalan and Browning, 1992), Canada (Petrie, 1974), South Africa (Holtzhausen, 1974) and Taiwan (Wu, 1979). In United States about 79% of fields for

seed production was contaminated with this pathogen (Babadoost M, 1979). Environmental conditions play a very important role in the manifestation of this pathogen. High humid conditions are the most favorable conditions for growth (Humpherson-Jones and Phelps, 1989). The availability of nutrients also significantly influences the virulence of *A. brassicicola* during its infection of cabbage leaves (Ding, 2005).

The infection process starts with germination of conidia approximately 4 hours after conidia come in contact of host plant, followed by formation of a specialized structure called an appresorium that leads to penetration and colonization of host plant (fig1.1A-D). The typical symptom of disease is marked by the appearance of small necrotic black spots on leaves, stem and other aerial parts of the plants. The spots are sooty black in texture due to presence of conidial spores on the surface. On large leaves the black spores form a typical pattern of concentric rings giving the spot a target like appearance. This happens because the pathogen does not grow with a uniform rate in response to the fluctuation in environment (Fig 1.2 A). On seedlings, symptoms include dark stem lesions immediately after germination results in damping-off, or stunted seedlings.

A. brassicicola is also known to secrete certain toxins which results in production of yellow halo regions preceding the black spots (Fig. 1.2 B), AB-toxin- the host specific toxin (Otani *et al.*, 1995) and certain cyclic despipeptide and fucicoccin based toxins have been known to be secreted by *A. brassicicola* (Lawrence *et al.*, 2008). Brassicolin A is another antibiotic compound isolated from the liquid cultures of *A.brassicicola* (Pedras *et al.*, 2009a).

World-wide economic loss due to black spot disease has been very large and tailoring of proper control of this pathogen is one of the most important priorities of research in plant pathology. Several chemical and biotic control methods have been tested for decreased growth and spore production of *A. brassicicola*. Examples of synthetic compounds which were effective are Biltox, Mancozeb (Dithane M-45), Maneb and Thiram. Since, long lasting synthetic chemicals are generally hazardous to the environment and contribute to pollution, their use should be carefully evaluated and new alternatives should be explored. Extracts from potato sprouts and *Solanum nigrum* roots have shown complete inhibition of conidial germination of *A. brassicicola* at a concentration of 10% (w/v) from fresh tissues, and 1% (w/v) from dry tissues (Lin *et al.*, 2011). The ambruticins, another class of natural antifungal polyketides originating from the myxobacterium *Sorangium cellulosum* have shown anti-fungal activity against *A. brasicicocla* (Dongo, 2009). Root extracts of black nightshade *(S. nigrum*) have also shown inhibition of growth of *A. brassicicola* (Muto *et al.*, 2006). In a recent study, the human pathogen *Pseudallescheria boydii* has been shown to secrete chemicals that are fungistatic, killing *A. brassicicola* (Ko *et al*., 2010) However, no known source of genetic resistance has been identified among Brassica cultivars (Sigareva and Earle, 1999). Direct losses plus the cost of control strategies contribute to the huge agricultural significance of this organism and the genus overall.

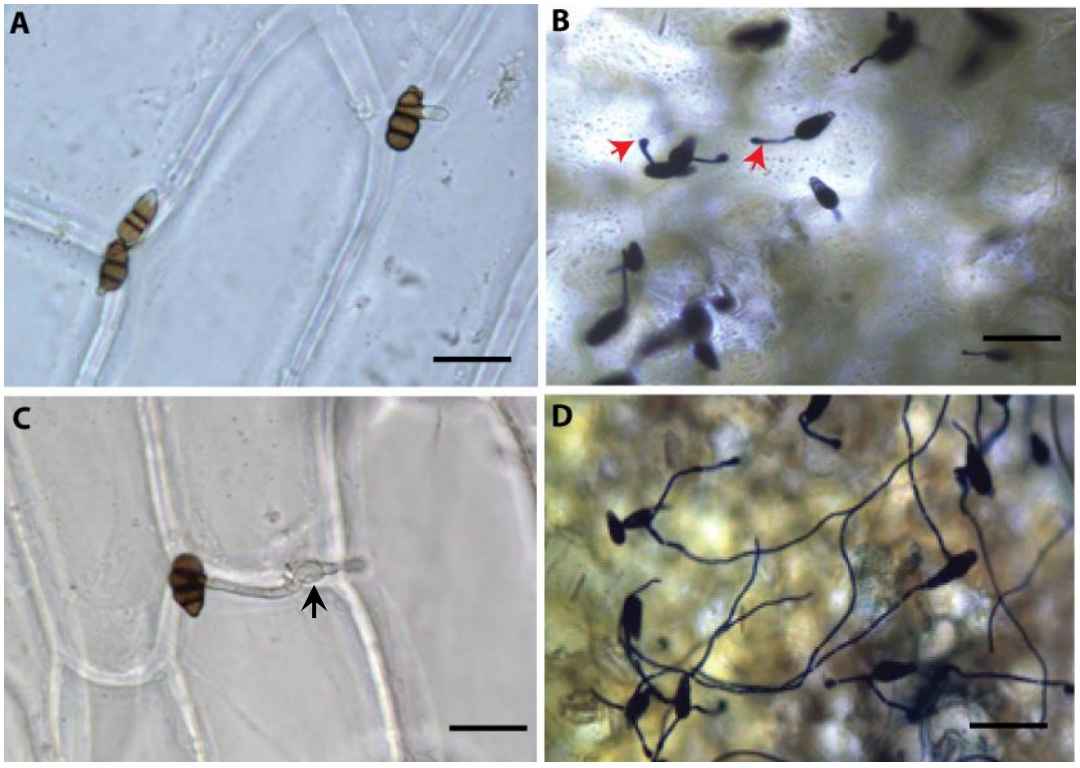

Fig. 1.1 A-D shows the various stages of pathogenesis of *A.brassicicola* on two hosts, viz. onion (**A and C**) and cabbage leaf (**B and D**). **A**. Germ tubes are emerging from the conidia after 4 hours of inoculation. **B**. The tip of germ tube forms an appresorium (marked by red arrows) are used for penetration 8 hours after inoculation. **C**. The germ tube penetrating host surface after 12 hours of inoculation. **D.** Colonization and development of mycelial network on the leaf 24 hours after inoculation. **B and D** images were recorded after staining with tryphan blue, while **A** and **C** are images without any stains. Bar shows100µm.

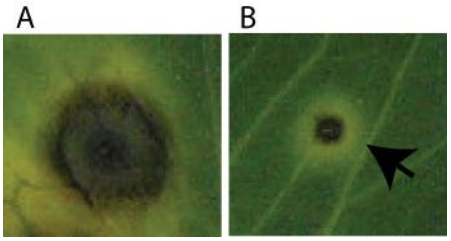

Fig. 1.2: Typical symptoms of *A. brassicicola* on cabbage leaf. **A**. Apperaence of concentric rings of conidia due to variable growth rate gives a typical target like structure. **B**. The yellowing of leaf preceeds necrosis due secretion of phytotoxins.

1.3.1 Current research status in *Alternaria brassicicola*:

Alternaria brassicicola is pathogenic to almost all members of brassica spp and *Arabidopsis thaliana*. Currently the genomes of many brassicas such as Chinese cabbage, broccoli and *A.thaliana* have been sequenced and are available in the public domain. This makes *A. brassicicola* an attractive model for studying host pathogen molecular interactions. A team led by Christopher Lawrence of Virginia Bioinformatics Institute, VA and Washington University Genome Center St. Louis, MO completed whole genome sequencing of ATCC 93866 strain (American type culture collection, VA) of *A. brassicicola*. The draft sequence is available in public domain -JGI fungal portal MycoCosm at http://jgi.doe.gov/Abrassicicola. (Grigoriev *et al.*, 2012; Ohm *et al.*, 2012). The genome of *A. brassicicola* is ~31 Mb distributed in 838 supercontigs (Lawrence personal communication). The availability of the draft sequence opened a door to functional genomics and a reverse genetics approach to initiate deep rooted molecular studies of physiology of this organism. Discovery and characterization of genes involved in pathogenesis and identification of regulatory genes are one of the most obvious applications of genome sequence to understand the molecular basis of pathogenesis.

Cramer and Lawrence did the earliest study in this direction where they tried to identify the differentially expressed genes of *A.brassicicola* during their infection on *A.thaliana* using the subtractive hybridization method (Cramer and Lawrence, 2004). Cho et al came up with a very robust and efficient, high throughput fungal transformation method and developed Linear Minimal Element constructs (LME) for generation of gene disruption mutants. A similar protocol with little modification was later used for making knockout mutants by deleting the target gene from the genome and replacing it with the selection marker gene (Cho *et al.*, 2006;

Cho *et al.*, 2009). Such high throughput method proved a vital tool for identification and understanding of various genes involved in pathogenesis. Many similar studies tend to enhance the knowledge related not only to the pathogenesis mechanism of *A. brassicicola* but of many other fungal necrotrophs

1.4 Host pathogen interactions:

Plants do not have a well-established immune system and are also sessile in nature; hence they cannot escape from the various biotic stresses from attacks from microbes, insects and nematodes. To thrive the plants must produce a defense response to potential pathogens (Chisholm *et al.*, 2006). Plants have evolved on earth over 700 million years (Heckman *et al.*, 2001) and it is suggested that molecular interactions between early land plants and epiphytic, symbiotic and pathogenic microbes played crucial role in the successful establishment of modern day land plants. (Chisholm *et al.*, 2006). During the disease cycle, interactions between a host and its pathogen are very complex and largely not well understood. The various activities occurring at the interface during initiation of infection and pathogen invasion is very critical as it decides the fate of the pathogen and the host. Therefore, it is imperative to have a detailed understanding of this interaction.

1.4.1 Pathogenicity Genes:

A pathogen must gain access to the interior of plant either by penetrating the epidermal layer or through natural openings such as stomata, lenticels, hydathodes or through wounds. Plant pathogens mainly fungi, bacteria and nematodes employ diverse strategies to invade plant and cause disease (Agrios, 2004). Pathogenesis is a multistep process that is hypothesized to be regulated by one or more genes, known as 'pathogenicity' genes (Cho, personal communication). The initial step of pathogenesis begins as soon as conidia land on the surface of the host plant. There are various mechanisms a fungus can employ to penetrate the host surface and colonize it. Appresorium facilitates penetration in many genera of fungi. An appresorium is a specialized club shaped structure needed for penetration though plant cuticle (Fig 1.1B). Melanin is the essential component of appresorium that generates high turgor pressure needed for penetration by the plant pathogenic fungi. Genes for melanin synthesis and crucial for pathogenesis have been identified in *M. griesa* (de Jong, 1997; Talbot, 2003), *Collectrotricum lagenarium, C. lindemuthianum and C. graminicola* (Y. Kubo, 1982; Rasmussen and Hanau, 1989; Kubo *et al.*, 1998) and among animal pathogens *Crypotococcus neoformans, Aspergillud fumigatus and Wangiella dermatitidis* (Salas *et al.*, 1996; Feng *et al.*, 2001; Feng *et al.*, 2008; Tsai *et al.*, 2008). However, surprisingly in *A. brassiciola* deletion of transcription factor encoding gene involved in melanin synthesis (*Amr1*) has been found to increase the pathogenesis (Cho *et al.*, 2012). To obtain nutrients from cell the infection structures of pathogen needs to enter inside cell by penetration of the cell wall. A battery of cell wall degrading enzymes (CWDE) involving several cutinase, pectinase, hydrolytic enzymes are involved in this step of pathogenesis (Srivastava *et al.*, 2012). Most of the CWDE encoding genes belongs to multigene families and are redundant

in function therefore, identification of a single gene among them as pathogenicity factor remains elusive. In *A. brassicicola* seven cutinase is present and gene disruption of individual gene has resulted in no change pathogenicity such as *CutAb1* (Chenglin Yao, 1995).

Recent data suggests that although up to present day various pathogenesis related genes have been characterized in many fungal genera still no common gene regulating pathogenesis across all genera of pathogenic fungi has been reported i.e. there is no universal pathogenicity or virulence factor (Idnurm and Howlett, 2001). In case of fungal phytopathogens till 2001, a total of 79 pathogenesis genes has been identified in various pathogenic fungi (Idnurm and Howlett, 2001). This number is expected to increase further due to availability of advanced molecular biology and genomics techniques (Islam *et al.*, 2012). Table 1.2 describes the identification and characterization of pathogenesis related genes. This is list is not a comprehensive collection of pathogenicity genes, as reports related to identification of newer genes are added almost every month, still the table describes about 178 genes that were described between 2001 and 2012. Though there is no universal criterion for classification of pathogenesis associated genes, we have classified genes into four major classes based on their position in the molecular pathogenesis pathway (fig.1.3)

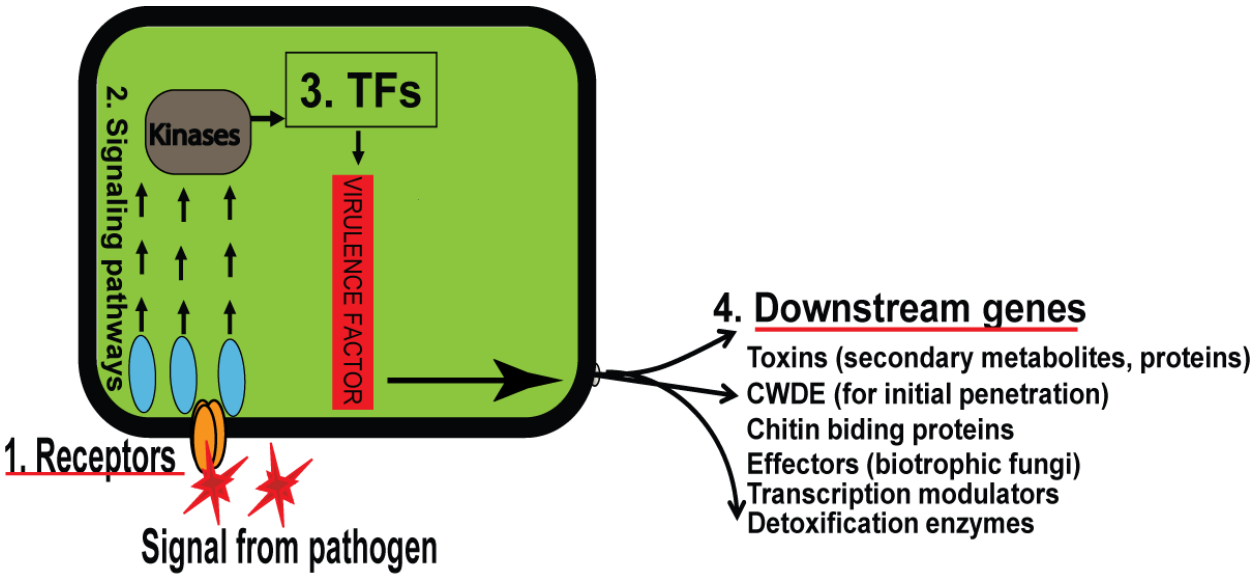

Fig1.3. **Four groups of genes having pivotal role in host pathogen interactions** and during pathogenesis in various pathogenic fungi. Abbreviations: TF= Transcription factors, CWDE= Cell wall degrading enzymes. Image: Modified from Cho personal communication

1.4.1.1 Receptors

G-Protein and GPCR (G-Protein Coupled Receptors)

GPCRs are trans plasma membrane proteins that respond to the external cues and communicate with the intracellular heterotrimeric G proteins (Guanine nucleotide binding Protein). In recent years G proteins have been found to be associated with diverse processes such as differentiation, mating and pathogenicity in a number of phytopathogenic fungi. The knockout mutants of genes listed in following table have shown reduction in virulence along with defects in other features.

Table 1.2 G-protein signaling genes associated with pathogenesis in plant pathogenic fungi.

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
AGA1	*Alternaria alternata*	Disruption	Change in colony and conidial morphology and sporulation, poor germination.	G protein signaling	Alternaria blotch	(Yamagishi *et al.*, 2006)
BcCdc42	*Botrytis cinerea*	Deletion	Reduced growth rate and hyphal branching, fewer, abnormal conidia, poor germination.	G protein signaling	Gray mold of grapes	(Kokkelink *et al.*, 2011)
BDM1	*Fusarium graminearum*	Deletion	Sexual and asexual sporulation, germ tube development, mycelial morphology.	G protein signaling	Wilt	(Horevaj and Bluhm, 2012)
ERL1	*Magnaporthe oryzae*	Disruption	Slow invasive growth.	G protein signaling	Rice blast	(Heupel *et al.*, 2009)
FgFlbA	*Fusarium graminearum*	Deletion	Abnormal conidia morphology, slow growth, poor germination and low mycotoxin production.	G protein signaling	Fusarium head blight	(Park *et al.*, 2012a)

Table 1.2 continued...

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
Gna1	*Stagonospora nodorum*	Disruption	Sporulation, albino phenotype, and secreted brown pigments in media.	G-protein and cAMP	Wheat leaf bloch	(Solomon *et al.*, 2004)
MAGB	*Magnaporthe grisea*	Mutagenesis	Autolysis of aged colonies, melanization, reduction in both sexual and asexual reproduction.	G protein signaling	Blast on rice	(Fang and Dean, 2000)
MoRIC8	*Magnaporthe oryzae*	Mutagenesis	Cellular differentiation, sporulation, sexual development.	G Protein signaling	Rice blast	(Li *et al.*, 2010b)
PTH11	*Magnaporthe oryzae*	Micro array	DelaysAppresorium maturation.	G Protein signaling	Rice blast	(Odenbach *et al.*, 2007)
RAS2	*Fusarium graminearum*	Deletion	Morphological defects, slower growth, delayed spore germination	G protein signaling	Fusarium head blight	(Bluhm *et al.*, 2007)
VdPKAC1	*Verticilium dhaliae*	Disruption	Poor spore germination, reduced conidia production.	G protein signaling	Vascular wilt of tomato	(Tzima *et al.*, 2010a)

1.4.1.2. Signaling:

Signal transduction is the central theme of organismic physiology and for this reason it is expected that genes of the signal transduction pathway should have a pivotal role in pathogenesis. It is crucial for the pathogen to perceive intracellular signals emanating from the host plant in order to mobilize various genes needed for pathogenesis and abatement of the plant defense response. Many homolog signal transduction genes like, G- protein signaling, mitogen activated kinases (MAPK) and cAMP signaling genes have been knocked out. Though they often resulted in a decrease in virulence, but simultaneously they also showed many pleiotropic effects like loss of conidiation, appresorium formation, slow vegetative growth etc. Such multiple effects in null mutants make it difficult to categorize the signal transduction gene as pathogenicity gene. Until recently no gene related to signal transduction has been reported to show exclusively loss of pathogenicity or reduction in virulence. Although at present, a number of genes associated with signal transduction and pathogenesis have been cloned and characterized. We still lack the complete understanding of extracellular signals and their transduction into intercellular signals are not well understood (Tudzynski and Sharon, 2003).

1.4.1.2.1 cAMP signaling

The cAMP signaling pathway is known to function downstream of G-proteins in filamentous fungi. It has been shown to have a crucial role during pathogenic development in

many phytopathogenic fungi. Fungal strains in which cAMP signals are blocked at different levels are disturbed at different stages of the infection process *in planta* (Tudzynski and Tudzynski, 1999; Tudzynski and Tudzynski, 2001). Apart from showing reduction in virulence the knockout mutants for genes encoding for cAMP pathway in different phytopathogenic fungi also shows reduction in growth, abnormalities in conidia morphology and in certain cases delayed spore germination on the host plant. Table 1.3 presents a list of cAMP genes involved in pathogenesis.

Table 1.3. cAMP signaling genes associated with pathogenesis in plant pathogenic fungi.

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
bcpka1, bcpkaR	*Botrytis cinerea*	Deletion	Reduction in growth, conidiation, germination	cAMP-PKA	Gray mold of grapes	(Schumacher *et al.*, 2008b)
CID1	*Fusarium graminearum*	Deletion	Reduction in conidiation and vegetative growth and DON production but an increase in pigmentation	cAMP-PKA	Fusarium head blight on wheat	(Zhou *et al.*, 2010)
cpk1,cac1	*Colletotrichum lagenarium*	Disruption	Slow growth and poor germination	cAMP-PKA	Anthracnose on cucumber	(Yamauchi *et al.*, 2004)
FAC1	*Fusarium verticillioides*	Disruption	Reduced vegetative growth reduced macroconidiation, increased microconidiation and heat and salt tolerant	cAMP-PKA	Stalk and ear rot in maize	(Choi and Xu, 2010)
FoCPKA	*Fusarium oxysporum*	Disruption	Reduced sexual reproduction, conidiation, penetration, Pleiotropic.	cAMPK MAPK	Vascular wilt	(Kim *et al.*, 2011)
MCC1	*Magnaporthe graminicola*	Deletion	Reduced vegetative growth, increased melanin on hypha ROS tolerance, Pleiotropic	cAMP-PKA	Septoria tritici blotch	(Choi and Goodwin, 2010)

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
SAC1	*Sclerotinia sclerotiorum*	Deletion	Slow growth aberrant sclerotia formation.	cAMP-PKA	White mold and cottony rot	(Jurick Ii and Rollins, 2007)
PIC5	*Magnaporthe grisea*	Deletion	Reduced appresorium formation and infectious growth	cAMP-PKA signaling	Rice Blast	(Zhang *et al.*, 2011)
Smu1	*Ustilago maydis*	Deletion	Impaired pheromone response and the regulation of cell polarity and cell cycle	cAMP-PKA	Smut of Maize	(Lovely *et al.*, 2011)
UCN1	*Ustilago maydis*	Disruption	Reduced mating	cAMP-PKA	Smut of Maize	(Egan *et al.*, 2009)
VdPKAC1	*Verticilium dahliae*	Disruption	Reduced conidiation, microsclerotia formation, and ethylene biosynthesis,	cAMP-PKA	Wilt	(Tzima *et al.*, 2012)

1.4.1.2.2 MAPK signaling

Mitogen activated protein kinases are one of the two signaling pathways other than cAMP pathway functioning downstream of G-protein signaling. MAP kinases belong to a family of serine/ threonine protein kinase and are known to mediate the changes in the intracellular activities of eukaryotic cells in response to the external environmental cues. (Tudzynski and Sharon, 2003). MAPK are activated by cascade of three kinases that are sequentially dephosphorylated, from MAPK kinase kinase, to MAPK kinase, to the terminal MAPK. The activated MAPK regulates various transcription factors and other downstream genes. In *Saccharomyces cerevisiae* five MAP kinase mediated signal transduction pathways have been identified (Zhao *et al.*, 2007). Three MAP kinase signaling pathway are shown to be active on filamentous fungi namely: (a) Fus3/Kss1- for mating responses and filamentous growth, (b) Slt2- responsible for cell integrity and (c) Hog- for osmoregulation and environmental stresses including oxidative and pH. Table 1.4 describes MAPK associated genes involved in fungal pathogenesis. The list includes almost all important fungal phytopathogens, the mutants for each gene shows a pleiotropic effect apart from reduction in virulence such as reduction in vegetative growth, reduction in conidia production and maturation and appresorium.

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Table 1.4. MAPK signaling genes associated with pathogenesis in plant pathogenic fungi.

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
AaFus3	*Alternaria alternata*	Disruption	Reduced vegetative growth, conidiation.	MAPK-FUS3/KSS1	Spot on citrus	(Lin *et al.*, 2009)
Amk1	*Alternaria brassicicola*	Disruption	Reduced in growth, lack of conidia formation, pleiotropic effect.	MAPK-FUS3/KSS1	Black spot of cabbage	(Cho *et al.*, 2007)
Bmp1	*Botrytis cinerea*	Deletion	Reduced in growth, failed penetration.	MAPK-FUS3/KSS1	Gray mold of grapes	(Zheng *et al.*, 2000)
Bmk1	*Bipolaris oryzae*	Disruption	Reduced, conidiation appresorium formation, vegetative growth.	MAPK-FUS3/KSS1	Brown spot disease on rice	(Moriwaki *et al.*, 2007)
chk1, mps1, hog1	*Cochliobolus heterostrophus*	Disruption Deletion	Female sterile, lack of conidia, appresoria.	MAPK-FUS3/KSS1	Southern corn blight of maize	(Igbaria *et al.*, 2008)
CMK1	*Colletotrichum lagenarium*	Deletion	Pleiotropic.	MAPK-FUS3/KSS1	Anthracnose on cucumber	(Takano *et al.*, 2000)
Cpmk2	*Cryphonectria parasitica*	Disruption	Reduced vegetative growth, conidiation.	MAPK-FUS3/KSS1	Chestnut blight	(Choi *et al.*, 2005)
Fmk1	*Fusarium oxysporum*	Deletion	Reduced appresorium growth.	MAPK-FUS3/KSS1	Wilt of tomato	(Di Pietro *et al.*, 2001)
FvMk1	*Fusarium verticillioides*	Deletion	Pleiotropic.	MAPK-FUS3/KSS1	Ear and stalk rots in maize	(Zhang *et al.*, 2011)

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
Kpp6	*Ustilago maydis*	Disruption	Reduced penetration, mating.	MAPK-FUS3/KSS1	Smut on Maize	(Brachmann *et al.*, 2003)
Gpmk1	*Fusarium graminearum*	Disruption	Reduced vegetative growth, conidiation, CWDE	MAPK-fuss3/KSS1	Wilt	(Jenczmionka and Schafer, 2005)
Mak2	*Phaeosphaeria nodorum*	Disruption	Reduced vegetative growth.	MAPK-FUS3/KSS1	Necrosis on wheat	(Solomon *et al.*, 2005)
Map1	*Fusarium graminearum*	Deletion	Multiple events in disease progression.	MAPK-FUS3/KSS1	Fusarium head blight	(Urban *et al.*, 2003)
MgFus3	*Mycosphaerella graminicola*	Disruption	Unable to differentiate pycnidia on plant-derived media.	MAPK-FUS3/KSS1	Septoria tritici blotch	(Cousin *et al.*, 2006)
MgSte50	*Mycosphaerella graminicola*	Deletion	Multiple events in disease progression.	MAPK-FUS3/KSS1	Leaf blotch	(Kramer *et al.*, 2009)
PTK1	*Pyrenophora teres*	Deletion	Reduced conidiation appresorium formation, vegetative growth.	MAPK-FUS3/KSS1	Blotch on barley	(Ruiz-Roldán *et al.*, 2001)

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
RPK1	*Colletotrichum lagenarium*	Deletion	Multiple events in disease progression.	MAPK-FUS3/KSS1	Anthracnose on cucumber	(Takano *et al.*, 2000)
Vmk1	*Verticilium dahliae*	Disruption	Reduced conidiation, microsclerotia formation	MAPK-FUS3/KSS1	Wilt	(Rauyaree *et al.*, 2005)
AbHog1	*Alternaria brassicicola*	Deletion	Sensitivity for osmotic, oxidative toxic chemical stress, resistance to phytoalexins.	MAPK-Hog	Black spot disease	(Joubert *et al.*, 2011)
AaHog1	*Alternaria alternata*	Disruption	Sensitivity for osmotic, oxidative and toxic chemical stress	MAPK-Hog	Spot on citrus	(Lin *et al.*, 2011)
BcSAK1	*Botrytis cinerea*	Gene deletion	Sensitivity for osmotic specific fungicides and oxidative stress.	MAPK-Hog	Gray mold of grapes	(Segmüller *et al.*, 2007)
Cpmk1	*Cryphonectria parasitica*	Deletion	Reduced conidiation, pigmentation.	MAPK-Hog	Chestnut blight	(Park *et al.*, 2012b)
Fhk1	*Fusarium oxysporum*	Deletion	Sensitivity for osmotic stress.	MAPK-Hog	Wilt of tomato	(Rispail and Di Pietro, 2010)

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
MgHog1	*Mycosphaerella graminicola*	Disruption	Sensitivity for osmotic stress.	MAPK-Hog	Septoria tritici blotch	(Mehrabi *et al.*, 2006b)
SSk1 and Hog 1	*Fusarium graminiarum*	Deletion	Sensitivity for osmotic stress.	MAPK-Hog	Wilt	Oide (Oide *et al.*, 2010) et al, 2010
AaSLT2	*Alternaria alternata*	N.A.	Sensitivity to fungicides growth reduction.	MAPK-Slt2	Spot on citrus	(Yago *et al.*, 2011)
AbSLT2	*Alternaria brassicicola*	Deletion	Osmotic, oxidative toxic chemical stress.	MAPK-Slt2	Black spot disease	(Joubert *et al.*, 2011)
Bmp3	*Botrytis cinerea*	Deletion	Reduced growth, conidiation.	MAPK-Slt2	Gray mold of grapes	(Rui and Hahn, 2007)
maf1	*Colletotrichum lagenarium*	Deletion	Reduction in appresorium formation and penetration.	MAPK-Slt2	Anthracnose on cucumber	(Kojima *et al.*, 2002)
cmpk1 and cmpk 2	*Claviceps purpurea*	Deletion	Reduction in appresorium formation and penetration	MAPK-Slt2	Ergot in rye and other cereals	(Mey *et al.*, 2002)
MgSlt2	*Mycosphaerella graminicola*	Disruption	Increased sensitivity to fungicides, growth	MAPK-Slt2	Septoria tritici blotch	(Mehrabi *et al.*, 2006a)

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
MGV1	*Fusarium graminearum*	Deletion	Abnormal sexual reproduction, lack of cell wall integrity	MAPK-Slt2	Scab disease on wheat	(Hou *et al.*, 2002)
MoMps1	*Magnaporthe oryzae*	Deletion	slt2 signaling	MAPK-Slt2	Rice blast	(Qi *et al.*, 2012a)
Mps1	*Cochliobolus heterostrophus*	Deletion	Reduced melanin biosynthesis, conidiation and fertility	MAPK-Slt2	Southern corn blight of maize	(Eliahu *et al.*, 2007b)
BOS5	*Botrytis cinerea*	Deletion	Reduced growth and conidiation.	MAPKK	Gray mold of grapes	(Yan *et al.*, 2010)
CpSte11	*Cryphonectria parasitica*	Disruption	Reduced mating response.	MAPKKK	Chestnut blight	(Park *et al.*, 2012b)
CoMEKK1	*Colletotrichum orbiculare*	Disruption	Slow hyphal growth and abnormal appresorium formation sensitive to high osmotic stress.	MAPKK	Anthracnose in Cucumber	(Sakaguchi *et al.*, 2010)
MCK1	*Magnaporthe oryzae*	Disruption	Reduced numbers of conidia and appresoria formation.	MAPKKK	Rice blast	(Jeon *et al.*, 2008)

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
CZK3	*Cercospora zeae-maydis*	Disruption	Reduced cercosporin biosynthesis and conidiation	MAPKKK	gray leaf spot on maize	(Shim and Dunkle, 2003)
PP2A and rgb 1	*Sclerotinia sclerotiorum*	RNAi	Reduced melanin biosynthesis, infection-cushion production.	MAPKK	White mold and cottony rot	(Erental *et al.*, 2007)

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1.4.1.3. Transcriptional regulation:

Fungi perceive various signals from the external environment by the receptors. Signals are further processed by the signal transduction machinery and finally transmitted to the downstream genes through regulatory molecules called transcription factors. Transcription factors are considered as the last link between the signal transduction pathway and the downstream genes and are described as a separate section later in this chapter. In fungal pathogenesis transcription factors plays very critical role in pathogenesis and may activate or deactivate genes as per the requirement of pathogenic stage. Many of transcription factors and their homologs have been discovered across all the major fungal phytopathogens (Son *et al.*, 2011; Cho *et al.*, 2012). The knockout mutants of transcription factor encoding genes that have shown reduction in virulence generally also show other abnormalities in some other characteristics. A few transcription factors have been discovered and characterized that show exclusively reduction in virulence traits only. The mutants of such genes were similar to wild types in all morphological and physiological properties except for their growth on host plants.

Transcription factors have been attractive targets for identification of novel pathogenicity genes as they are in between signaling genes that are usually pleiotropic and the downstream genes that often shows redundancy in function. In last few years many publications have emerged describing pathogenesis related transcription factors. Table 1.5 enlists pathogenesis associated transcription factors sub-grouped on the basis of DNA binding motifs.

Table 1.5. Transcription factors encoding genes associated with pathogenesis in plant pathogenic fungi.

Gene	Fungus	Method	Effect of inactivation	Class	Disease	Reference
FgStuA	*Fusarium graminearum*	Deletion	Developmental processes, secondary metabolite and spore production	APSES	Head blight or scab on wheat and barley	(Lysøe *et al.*, 2010)
GcSTUA	*Glomerella cingulata*	Deletion	Turgor pressure, glycogen and triacylglycerol synthesis in appresoria	APSES	Apple and onion skin rot	(Tong *et al.*, 2007)
MoCDTF1	*Magnaporthe oryzae*	Mutation	Downstream of the cAMP/PKA signaling, appresorium formation	APSES	Rice blast	(Yan *et al.*, 2011a)
MoSom1	*Magnaporthe oryzae*	Mutation	Downstream of the cAMP/PKA signaling, appresorium formation	APSES	Rice blast	(Yan *et al.*, 2011b)
MoSwi6	*Magnaporthe oryzae*	Disruption	Cell wall integrity, stress responses	APSES	Rice blast	(Qi *et al.*, 2012b)
Med 1	*Ustilago maydis*	Deletion	Mating	APSES	Smut of maize	(Chacko and Gold, 2012)

Gene	Fungus	Method	Function	Class	Disease	Reference
Ust1	*Ustilago maydis*	Deletion	Sporulation	APSES	Smut of maize	(García-Pedrajas *et al.*, 2010)
BcAtf1	*Botrytis cinerea*	Deletion	Response to physical or chemical stress	b-ZIP	Gray mold of grapes	(Temme *et al.*, 2012)
CPTF1	*Claviceps purpurea*	Deletion	Reactive oxygen species (ROS) regulation	b-ZIP	Ergot in rye	(Nathues *et al.*, 2004)
ZIF1	*Fusarium graminearum*	REMI	Deoxynivalenol (DON) production and sexual development	b-ZIP	Head blight or scab on wheat and barley	(Wang *et al.*, 2011)
MoZif1	*Magnaporthe oryzae*	REMI	Reduction in vegetative growth	b-ZIP	Rice Blast	(Wang *et al.*, 2011)
Moatf1	*Magnaporthe oryzae*	Deletion	Oxidative stress	b-ZIP	Rice Blast	(Guo *et al.*, 2010)
Abvf19	*Alternaria brassicicola*	Disruption	Carbon utilization	C2H2	Black spot disease	(Srivastava *et al.*, 2012)
AbVf8	*Alternaria brassicicola*	Deletion	Pathogenesis only	C2H2 Zinc finger	Black spot disease	(Cho *et al.*, 2012)
AbPacC	*Alternaria brassicicola*	Deletion	pH regulation	C2H2 Zinc finger	Black spot disease	(Cho *et al.*, 2012)

Gene	Fungus	Method	Function	Class	Disease	Reference
AbSte12	*Alternaria brassicicola*	Deletion	Conidiation and carbon source utilization	C2H2 Zinc finger	Black spot disease	(Cho *et al.*, 2009)
BcCRZ1	*Botrytis cinerea*	Deletion	Ca(2+)-dependent signaling pathway,	C2H2 Zinc finger	Gray mold of grapes	(Schumacher *et al.*, 2008a)
PacC^{KLAP2}	*Colletotrichum acutatum*	Disruption/ ATMT	pH regulation	C2H2 Zinc finger	Postbloom fruit drop of citrus	(You *et al.*, 2007)
KLAP1	*Colletotrichum acutatum*	Deletion	Penetration	C2H2 Zinc finger	Key lime anthracnose (KLA)	(Chen *et al.*, 2005)
FoST12	*Fusarium oxysporum*	Disruption	Virulence only	C2H2 Zinc finger	Vascular wilt of bean	(Rispail and Di Pietro, 2009; Asunción García-Sánchez *et al.*, 2010)
Ste12	*Fusarium. oxysporum*	Disruption	MAPK Fus3/Kss1 downstream gene, Pleiotropic	C2H2 Zinc finger	Wilt in Tomato	(Rispail and Di Pietro, 2009)
CON7	*Magnaporthe oryzae*	NA	Pathogenesis associated signal transduction.	C2H2 Zinc finger	Rice blast	(Odenbach *et al.*, 2007)

Gene	Fungus	Method	Function	Class	Disease	Reference
pac1	*Sclerotinia sclerotiorum*	Deletion	pH regulation	C2H2 Zinc finger	White mold cottony rot	(Rollins, 2003b)
MST12	*Magnaporthe grisea*	Disruption	MAPK Fus3/Kss1 downstream gene, Pleiotropic	C2H2 Zinc finger	Rice blast	(Park *et al.*, 2002)
pac1	*Sclerotinia sclerotiorum*	Site directed mutation	pH regulation	C2H2 Zinc finger	White mold and cottony rot	(Kim *et al.*, 2007b)
MoCRZ1	*Magnaporthe oryzae*	Deletion	Ca(2+)-dependent signaling pathway, slow growth	C2H2 Zinc finger	Rice blast	(Choi *et al.*, 2009)
CST1	*Colletotrichum lagenarium*	Deletion	MAPK Fus3/Kss1, conidial germination, appressorium formation, and invasive growth	C2H2 Zinc finger	Anthracnose on cucumber	(Tsuji *et al.*, 2003)
Snt2	*Fusarium oxysporum*	Deletion	Conidia production, vegetative growth	Cys4HisCys3	Wilt/ Muskmelon	(Denisov *et al.*, 2011)
AbCre	*Alternaria brassicicola*	Deletion	Carbon utilization	Zn(II)2Cys6	Black spot disease	(Cho *et al.*, 2009b)
AbPro	*Alternaria brassicicola*	Deletion	Carbon utilization	Zn(II)2Cys6	Black spot disease	(Cho *et al.*, 2009)

Gene	Fungus	Method	Function	Class	Disease	Reference
Amr1	*Alternaria brassicicola*	Deletion	Conidiation and carbon source utilization	Zn(II)2Cys6	Black spot disease	(Cho *et al.*, 2012)
Abpf1	*Alternaria brassicicola*	Deletion	Pathogenesis only	Zn(II)2Cys6	Black spot disease	Cho et al, 2012
bcreg1	*Botrytis cinerea*	Deletion	Conidia production and penetration	Zn(II)2Cys6	Gray mold of grapes	(Michielse *et al.*, 2011)
Nir1	*Colletotrichum acutatum*	Deletion	Nitrogen metabolism	Zn(II)2Cys6	Key lime anthracnose (KLA)	(Horowitz *et al.*, 2006)
EBR1	*Fusarium graminearum*	Disruption	Growth and spore germination	Zn(II)2Cys6	Wheat and other cereals, Fusarium head blight	(Zhao *et al.*, 2011)
fosge1/SGE1	*Fusarium oxysporum*	Deletion	Pathogenesis only	Zn(II)2Cys6	Wilt of tomato	(Michielse *et al.*, 2009)
xlnR	*Fusarium oxysporum*	Deletion	cell wall integrity	Zn(II)2Cys6	Wilt	(Calero-Nieto *et al.*, 2007)

Gene	Fungus	Method	Function	Class	Disease	Reference
Fow2	*Fusarium oxysporum*	REMI	Pathogenesis only	Zn(II)2Cys6	Wilt/ Tomato	(Imazaki *et al.*, 2007)
ZFR1	*Fusarium verticillioides*	Deletion	Fumonisin biosynthesis	Zn(II)2Cys6	Ear and stalk rots in maize	(Bluhm *et al.*, 2008)
Ctf1 alpha	*Fusarium solani*		Cutinase regulation	Zn(II)2Cys6	Wilt	
MIR1	*Magnaporthe grisea*	Deletion	Pathogenesis only	Not defined	Rice Blast	(Li *et al.*, 2007)
Com1	*Magnaporthe oryzae*	Deletion	Conidium morphology	H-L-H	Blast on rice	(Lu *et al.*, 2007; Yang *et al.*, 2009)
MNH6	*Magnaporthe grisea*	Deletion	Chromosomal modification	HMG box	Rice blast	(Lu *et al.*, 2007)
Fox1	*Ustilago maydis*	Deletion	Oxidative stress	Forkhead	Smut Maize	(Zahiri *et al.*, 2010)
FgVe1	*Fusarium graminearum*	Disruption	Pleiotropic	Velvet gene regulators	Fusarium head blight	(Merhej *et al.*, 2012)
FgVELB	*Fusarium graminearum*	Deletion	Pleiotropic	Velvet gene group of regulators	Fusarium head blight	(Merhej *et al.*, 2012)

Abbreviation: APSES: **A**sm1p, **S**ok2p, **P**hd1p, **E**fg1p, and **S**tuAp (named on a group of transcription factor). Zn(II)2Cys6: Zinc binuclear Cysteine 6 transcription factor. C2H2: Two cysteine and two histidine containing transcription factor. REMI: Restriction enzyme mediated integration; it is a method of fungal transformation. Pleiotropic: multiple effect e.g. conidia production, vegetative growth, appresorium formation, penetration, response to abiotic stresses and colony texture.

1.4.1.4 Downstream genes:

Downstream genes are the genes, which are actually involved in performing different functions during pathogenesis. They act in response to the stimulus they receive from the upstream genes like transcription factors and signal transduction genes. Different downstream genes are critically involved in each stage of pathogenesis. Genes of early stages of infection are those which are involved in adhesion, surface sensing, spore germination, germ tube elongation and appresorium formation. Most of the fungal phytopathogens have a necrotrophic stage in their life cycle which involves destroying the plant structure and living on the biomass of dead plant biomass. A battery of genes is involved in digestion of plant cell wall collectively termed as cell wall degrading enzymes (CWDE). The CWDE encoding genes are a big group of downstream gene coding for several cutinase, pectinase, hydrolytic enzymes that are involved in pathogenesis (Srivastava *et al.*, 2012). Though mostly downstream gene shows redundancy there are few CWDE encoding genes whose mutants have shown reduction in virulence in phytopathogenic fungi (table 1.6). Pathogenic fungi are also known to secrete mycotoxins and secondary metabolites that further help fungi in killing plant cells and induce colonization. The toxins can be non-host specific or host specific. The host specific toxins are usually also virulence factors (Table1.6).

Plants secrete many antimicrobial compounds to defend themselves from invading pathogens. These compounds are basically secondary metabolites, phytoanticipins and phytoalexins. Pathogens in order to succeed in colonization must have the ability to detoxify

them. A number of genes involved in such detoxification have been reported from different fungi that also function as pathogenicity or virulence factors e.g. *Avenacin A-1* from *Gaeumannomyces graminis* var. avenae (Bowyer *et al.*, 1995) tomatinase gene of *Septoria lycopersici* (Martin-Hernandez *et al.*, 2000). PDA-1 gene encoding pisatin demethylase from *Necteria haematococca* detoxifies the infamous phytoalexin of pea: pisatin. Disruption mutants made after disruption of pisatin have shown reduction in virulence. Other than those genes involved in detoxification there are numerous genes that are critical for detoxification and survival of pathogen on the host plant. One such group of genes includes transporter genes. These genes are involved in pumping out plant toxins and influx and efflux of molecules from fungi (Del Sorbo G *et al.*, 1997). ABC1 transporter gene is one such gene disruption of which in *M.griesa* has resulted in loss of pathogenicity (Urban *et al.*, 2003). Similarly, disruption of ABC transporter gene in *B.cinerea* has shown sensitivity for phytoalexin resveratrol and reduction in pathogenesis (Schoonbeek *et al.*, 2001). *Alternaria alternata* pathotypes synthesizes host specific toxins – AK-toxin. Disruption of gene encoding for biosynthesis of this resulted in decrease in pathogenesis in Japanese pear and in Apple (Tanaka *et al.*, 1999).

There are certain unique genes that have been identified to show decrease in pathogenicity but are still not annotated. With advent of new technologies of functional genomics and sequencing of new genomes, new genes related to pathogenicity and virulence is expected to be discovered, which will further our knowledge of fungal patho-physiology.

1

Table 1.6. Various downstream genes associated with pathogenesis in plant pathogenic fungi.

Gene	Fungus	Method	Function	Disease	Reference
			Cell wall degradation		
GH 6 and 7c	*Magnaporthe oryzae*	Silencing	Cell wall degradation (CWDE)	Rice Blast	(Van Vu *et al.*, 2012)
VdSNF1	*Verticilium dahliae*	Disruption	Cell wall degradation	Wilt in herbaceous plants	(Tzima *et al.*, 2012)
Bcchs3a	*Botrytis cinerea*	Disruption	Fungal cell wall integrity	Gray mold of grapes	(Arbelet *et al.*, 2010)
MfCUT1	*Monilinia fructicola*	Disruption	Cell wall degradation (CWDE)	Brown rot blossom blight	(Lee *et al.*, 2010)
LIP1	*Blumeria graminis*	NA	Degradation of lipid/wax on plant leaves	Powdery mildew	(Feng *et al.*, 2009)
Ssaxp	*Sclerotinia sclerotiorum*	Disruption	Degradation of lipid/wax on plant leaves	Necrosis on canola	(Yajima *et al.*, 2009)
Frp-1	*Fusarium oxysporum*	Deletion	Carbon and sugar utilization and cell wall degradation (CWDE)	Wilt on tomato	(Jonkers *et al.*, 2009)
CgChsV	*Colletotrichum graminicola*	Disruption	Cell wall degradation (CWDE)	Maize leaf anthracnose	(Werner *et al.*, 2007)
gas1	*Fusarium oxysporum*	Disruption	Vegetative growth, osmoregulation	Vascular wilt	(Caracuel *et al.*, 2005)

Gene	Fungus	Method	Function	Disease	Reference
ARG1	*Fusarium oxysporum*	Disruption	Arginine biosynthesis	Wilt	(Namiki *et al.*, 2001)
CcPelA	*Colletotrichum coccodes*	Disruption	Cell wall degradation (CWDE)	Black rot on potato	(Ben-Daniel *et al.*, 2012)
Pel B and PacC	*Colletotrichum gloeosporioides*	Deletion	CWDE and pH related effect	Anthracnose of Yam	(Miyara *et al.*, 2008)
FgATG15	*Fusarium graminearum*	Disruption	Ariel hyphae production, conidia structure	Fusarium head blight	(Nguyen *et al.*, 2011)
CHS1	*Fusarium asiaticum*	Deletion	Osmotic stress, conidia and mycelium morphology	Fusarium head blight	(Xu *et al.*, 2010)
MoSNF1	*Magnaporthe oryzae*	Deletion	Cell wall degradation (CWDE)	Rice blast	(Yi *et al.*, 2008)
Bcpme1	*Botrytis cinerea*	Disruption	CWDE, Signaling, impaired pectin degradation	Gray mold of grapes	(Valette-Collet *et al.*, 2003)
			Mycotoxin and secondary metabolite formation		
PDA1	*Nectria haematococca* MPVI	Insertion mutation	CDC conditionally dispensable chromosome	Root and stem rot of pea	(Garmaroodi and Taga, 2007)
DES1	*Magnaporthe oryzae*	Insertion mutation	Expression of peroxidase	Rice Blast	(Chi *et al.*, 2009)

Gene	Fungus	Method	Function	Disease	Reference
LAM1, RED2, RED3, OX11, TOX9 and RED1	*Cochliobolus heterostrophus*	Deletion	(T-)toxin synthesis	Southern corn blight of maize	(Inderbitzin *et al.*, 2010)
ACTTS3	*Alternaria alternata*	Disruption	Polyketide synthase	Alternaria brown spot	(Miyamoto *et al.*, 2010)
AbPKS9	*Alternaria brassicicola*	Deletion	Polyketide synthase depudecin biosynthesis	Leaf spot	(Wight *et al.*, 2009b)
ACTT2	*Alternaria brassicicola*	Silencing	Toxin biosynthesis	Citrus brown spot	(Miyamoto *et al.*, 2008)
Tom1	*Fusarium oxysporum*	Deletion	Tomatinse degradation gene	Wilt	(Pareja-Jaime *et al.*, 2008)
FUM	*Fusarium verticillioides*	N.A.	Fumonisin synthesis	Rots on maize	(Glenn *et al.*, 2007)
CTB1	*Cercospora nicotianae*	Disruption	PKD synthase	Wide range of disease	(Choquer *et al.*, 2005)
Deck-1	*Cochliobolus heterostrophus*	Disruption	PKD - Toxin synthase	Virulent to TMS	(Rose *et al.*, 2002)
AaNox A	*Alternaria alternata*	Deletion	Reactive oxygen species detoxification	Spot on citrus	(Yang and Chung, 2012)
PPT1	*Cochliobolus sativus*	Deletion	PKD/NRPS	Root rot in Wheat and Barley	(Leng and Zhong, 2012)

Gene	Fungus	Method	Function	Disease	Reference
TRI6	*Fusarium culmorum*	Deletion	Mycotoxin gene	Crown and foot rot of potato	(Schmelz *et al.*, 2011)
Cpnox1	*Claviceps purpurea*	Deletion	Reactive oxygen species detoxification	Ergot of rye	(Giesbert *et al.*, 2008)
Sid1	*Fusarium graminearum*	Deletion	Sidophore uptake gene	Fusarium head blight	(Greenshields *et al.*, 2007)
SSM1	*Magnaporthe grisea*	Disruption	NRPSs for sidophore synthesis	Rice blast	(Anke *et al* 2007)
AbNPS2	*Alternaria brassicicola*	Disruption	NRPSs for conidia structure	Black spot in brassica	(Kim *et al.*, 2007a)
			Growth and Metabolism		
fst1	*Fusarium verticillioides*	Deletion	Sugar transporter	Necrosis in wheat and rice	(Kim and Woloshuk, 2010)
Top1	*Fusarium graminearum*	Random insertion	Topoisomerase	Fusarium Ear blight	(Baldwin *et al.*, 2010)
GDH2	*Colletotrichum gloeosporioides*	Disruption	Glutamate dehydrogenase	Broad host range	(Miyara *et al.*, 2010)
Lmepi	Leptosphaeria *maculans*	Disruption	Encodes for enzymes for galactose metabolism	Spots on brassica	(Remy *et al.*, 2009)

Gene	Fungus	Method	Function	Disease	Reference
bcnoxa and bcnoxb	*Botrytis cinerea*	Deletion	NADPH oxidase, Penetration and colonization	Gray mold of grapes	(Segmüller *et al.*, 2007)
catB	*Magnaporthe grisea*	Deletion	Catalase, appresorium formation	Rice Blast	(Skamnioti *et al.*, 2007)
CND5	*Botrytis cinerea*	Deletion	P450 monooxygenase, botrydial production	Gray mold of grapes	(Siewers *et al.*, 2005)
MgAtr4	*M.graminicola*	Disruption	ABC transporter	Septoria blotch	(Stergiopoulos *et al.*, 2003)
Gabc1	*Gibbrella. pulicaris*	NA	ABC transporter, phytoalexin degradation	Dry rot of potato	(Fleiβner *et al.*, 2002)
BCAtrb	*Botrytis cinerea*	Deletion	ABC transporter, phytoalexin degradation	Gray mold of grapes	(Schoonbeek *et al.*, 2001)
ATG8	*Ustilago maydis*	Deletion	Autophagy gene	Smut of maize	(Nadal and Gold, 2010)
FgTEP1	*Fusarium graminearum*	Deletion	Tensin-like phosphatase 1, conidia production	Wilt	(Zhang *et al.*, 2010)

Gene	Fungus	Method	Function	Disease	Reference
FgPtc1P	*Fusarium graminearum*	Deletion	Phosphatase, mycelium growth	Head blight	(Jiang *et al.*, 2010)
TasHyd1	*Tricoderma asperellum*	Deletion	Hydrophobin gene, colonization	Green mold	(Viterbo and Chet, 2006)
Adh1	*Fusarium oxysporum*	Deletion	Alcohol dehydrogenase	Wilt of tomato	(Corrales Escobosa *et al.*, 2011)
MoCDC15	*Magnaporthe oryzae*	Deletion	Cell cycle gene, conidia formation, plant surface attachment	Rice blast	(Goh *et al.*, 2011)
MoCYP51A	*Magnaporthe oryzae*	Deletion	Sterol methylase, conidia formation and germination	Rice Blast	(Yan *et al.*, 2011b)
AaPex6	*Alternaria alternata*	Disruption	Peroxysome synthase	Citrus disease	(Imazaki *et al.*, 2010)
Aso1	*Alternaria brassicicola*	Deletion	Anastomosis	Black spot disease	(Craven *et al.*, 2008)
MoHYR1	*Magnaporthe oryzae*	Deletion	Peroxidase	Rice blast	(Huang *et al.*, 2011)
NhABC1 and PDA1	*Nectria haematococca MPVI*	Disruption	Transporter and cytochrome P450 respectively	Root and stem rot of pea	(Coleman *et al.*, 2010)
PEP genes	*Nectria haematococca MPVI*	NA	Cytochrome P450	Root and stem rot of pea	(Han *et al.*, 2001)

Gene	Fungus	Method	Function	Disease	Reference
			Novel and Miscellaneous		
CoPex13	*Colletotrichum orbiculare*	Disruption	Downstream gene, peroxisome and melanin synthesis	Anthracnose in Cucumber	(Fujihara *et al.*, 2010)
SIX	*Fusarium oxysporum*	Deletion	Avr genes	Tomato vascular wilt	(Thatcher *et al.*, 2011)
MoLDB1	*Magnaporthe oryzae*	Insertion mutation	Spore production. hydrophobic interactions	Rice blast	(Li *et al.*, 2010a)
CoKEL1	*Colletotrichum orbiculare*	Disruption	Kelch proteins, appresorium formation, turgor pressure generation	Cucumber anthracnose	(Sakaguchi *et al.*, 2009)
Avr4	*Cladosporum fulvum*	silencing	Avr protein, Prevents chitin from digesting by plant	Tomato leaf mold	Esse et al and Burg et al, 2006, 2007
FgFSR1	*Fusarium graminearum*	Disruption	Virulence only	Head blight	(Shim *et al.*, 2006)
CPR1	*Colletotrichum graminicola*	Disruption	Colonization	Anthracnose leaf blight	(Thon *et al.*, 2002)
CgDN3	*Colletotrichum gloeosporioides*	Disruption	Pathogenicity gene, lack of penetration	Anthracnose of Yam	(Stephenson *et al.*, 2000)
MGA1	*Magnaporthe grisea*	Insertion mutation	Appresorium formation	Rice blast	(Gupta and Chattoo, 2007)

1.4.2. Plant defense system

Broadly plant resistance can be divided into two major types: non-host resistance and host resistance (Agrios, 2004). Non-host resistance, also known as basic resistance or basic incompatibility or heterologous interaction is the first level of defense and is non-specific to the host (Agrios, 2004). It can be further of two types- passive and active basic resistance. The passive basic or pre-existing resistance largely consists of preexisting barriers like: wax, cuticle layer on plant surface or presence of tannins pectolytic enzymes (in young plants) and saponins. The active basic resistance or induced resistance is not a pre-existing resistance and comes into action in response to a pathogen attack. Some common examples are formation of callose papillae, cork layer and tyloses on xylem tissue.

Host resistance is also known as host specific resistance. This type of resistance is selective against a particular pathogen. The resistance can be a non-race specific or horizontal resistance which is polygenic in nature, or vertical resistance which is against a specific race of pathogen or in other words the vertical resistance is the defense response against a specific race of a pathogen and is usually monogenic in nature. It forms the basis of classical theory of gene-for- gene interactions and the genesis of *R* and *Avr* genes

1.5. Current model of Pathogenicity:

Plant pathogens and host plants have evolved a complicated relationship during pathogenesis. First study to explain genetics of host resistance was illustrated by Harold Henry Flor in his study with flex rust (Flor, 1971). He coined the phrase gene-for-gene interactions, which is said to be the first model that explains host pathogen interactions during pathogenesis. According to this model, host resistance occurs when pathogen derived race specific elicitors (encoded by *Avr* or Avirulence gene) is recognized by their corresponding resistance or R genes present in plants. As a result of this compatible recognition the plant defense mechanism is activated results in expression of hyper sensitivity (HR) response culminates in programmed cell death or apoptosis. On the contrary when the intersection between *Avr-R* genes is incompatible, the plant defense mechanism is not activated resulting in disease or successful colonization by pathogens. The first Avr gene was isolated from *Pseudomonas syringae*, (Staskawicz *et al.*, 1984)), followed by one in fungus and recently in an oomycete (Jan A. L. van Kan, 1991; Shan *et al.*, 2004).

1.5.1 PAMPS and MAMPS

The classic phenomenon of gene-for-gene interaction holds only for biotrophs which colonize living plant tissue before consuming the plant biomass. This gives the biotrophic pathogen sufficient time to develop gene-for-gene phenomenon. Necrotrophs possess a very

aggressive life style. Immediately upon their infection on host plant they start killing host tissues (Zuccaro A. *et al.*, 2011), therefore have very limited time for establishing any host pathogen interactions. Nevertheless, necrotrophs like B. *cinerea* and *S.sclerotiorum* are known to induce a HR-like symptom which ends with programmed cell death in host plants. The death of cells or tissues acts a starting point of infection for necrotrophs as it provides an ideal niche and advantage for these pathogens to initiate and perpetuate their infection, ultimately making the host susceptible to them (Govrin and Levine, 2000). Whether this kind of response is extended to other necrotrophs is still unknown (Govrin and Levine, 2000; Laluk, 2010). There are many other differences that exist between interactions of necrotrophs and biotrophs with their host during infection (Laluk, 2010). Thus, in addition to *Avr-R* based plant resistance, plants have another broader defense mechanism that is effective against both biotrophs and necrotrophs. It is based more on the surveillance and recognition of specific molecular patterns associated with microbes. This mode of resistance is activated after the plant interior is breached and the cell wall is penetrated exposing the host cell membrane to the microbes, where they encounter extracellular surface receptors that recognize the molecular patterns. These specific molecular patterns are termed as PAMPs (Pathogen Associated Molecular Patterns) or MAMPS (Microbe Associated Molecular Patterns) (Chisholm *et al.*, 2006). The degradation products generated from the activity of microbial activity, such as cell wall fragments generated by CWDEs are termed as DAMPs or Damage Associated Molecular Patterns (Mengiste, 2012). The resistance initiated in response to PAMPS/MAMPS/DAMPS is termed as PTI or PAMP triggered Immunity. PTI is a general plant response that is effective against all kind of pathogens irrespective of their immunological characteristics (Mengiste, 2012).

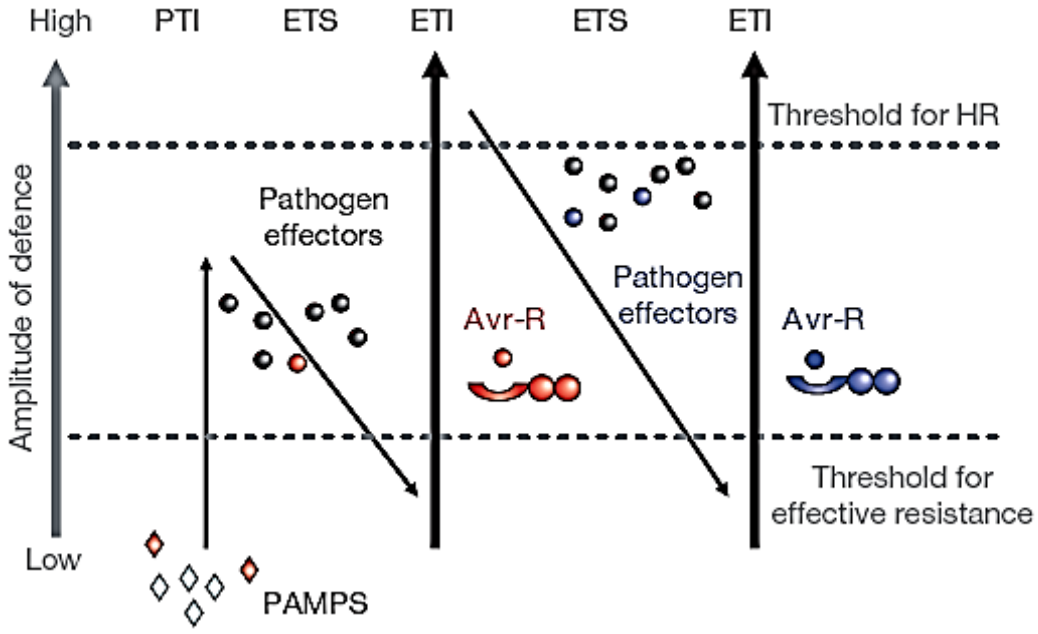

Fig 1.4: The zig zag model of pathogenicity as proposed by Jones and Dangl. The various phases of plant microbe interactions during pathogenesis is described using this model. (Jones and Dangl 2006) Abbreviations: PAMPS: Pathogen Associated Molecular Patterns, PTI: PAMP Triggered Immunity, ETS: Effector Triggered Immunity, Avr-R: Avirulence-Resistance gene interaction. ETI: Effector triggered Immunity.

The common examples of P/M/DAMPs are chitin from fungi, bacterial flagellin, lipo-polysaccharides and arachidoic acid from oomycetes. In 2006 Jones and Dangl proposed a zig-zag model to explain the PAMP-PTI triggered Immunity (fig 1.3) and described a four phased immune response mechanism. In phase I the specialized receptors on plant cell membrane called PRRs (Pattern Recognition Receptors) recognizes the PAMPs and initiates PTI (PAMP Triggered Immunity) and checks the colonization of intruding pathogen. In second phase some pathogens secrete effectors which could be a pathogenicity or virulence factor, and other agent that can interfere with PTI and help them to escape resulting in ETS or Effector Triggered Susceptibility. The third phase involves the recognition of effectors by NB-LRR (Nuclear

Binding- Leucine Rich Repeats) containing protein resulting in effector-trigger immunity or ETI. In the fourth phase the pathogen undergoes genetic variation and tries to evade ETI either by acquiring new effectors or modifying the existing one so that they cannot be recognized by the NB-LRR proteins (Jones and Dangl 2006). Several PRRs like membrane localized receptor like kinase (RLKs), chitin receptors (LysM/CERK1), (Miya *et al.*, 2007; Wan *et al.*, 2008) peptide receptors (Yamaguchi *et al.*, 2006; Yamaguchi *et al.*, 2010) and wall associated kinase (WAK1) (Brutus *et al.*, 2010) have been characterized as related to necrotrophic immunity. Loss of these PRRs attenuates the basal resistance to the corresponding pathogens. (Mengiste, 2012).

1.6 Transcription Factors:

Fungal genomes are usually small in size. For example Dothideomycetes, which is one of the most important classes of fungal phytopathogens, have genome sizes ranging from 21.88 Mb in *Baudoinia compniacensis* to 74.14 Mb in *Mycosphaerella fijiensis,* with an average genome size of 39Mb (Ohm et al 2012). The average number of predicted protein coding genes among fungi is ~12000 (Kupfer *et al.*, 1997). Some of these thousands of genes are constantly needed to express and encodes for proteins that are needed for daily survival; they are known as Housekeeping genes. While there are others, which are conditionally expressed and are differentially and coordinately regulated for their expression. Gene expression is a multi-step process that starts from transcription and ends after protein translation. Regulation of gene expression can occur any of the five stages: activation of gene structure, initiation of transcription, processing the transcript, transport to cytoplasm and translation of mRNA.

However, in eukaryotes gene expression is usually controlled at the level of initiation of transcription that is during the mRNA synthesis step. Three different RNA polymerases (RNA Pol) are required to complete this step (Lewin, 2004). Each RNA Pol is responsible for a different class of transcription: PolI transcribes rRNA (ribosomal RNA), PolII mRNA (messenger RNA), and PolII tRNA (transfer RNA) and other small RNAs. Any protein that is needed for the initiation of transcription is defined as a transcription factor. Many transcription factors act by recognizing *cis*-acting sites that are parts of promoters or enhancers of a gene. However, binding to DNA is not the only means of action for a transcription factor. A factor may recognize another factor, or may recognize RNA Polymerases. In Eukaryotes, transcription factors, rather than the enzymes themselves, are principally responsible for recognizing the promoter. Transcription factors are able to bind to specific sets of short conserved sequences contained in each promoter. Some of these elements and factors are common, and are found in a variety of promoters and used constitutively; others are specific and their use is regulated (Guasconi *et al.*, 2004). The transcription factors that assist RNA polII can be divided into three groups or types:

- **The general factors**: They are non-specific in their binding capacity to the promoter. They form the basal transcription complex and are required for the initiation of RNA synthesis.
- **The upstream factors**: They are DNA-binding proteins that recognize specific short consensus elements located upstream the transcription start point. These factors are ubiquitous and act upon any promoter that contains the appropriate binding site on DNA. They increase the efficiency of initiation.

- **The inducible factors**, theses function in the same general way as the upstream factors, but have a regulatory role. They are synthesized or activated at specific times and in specific tissues. The sequences that they bind are called response elements. (Lewin, 2004) (Spitz and Furlong, 2012).

Transcription factors may either enhance or repress the expression of a gene. Currently, one of the major focuses of scientific research is the structure and mechanism of action for transcription factors. Researchers are interested in understanding the complex regulatory programs that allows single a genome to regulate thousands of different cell types. Supporting this fact in the year 2009 alone, more than 8000 scientific papers published were on transcription factors (Yusuf *et al.*, 2012).

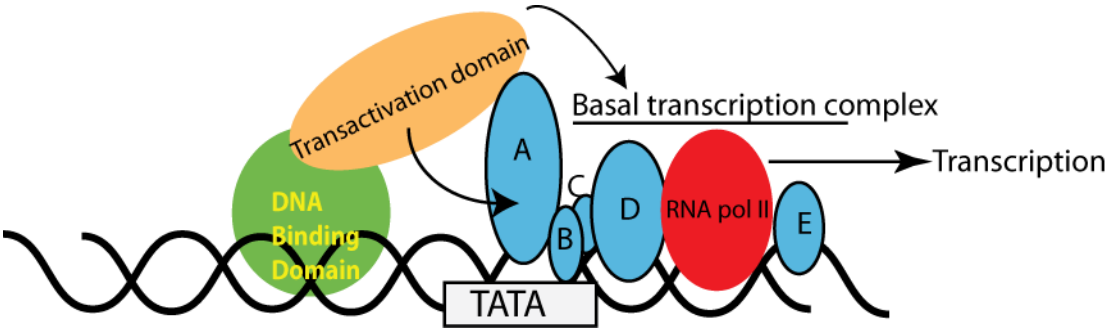

Fig. 1.5 A simplistic view of regulatory mechanisms of gene transcription. The RNA transcribing enzyme, RNA polymerase II (red), requires general transcription factors (TFII) A,B,C,D and E (blue), which themselves consist of multiple subunits, to recognize the transcription start site via the TATA box or related sequences in the core promoter.The sum of these factors, forms the basal transcription complex. Transcription factors (green) bind to specific DNA sequences via their DNA-binding domain (DBD) and modulate the rate of transcription via their transactivation domain(s) (TAD).

1.6.1 Structure:

1.6.1.1. Transcription factor domains:

Traditionally, transcription factors have been described as modular proteins made of two-three components that are termed as domains, for example: DNA binding domain (DBD) and Transactivation domain (TAD), sometime a third dominion is also reported which is known as signal sensing domain or (SSD) (Spitz and Furlong, 2012). Usually a conserved nuclear localization signal (NLS) motif is also present that regulates import of transcription factors to nucleus. Presence of modulatory structure permits the combination of different domains to form transcription factors with discrete functions from a relatively small number of components (Schwechheimer *et al.*, 1998). Therefore functionally several transcription factor families can be described on the basis of common types of motifs that are responsible for binding to DNA

Also, not all transcription factors belong to any of the known families e.g. p53 (Pabo and Sauer, 1992). In the following section important groups of transcription factors that uses specific motifs to bind DNA are described:

Fig. 1.6 Modular structure of transcription factor: The DNA binding domain is used for binding to the specific DNA sequence of the gene. Several transcription factors are classified on the basis of these DNA binding motifs. The other compartments are transactivation domain and signal sensing domain that have regulatory role during transcription. The signal sensing role is always present as a component of transcription factor.

1.6.1.1.1. Zinc Finger Proteins:

Zinc fingers (PF0096), were first discovered in the *Xenopus* transcription factor IIIA (TFIIIA).The zinc finger transcription factor was originally found in the factor TFIIIA, which is required for RNA PolIII to transcribe 5S rRNA genes (Miller *et al.*, 1985). It is one of the major structural motifs involved in DNA protein interactions. Zinc finger domains are relatively small protein motifs containing multiple fingers like protrusions and bind to zinc element. Proteins in this family usually contain tandem repeats of the 30-residue, with each motif containing the sequence pattern: Cys-X2-4-Cys-X3-Phe-X3-Leu-X2-His-X3-His

The motif takes its name from the loop of amino acids that protrudes from the zinc binding site and is described as the Cys2/His2 finger. In general, zinc fingers coordinate zinc ions with a combination of cysteine and histidine residues (fig 1.1b). Originally, the number and order of these residues was used to classify different types of zinc fingers (e.g., Cys_2His_2, Cys_4, and $(Zn(II)_2Cys_6Hys_6)$. More recently, a more systematic method has been used to classify zinc finger proteins instead. This method classifies zinc finger proteins into "fold groups" based on the overall shape of the protein backbone in the folded domain. The most common "fold groups" of zinc fingers are the Cys_2His_2-like (the "classic zinc finger"), treble clef, and zinc ribbon. (Krishna *et al.*, 2003; Brayer and Segal, 2008)

1.6.1.1.2. Leucine Zipper:

The basic leucine zipper domain (bZip; PF00170) was first discovered as conserved sequence motif in many eukaryotic transcription factors (Landschulz *et al.*, 1988). The DNA binding domain consists of 60-80 amino acid residues and consists of two distinct subdomains the leucine zipper region mediates dimerization and the basic region that binds with the DNA binding region comprises a number of basic amino acids such as arginine and lysine (Landschulz *et al.*, 1988; Pabo and Sauer, 1992)

1.6.1.1.3. Helix-Loop-Helix:

The HLH (HLH; PF0010) is also an important eukaryotic regulator that is very similar in function and structure to the leucine zipper, and has a motif that can bind to DNA and also dimerize. The HLH proteins have conserved sequence of 40-50 amino acids containing two amphipathic α helices separated by a linker region (the loop) of varying length.

1.6.1.2 Transactivation Domains

Following DNA binding, a transcription factor exerts an influence over gene expression. This is done through interaction with other transcription factors or with the basal transcriptional machinery in order to affect the efficiency of formation or binding of the transcription complex. These associations are often mediated through a transactivation domain, which in most cases

ranges from 30-100 amino acids in length and contain variable functional amino acid arrangements such as glutamine- or proline-rich regions. Transactivation domains may act directly or they may recruit co-activator proteins that possess activation properties and an ability to interact with the basal transcription machinery, but these proteins lack any intrinsic DNA-binding capacity. For example, cAMP responsive element binding protein (CREB) associates with CREB binding protein (CBP), which in turn enhances gene transcription through activation of TFIIB. Alternatively, some transcription factors mediate their effects by simply binding DNA and transmitting conformational changes through chromatin structure. By doing this the transcription factor improves the accessibility of proteins to DNA binding sites.

1.7 Objectives of the study:

General goal: Discovery of novel transcription factors regulating pathogenesis in the plant pathogenic fungi *Alternaria brassicicola.*

Hypothesis: Zinc finger domains containing transcription factors are involved in regulation of pathogenesis in *A.brassiciola*.

Objectives:

1. Creation of targeted gene disruption- or gene deletion- mutants for Cys_2His_2 and $Zn(II)_2Cys_6$ zinc binuclear fungal specific transcription factor.
2. Identification of transcription factors regulating genes associated with pathogenesis in host plant green cabbage (*Brassica oleracia*) and *Arabidopsis thaliana*.

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CHAPTER 2

Targeted Gene Knock Out of Zinc Finger Domain Transcription Factor Encoding Genes by Gene Disruption.

Abstract

The necrotrophic fungus *Alternaria brassicicola* causes black spot disease of brassica plants. The *de novo* synthesis of various catabolic enzymes and secondary metabolites needed for pathogenesis are under the regulation of transcription factors. In this study we have knocked out 184 zinc finger domain containing transcription factor encoding genes using a targeted gene disruption method to screen the pathogenesis associated genes in *Alternaria brassicicola.* A total of 173 genes were successfully disrupted. The knock out mutants of the remaining eleven genes failed to produce any viable colonies after two transformation experiments. Our bioassays on cabbage leaves identified one pathogenicity factor with mutants that were nonpathogenic, and four strong virulence factors whose mutants showed 50-90% reduction in disease symptoms compared to the wild type. We also discovered a unique gene whose mutants showed a 100% increase in virulence and one mutant that shows complete loss of conidia yet no effect on virulence. Five of six genes having effect on virulence were novel virulence factors and only one gene (*PacC*) was previously identified as a pathogenicity factor in other fungal species. The discovery of these genes and detailed understanding of their mechanism of action will enhance our knowledge of molecular pathogenesis and host pathogen interactions of *A. brassicicola* as well as of other related fungi.

2.1. Introduction

Transcription factors are historically classified according to their DNA binding domains (DBD) (Yusuf *et al*., 2012). Among all known transcription factor families, the zinc finger DNA binding motif containing transcription factors forms the largest family which is further classified into 10 different classes (Ganss and Jheon, 2004). The transcription factors containing two cysteine and two histidine residues abbreviated as Cys_2His_2 or C2H2 forms the largest class of transcription factors among zinc finger as well as among all classes of known transcription factors (Tupler *et al.*, 2001) The C2H2 motif is generally present in tandem arrays, and consists of the sequence Y/F-X-C-X2-4-C-X3-F-X5-L-X2-H-X3-5-H, where X represents variable amino acid residues (Pavletich and Pabo, 1993). The two conserved cysteine and histidine side-chains (C2H2) form coordinate bonds with the zinc ion. Being the largest class in the transcription factor family, C2H2 transcription factors are expected to participate in regulation of almost all major traits in fungi including different steps of pathogenesis.

Pathogenicity mechanisms like secretion of cell wall degrading enzymes, effector molecules and toxin production in a fungal pathogen becomes active as soon as the pathogen comes into the contact of host plant. On the surface of the host plant, the fungal cell encounters unfavorable environment created by the plant defense machinery mainly through secretion of phytotoxins, antimicrobial compounds such as phytoalexins and phytoanticipins, oxidative radicals and structural and chemical barriers. For successful colonization fungi must be able to rapidly sense these unfavorable environments and elicit a coordinated response to such cues

(Naar and Thakur, 2009). Many different types of cellular sensors signal transduction pathway components and effectors are present in the fungal system that rapidly sense and respond to the external signals. Transcription factors being the last link between signal molecules and downstream effector molecules are expected orchestrate the sensor and response phenomenon and play a pivotal role in regulation of pathogenesis. Until now only one study has been reported to systematically screen the transcription factors for their putative role in fungal pathogenesis where all transcription factor encoding genes of *Fusarium graminearum* were knocked out using a non-targeted method which is very tedious and time taking.

Alternaria brassicicola is an important plant pathogen that causes black spot disease of cultivated brassicas. Several physiological and structural changes takes place during interactions of fungal pathogens and their hosts. Being a necrotrophs *A. brassicicola* establishes interactions with its hosts for a very brief period of time providing very limited opportunities to study the associated mechanism of pathogenesis.

The recent availability of draft genome sequence of *A. brassicicola* (strain ATCC 93866) has paved the way for using a functional genomics approach for understanding the molecular mechanism of pathogenesis. The development of novel methodologies of reverse genetics, such as development of highly efficient gene knock out strategy for creating gene disruption mutants (Cho *et al.*, 2006) has created opportunities to do massive, high throughput gene knockout studies for identification of pathogenesis related gene in *A. brassicicola*. Knockout mutants were made by disrupting *Fus3/Kss1 MAP kinase* homolog in *A. brassicicola Amk1 (map kinase)* and were found to be non-pathogenic (Cho *et al.*, 2007). Similarly in another study three novel

pathogenicity genes were identified by making deletion mutants of AbSte12 (Δ*abste12), AbNIK (*Δ*abnik)* and *AbPro1 (*Δ*abpro1*). All these genes were regulatory genes and therefore apart of having role in pathogenesis, they also showed pleiotropic effects such as: loss of conidiation and reduced vegetative growth (Cho *et al.*, 2009). Knock out deletion mutant of homolog of Sugar Non fermenting 1 (SNF1) gene *AbSNF1 (*Δ*absnf1)* and *gene* involved in carbon catabolite repression *AbCre1 (*Δ*abcre1)in A. brassicicola* have shown either very marginal decrease in pathogenicity or no change in virulence (Tonukari *et al.*, 2000; Ospina-Giraldo *et al.*, 2003; Tzima *et al.*, 2010b). Mutation of the five genes abolished secondary metabolite depudecin synthesis and caused a small (10%) reduction in virulence compared to wild-type *A. brassicicola* (Wight *et al.*, 2009). A number gene knockout mutant were made of genes encoding for CWDE that were highly expressed during plant infection but did not resulted in a change in virulence (Cho, unpublished), suggesting the functional redundancy among gene families and therefore identification of pathogenesis related genes among them is difficult. The kinases of major signal transduction pathways are involved in diverse cellular processes including pathogenesis. Therefore a pathogenicity gene is difficult to identify by making knock out mutants of signal transduction pathway gene because the knock out mutants will show pleiotropic loss of functions.

Considering the facts of above mentioned studies we decided to focus on transcription factors (TFs) as they are key molecules that switch for diverse physiological responses to various signals and are found functioning below the signal molecules but just above the downstream genes. Cho et al (personal communication) previously mined 421 number of putative transcription factors encoding genes from the genome sequence of *A. brassicicola* using Pfam

searches (Finn *et al.*, 2010). In this study zinc finger domain containing transcription factors with the C2H2 motifs were knocked out targeted through gene disruption. A set of another thirty two disruption mutants of C3HC4 motif zinc finger transcription factor was kindly provided by Dr.Yangrae Cho for further studies. This chapter describes systematic creation of gene disruption mutant library of zinc finger domain containing transcription factor encoding genes and identification of pathogenicity associated genes among them in the plant pathogenic fungus *A. brassicicola*.

Table 2.1. Summary of *A. brassicicola* transcription factor domains based on PFAM scan

Description [a]	**No. of gene**
Zn-finger domain	221
Fungal specific TF	72
phd finger	1
Helix-turn-helix	29
Regulatory receiver	23
Leucine zipper	21
High mobility box group	15
Helix-loop-helix	13
DNA binding myb	10
Jumonji	7
Homeobox	4
B-Regulatory protein	3
T-Regulatory protein	2
Total	421

a. Putative transcription factors identified in *A. brassicicola* genome. (Cho *et al.*, 2012)

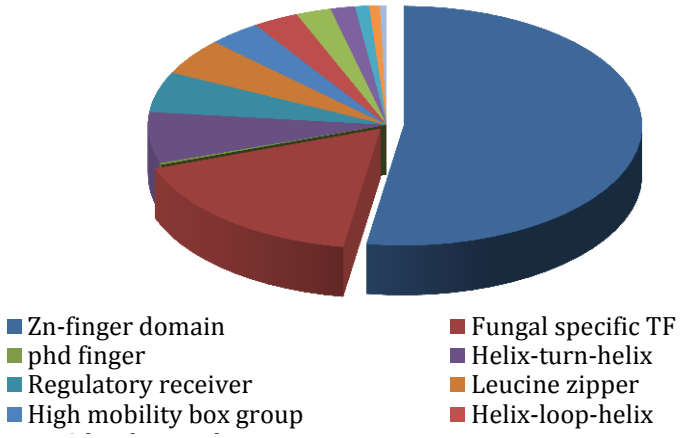


Fig. 2.1: The distribution of putative TFs in *A. brassicicola* genome. The transcription factors were identified through HMMER analysis from the database derived from the whole genome sequencing of *A.brassicicola*. Based on the PFAM domain the putative TFs were classified into 12 groups (Cho *et al.*, 2009). The Zinc finger domain containing TF formed the largest group followed by the fungal specific TFs. In this study we attempted to make targeted KO mutants of these two largest groups. Table 2.1 describes the status of work carried out during this dissertation research.

2.2 Materials and Methods

2.2.1 Fungus, host plants and culture media

In the present study we used the plant pathogen *Alternaria brassicicola* (Schweinitz, Wiltshire) the same strain (ATCC 93866) which was used for sequencing the whole genome. Cabbage (*Brassica oleracia (capitata group)*) and *Arabidopsis thaliana* were used as the host plants for pathogenicity assays. All wild type fungal cultures were grown on potato dextrose agar (PDA) (Difco™ PDA, BD bioscience MD, USA), while mutants that had hygromycin resistance gene inserted in their genome were grown on PDA supplemented with 30ng/µl Hygromycin B (Roche Biosciences, Mehemm, Germany). For growing in liquid culture 1% glucose in 0.5% yeast extract (GYEB) was used. For growth assays of selected mutants we added appropriate reagents at different concentrations determined by the EC_{50} values in PDA as described in the growth assay section. All cultures were incubated at 27°C in the dark on PD. When in liquid media they were grown at 25°C in dark at 100 r.p.m. in a rotatory shaker (New Brunswick Scientific, NJ, USA). For long term storage 20% (v/v) glycerol stocks of conidia were made.

2.2.2 Disruption constructs production and transformation

Disruption construct were made in a three PCR reactions (Cho *et al*., 2006). In the first PCR reaction, genomic DNA of *A. brassicicola* was used as template to amplify and two primers (FG and RG in fig 2.2) were designed for amplification of 200-500bp fragments within the coding region of the target gene. Hygromycin phosphotranferase (*hph*) gene was amplified from plasmid pCB1636 using primers FH and RH (fig 2.2). A tail of complementary nucleotide (10nt) is attached to RG and FH primers. The two amplicons were then mixed in equal molar concentration ratio and used as template for third PCR reaction; this PCR reaction uses primers FG and RH that stiches the two amplicons to form a disruption construct, containing 200-500bp of partial DNA sequence with *hph* gene as a selective marker.

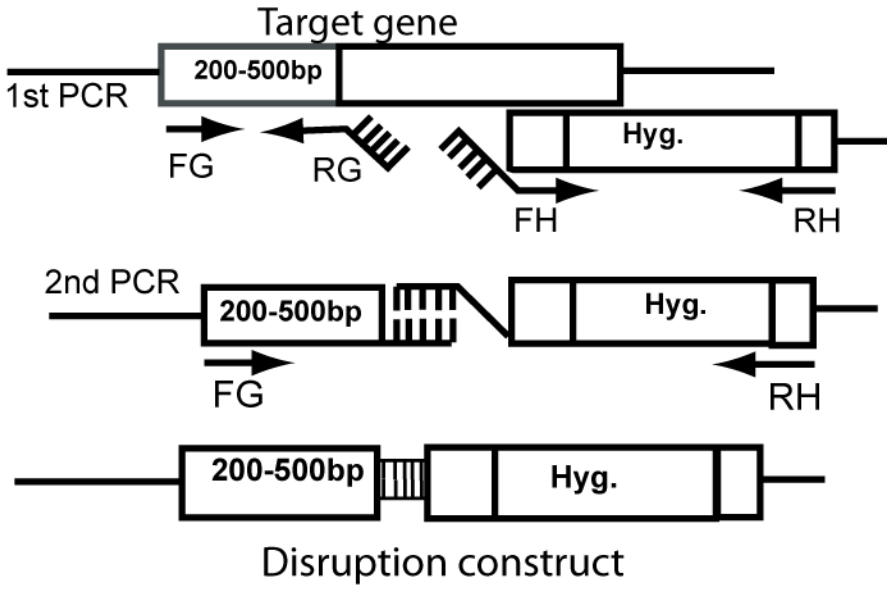


Fig. 2.2. Schematic diagram of production of disruption constructs by PCR. 1st PCR reaction shows amplification of partial sequence of target gene (i.e. gene to be disrupted) and amplification of hygromycin resistance gene cloned in a plasmid. Both reactions are done in two separate tubes. The 2nd PCR shows stitching of partially amplified target gene and hygromycin through the complementary tails attached to the primers RG and FH. Primers were designed using freely available program Primer 3 (version 0.4.0) http://frodo.wi.mit.edu/. Abbreviations: Hyg. Hygromycin resistance gene, FG and RG, FH and HR are primer pairs designed to amplify partial targeted gene sequence and selectable marker gene respectively.

2.2.3 Transformation

Transformation was carried out on the protoplast prepared from *A. brassicicola* cultures grown for 5-7 days on PDA plates. The transformation method was originally described by (Tsuge *et al.*, 1990; Shiotani and Tsuge, 1995) for *A.alternata* transformation, later Cho et al suggested modification of the method for *A. brassicicola* transformation (Cho *et al.*, 2006). Conidia was gently removed from the surface of colony and underlying vegetative hyphae were

scrapped out and inoculated on liquid GYEB medium and grown for 72 hours at 25°C at 100rpm. The mycelial balls were harvested by centrifuging the culture at 3500 rcf at 4°C for 5 minutes (Eppendorf, 5810, Eppendorf AG, Hamburg, Germany). The collected mycelial balls were washed twice with ice cold 0.7N NaCl and later digested with enzyme kitalase (Wako Chemical Inc. VA, USA) dissolved in 0.7N NaCl. at 28°C for 2-3 hours shaking at 100 rpm. The condition of digestion was frequently checked by taking out 10µl of digesting solution and visualizing under microscope. After most of the tissue was digested into protoplast (Figure) it was filtered through two layers of miracloth and collected in oak ridge tube. The protoplasts were collected by centrifugation at 700 rcf for 10 min at 4ºC, washed once with 10 ml of 0.7 M NaCl and then with 10 ml of STC buffer (1 M Sorbitol, 50 mM Tris-HCL,pH 8.0, and 50 mM $CaCl_2$). The protoplasts were suspended in STC at a concentration of 7×10^6 in 70 µl, after which 10 µg/10µl ddH_2O of disruption construct was added to the protoplast and gently mixed by tapping. The transformation mix was incubated on ice for 10 min. Heat shock transformation was performed by incubating the transformation mixture at 42ºC for 2 min followed by incubating it for 10 minutes on ice. Later 800 µl of 40% PEG solution was added to the transformation mixture and incubated for 15 minutes at room temperature. Then, 400 µl of the transformation mixture was added to 50 ml of molten regeneration medium (1 M sucrose, 0.5% yeast extract, 0.5% casein amino acids, and 1% agar,) in a 50-ml tube similarly one more tube was prepared and subsequently each tube was poured into two 15-mm petri dish thus over all four transformation plates for each gene was prepared. After 24 hours, the plates were overlaid with 25 ml of hygB (Invitrogen, Carlsabad CA) containing PDA at a concentration of 30 µg/ml. Ten genes constructs were used for transformation in single batch of transformation and in most of the cases more than 30 colonies were obtained growing on each transformed plates (Fig.2.3)

from one out four plates 6 colonies were selected and transferred to PDA plates on the basis of their growth pattern, spores were collected and stored as glycerol stocks in -80°C for later use.

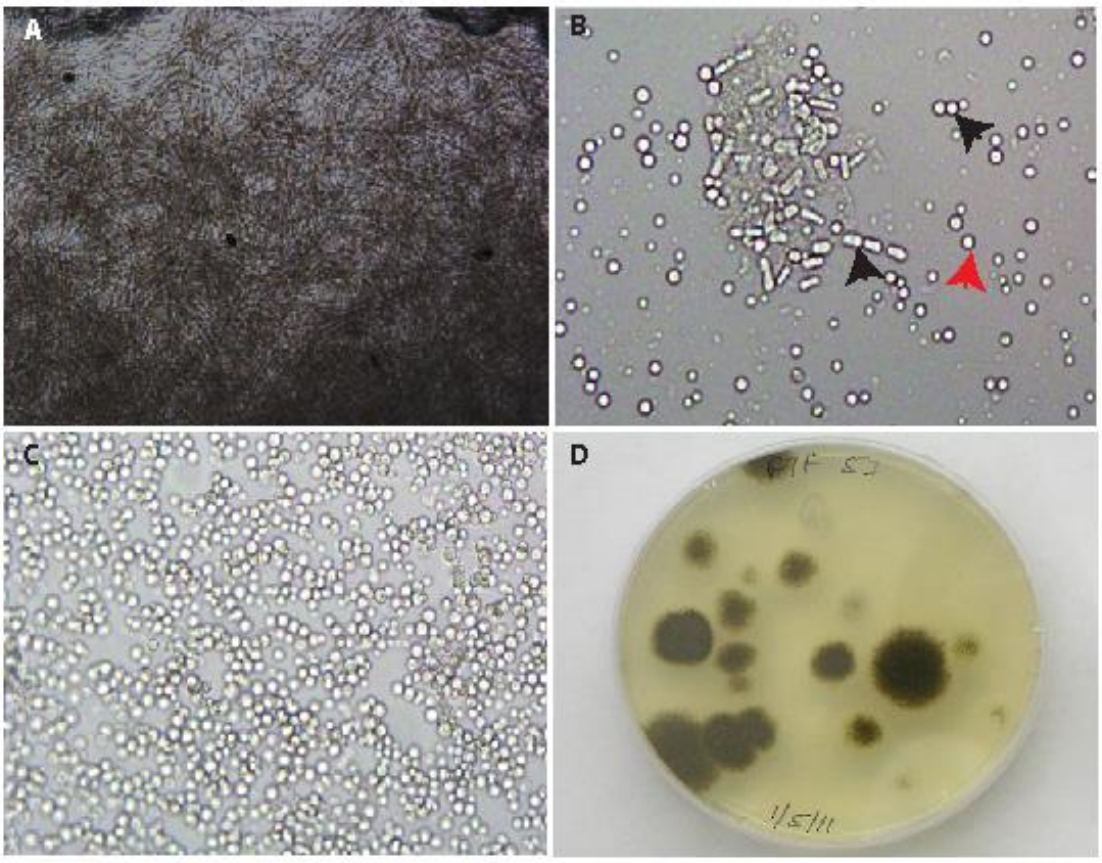


Fig. 2.3 Stages of protoplast formation. The mycelium of fungus is treated with kitalase enzyme to digest the fungal cell wall and produce protoplasts. **A.** Shows the intense network of mycelium before addition of kitalase. **B**. Shows the mycelium producing protoplasts after 1.5 hours after the addition of kitalase. Note that **black** arrows show mycelium actively being digested to produce protoplasts and the **red** arrows show formed protoplasts. **C.** Millions of protoplasts are produced after 2-3 hours of enzymatic digestion. 7-10 million protoplasts are used for transformation of a single construct. **D**. Shows the appearance of transformed colonies in the selection medium.

2.2.4 Mutant purification:

The heterokaryotic nature of conidia is one of the limitations in obtaining true mutants. This drawback was circumvented by doing single spore isolations of each mutant clone. From the transformation plates 5-9 individual hygB resistant transformants were transferred to a fresh hygB-containing plate and each transformants was purified further by transferring a single spore to a fresh hygB-containing plate. This process was done two times to ensure the purity of the mutant and absence of any contamination from wild type nuclei. After two rounds of single spore isolations conidia were collected for making glycerol stock and the mycelium was scrapped to inoculate the GYEB medium for DNA isolation.

2.2.5 DNA isolation:

Total genomic DNA was isolated from mycelial tissues using the phenol-chloroform extraction method. Cultures containing mutant colonies were grown for 3 days in 50 ml of GYEB media. Approximately 0.2 g of mycelium was harvested, filtered and semi-dried with paper towels, and ground into fine powder with a mortar and pestle in the presence of liquid nitrogen. The samples were mixed with 900 µl of extraction buffer (0.7 M NaCl, 50 mM Tris-HCL, pH8.0, and 10 mM EDTA) and 10 µl of RNAse A (Sigma-Aldrich) at 100 mg/ml and incubated at 60ºC for 30 min with intermittent mixing. Water (900µl) was added to the tube and DNA was extracted with 2 ml of Tris-saturated phenol, followed by chloroform extraction. DNA was separated from the solution by adding a 0.75v/v of volume of isopropanol at room

temperature followed by centrifuging at 1200 rpm for 10 min at room temperature. The pellet containing DNA was washed with 70% ethanol. After drying DNA samples are dissolved in80-100µl ultrapure nuclease free water (Gibco, Invitrogen Inc. Carlsbad, CA, U.S.A.). The quality and quantity of DNA was estimated by running them in 0.8% gel and taking spectrophotometric readings (Eppendorf AG, Germany) respectively.

2.2.6 Verification of mutants by PCR:

The genomic DNA was used for PCR verification of the disruption mutants with gene specific primers and hygromycin B gene specific primers. The location of primers is shown in the figure 2.4. A total of 50ng of genomic DNA was used in each 20ul PCR reaction using ABI thermo cycler (ABI Foster city CA USA). The PCR reaction consisted of 94ºC denaturation for 5 min followed by 30 cycles of 30s of denaturation at 94ºC, 30 s of annealing at 55ºC, and 2 min of elongation at 72ºC using high fidelity *taq* polymerase (Invitrogen, Carlsbad, CA, USA.). After the cycling reactions, the final elongation was performed at 72ºC for 7 min. PCR products were size fractionated on 1% agarose gel ethidium bromide added to it.

2.2.7 Pathogenicity assays:

One colony of true mutant for each gene was selected for pathogenicity assays. Conidial spores were collected from wild type growing on PDA and mutant spores are collected from true mutant cultures growing on PDA + 30ng/µl hygromycin B plates. The spores were washed thoroughly to remove any part of nutrient in deionized water and counted using a hemocytometer (Hausser Scientific Co., Horsham, PA USA). Detached leaves from 8-10 week-old green

cabbage (*Brassica oleracia var. capitata*) were inoculated on the left and right sides of mid rib by wild-type and mutant conidia respectively. Each assay was performed in two sets by inoculating with 2000 and 1000 conidia in 10µl of distilled water. The inoculated leaves were placed in petri plates where 100% humidity is maintained. Lesion diameters were measured on the fifth day of inoculation. The relative virulence of each mutant was quantified and compared with wild type using lesion size developed by mutants and wild type under the same test condition by a formula [(*D*w - *D*m)/*D*w; where *D*w= lesion diameter for the wild-type infection, *D*m= lesion diameter for mutant]. Paired t test was used to determine the virulence difference between mutants and wild type.

2.2.8 Growth assays

Wild type and mutants were inoculated by spotting equal numbers of conidia using sterile 10µl pipette tips on PDA and PDA hygromycin containing plates. Growth pattern, mainly colony morphology, growth rate were observed and compared with wild type to find out the growth patterns of mutants under saprophytic conditions. Colony diameter was measured five days post inoculation.

2.3. Results

2.3.1 Generation of disruption mutants

The draft sequence of the whole genome of *A. brassicicola* is already available in the public domain. The genomic DNA sequences of 431 predicted transcription factors encoding genes were manually mined from the whole genome sequence database. Transcription factor containing zinc finger C2H2 motifs were knocked out by targeted gene disruption though protoplast transformations of A. brassicicola strain ATCC 93866. The success rate of transformation was extremely high; we were able to produce mutants for 173 out 184 targeted genes that were knocked out. The remaining 11 gene knockouts failed to produce any viable mutants, though initially we saw some colonies which did not survive later to produce fully mature colonies. We repeatedly attempted to transform them and each time they produced similar result. We thus suspected that they were encoding for essential genes necessary for survival. A total of 1 to 30 mutant colonies were produced per gene transformed and the average number of mutant colonies produced was 7.1. 4 colonies per gene were selected from the plates for further purification and verification. In instances where numbers of colonies were less than 4, all colonies were taken for further analysis. PCR was done for verification and showed that 80.4% the colonies were true mutants and 20% were ectopic. Table 2.2 describes the summary of the knock results that were obtained after disrupting transcription factor encoding genes.

Table 2.2. List of Zinc finger transcription factor genes whose mutants were screened in this study.

Gene ID	Locus [a]	Location [b]	BlastP (GenBank No.)	E-value	PFAM domain	D [c]	T[d]	Importance
9606	AB00218.1	Contig0:603748-607339	XP_001940152.1	0.0E+00	PF00096	3	3	No change
9607	AB03110.1	Contig2:1024104-1026406	XP_003296366.1	0.0E+00	PF00096	3	3	No change
9608	AB03112.1	Contig2:1029347-1032084	XP_003296367.1	0.0E+00	PF00096	4	5	No change
9609	AB03945.1	Contig3:882453-884672	XP_001934449.1	0.0E+00	PF00096	1	2	No change
9610	AB09849.1	Contig15:625649-628108	XP_001941788.1	0.0E+00	PF00096	6	6	No change
9611	AB06726.1	Contig6:1349660-1353040	XP_003306903.1	0.0E+00	PF00096	3	3	No change
9612	AB02276.1	Contig1:2621302-2624583	AEN02471.1	0.0E+00	PF00096	4	4	*Amr1*
9614	AB04090.1	Contig3:1258023-1260004	BAI44433.1	0.0E+00	PF00096	3	3	*AbPacC*
9615	AB04255.1	Contig3:1676712-1679474	XP_003300534.1	0.0E+00	PF00096	1	1	No change
9617	AB00853.1	Contig0:2390167-2391110	XP_003302358.1	2.0E-163	PF00096	2	2	No change
9619	AB08163	Contig9:1035815-1036929	JN835469	0.0E+00	PF00096	3	3	*AbVf19*
9622	AB02113.1	Contig1:2168156-2169840	XP_001802446.1	0.0E+00	PF00096	3	3	No change
9624	AB07477.1	Contig8:563569-565771	XP_003295512.1	0.0E+00	PF00096	1	1	No change
9625	AB01819.1	Contig1:1379994-1381233	XP_003300268.1	0.0E+00	PF00096	1	1	No change
9627	AB07424.1	Contig8:416693-418163	XP_003304533.1	0.0E+00	PF00096	1	1	No change
9629	AB07415.1	Contig8:375257-376416	CBX92496.1	4.0E-11	PF00096	4	4	No change
9630	AB07412.1	Contig8:365481-367155	CBX92496.1	3.0E-09	PF00096	2	2	No change
9631	AB00691.1	Contig0:1925856-1927071	XP_003295272.1	0.0E+00	PF00096	2	2	No change
9632	AB10302.1	Contig21:1116-2558	XP_003298351.1	0.0E+00	PF00096	2	3	No change
9634	AB09574.1	Contig14:749794-752044	XP_001932104.1	0.0E+00	PF00096	1	1	No change
9635	AB05424.1	Contig4:2391531-2393128	XP_001940496.1	6.0E-81	PF00096	1	1	No change
9637	AB09078.1	Contig12:217862-219071	XP_003306002.1	0.0E+00	PF00096	NA	5	No Conidia
9638	AB10053.1	Contig16:359908-361704	XP_003301582.1	2.0E-128	PF00096	1	1	No change
9639	AB10138.1	Contig16:591340-592695	XP_003299982.1	0.0E+00	PF00096	4	4	No change
9640	AB10000.1	Contig16:228079-230139	CBX93118.1	3.0E-161	PF00096	3	3	No change
9641	AB04555.1	Contig3:2516801-2518084	XP_001795924.1	7.0E-135	PF00096	1	1	No change
9642	AB09132.1	Contig12:403691-406054	XP_001931428	0.0E+00	PF00096	4	4	*AbSte12*
9704	AB00266.1	Contig0:745420-747735	XP_003304913.1	9.0E-35	PF00097	3	3	No change

Table 2.2 Continued….

Gene ID	Locus [a]	Location [b]	BlastP (GenBank No.)	E-value	PFAM domain	D [c]	T [d]	Importance
9707	AB08006.1	Contig9:637184-640414	XP_001931585.1	0.0E+00	PF00097	3	3	No change
9708	AB08669.1	Contig11:68678-70783	XP_003305645.1	0.0E+00	PF00097	4	4	No change
9710	AB03852.1	Contig3:639740-641848	XP_001934552.1	0.0E+00	PF00097	4	4	No change
9711	AB04451.1	Contig3:2208031-2211408	XP_003298357.1	0.0E+00	PF00097	3	3	No change
9712	AB09010.1	Contig11:989842-992093	XP_001937534.1	0.0E+00	PF00097	2	2	No change
9715	AB01036.1	Contig0:2898423-2899596	XP_001393115.2	2.0E-07	PF00097	2	3	No change
9716	AB01723.1	Contig1:1094055-1096231	XP_003299366.1	0.0E+00	PF00097	3	6	No change
9717	AB03215.1	Contig2:1363346-1363938	XP_003144703.1	1.0E-04	PF00097	4	4	No change
9802	AB03094.1	Contig2:981791-982773	XP_001935125.1	4.0E-92	PF00098	2	2	No change
9804	AB09786.1	Contig15:464644-468111	XP_003299085.1	7.0E-150	PF00098	2	2	No change
9806	AB02616.1	Contig1:3586493-3588690	XP_001938705.1	0.0E+00	PF00098	3	3	No change
9807	AB03863.1	Contig3:672224-673984	XP_001934527.1	2.0E-109	PF00098	2	2	No change
9813	AB08270.1	Contig10:13-2970	XP_003298630.1	2.0E-89	PF00098	3	3	No change
9811	AB02531.1	Contig1:3378762-3380052	XP_003300585.1	4.0E-74	PF00098	3	3	No change
9812	AB08869.1	Contig11:613365-614795	XP_003301685.1	0.0E+00	PF00098	4	4	No change
32001	AB02095.1	Contig1:2103669-2107046	XP_003299173.1	0.0E+00	PF00320	3	3	No change
32003	AB07759.1	Contig8:1383029-1384942	XP_001931005.1	0.0E+00	PF00320	1	2	No change
32006	AB05899.1	Contig5:1467601-1470725	XP_003302597.1	0.0E+00	PF00320	1	1	No change
32007	AB00430.1	Contig0:1184967-1186632	XP_003299406.1	0.0E+00	PF00320	4	4	No change
56901	AB04148.1	Contig3:1395515-1398596	XP_003299697.1	0.0E+00	PF00569	4	4	No change
56902	AB06478.1	Contig6:636058-639195	XP_003301663.1	0.0E+00	PF00569	4	4	No change
56903	AB01653.1	Contig1:694998-696539	XP_001941567.1	0.0E+00	PF00569	4	4	No change
62801	AB01205.1	Contig0:3431097-3436600	XP_003297343.1	0.0E+00	PF00628	4	4	No change
62802	AB01944.1	Contig1:1704876-1706459	XP_003306725.1	0.0E+00	PF00628			No change
62804	AB03636.1	Contig3:50265-52547	XP_001804886.1	0.0E+00	PF00628	2	2	No change
62806	AB09505.1	Contig14:546424-551177	XP_003303689.1	0.0E+00	PF00628	4	4	No change

Table 2.2 Continued….

Gene ID	Locus [a]	Location [b]	BlastP (GenBank No.)	E-value	PFAM domain	D [c]	T[d]	Importance
62808	AB05523.1	Contig5:125470-128127	XP_001934105.1	0.0E+00	PF00628	3	3	No change
62809	AB04844.1	Contig4:735851-737297	XP_003299102.1	0.0E+00	PF00628	2	3	No change
62810	AB04726.1	Contig4:412142-414271	XP_003296597.1	0.0E+00	PF00628	2	2	No change
62811	AB07549.1	Contig8:779675-782333	XP_003300540.1	0.0E+00	PF00628	3	3	No change
62812	AB09605.1	Contig14:827570-829523	XP_003302258.1	0.0E+00	PF00628	2	2	No change
62813	AB09588.1	Contig14:783921-787644	XP_003305170.1	0.0E+00	PF00628	4	4	No change
62814	AB06830.1	Contig7:212601-214010	XP_001936061.1	5.0E-65	PF00628	4	4	No change
64201	AB06290.1	Contig6:108317-109387	XP_001940035.1	2.0E-45	PF00642	2	2	No change
64202	AB03443.1	Contig2:2081713-2083341	XP_001938229.1	1.0E-158	PF00642	3	3	No change
64206	AB03356.1	Contig2:1837438-1838609	CBX95620.1	1.0E-96	PF00642	4	4	No change
64207	AB03343.1	Contig2:1804735-1805527	XP_001937849.1	5.0E-141	PF00642	4	4	No change
64209	AB04679.1	Contig4:294507-297213	CBY01757.1	6.0E-13	PF00642	4	4	No change
64212	AB02070.1	Contig1:2030227-2032558	XP_003305409.1	0.0E+00	PF00642	4	4	No change
64213	AB03376.1	Contig2:1886699-1889030	XP_001937882.1	0.0E+00	PF00642	4	4	No change
64216	AB04679.1	Contig4:294507-297213	XP_003303480.1	0.0E+00	PF00642	4	4	No change
65101	AB07023.1	Contig7:729536-731616	XP_003301333.1	0.0E+00	PF00651	4	4	No change
65102	AB03676.1	Contig3:168781-170063	XP_003306538.1	1.0E-64	PF00651	4	4	No change
65103	AB03044.1	Contig2:843141-845379	XP_001934912.1	0.0E+00	PF00651	4	4	No change
65104	AB09376.1	Contig14:133291-134557	XP_003302754.1	2.0E-120	PF00651	1	1	No change
65106	AB04490.1	Contig3:2317241-2318396	XP_001936292.1	0.0E+00	PF00651	2	3	No change
78101	AB10310.1	Contig24:1533-3186	CBX92315.1	0.0E+00	PF00781	3	3	No change
85603	AB04608.1	Contig4:78402-79910	XP_003002885.1	0.0E+00	PF00856	2	3	No change
85604	AB07709.1	Contig8:1219957-1221670	XP_003300216.1	0.0E+00	PF00856	4	4	No change
85605	AB04278.1	Contig3:1741567-1742686	XP_003302442.1	0.0E+00	PF00856	4	4	No change
85606	AB10176.1	Contig17:68194-69174	XP_001795908.1	1.0E-134	PF00856	3	4	*AbVf8*
85607	AB08122.1	Contig9:927454-930236	XP_003295568.1	0.0E+00	PF00856	1	1	No change
85608	AB08812.1	Contig11:451846-455476	XP_001937463.1	0.0E+00	PF00856	4	4	No change

Table 2.2 Continued….

Gene ID	Locus [a]	Location [b]	BlastP (GenBank No.)	E-value	PFAM domain	D [c]	T[d]	Importance
85611	AB03124.1	Contig2:1060023-1062717	XP_003297760.1	0.0E+00	PF00856	1	1	No change
85615	AB08906.1	Contig11:715092-717604	XP_003299046.1	0.0E+00	PF00856	3	4	No change
85616	AB06168.1	Contig5:2240600-2242763	XP_001934285.1	0.0E+00	PF00856	3	3	No change
85617	AB00131.1	Contig0:350570-353319	XP_001800171.1	2.0E-59	PF00856	4	4	No change
85618	AB08932.1	Contig11:786016-787226	CBX94944.1	5.0E-180	PF00856	4	4	No change
85619	AB05603.1	Contig5:397959-399167	XP_003303322.1	2.0E-138	PF00856	3	4	No change
85620	AB00329.1	Contig0:904316-906286	XP_003306372.1	0.0E+00	PF00856	4	4	No change
85621	AB09321.1	Contig12:928587-930117	XP_001930941.1	0.0E+00	PF00856	4	4	No change
85622	AB05741.1	Contig5:963883-967043	XP_001803285.1	2.0E-170	PF00856	2	2	No change
85624	AB07767.1	Contig8:1414492-1415564	XP_003298636.1	1.0E-155	PF00856	2	2	No change
141201	AB06607.1	Contig6:1022728-1025936	XP_001939955.1	0.0E+00	PF01421	1	1	No change
141202	AB07835.1	Contig9:166284-170085	XP_001933214.1	0.0E+00	PF01421	1	1	No change
141203	AB04846.1	Contig4:739156-740894	XP_001936862.1	0.0E+00	PF01421	3	3	No change
141204	AB01506.1	Contig1:290072-291716	XP_001941629.1	0.0E+00	PF01421	2	2	No change
141205	AB08255.1	Contig9:1281578-1288066	XP_003301105.1	0.0E+00	PF01421	3	4	No change
152901	AB01677.1	Contig1:766112-771064	XP_001933733.1	0.0E+00	PF01529	1	1	No change
152902	AB05099.1	Contig4:1455353-1457606	XP_001936959.1	0.0E+00	PF01529	4	4	No change
152903	AB02967.1	Contig2:645656-646688	XP_001938276.1	8.0E-151	PF01529	4	4	No change
152904	AB10328.1	Contig34:3546-4558	XP_001932503.1	4.0E-148	PF01529	1	2	No change
187302	AB08672.1	Contig11:75887-76966	XP_003305639.1	0.0E+00	PF01873	2	2	No change
217601	AB07745.1	Contig8:1345646-1348267	XP_003305269.1	0.0E+00	PF02176	4	4	No change
220701	AB09555.1	Contig14:689988-691795	CBY02261.1	0.0E+00	PF02207	3	4	No change
220702	AB08275.1	Contig10:78529-85026	XP_003303019.1	0.0E+00	PF02207	3	3	No change
295301	AB09270.1	Contig12:792943-794916	XP_001931322.1	3.0E-174	PF02953	4	4	No change
43661	AB05256.1	Contig4:1920585-1922047	XP_001937125.1	0.0E+00	PF04366	3	3	No change
43662	AB03525.1	Contig2:2360240-2362182	XP_003297123.1	0.0E+00	PF04366	4	4	No change
44341	AB08088.1	Contig9:833008-834546	XP_003295715.1	0.0E+00	PF04434	4	4	No change

Table 2.2 Continued….

Gene ID	Locus [a]	Location [b]	BlastP (GenBank No.)	E-value	PFAM domain	D [c]	T [d]	Importance
44383	AB01851.1	Contig1:1458194-1459126	XP_003304788.1	1.0E-157	PF04438	1	1	No change
48101	AB03794.1	Contig3:487339-490399	XP_003298437.1	0.0E+00	PF04810	4	4	No change
48102	AB09445.1	Contig14:372038-375217	XP_001932079.1	0.0E+00	PF04810	3	3	No change
51311	AB09155.1	Contig12:460620-462868	XP_003305851.1	0.0E+00	PF05131	1	3	No change
51911	AB02769.1	Contig2:80061-80913	XP_003298509.1	2.0E-173	PF05191	2	2	No change
51912	AB06054.1	Contig5:1924363-1928595	CBX96787.1	0.0E+00	PF05191	4	4	No change
62201	AB07732.1	Contig8:1299098-1299969	XP_003304545.1	4.0E-153	PF06220	2	2	No change
62211	AB07282.1	Contig7:1499779-1501365	XP_001936439.1	0.0E+00	PF06221	2	2	No change
68391	AB09418.1	Contig14:261940-263341	XP_003295916.1	0.0E+00	PF06839	4	4	No change
75351	AB04753.1	Contig4:489281-491190	XP_003298785.1	0.0E+00	PF07535	3	3	No change
79751	AB06132.1	Contig5:2150055-2151180	XP_003301194.1	0.0E+00	PF07975	2	2	No change
68271	AB08876.1	Contig 11: 643934-649126	CBX95050.1	0.0E+00	PF6827	2	3	No change
39701 [e]	AB01194.1	Contig0:3407266-3408753	XP_003296745.1	1.00E-134	PF00097	0	0	No change
39703 [e]	AB00267.1	Contig0:748469-749161	XP_001937331.1	0.00E+00	PF00097	3	3	No change
39704 [e]	AB00266.1	Contig0:745420-747735	XP_002340191.1	1.00E-79	PF00097	4	4	No change
39706 [e]	AB03080.1	Contig2:946685-947849	XP_003305224.1	0.00E+00	PF00097	3	3	No change
39707 [e]	AB08006.1	Contig9:637184-640414	XP_001931585.1	0.00E+00	PF00097	2	2	No change
39708 [e]	AB08669.1	Contig11:68678-70783	XP_003305645.1	0.00E+00	PF00097	1	1	No change
39710 [e]	AB03852.1	Contig3:639740-641848	XP_001934552.1	0.00E+00	PF00097	2	2	No change
39711 [e]	AB04451.1	Contig3:2208031-2211408	XP_003298357.1	0.00E+00	PF00097	5	5	No change
39712 [e]	AB09010.1	Contig11:989842-992093	XP_001937534.1	0.00E+00	PF00097	2	2	No change
39713 [e]	AB04992.1	Contig4:1160666-1161238	XP_001796522.1	3.00E-39	PF00097	0	0	No change
39714 [e]	AB08113.1	Contig9:905185-908735	XP_003296460.1	0.00E+00	PF00097	1	1	No change
39715 [e]	AB01036.1	Contig0:2898423-2899596	XP_001393115.2	2.00E-07	PF00097	0	0	No change
39716 [e]	AB01723.1	Contig1:1094055-1096231	XP_003299366.1	0.00E+00	PF00097	4	4	No change
39717 [e]	AB03215.1	Contig2:1363346-1363938	XP_003144703.1	0.00E+00	PF00097	4	5	No change
39718 [e]	AB01089.1	Contig0:3062508-3063705	XP_001941858.1	4.00E-120	PF00097	4	5	No change

Table 2.2 Continued….

Gene ID	Locus [a]	Location [b]	BlastP (GenBank No.)	E-value	PFAM domain	D [c]	T[d]	Importance
39719 [e]	AB01919.1	Contig1:1640168-1641586	XP_001938514.1	0.00E+00	PF00097	2	2	No change
39720 [e]	AB03199.1	Contig2:1317132-1320275	XP_003295534.1	0.00E+00	PF00097	4	4	No change
39722 [e]	AB06296.1	Contig6:119910-122904	XP_001940029.1	1.00E-128	PF00097	3	3	No change
39723 [e]	AB02114.1	Contig1:2170675-2172190	XP_003296080.1	0.00E+00	PF00097	4	4	No change
39724 [e]	AB04972.1	Contig4:1103364-1104112	XP_003296618.1	1.00E-19	PF00097	1	1	No change
39725 [e]	AB01822.1	Contig1:1392278-1393621	XP_001802949.1	1.00E-11	PF00097	2	2	No change
39726 [e]	AB05328.1	Contig4:2127741-2128836	XP_001940911.1	5.00E-166	PF00097	1	1	No change
39727 [e]	AB08161.1	Contig9:1024734-1027685	XP_001823065.2	0.00E+00	PF00097	2	2	No change
39728 [e]	AB02886.1	Contig2:437689-438507	XP_003297036.1	5.00E-98	PF00097	2	2	No change
39729 [e]	AB01427.1	Contig1:71556-73294	XP_001938467.1	0.00E+00	PF00097	4	4	No change
39731 [e]	AB03160.1	Contig2:1206375-1207219	CBX99588.1	1.00E-09	PF00097	2	3	No change
39732 [e]	AB08161.1	Contig9:1024734-1027685	XP_001794244.1	0.00E+00	PF00097	3	4	No change
39733 [e]	AB07745.1	Contig8:1345646-1348267	XP_003305269.1	0.00E+00	PF00097	3	4	No change
39734 [e]	AB06332.1	Contig6:264003-264929	XP_003306621.1	1.00E-176	PF00097	1	1	No change
39735 [e]	AB06820.1	Contig7:184097-185131	XP_001936051.1	2.00E-148	PF00097	2	2	No change
39736 [e]	AB08547.1	Contig10:905639-907345	XP_001932158.1	0.00E+00	PF00097	0	3	No change

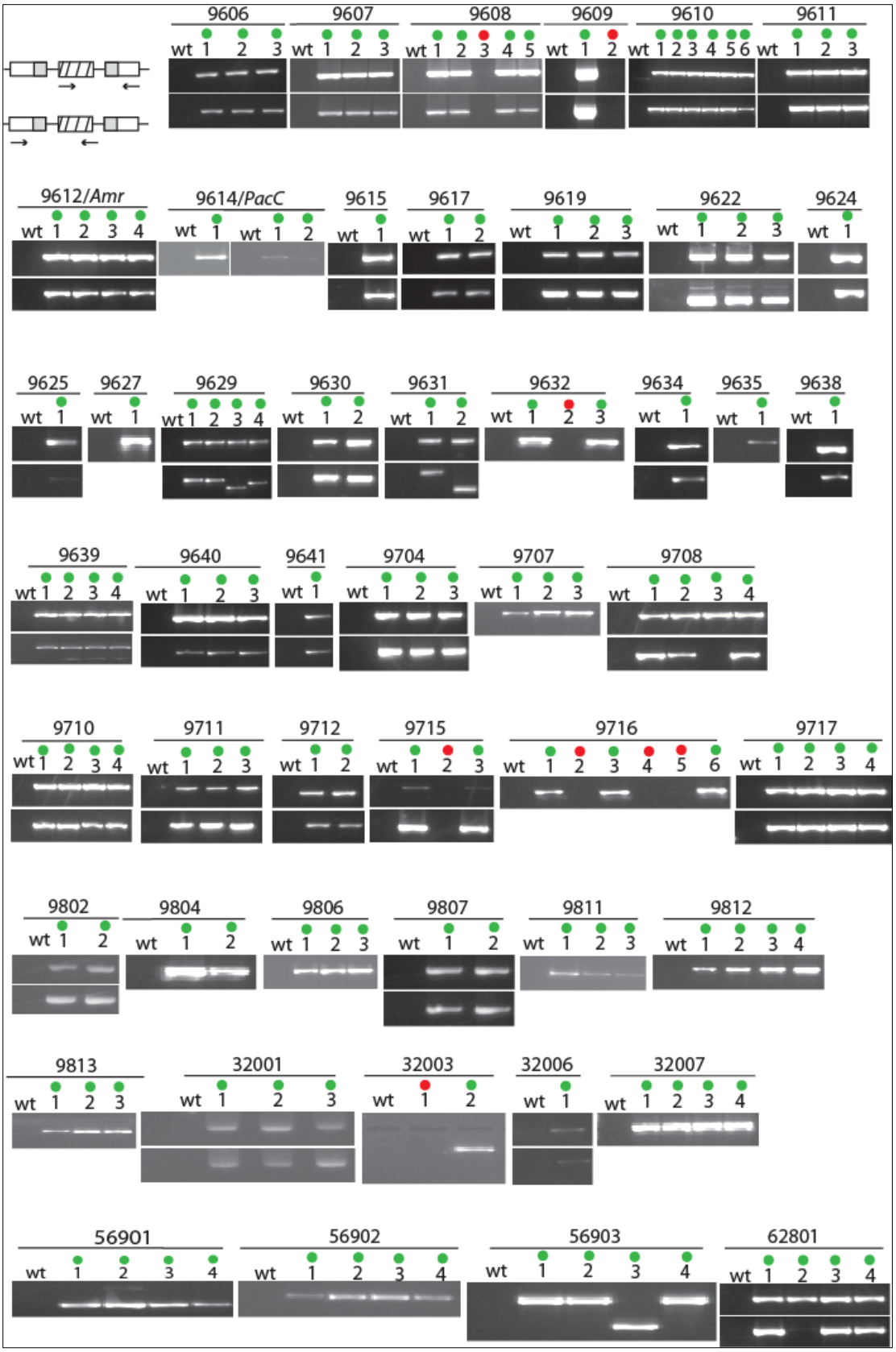
a: Locus number corresponds to that assigned to individual protein-coding genes in the *A. brassicicola* genome assembly in JGI portal.

b: Corresponds to location on individual scaffolds in the machine-annotated *A. brassicicola* genome assembly.

c: Total number of true mutant disruption colonies verified through PCR.

d: Total number of colonies appeared after transformation.

e: Transformation was done in a different study and putative mutant colonies were kindly provided by Yangrae Cho. The downstream work of true mutant verification and pathogenicity assays were done in this study.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wt 1 2 3
9608
wt 1 2 3 4 5
9609
wt 1 2
9610
wt 1 2 3 4 5 6
9611
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9612/Amr
wt 1 2 3 4
9614/PacC
wt 1 wt 1 2
9615
wt 1
9617
wt 1 2
9619
wt 1 2 3
9622
wt 1 2 3
9624
wt 1
9625
wt 1
9627
wt 1
9629
wt 1 2 3 4
9630
wt 1 2
9631
wt 1 2
9632
wt 1 2 3
9634
wt 1
9635
wt 1
9638
wt 1
9639
wt 1 2 3 4
9640
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9641
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9708
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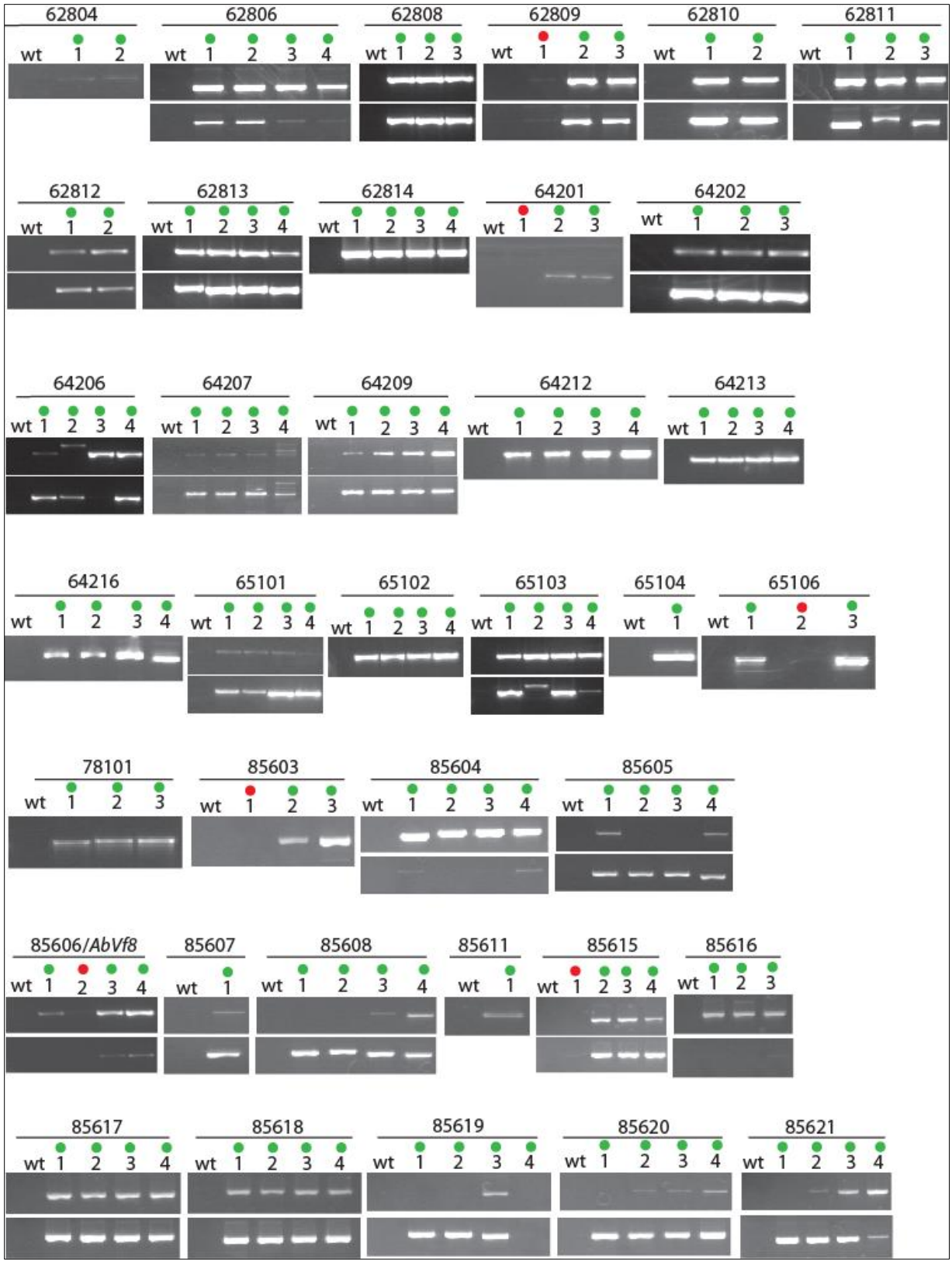
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wt 1 2 3 4
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85605
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wt 1 2 3 4
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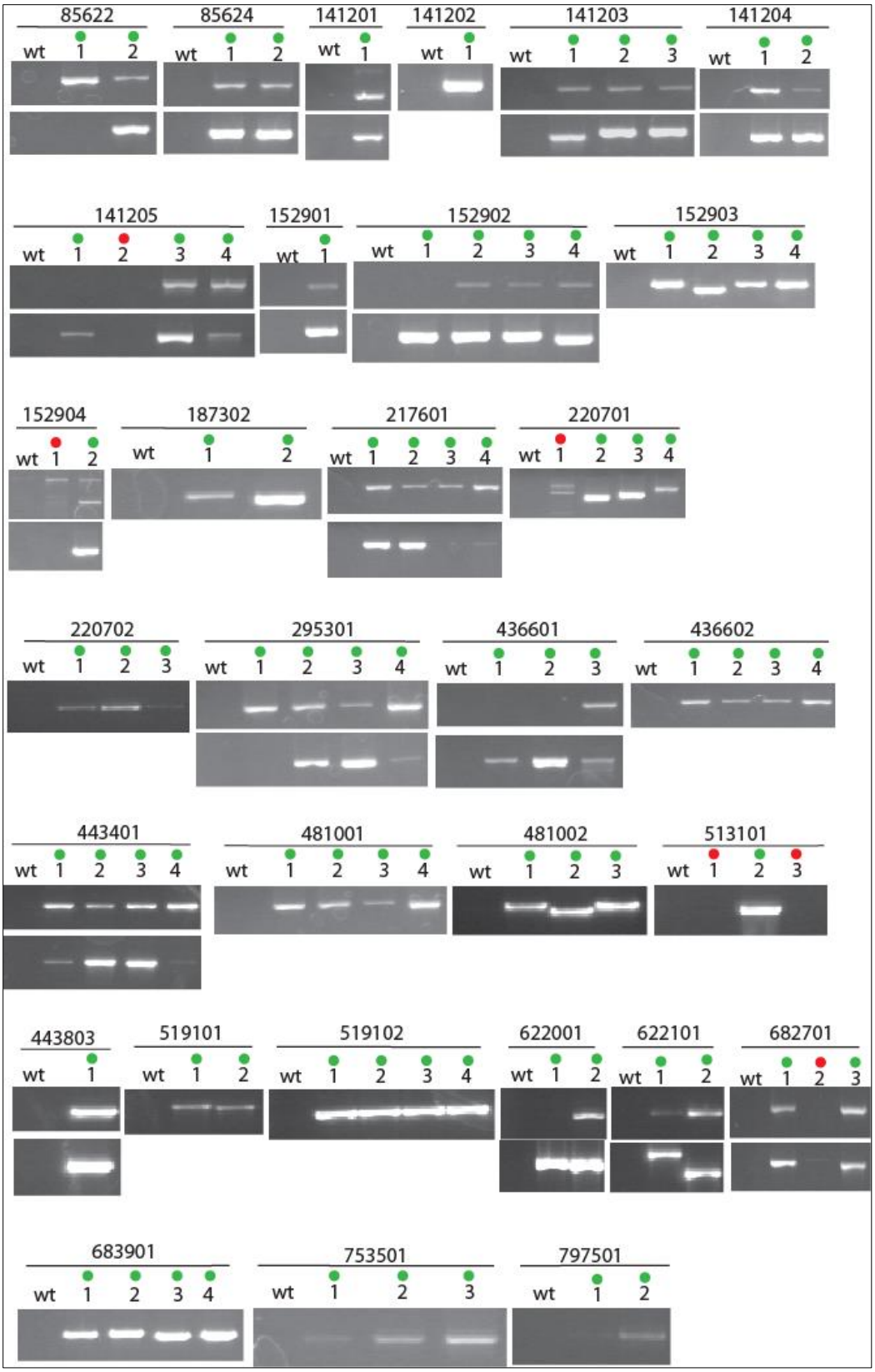
wt 1 2 3 4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wt 1 2
85624
wt 1 2
141201
wt 1
141202
wt 1
141203
wt 1 2 3
141204
wt 1 2
141205
wt 1 2 3 4
152901
wt 1
152902
wt 1 2 3 4
152903
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152904
wt 1 2
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wt 1 2
217601
wt 1 2 3 4
220701
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wt 1 2 3
797501
wt 1 2

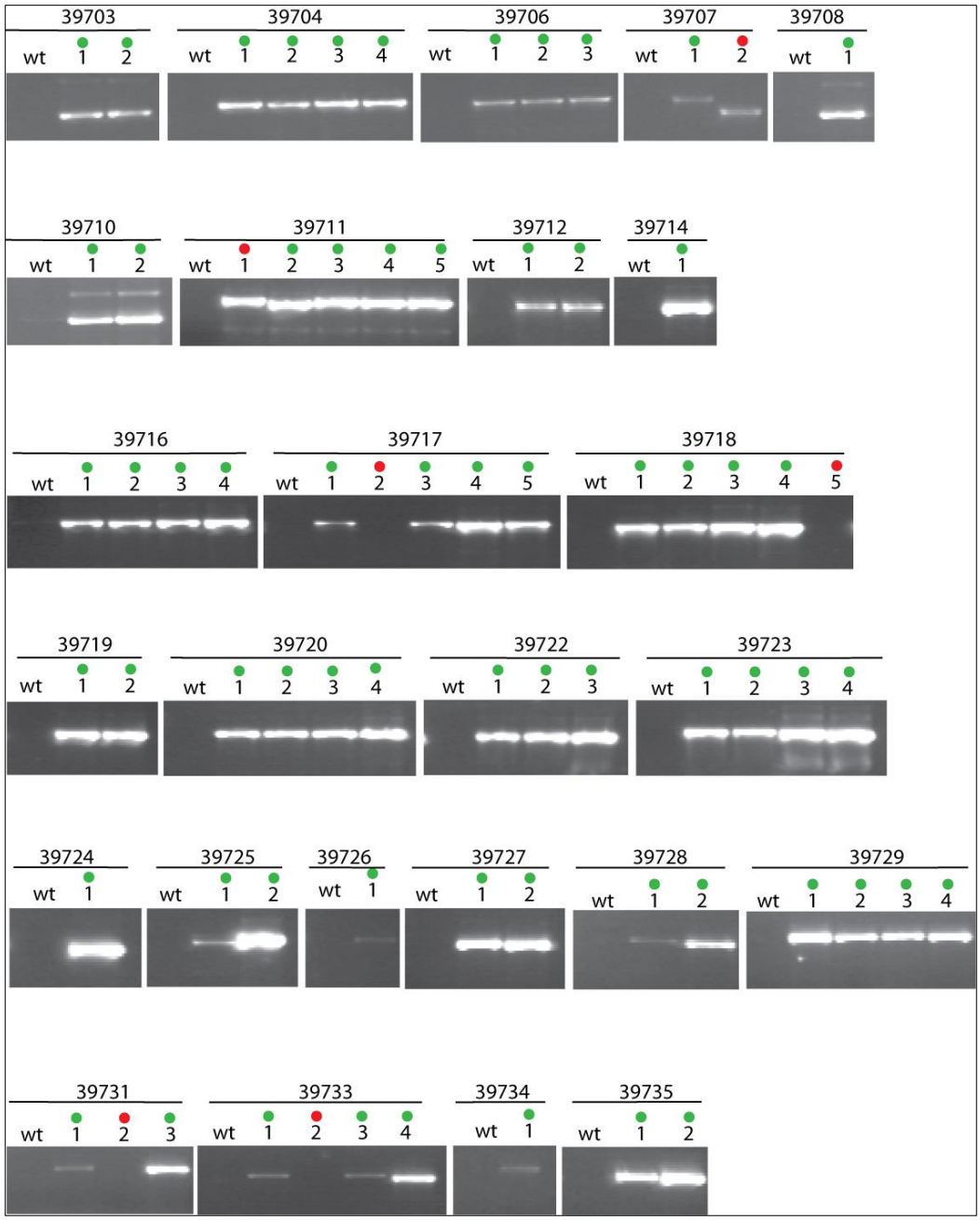

Fig. 2.4 Verification of targeted gene disruption by polymerase chain reaction (PCR). PCR amplification was performed with two pairs of target gene specific and HygB resistance gene primers. Relative primer binding locations are marked below the schematic diagram of the disrupted target gene on the left side of the gel images at the top row of page one. Hatched box = a selectable marker gene, white box = a target genomic locus, and gray box = partial target gene. The green colored dots over each lane represents true mutants, while red colored show ectopic mutants.

2.3.2 Identification of pathogenesis related genes:

Lesion diameter was measured to quantify the changes in virulence caused by knocking out the gene. Only six of the knockout mutants showed changes in virulence. We hypothesize that these six genes have positive (non-pathogenic, avirulent) or negative (more virulent) regulatory control of pathogenesis. We also found one gene mutant which, showed no difference in virulence but did not produce conidia. Among the seven genes only one (*PacC)* has been previously described in other fungi. The rest were novel genes with no functional description. Table 2.3 summarizes the results of pathogenicity and growth assays. Individual genes are discussed in following sub sections.

Table 2.3 Genes important for regulation of pathogenesis identified as a result of gene disruption KO study.

Gene ID[a]	Locus[b]	Colony diameter		Lesion size	Cellular localizationc[c]
		PDA	PDA + Hyg		
9619/ *AbVf19*	AB08163	Reduced	Reduced	Reduced by 75%	Nucleus (0.98)
62808/*AbVf8*	AB10176.1	Reduced	Reduced	Reduced by 73%	Cytoplasm(0.47), Nucleus (0.45**)**
397031	AB03160.1	No change	Reduced	Reduced by 73%	Nucleus (0.9)
397032	AB08161.1	No change	Reduced	Reduced by 100%	Peroxisomes (0.99)
9614/ *AbPacC*	AB04090.1	No change	Reduced	Reduced by 100%	Nucleus (0.99)
9612/*Amr1*	AB02276.1	No change	No change	Increased by 100%	Nucleus (0.92)
9637	AB09078.1	No change	No change	No change	Nucleus (0.99)

a: The name assigned to each gene in the laboratory based on the transcription factor list. New and formal names were assigned according to published results e.g. *AbVf19* and *Amr 1*.
b: Locus number corresponds to that assigned to individual protein-coding genes in the *A. brassicicola* genome assembly.
c: Cellular localization of transcription factor predicted *in silico* by using SherLoc program.

2.3.2.1 *Ab9619 or AbVf19*

The pathogenicity assays on detached leaves of green cabbage with the disruption mutant of *AbVf19* produced 75% reduction in lesion size as compared with wild type (Table2.3 and 2.4). The gene *loci* encoding *AbVf19* was deleted from the *A. brassicicola* genome. The deletion mutant *(*Δ*abvf19*) was then used for pathogenesis assays; it also showed reduction in virulence as in the previous results (Fig. 2.5 and table 2.5). The clarify that the loss of virulence in the knockout mutants is because of absence of *AbVf19* gene, the complementation mutants were made by reinserting the wild type allele into the deletion mutant. The pathogenesis assays done with the complementation strain showed complete restoration of virulence equivalent to wild type strain (Fig 2.6 and table 2.5). The growth assays showed that in general *AbVf19* colonies were slow growing on PDA and PDA containing hygromycin. No change in growth rate was observed when pharmacological agents like 30ng/µl of antibiotics kanamycin and ampicillin were added to the PDA and Δ*abvf19* was cultured (Fig 2.7). Using SherLoc (http://abi.inf.uni-tuebingen.de/Services/SherLoc) the *in silico* localization the protein inside the cell was predicted to be in the nuclei with a probability of 98%. AbVF19 protein is found inside the nucleus indicating their possible role in transcriptional regulation (Shatkay *et al.*, 2007). This observation was further substantiated when we tagged the AbVF19 protein with GFP and observed under the confocal microscopy to show that it was localized in nucleus (fig 2.8). (Srivastava *et al.*, 2012).

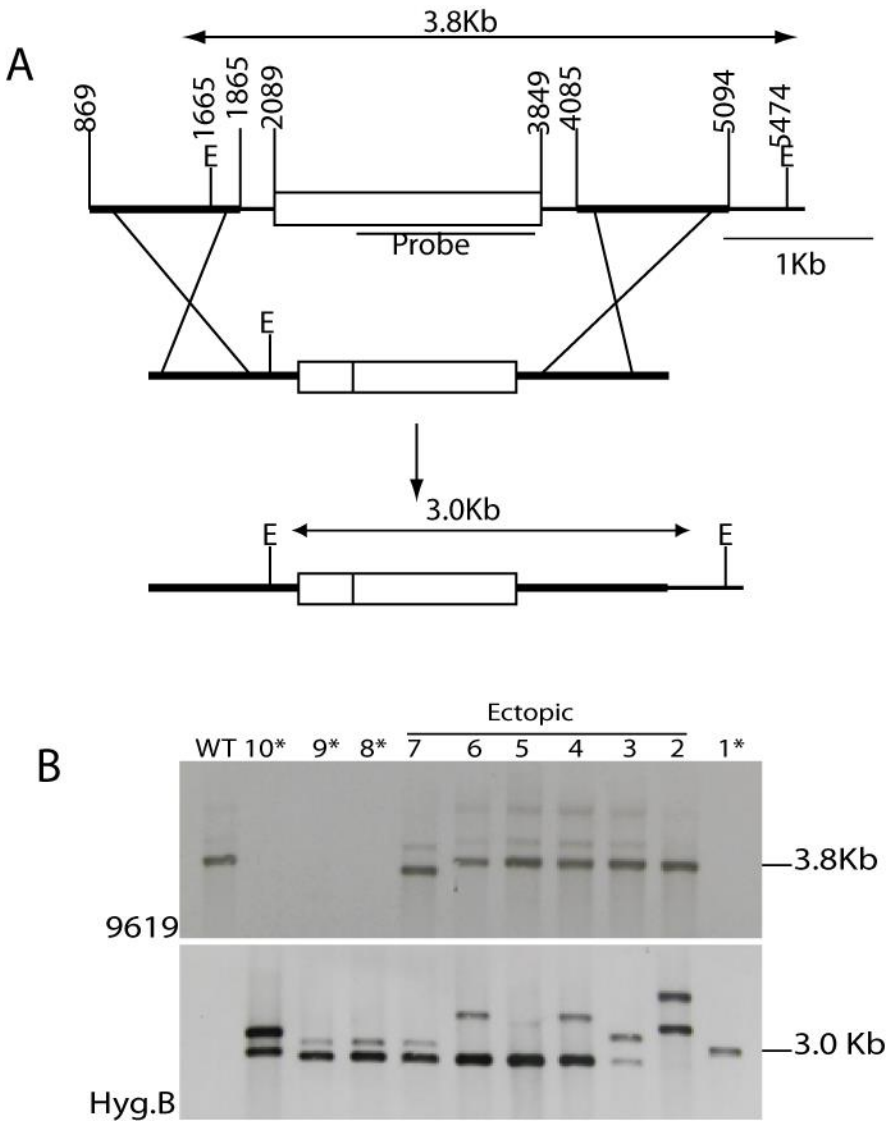

Fig2.5. Targeted deletion of *AbVf19 gene*. Replacement of the *AbVf19* coding region with a selectable marker *Hygromycin B transferase* (Hyg B) cassette. **A**. The upper panel is a schematic diagram of wild-type locus, replacement construct, and mutant locus in order. **B** The lower panel is a collection of southern blots displaying loss of the *AbVf19* gene in selected mutants. Abbreviations: E = Restriction enzyme BamH1 digestion site. Asterisk (*) indicate DNA lanes of mutants which were used for pathogenicity assays fig.2.6. The details of southern hybridization methodology are described in Chapter3.

Table 2.4 Decreased virulence of disruption mutants of *Abvf19* compared to wild-type *Alternaria brassicicola.*

Mutant	d.f.	Lesion diameter (in mm)		Virulence decrease	p-value
		wt	mutant		
abvf19-2	10	11.2±3.3	2.8±2.1	75%	1.54E-05
abvf19-2	8	9.2±4.6	2.1±3.0	77%	1.0E-04
abvf19-2	8	11.1±4.5	5.4±3.1	51%	2.0E-04

d.f. = degrees of freedom, wt = wild type, p-value = probability

Table 2.5 Decreased virulence of two strains of *Δabvf19* deletion mutants compared to wild-type *A. brassicicola.*.

Mutants	d.f.	Lesion size (mm)		Virulence decrease	p-value
		wt	Mutant		
Δabvf19-1	8	19.6±2.6	4.3±3.2	78%	4.0E-09
*Δabvf19-1**	8	10.4±5.3	0.9±0.9	92%	7.0E-05
Δabvf19-10	8	9.4±4.5	1.8±1.9	81%	8.0E-04

wt = wild type, d.f. = degrees of freedom, P = probability, lesion size indicates the average lesion diameter. Result marked with an asterisk (*) was from pathogenicity assays with whole plants, others were from assays with detached leaves. Also see fig. 2.6

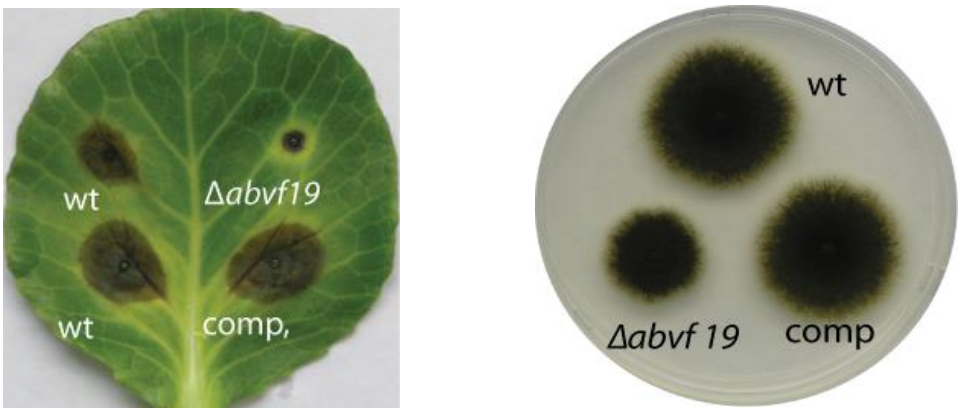

Fig. 2.6: Pathogenicity and growth assays of wt, Δ*abvf19* and complementation strain. The pathogenicity assays were done on green cabbage leaves by using equal numbers of conidia and measuring lesion size. The lesion size produced by the Δ*abvf19* was severely reduced in comparison to the wt which shows large lesion. The mutant when complemented with wt allele (comp.) showed restoration of virulence equivalent to wt. Similar results were also observed during the growth assay done on PDA plate. The size of mutant is less than the wt however the complemented one shows restoration of growth potency. Abbreviations: wt= wild type, comp= complemented strain.

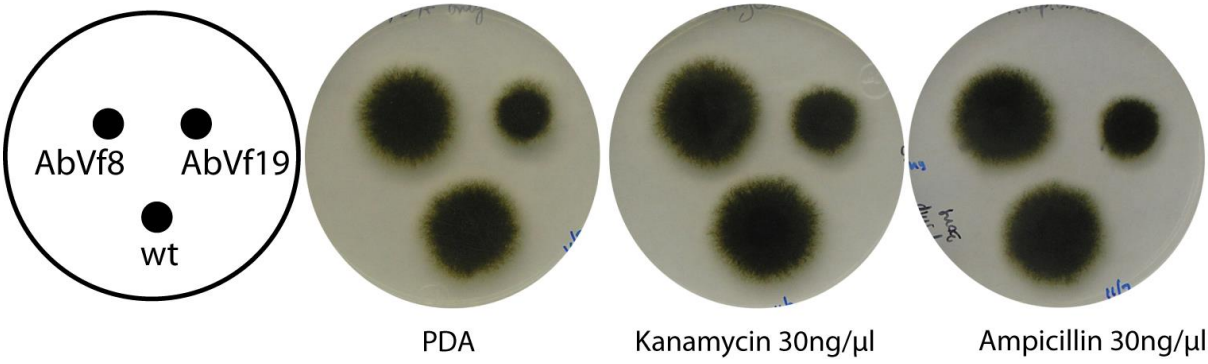

Fig. 2.7: Growth assays of wt, Δ*abvf19* and AbVf8 in presence of pharmacological agents. The growth assays were done by spotting equal number of conidia on PDA plates supplemented with antibiotics Kanamycin and Ampicillin using equal number of conidia.

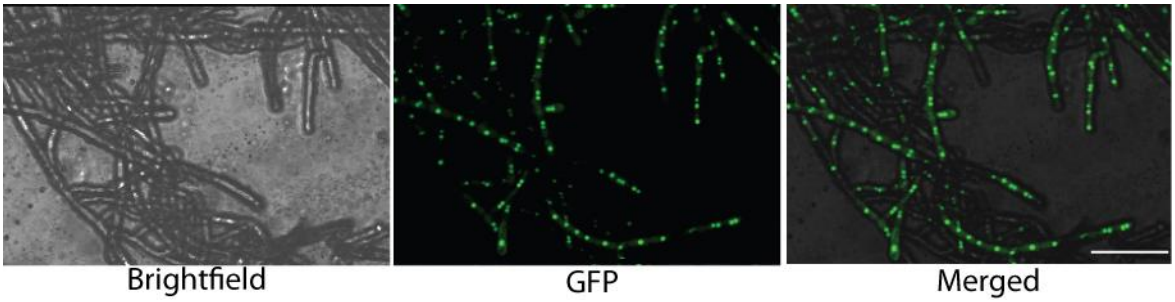

Fig 2.8. Confocal microscope images showing localization of AbFv19 protein in nucleus. A mutant was made by tagging GFP on the c-terminus of Abvf19 protein. The mutant was grown on PDA mediium for 4 days. The detailed procedure of GFP tagging and miscroscopy is described in the methods section of chapter 4.

2.3.2.2 *Ab62808* or *Abvf08*

Targeted gene disruption mutants *Ab62808* or *AbVf8* gene encoding for a putative transcription factor shows a 73% reduction in lesion size in comparison to wild type (Table 2.6). Further experiments were done by creating deletion mutants for *AbVf8* genes and complementing the deletion strain with the wild type allele of *AbVf8*. The pathogenicity assays with deletion mutant also showed reduction of virulence and complete restoration of virulence with complementation strains (Table 2.7 and fig.2.9). Growth rate with osmotic stressor 3 and 6 mM NaCl was reduced. No change in growth rate was observed with 0.4 and 0.6 mM H_2O_2 (table 2.8 and fig. 2.10).

Table 2.6 Decreased virulence of *disruption abvf8* compared to wild-type *A. brassicicola*

Mutants	d.f.	Lesion diameter (in mm)		Decrease in lesion diameter	p-value
		wt	mutant		
abvf8	13	9.2±4.1	2.5±3.4	72.8%	3.45E-06

Table 2.7 Decreased virulence of Δ*abvf8* compared to wild-type *Alternaria brassicicola*

Mutants	d.f.	Lesion diameter (mm)		Decrease in lesion diameter	*p*-value
		wt	mutant		
Δ*abvf8-2*	11	11.5±4.1	7.7±4.6	55.4%	1.05E-05
Δ*abvf8-3*	11	15.1±4.4	10.6±5.5	55.1%	2.00E-04
*AbVf8-10**	11	15.8±4.2	15.8±3.9	0.0%	0.72

d.f. = degrees of freedom, wt = wild type, p-value = probability, *AbVf8-10** is an ectopic mutant.

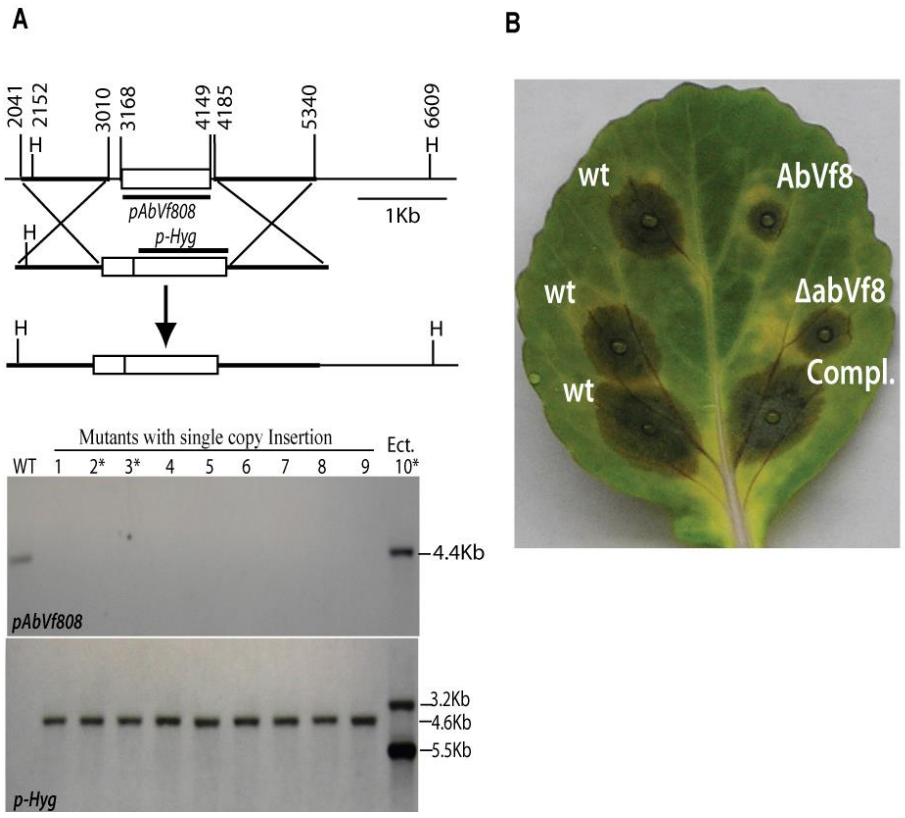

Fig.2.9 Targeted deletion of *AbVf808* gene and reduction in virulence. **A.** Replacement of the *AbVf808* coding region with a selectable marker *Hygromycin B transferase* (Hyg B) cassette. the upper panel is a schematic diagram of wild-type locus, replacement construct, and mutant locus in order and the lower panel is a collection of Southern blots displaying loss of the *AbVf808* gene in selected mutants. Probe regions are marked by P-AbVf01, P-3', and P-Hyg. Abbreviations: H = *HindIII* enzyme digestion site. Asterisks (*) indicate DNA lanes of mutants whose pathogenicity assay results presented in fig **B**. Reduced virulence of *Δabvf808* mutants on host plants (*Brassica oleracea*).

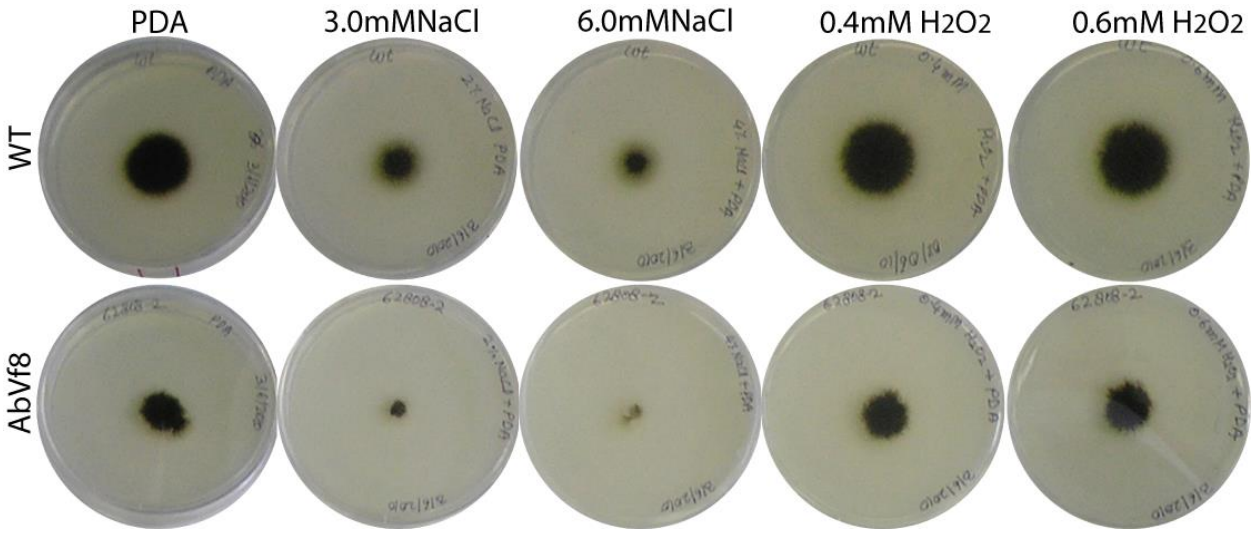

Figure 2.10 Growth responses in the presence of osmotic and oxidative stress. PDA plates containing the indicated chemicals were inoculated with same number of conidia of wt and mutant *AbVf8* to assess the response of mutant towards these stressors.

Table 2.8 Decreased in the colony size in the presence of osmotic and oxidative stress inducing agents in *A.brassicicola* and Δ*abvf8.* All measurement is of colony diameter and in millimeter (mm) units.

	PDA	**NaCl**				**H_2O_2**			
		3mM	Change	6mM	Change	0.4mM	Change	0.6mM	Change
WT	40	25	37%	19	52%	43	-7%	39	2%
Δ*abvf8*	23	9	60%	6	73%	25	-8%	25	-8%

2.3.2.3 *Ab39731*

The pathogenicity assays on the detached leaves of green cabbage with deletion mutant of Ab39731 gene showed reduction in lesion size by 73.4 % and by 30.0%. No changes were observed when mutant strain was cultured on PDA.

Table 2.9 Decreased virulence of *Ab39731* compared to wild-type *Alternaria brassicicola*

Mutants	d.f.	Lesion diameter (in mm)		Virulence decrease	p-value
		wt	mutant		
ab39731-1	10	9.4±3.5	2.6±2.2	73.40%	3.75E-05
ab39731-1	10	8.0±3.4	5.3±4.0	30.00%	6.80E-03

d.f. = degrees of freedom, wt = wild type, p-value = probability

2.3.2.4 *Ab397032*

The disruption mutants of *Ab397032* gene showed complete loss of virulence in pathogenicity assays. They showed no reduction in vegetative growth on PDA but growth reduction was observed in the presence of hygromycin. This reduction can be attributed to truncation of hygromycin resistance gene due to exonuclease activity during incorporation into genome at the time of transformation. This gene is predicted to be localized in peroxisomes and the BLAST results also predicts that it is a peroxisome associated gene (*Pex*). Interestingly, Kim et al in 2009 deleted *Pex10* and *Pex 12* genes but they failed to notice any changes in virulence (Kim *et al.*, 2009). Hence, this gene will be the first report of a pex class gene involved in pathogenesis in *A. brassicicola*.

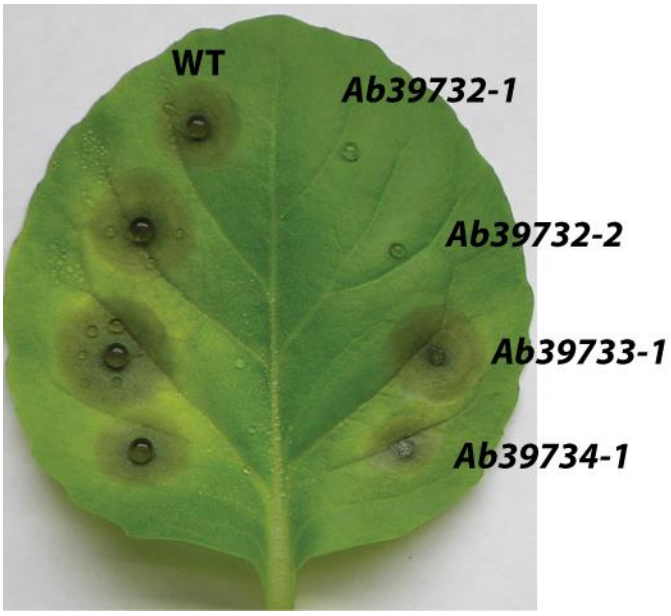

Fig.2.11. Pathogenicity assay of Ab39732 mutant. For pathogenicity assays equal numbers of conidia from wild type and mutants were inoculated on left and right hand side of mid rib, respectively.

Table 2.10 Decreased virulence of *Ab397032* compared to wild-type *Alternaria brassicicola*

Mutants	d.f.	Lesion diameter (in mm)		Virulence decrease	p-value
		wt	mutant		
Δab49732-1	9	9.1±4.7	0.5±1.8	94.60%	1.90E-04
Δab49732-2	9	10.3±4.9	0.0±0.0	100.00%	9.74E-05

d.f. = degrees of freedom, wt = wild type, p-value = probability

2.3.2.5 9614/*AbPacC*

Pathogenicity assays with disruption mutant strain of AbPacC gene showed complete loss of pathogenicity. The mutant strain grew slowly on PDA as well as on PDA supplemented with osmotic stress inducing reagent 3mM and 6mM NaCl 55% and 63% respectively. The growth rate remained unaffected in the presence of an oxidation reagent H_2O_2. The *AbPacC* gene has a single copy in the genome sequence of *A. brassicicola* and shows a high sequence similarity (E=0.0) with the *PacC* gene in other fungi.

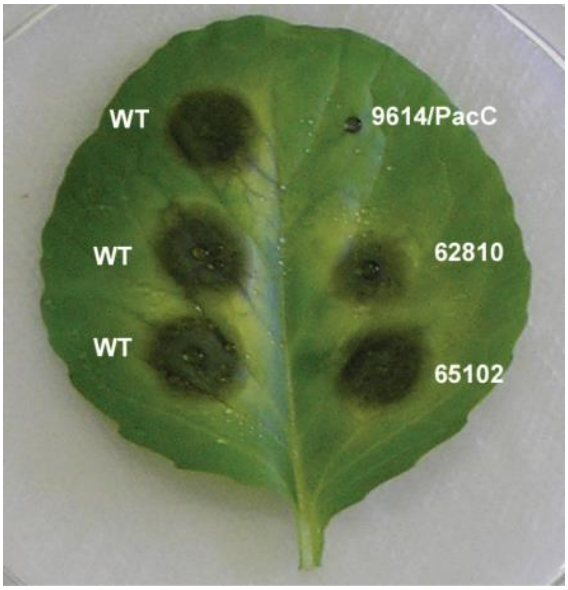

Fig.2.12. Pathogenicity assay of AbPacC mutant. For pathogenicity assays equal numbers of conidia from wild type and mutants were inoculated on left and right hand side of mid rib, respectively. The mutant strain were non- pathogenic.

Table 2.11 Loss of pathogenicity of the *abpaCc* mutant

Mutants	d.f.	Lesion diameter (mm)		Decrease in lesion diameter	*p*-value
		wt	mutant		
abpacc-2	8	11.6±3.7	0.0±0.0	-	1.39E-05

d.f. = degrees of freedom, wt = wild type, p-value = probability.

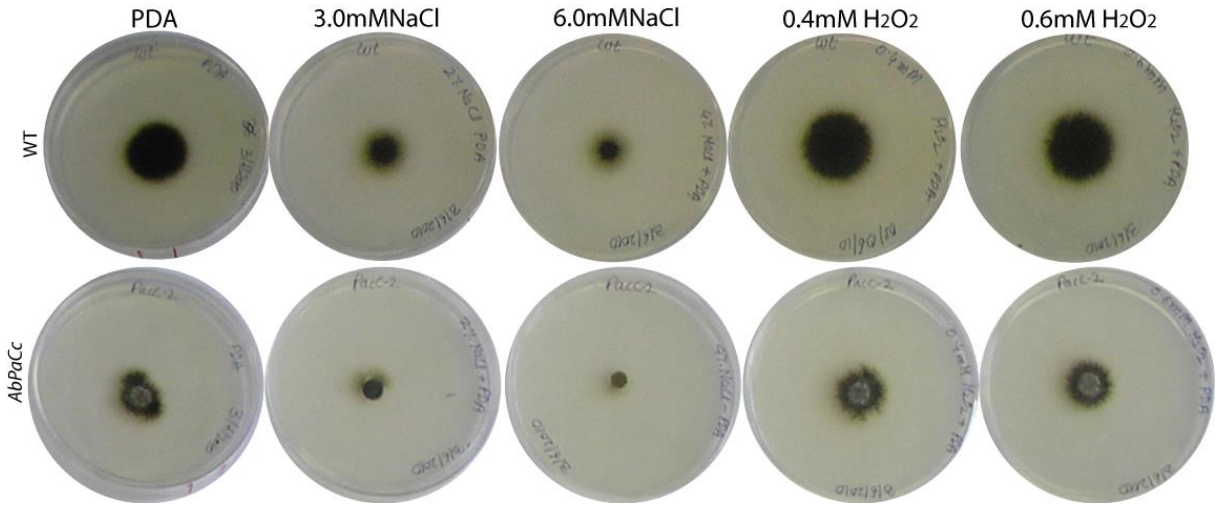

Fig. 2.13 Growth responses in the presence of Osmotic and oxidative stress. PDA plates containing the indicated chemicals were inoculated with same number of conidia of wt and mutant *AbPacC* to assess the response of mutant towards these stressors.

Table 2.12 Decreased in the colony size in the presence of osmotic and oxidative stress inducing agent in *Alternaria brassicicola* and Δ*abvf8.* All measurement are of colony diameter and in millimeter (mm) units

	PDA	NaCl				H_2O_2			
		3mM	%Change	6mM	%Change	0.4mM	%Change	0.6mM	%Change
WT	40	25	37	19	52	43	-7	39	2
abpaccc	26	11	55	10	63	28	-8	25	5

2.3.2.6 9612/*Amr1*

The knockout mutants of transcription factor encoding gene *Amr1* produced visibly different phenotypes consisting of albino colonies mainly due to loss of melanin from the conidia (fig 2.14 A and B). We named this gene as *Amr1* or *Alternaria brassicicola* melanin regulation gene. The mutants also produced a pink color pigment when grown on PDA (Fig 2.14C). We collected this pigment and injected it into the host plant to see if the pigment has any role in pathogenesis. The plants showed no sign of hypersensitivity or any other abnormality and we concluded that the pigment has no toxic effect. We also produced gene deletion knockout mutants for *Amr1* gene (*Δamr1*), and later one of the deletion mutant strain was also complemented with the wild type allele of *Amr1* gene (Δamr1::Amr1::NAT) . We performed comprehensive pathogenicity assays using three different strains and complemented mutants. The knockout mutants surprisingly showed ~50-80% increase in lesion size in comparison to lesions made by the wild type strain indicating that due to loss of Amr1 gene *A. brassicicola* has become more virulent (table 2.13 and fig 2.14D). The complemented strain showed lesion of same size as the lesions produced by wild type *A.brassicicola*. The growth assays were also done with different stress-inducing reagents such as NaCl (3mM), H_2O_2 (2.5mM and 5.0mM) and sorbitol (1.2M). No difference was observed in growth rate with any of these stressors (data not shown).

Table 2.13. Increased virulence of four strains of *Δamr1* deletion mutants compared to wild-type *Alternaria brassicicola*.

Mutants	Number of spores	d.f.	Lesion size (mm)		Increase in lesion size	P- value
	wt/mut		wt	Mutant		
Δamr1-1	2,500/2,200	11	12.4±3.4	18.4±1.9	49%	1.78E-05
Δamr1-4	2,000/2,000	11	9.4±7.4	17.0±5.6	82%	1.40E-04
Δamr1-5	1,200/1,200	8	6.9±4.6	10.9±7.4	58%	1.80E-02

wt = wild type, mt = mutant, d.f. = degrees of freedom, P = probability, lesion size indicates the average lesion diameter.

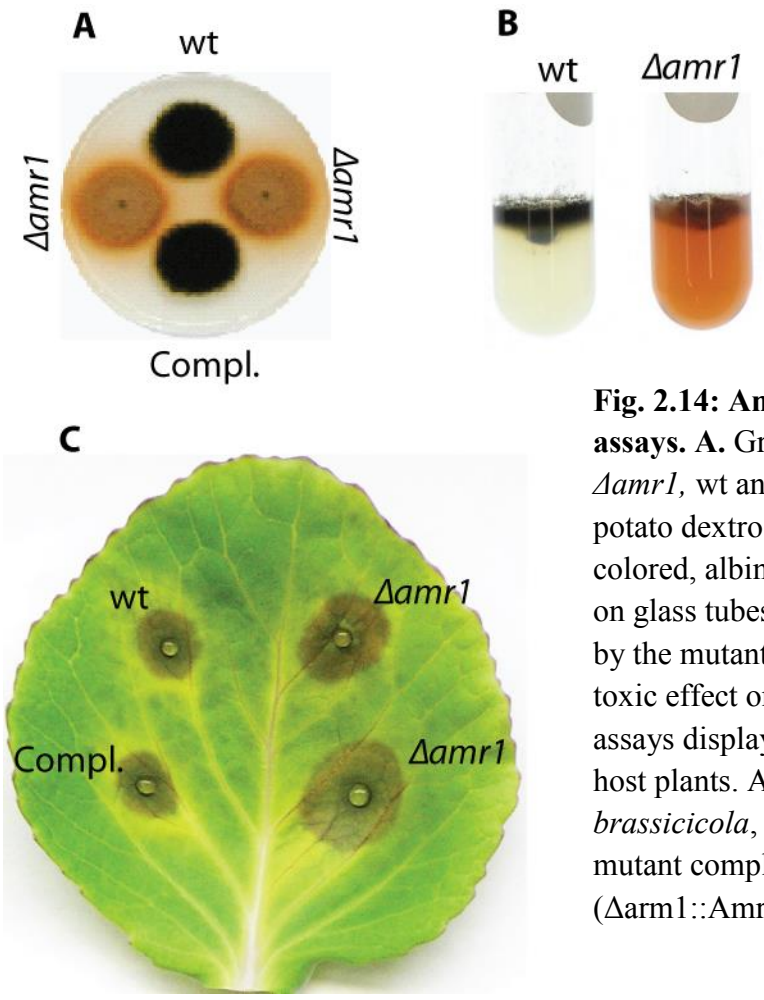

Fig. 2.14: Amr1 mutant phenotype and pathogenesis assays. A. Growth and comparison of texture between *Δamr1,* wt and complementation strain mutant on a potato dextrose agar plate. The mutant show orange colored, albino colony. **B**. wt and mutants were grown on glass tubes to show the colored secretion produced by the mutant. The colored secretions had no apparent toxic effect on host plants. **C** Results of pathogenicity assays displaying increased virulence of the mutants in host plants. Abbreviations: wt; wild type *A. brassicicola*, *Δamr1*: Amr1 deletion mutant, compl; mutant complemented with a native allele of Amr1 gene (Δarm1::Amr1::NAT).

2.4 Discussion

In the present study 184 *Alternaria brassicicola* transcription factor encoding genes with the zinc finger domain were analyzed. Only six genes were strongly associated with pathogenesis. Apart from these six genes two more transcription factor genes *AbSte12* and *AbPro1* have been identified in previous studies (Cho *et al.*, 2012). The discovery of only eight genes regulating pathogenesis trait out of more than 200 genes studied suggests that pathogenesis is a very tightly regulated trait under the control of fewer numbers of transcription factors. This result is comparable to the knock out study of 693 putative transcription factors where 62 genes affected virulence (Son *et al.*, 2011). These results identify the prospective targets that can be utilized for designing of anti-fungal agents for effective management of fungal associated diseases. Our present study can be a perfect foundation for undertaking such studies in future.

AbVf19 is present in single copy in the genome of *A. brassicicola*. The predicted amino acid sequence of the *AbVf19* protein contains nuclear localization signals and tandem C2H2- zinc finger domains (Pfam ID: PF00096) (Bateman *et al.*, 2004). The nuclear localization of *AbVf19* protein was also confirmed by accumulated green fluorescence signals from AbVf19-GFP fusion proteins (Srivastava *et al.*, 2012). *AbVf19* gene displayed approximately 90% reduction in virulence, hence named as *Alternaria brassicicola* virulence factor 19 (AbVf19). Similar reduction in virulence was also observed with multiple strains of deletion mutants of *AbVf19* during the pathogenicity assays on detached leaves as well as on whole leaves of green cabbage (*Brassica oleracia*) and wild crucifer *Arabidopsis thaliana.* The reduction in virulence and its restoration in pathogenicity assays by mutants and complementation strains provides substantial evidence of involvement of *AbVf19* in pathogenesis. Their function in the fungal physiology

especially during pathogenesis was characterized by mRNA expression profiling of whole trancriptome of AbVf19 mutant. The gene expression profile analysis suggests that AbVF19 protein is involved in depolymerization of cell wall components or extracellular proteins during the late stages of plant infection (Srivastava *et al.*, 2012). Relatively less little is known about the regulation of major group of enzymes involved in digestion of plant cell wall components during pathogenesis. Such enzymes are termed as cell wall degrading enzymes (CWDE). SNF1 homologs are known to regulate CWDEs (Tonukari *et al.*, 2000; Ospina-Giraldo *et al.*, 2003). The mutant of SNF1 in *Cochlilobolus carbonum* mainly showed reduction in virulence due to lack of penetration by the mutant. However in case of *AbVf19* the penetration and appresorium like structure formation was not different from the wild type *A. brassicicola* and the knockout mutant showed only reduction in lesion size on the host leaf (Srivastava *et al.*, 2012). *Cbh7* (cellobiohydrolase) is an important CWDE which known to be induced during pathogenesis (Cramer *et al.*, 2006; Cho *et al.*, 2007). The genes encoding for *cbh7* is considered important for pathogenesis. However the knockout mutant of *cbh7* was did not show any reduction in virulence (Cho unpublished). Similarly cutinase gene (*CutAb1*) transcripts were also reduced in Δ*abvf19* but its individual knock out did not produce any changes in the virulence (Chenglin Yao, 1995). These results support the notion that *AbVf19* coordinately regulates the various downstream hydrolase genes needed for degradation of plant materials during pathogenesis.

The gene *AbVf8* encodes for 371 amino acids. The protein has 0.47 and 0.45 probability to be localized in cytoplasm or nucleus, still presence of C2H2 zinc finger motif strongly suggests this gene to be a transcription factor. The *AbVf8* gene shown no sequence similarities to known pathogenesis-associated genes in any fungus and is annotated as either a hypothetical

protein or a predicted transcription factor. Vegetative growth rates and the formation of conidia were comparable to the wild type on PDA. However, the slower growth in the presence of osmotic stress inducing reagent indicates its possible involvement in osmoregulation. Predicted domains in the protein sequence of the *AbVf8* genes were somewhat informative regarding their functions. The predicted protein encoded by *AbVf8* also contained a putative SET domain. SET domains are thought to be an epigenetic regulator of gene expression during development and modulate chromatin structure. The reduction in virulence by the knockout mutants and restoration of virulence by the complementation strain during pathogenesis assays on green cabbage strongly supports that *AbVf8* is involved in the regulation of pathogenicity (Cho *et al.*, 2012).

Amr1 knockout mutant produces albino colonies, which is mainly because of absence of pigment melanin. Melanin is a ubiquitous pigment that plays an important role in protecting fungi from the damaging effects of environmental stress. It accumulates in the cell walls of hyphae and conidia during the late stationary phase of mycelial growth. The homologs of *Amr1* also produced similar albino colonies when the respective genes were knocked out in other fungi (Tsuji *et al.*, 2000b; Eliahu *et al.*, 2007a; Kihara *et al.*, 2008). Normally in a knock out mutant we see a loss of function phenomenon but unexpectedly, the *Amr1* gene knockout mutants were more virulent than the wild type, producing larger lesions (Table 1 and figure). However, mutation of *Amr1* homologs in the phytopathogenic fungi *Magnaporthe grisea* and *Colletotrichum lagenarium* produced melanin-deficient conidia, but their pathogenicity was not affected (Tsuji *et al.*, 2000b). (Tsuji *et al.*, 2000b). Melanin deficiency caused by a mutation of other genes in the melanin biosynthesis pathway was associated with a loss of pathogenicity in *M. grisea* and *C. lagenarium* (Chumley and Valent, 1990; Howard *et al.*, 1991; Kubo *et al.*,

1991) and a decrease in virulence in the opportunistic human pathogens *Aspergillus fumigatus* and *Waggiella dermatitidi*s (Langfelder *et al.*, 1998; Feng *et al.*, 2001). In most other plant-pathogenic fungi, melanin deficiency usually had little effect on pathogenesis under controlled laboratory conditions (Guillen *et al.*, 1994 ; Kawamura *et al.*, 1999; Kihara *et al.*, 2004; Moriwaki *et al.*, 2004), but the fungi were nonpathogenic under field conditions (Guillen *et al.*, 1994). In summary, the increased virulence of the *Δamr1* mutants was unexpected and unique among all melanin-deficient mutants of other pathogenic fungi (Cho *et al.*, 2012)

The *AbPacC* gene has a single copy in the genome sequence of *A. brassicicola* and shows a high sequence similarity (E=0.0) with the *PacC* gene in other fungi. *PacC* is the transcription factor in the pH regulation pathway of *Aspergillus nidulans* (Penalva and Arst, 2004) and controls the expression of the fumonisin toxins in *Fusarium verticillioides* (Flaherty *et al.*, 2003) and endopolygalacturonase and oxalic acid in *Sclerotinia sclerotiorum* (Rollins and Dickman, 2001; Rollins, 2003a). Mutants of homologous genes in *S. sclerotiorum, Candida albicans*, and *F. oxysporum* show reduction in virulence. (Caracuel *et al.*, 2003; Davis, 2003; Rollins, 2003a). Though the *PacC* knockout mutant showed slow growth in presence of NaCl which induces ionic osmotic stress, its exact function in patho-physiology of *A. brassicicola* remains elusive. *Ab397031*and *Ab 397032* showed reduction in virulence and good candidates for further studies of pathogenicity associated genes. The *Ab39732* is annotated as *Pex10* gene. The *Pex* family of genes is essential components of peroxisome import machinery. In *A. brassicicola* Kim et al knocked out, another member of *Pex* gene –*Pex 14* but the mutants did not show any effect on virulence (Kim et al 2009). Interestingly the pex10 gene discovered in this study were nonpathogenic that is they did not produced any disease symptom or lesion. *Ab9637* is also a very interesting gene discovered in this study that though had no effect on virulence but the

mutants did not produce conidia from protoconidia. Similar phenotypes were also observed when *Amk1* gene a homolog of MAPK Fuss3/Kss1 was knocked out in *A. brassicicola* (Cho *et al*., 2007). Disrupted *amk1* mutants also produced no conidia, but unlike *Ab9637*, *Amk1* mutants were nonpathogenic. We speculate that *Ab9637* could be a downstream transcription factor for *Amk1*. Establishment of this notion will enhance our understanding of the signal transduction pathway involved in pathogenesis in *A. brassicicola*. In summary, this study generates a wealth of information regarding the putative regulatory roles of zinc finger domain transcription factors that can serve as a starting point for the detailed study of pathogenesis mechanisms in *A. brassicicola* and many other necrotrophic fungi.

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CHAPTER 3

Targeted Gene Deletion of Zn(II)2Cys6 Transcription Factors for Identification of Pathogenesis Associated Genes.

Abstract

$Zn(II)_2Cys_6$ transcription factors also known as Zinc binuclear transcription factors or C_6 transcription factors are exclusively present among members of fungal kingdom. The identification of pathogenesis associated transcription factors among $Zn(II)_2Cys_6$ transcription factors makes them active target for developing control measures and to check the proliferation of fungi as a plant pathogen. In the present study we made knockout mutants 35 such transcription factors by systematically deleting them from the genome of *Alternaria brassicicola.* Out of the 35 gene deleted only one did not produced any viable colonies. Of the remaining 34 genes more than 100 transformed colonies were obtained per plate. The southern hybridization verified 32 genes showing true gene deletion mutants while 2 showed presence of 5' or 3' insertion events. The pathogenesis assays on detached leaves of green cabbage resulted in identification of 6 genes associated with pathogenesis. All the 6 genes were novel and are annotated as hypothetical proteins. 1 gene out of the 6 gene identified whose mutants were nonpathogenic was involved in the penetration or early colonization of host plants. This gene switched the life cycle of the fungus from saprophytic to parasitic. This gene is referred as a pathogenicity factor. It is the only second pathogenicity factor known in *Alternaria brassicicola*. Another gene whose mutants had >70% reduction in virulence was associated with detoxification of phytoalexins, which is important for early plant colonization. Remaining 4 genes also showed severe reduction in lesion diameters and not characterized for their function. The results from the present study can serve as future targets for disease management.

3.1 Introduction

The structural diversity among the zinc finger domains containing transcription factors is because of the presence of varied DNA binding motifs which are conserved among members of different families (Miller *et al.*, 1985; Shelest, 2008). The $Zn(II)_2Cys_6$ or zinc cluster or zinc binuclear cluster is an important family of zinc finger transcription factors. The members of this transcription factor family have the conserved motif CysX2CysX6CysX5–12CysX2CysX6–8Cys and are found exclusively among members of fungal kingdom (MacPherson *et al.*, 2006). The cysteine residue binds two zinc atoms to coordinate folding and secondary structure formation of the zinc cluster protein (Klug, 2010). Zinc cluster proteins have a modular structure and contain several functional domains apart from the cysteine-rich DNA binding domain (DBD), including the regulatory and activation domains (fig. 3.1). The metal binding region of DBD consists of cysteine rich region where two zinc atoms are nested and is bridged by a pair of alpha helices consisting of 6 cysteine residues (Gardner *et al.*, 1991; Rodgers and Coleman, 1994). The cysteine rich DBD is commonly located at the N terminus of the protein. The zinc cluster protein has been found in many ascomycetes: *Fusarium* (Khan *et al*., 2003; Son *et al*., 2011), *Magnoporthe* (Dean *et al.*, 2005), *Trichoderma* (Aro *et al.*, 2001), *Saccharomyces, Aspergillus, Kluyveromyces, Neurospora, Schizosaccharomyces* (MacPherson *et al.*, 2006), *Candida* (Maicas *et al*., 2005), *Colletotrichum* (Tsuji *et al*., 2000a), *Cercospora* (Chung *et al.*, 2003) and *Penicillium* (Abe *et al.*, 2002) species. The zinc cluster proteins have been identified exclusively in fungi (Drobna *et al.*, 2008) and the first- and best-studied in this cluster is Gal4p, a transcriptional activator of genes involved in the catabolism of galactose in *S. cerevisiae* (MacPherson *et al.*, 2006). Most transcription factors that contain a $Zn(II)_2Cys_6$ binuclear zinc

cluster DNA binding domain recognize a CGG motif (Schjerling and Holmberg, 1996; Todd and Andrianopoulos, 1997). CGG with some variations is the common motif used by the zinc binuclear protein as recognition site. The variation in the CGG triplets can produce three different patterns; palindromic repeats or inverted repeats (CGG-CCG), direct repeats (CGG-CGG), or everted repeats (CCG-CGG) (Mamane *et al.*, 1998; MacPherson *et al.*, 2006). They can also interact with DNA as monomers, homodimers, or heterodimers (Fig.3.2) (Lohr *et al.*, 1995; Schjerling and Holmberg, 1996; Todd and Andrianopoulos, 1997; Shelest, 2008). However, each individual protein in this family displays variation in the number of nucleotides between the two CGG repeats. For example, GAL4p and LAC9p bind to CGGN11CCG, PPR1p and UAY bind to CGGN6CCG, and PUT3p binds to CGGN10CCG (Todd and Andrianopoulos, 1997).

Fig 3.1: Modular structure of $Zn(II)_2Cys_6$ TFs. Zinc cluster transcription factors can be separated into three functional domains: the DBD, the regulatory domain, and the acidic region. In addition, the DBD is compartmentalized into subregions: the zinc finger, the linker, and the dimerization domain. These regions contribute to DNA-binding specificity and to protein-DNA and protein-protein interaction. MHR, middle homology region (MacPherson *et al.*, 2006)

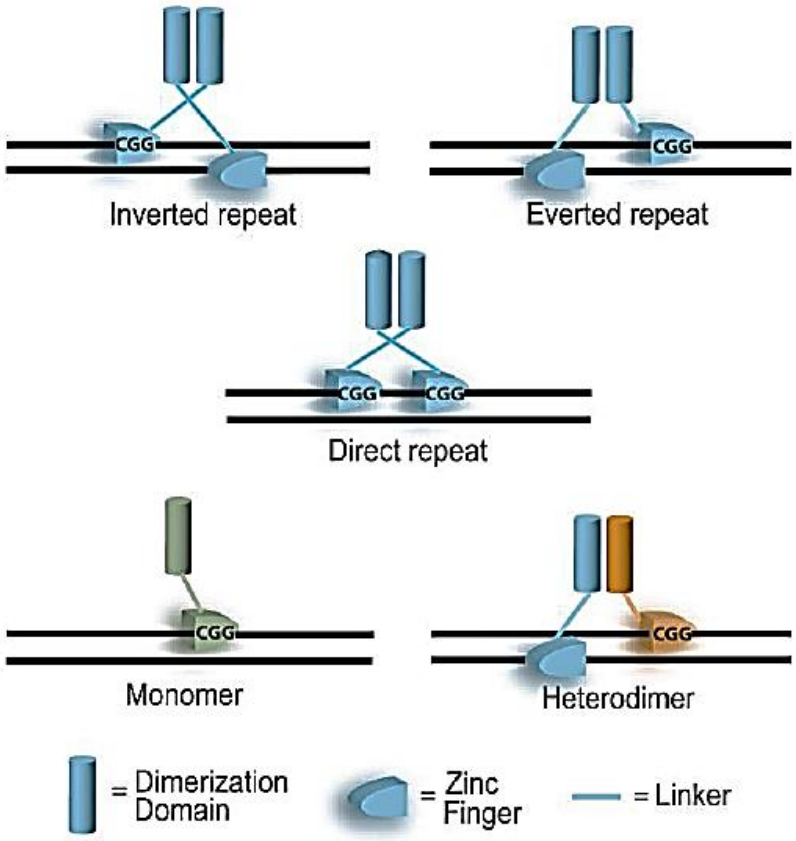

Fig. 3.2: A model for zinc cluster protein DNA recognition. Zinc cluster proteins preferentially bind to CGG triplets that can be oriented in three different configurations: the inverted, everted, and direct repeats. The orientation of CGG triplets and the nucleotide spacing between the triplets are the two major determinants of DNA-binding specificity. Zinc cluster proteins can also bind as monomers (in green) as well as homodimers (two molecules in blue) and heterodimers (one molecule in blue and one in orange) (MacPherson *et al.*, 2006)

Zinc binuclear transcription factor family is involved in plethora of cellular and metabolic functions. These processes include sugar metabolism, gluconeogenesis and respiration, amino acid metabolism, nitrogen utilization and vitamin synthesis. Also they orchestrate mitosis, meiosis, chromatin remodeling. A large proportion of zinc cluster proteins are involved in cell's response to stress and antifungal multi drugs or PDRs (pleiotropic drug response) (Nishimura *et al.*, 1992; Lohr *et al.*, 1995; Todd and Andrianopoulos, 1997; Huh *et al.*, 2003; MacPherson *et*

al., 2006). Many of these transcription regulators have one than one overlapping functions while some of them are exclusively controlling a specific function (Akache *et al.*, 2004).

Presently many zinc binuclear transcription factors have been characterized but most of them have shown to regulate either metabolic or cellular activity (Son *et al.*, 2011). Pathogenesis is an important mechanism that is essential for survival of many plant pathogenic fungi and a fungal specific transcription factor exclusively regulating pathogenesis can be a very attractive target for controlling pathogenesis without effecting other plants and animals.

In this study we made gene knockout mutants of 35 $Zn(II)_2Cys_6$ binuclear transcription factors to determine the TFs among this group involved in pathogenesis in plant pathogenic fungi *Alternaria brassicicola.* Also the knockout mutants were made by deletion or replacement of the target gene with selectable marker gene hygromycin B phosphotransferase-encoding gene (hph) gene. This method ensured that no part of the sequence is left in the genome that may make a partial polypeptide as well as also ensures that no wild type reversal occurs due to popping out of the mutant sequence.

3.2 Materials and Methods

3.2.1 Fungal strain, host plants media, genes used and transformation:

In this study also we used the same strain of *Alternaria brassicicola* isolate ATCC 93866 as was used in the previous chapter. Same media were used as described in chapter 2. We used cabbage (*Brassica oleracia var. capitata*) and *Arabidposis thaliana* ecotype Columbia and its mutant *pad-3* as host plants for pathogenesis assays. There are 73 zinc binuclear fungal specific transcription factors annotated in the *Alternaria brassicicola* genome. Out of these 35 were knocked out in this study. Protoplasts were generated and transformed by PEG mediated heat shock method as described in chapter 2.

3.2.2 Primer design and generation of targeted gene mutants

The genomic DNA sequence for each targeted gene was retrieved from the public database for each gene and putative start and stop codons were identified from the sequence. Two sets of primers P1, P2 and P5, P6 were designed to amplify flanking 5' and 3' flanking regions of the target gene locus respectively, using Primer3 (Fig3.3). Another set of primers P3 and P4 were designed to amplify the *Hyg B* selectable marker gene cassette (1,436 bp) from pCB1636 (Sweigard *et al.*, 1995). 20 nucleotide complementary tails were added to primer P2 and primer P3 and similar tails were added between primer P5 and P6. In first PCR reaction the 5' and 3' regions from the genomic DNA and *Hyg B* locus from pCB1636 were amplified in two separate reactions. The first PCR reaction was carried out in 25 µl reaction volume to amplify 1-

1.5 kb by 94°C, denaturation for 5 minutes followed by 30 cycles of 15 seconds denaturation at 94°C, 15 seconds annealing at 55°C and 2 minutes elongation at 72°C. After the cycling reactions, the final elongation was performed at 72°C for 7 minutes. The amplicons from first PCR reaction were mixed in equimolar ratio and used as template for the second PCR reaction using primer P1 and P6. The complementary tails pairs to form a bridge and replace the target gene with selection marker *Hyg. B* gene. The PCR condition for this reaction is 94°C denaturation for 1.5 minutes followed by 33 cycles of 10 seconds denaturation at 94°C, 10 seconds annealing at 55°C and 4 minutes of elongation at 68°C. The cycling reaction was followed by the final elongation at 68°C for 7 minutes. The PCR product was size fractionated on 1% agarose gel by electrophoresis. The successful pairs were scaled up to amplify a 200 µl reaction volume and the PCR products were purified with a PCR purification kit. (Qiagen, Palo Alto, CA, USA)

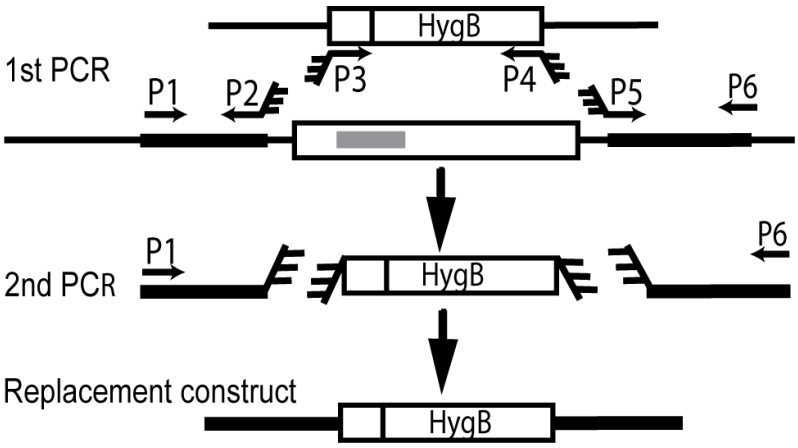

Fig3.3. Production of target gene replacement constructs by PCR. P1, P2, P3, P4, P5 and P6 represent primer pairs designed to amplify their respective position as described in the text. The *Hyg B* selectable marker gene is cloned in the plasmid pCB1636 and is amplified in a separate reaction.

3.2.3 Single spore purification, DNA extraction and southern verification of true mutants:

The 5-9 mutant colonies were selected and single spore isolated twice to ensure the purity of mutants. Purified colonies were grown in liquid cultures and DNA was extracted as described in the chapter 2. For verification of true mutants a total of 3 µg of DNA was digested with appropriate restriction enzyme (New England biolabs, Ipswich, MA, USA). The digested DNA was size fractionated on 0.7% agarose gel and transferred to Hybond N+ nylon membrane (Roche Diagnostics, Indianapolis, IN USA). Gene specific and hygromycin B specific DNA probes were synthesized by amplifying and labeling the amplicons in a PCR reaction using a PCR digoxigenin (DIG) probe synthesis kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). N+ nylon membrane was washed in a final solution of 0.1x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at 68ºC. Chemiluminescent detection was used to develop the blots with CDP-Star using the DIG detection kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Hybridization of membranes was performed at 45ºC using the Block and Wash Buffer set according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The blot was hybridized twice, first with gene specific and second with hygromycin specific probe. Before undergoing the second blotting the blot was stripped as per the manufacture's protocol.

3.2.4 Pathogenicity assays

Pathogenicity assays were performed with modifications as described previously using detached leaves harvested from 5- to 8-week-old plants or on the leaves of whole plants as described in chapter 2.

3.3 Results

3.3.1 Knock out and Screening of mutants

The linear minimal constructs for gene replacement were designed as described in the methods section and were used for transformation of fungal protoplasts. The transformation method used for disruption of gene encoding for Cys_2His_2 transcription factor (as described in the chapter 2, methods section) was also used for deleting a set of 35 Zn(II)Cys6 binuclear cluster domains containing transcription factors encoding genes from the *Alternaria brassiciola* genome and replacing them with a hygromycin B phosphotransferase (*Hyg B*) cassette. All the genes were successfully deleted producing mutant colonies except for one gene where in two different attempts the transformation failed producing no colonies. For rest of the genes more than 100 colonies were produced on average in each plate from transformed protoplasts in a selective medium containing hygromycin. A few colonies were selected (from 4 to 10) for two rounds of single spore purification to avoid selection of heterokaryons containing wild type and mutant nuclei. DNA isolated from them was used for southern verification. The southern hybridization results for 175 transformants showed 62% (108/175) efficiency of targeted gene deletion by replacement of the targeted gene with either single or multiple copies of *Hyg B* cassettes (table 3.1). Further 29.1% (51/175) of the transformants showed integration of *Hyg B* cassette at 5' or 3' end of the target gene by single homologous recombination event and were did not delete the targeted gene from the genome. Meanwhile 9.1% (16/175) transformants showed insertion of *Hyg B* cassette at some undefined location. To this point out of 35 attempted genes for making knocked out mutant we were successful with 32 genes which were replaced by

at least one single or multiple copies of HygB cassette two genes produced only 5' or 3' insertion mutants and one gene did not produce any colonies. The identified true mutants were later used for assessing their phenotypic effect on the pathogenesis trait. The pathogenicity assay performed on the detached leaves of green cabbage identified five genes that are virulence factors that is their knockout mutants showed severe reduction in virulence and one pathogenicity factor whose knockout mutant showed complete loss of virulence. Five of the six transcription factor encoding genes showing regulatory effects on pathogenesis are novel while one gene *Abftf14* is annotated as Cutinase TF1alpha in *Aspergillus oryzae* (E=0.0). In the following sub sections the details of each of genes are presented along with their pathogenicity results.

Table 3.1 Summary of the gene deletion knock out mutants produced in this study

Gene ID	Locus[a]	Location[b]	BlastP (GenBank No.)	E-value	PFAM[c]	D[d]	5'[e]	3'[f]	M[g]	U[h]	P[i]	Function[j]
AbVf01	AB07899.1	Contig9:337826-340517	XP_003833681.1	0.00E+00	PF00172	7	0	0	0	1	N	Hypothetical protein
Abpf02	AB06533.1	Contig6:804771-806133	XP_003295651.1	0.00E+00	PF00172, PF04082	7	0	0	0	0	R	Hypothetical protein
Abftf4	AB07997.1	Contig9:617522-619876	XP_003301831.1	0.00E+00	PF04082	9	0	0	0	0	P	Hypothetical protein
Abftf5	AB05282.1	Contig4:1982481-1983863	XP_003299229.1	0.00E+00	PF04082	6	0	0	0	0	R	Hypothetical protein
Abftf6	AB02626.1	Contig1:3612697-3616028	XP_001938725.1	0.00E+00	PF04082	7	0	1	0	0	P	Hypothetical protein
Abftf7	AB03485.1	Contig2:2191819-2194417	XP_003845285.1	1.00E-53	PF04082	5	0	0	0	0	P	Hypothetical protein
Abftf8	AB00227.1	Contig0:631307-633744	XP_001940163.1	0.00E+00	PF00172	8	0	1	0	0	R	C6 TF
Abftf9	AB03759.1	Contig3:398265-400830	XP_001934154.1	0.00E+00	PF04082	6	0	2	0	0	P	Fungal specific TF
Abftf11	AB03633.1	Contig3:37786-43168	XP_003305597.1	0.00E+00	PF00172, PF04082	0	4	0	1	0	P	Hypothetical protein
Abftf12	AB08440.1	Contig10:594364-596776	XP_003299884.1	0.00E+00	PF00172, PF04082	2	0	1	1	0	P	Hypothetical protein
Abftf13	AB08084.1	Contig9:821936-823728	XP_003833780.1	0.00E+00	PF04082	2	0	0	0	2	P	Hypothetical protein
Abftf14	AB02376.1	Contig1:2972695-2975173	XP_001941428.1	0.00E+00	PF00172, PF04082	4	0	0	0	0	R	Cutinase TF1 alpha
Abftf16	AB09807.1	Contig15:525057-527152	XP_001936803.1	0.00E+00	PF04082	1	0	2	1	0	P	Predicted protein

Table 3.1 continued....

Gene ID	Locus[a]	Location[b]	BlastP (GenBank No.)	E-value	PFAM[c]	D[d]	5'[e]	3'[f]	M[g]	U[h]	P[i]	Function[j]
Abftf18	AB05039.1	Contig4:1294390-1297964	XP_003299776.1	0.00E+00	PF00172, PF04082	2	1	1	1	0	P	Hypothetical protein
Abftf19	AB08099.1	Contig9:861728-864023	XP_001933261.1	0.00E+00	PF04082	2	2	0	1	0	P	Predicted protein
Abftf20	AB06115.1	Contig5:2101991-2106460	XP_001933942.1	0.00E+00	PF00171, PF04082	3	0	1	0	0	P	Aldehyde dehydrogenase
Abftf21	AB06774.1	Contig6:1484051-1486188	XP_003305899.1	0.00E+00	PF00172, PF04082	1	1	0	2	0	P	Hypothetical protein
Abftf22	AB06225.1	Contig5:2385650-2389002	XP_003303904.1	0.00E+00	PF00187, PF01522, PF00172	0	0	1	1	1	P	Hypothetical protein
Abftf24	AB03898.1	Contig3:757783-760271	XP_001934500.1	0.00E+00	PF04082	1	4	0	0	0	R	Hypothetical protein
Abftf25	AB03734.1	Contig3:329182-331468	XP_003299473.1	0.00E+00	PF04082	2	1	0	0	2	P	Hypothetical protein
Abftf26	AB01090.1	Contig0:3064316-3066045	XP_001941856.1	0.00E+00	PF04082	4	0	1	0	0	P	Fungal specific TF
Abftf28	AB08540.1	Contig10:875064-877422	XP_003302244.1	0.00E+00	PF00172, PF04082	0	0	4	0	1	X	Hypothetical protein
Abftf29	AB06017.1	Contig5:1799186-1804091	XP_001933904.1	0.00E+00	PF00172, PF04082	0	0	3	2	0	P	Zinc finger TF
Abftf30	AB07737.1	Contig8:1312294-1315789	XP_003302443.1	0.00E+00	PF04082	4	0	1	0	0	P	Hypothetical protein
Abftf31	AB02139.1	Contig1:2238879-2241341	XP_001938997.1	0.00E+00	PF04082	2	0	0	2	1	R	Quinic acid utilization

Table 3.1 continued….

Gene ID	Locus[a]	Location[b]	BlastP (GenBank No.)	E-value	PFAM[c]	D[d]	5'[e]	3'[f]	M[g]	U[h]	P[i]	Function[j]
Abftf32	AB04206.1	Contig3:1541369-1543321	XP_003306883.1	0.00E+00	PF03902, PF04082	1	0	2	1	0	P	Hypothetical protein
Abftf33	AB00953.1	Contig0:2657498-2658308	XP_003301165.1	0.00E+00	PF04082	0	0	1	2	2	P	Hypothetical protein
Abftf35	AB00608.1	Contig0:1703232-1704065	XP_003302917.1	1.00E-108	No PFAM Value	0	4	0	0	1	X	Hypothetical protein
Abftf36	AB00597.1	Contig0:1672628-1675502	XP_003302928.1	0.00E+00	PF00172, PF04082	1	1	0	0	4	P	Hypothetical protein
Abftf37	AB03600.1	Contig2:2587341-2589563	XP_003296110.1	0.00E+00	PF00172, PF04082	1	2	1	0	0	P	Hypothetical protein
Abftf38	AB08475.1	Contig10:683918-687157	XP_001932420.1	0.00E+00	No PFAM Value	2	1	2	0	0	P	Hypothetical protein
Abftf50	AB05941.1	Contig5:1589479-1591412	CCF33278.1	7.00E-84	No PFAM Value	4	0	0	0	0	P	Hypothetical protein
Abftf53	AB07610.1	Contig8:952146-955267	XP_001931163.1	0.00E+00	PF04082	2	0	1	0	1	P	Cutinase TF 1 alpha
Abftf55	AB00158.1	Contig0:431448-434309	XP_003302313.1	0.00E+00	PF00172, PF04082	0	3	1	1	0	P	Hypothetical protein

a: Locus number corresponds to that assigned to individual protein-coding genes in the *A. brassicicola* genome assembly.
b: Corresponds to location on individual scaffolds in the machine-annotated *A. brassicicola* genome assembly.
c: The domains assigned by the PFAM searches
d: The number of true single copy replacement mutant colonies produced as a result of double recombination events
e: The insertion of replacement construct into the 5' end of target gene as a result of single homologous recombination event.
f: The insertion of replacement construct into the 3' end of target gene as a result of single homologous recombination event.
g: colonies with incorporation of multiple copies of construct for replace of target gene.
h: Un explained southern pattern.
i: The pathogenicity status of the mutants. N= non-pathogenic, R= reduced virulence, X= no results because all mutants were ectopic insertions.

Following subsection describes each mutant obtained in this study.

3.3.2.1 *Abvf1*

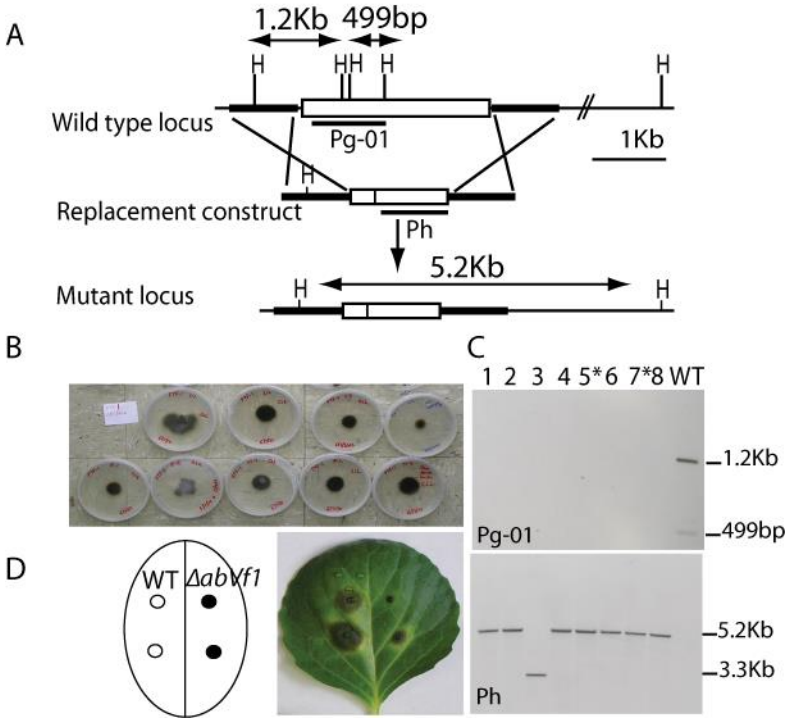

Fig.3.4: Targeted deletion and pathogenicity assay of *AbVf1* gene. **A**. Schematic diagram representing the wild-type locus, replacement construct, and mutant locus, it also shows the double recombination event that occurred to produce the 8 transformants that were verified. The details of this process is already discussed in the method section, upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-01 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 9 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** 8 clones (1 clone was deleted as the DNA quality was not optimum for southern) were used in southern hybridization with two probes as described above. When hybridized with gene specific probe only band of appropriate size appeared in wild type lane showing the presence of gene wild type while deletion from all mutant clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and have replaced the wt gene locus. Asterisks (*) indicate DNA lanes of mutants which were used for pathogenicity assays. **D** Decreased virulence of *Δabvf1* mutants on host plants (*Brassica oleracea*). The leaf was detached and photographed at the end of the assay (table 3.2). Abbreviations: H = *Hind*III enzyme digestion site, wt= wild type.

Table 3.2: Decreased virulence of two strains of *Δabvf1* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabvf1-7	2000	5	17.3±3.8	8.3±6.0	52%	0.001
Δabvf1-7	1000	5	18.6±2.1	9.0±6.0	52%	0.010
Δabvf1-5	2000	13	13.1±5.8	3.9±2.7	70%	6.2E-05
Δabvf1-5	3000	8	15.3±2.8	1.5±1.3	90%	4.8E-05
Δabvf1-5	1500	7	11.6±3.2	0.3±0.8	97%	6.2E-06
Δabvf1-5	3000	9	18.3±2.8	3.4±1.5	81%	1.5E-05
Δabvf1-5	1500	7	17.1±4.1	1.0±2.4	94%	0.0002
Δabvf1-5	3000	12	12.3±2.7	3.5±4.4	72%	0.0070
Δabvf1-5	1500	16	12.5±2.3	4.3±2.7	66%	0.0030
Δabvf1-5	3000	10	13.8±1.8	6.2±4.3	46%	6.2E-06
Δabvf1-5	1500	12	12.3±5.4	4.6±5.1	63%	4.4E-06
Δabvf1-5	3000	12	10.0±3.0	2.2±0.9	78%	4.0E-06
Δabvf1-5	1500	11	9.3±4.3	2.7±1.2	68%	1.2E-05

Legend for table: d.f. = degrees of freedom, P = probability, lesion size indicates the average lesion diameter.

Abvf1 gene is predicted to code for 808 amino acids with Zn_2Cys_6 DNA binding domain (PF00172, E-value 0.0007) at 18-57 amino acid residue. BLAST (Altschul *et al.*, 1997) survey annotates the gene to code for hypothetical protein and WOLF PSORT (Horton *et al.*, 2007) predicts its subcellular localization in the nucleus and cytosol. Over 100 colonies appeared from

transformation of *A. brassicicola* protoplasts done to create targeted gene knockout mutants. Eight colonies were selected for single spore purification and verification of true mutants i.e. mutants showing replacement of *AbVf1* gene loci with single copy of *Hyg B* cassette (Fig 3.4-B-D). Southern hybridization demonstrated that seven out of eight colonies were true single copy gene replacement insertions. While, remaining one was ectopic insertion (lane no.3 in fig. 3.4). A schematic diagram is shown as fig 3.4 that describes the locus of wild type and mutants and also indicates the location on probes along with the sizes of expected bands in the southern blot. Once the true mutant colonies were identified, two clones namely 5 and 7 were selected from them for pathogenesis assays on the basis of their growth pattern during single spore isolation stage (Fig. 3.4 C) and thirteen pathogenicity assays were performed with these selected clones (Table 3.2) as described in the methods section. Approximately 70% decrease in virulence was observed when lesion size produced by the wild type and the mutant were measured in these assays. This gene was further characterized for its role in virulence and found to be involved in detoxification of phytoalexin- brassinin during the early stages of pathogenesis (see chapter 4). To further substantiate its role in virulence the mutant was complemented with wild type allele of the gene and showed complete restoration of virulence comparable to wild type (fig 3.4 and table 3.2). Also several other deletion mutants were made with different construct and all of them showed similar reduction in virulence. These results are separately discussed in chapter 4.

In all we concluded from all assays that mutants are not virulent as wild types and categorized this gene as a strong virulence factor.

3.3.2.2 *Abpf2*

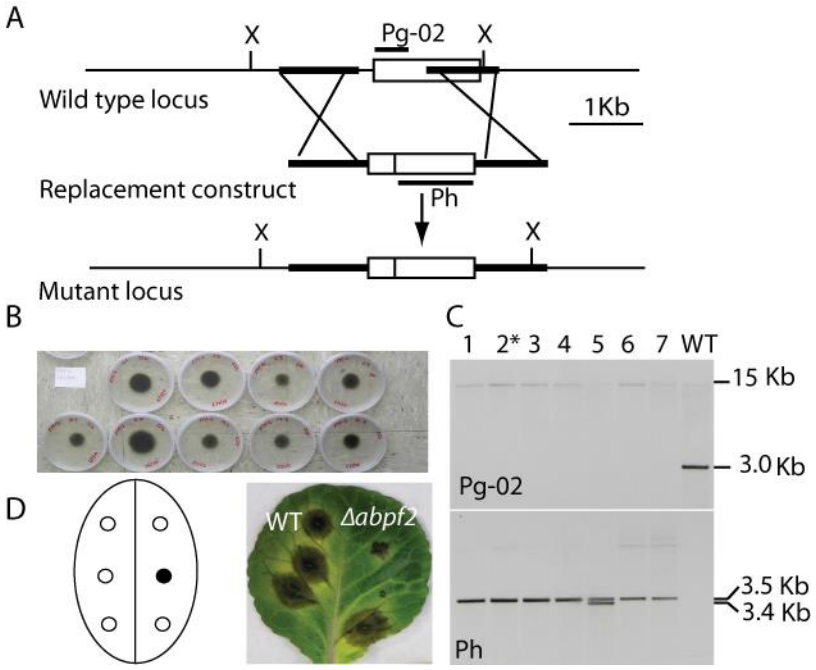

Fig.3.5: **Targeted deletion and pathogenicity assay of *Abpf2* gene**. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, it also shows the double recombination event that occurred to produce the seven transformants that were verified Pg-02 and Ph in the diagram is for the gene specific and Hyg cassette specific probes used for hybridization respectively. **B** nine clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** seven clones out of nine were used in southern hybridization with the two probes as described above. When hybridized with gene specific probe only band of appropriate size appeared in wild type lane showing the presence of wild type gene while it was deleted from all mutant clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and had replaced the wt gene locus. Asterisk (*) indicate DNA lanes of mutants which were used for pathogenicity assays. **D** Loss of virulence *Δabpf2* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with 2,000 conidia of the wild type and *Δabpf2* mutant on a detached leaf (table 3.3). Abbreviations: X = *XbaI* enzyme digestion site, WT= wild type

Table 3.3: Decreased virulence of two strains of *Δabpf2* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabpf2-1	2000	13	12.2±4.9	0.0±0.0	100%	4.24E-07
Δabpf2-2	1500	13	15.2±2.0	0.0±0.0	100%	3.44E-07
Δabpf2-2	3000	13	15.4±2.3	0.0±0.0	100%	5.00E-03

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

Gene *Abpf2* is predicted to encode 630 amino acids. It has Zn_2Cys_6 fungal-specific DNA binding domain (IPR001138, PF00172) predicted by Interpro (Bateman *et al.*, 2004) but not by PSORT (Horton and Nakai, 1997). Homologs of the gene were annotated as either a hypothetical protein or a C6 zinc finger domain protein in other fungal genomes. It is not found as single copy gene; rather we found a gene with 320 amino acids (960 nucleotides) whose nucleotides and predicted amino acids are 86% (797/928) and 88% (268/303) identical to *AbPf2* gene, respectively (Cho et al unpublished). The light bands on the with the gene specific probes shows the presence of homolog of this gene present in the genome as described earlier.

The results of pathogenicity assays done with two different clones of *Δabpf2* mutant shows complete loss of pathogenicity i.e no lesion was developed at the site inoculated by *Abpf2* deletion mutants. Pathogenicity was fully restored when the deletion mutant was complemented with wild type allele *(*Δ*abpf2:ABPf2*), confirming that *abpf2* is a pathogenicity factor. This gene

is the third pathogenicity factor discovered in *A.brassiciola* (previous two are *AbPacC* and *Amk1*), but its discovery is significant because unlike others it shows no influence on vegetative growth or other fungal trait and exclusively regulates pathogenicity. Secondly, coding for a fungal specific transcription factor it is an attractive target for designing anti-fungal agents.

3.3.2.3 *Abftf4:*

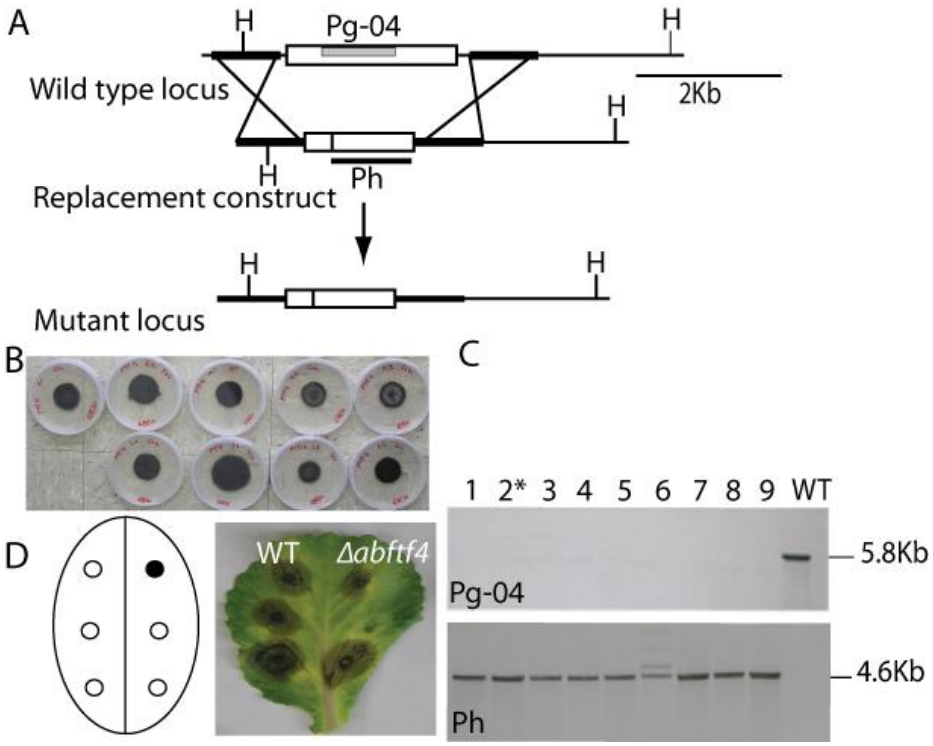

Fig.3.6: **Targeted deletion and pathogenicity assay of *Abftf4* gene**. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the nine transformants that were verified. Probes used for hybridization are indicated as Pg-04 and Ph in the diagram for gene specific and Hyg cassette specific probes respectively. **B**. Nine clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** All nine clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe only bands of appropriate size appeared in wild type lane showing the presence of gene wild type while deletion from all mutant clones, except for the clone in lane 6 shows double band indicating presence of multiple copy insertion. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and have replaced the wt gene locus. An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. **D** Loss of virulence *Δabftf4* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with 2,000 conidia of the wild type and *Δabftf4* mutant on a detached leaf (table 3.4). Abbreviations: H = HindIII

Table 3.4: Decreased virulence of two strains of *Δabftf4* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf4	2000	13	15.6±4.7	12.7±5.4	19%	0.05
Δabftf4-2	2000	5	16.8±3.5	14.0±2.5	17%	0.03
Δabftf4-2	1000	5	19.3±3.3	18.3±3.2	5%	0.42
**Δabftf4-2*	1500	9	12.5±1.2	12.4±1.3	1%	0.88
**Δabftf4-2*	3000	9	17.1±4.1	16.6±5.1	3%	0.35

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Abftf4 is a 670 amino acid encoding gene. Interpro predicts it to encode for Gal4 domain containing fungal specific transcription factor (IPR007219). The initial pathogenicity assays show very small reduction in pathogenicity. To confirm this trend of reduction in virulence we performed a controlled pathogenicity assay with two different concentrations of conidia on 50 days old green cabbage plant leaves. These pathogenicity assays showed negligible reduction in the virulence of mutants in comparison to wild type *A. brassicicola* (results shown as * in the pathogenicity assay table above). Hence we conclude *Abftf4* is not a virulence factor.

3.3.2.4 *Abftf5*

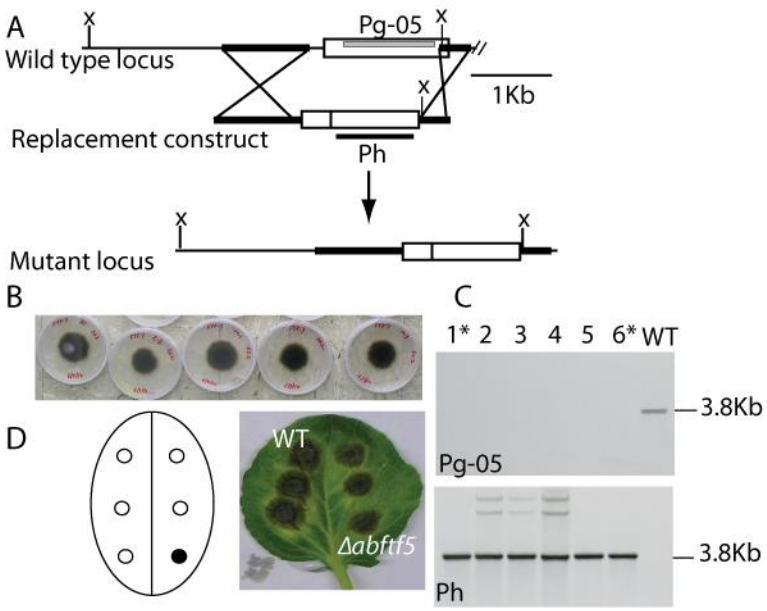

Fig.3.7: Targeted deletion and pathogenicity assay of *Abftf5* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the nine transformants that were verified. Probes used for hybridization are indicated as Pg-05 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. Nine clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** 6 of 9 clones was used in southern hybridization with two probes as described above. When hybridized with gene specific probe only band of appropriate size appeared in wild type lane showing the presence of gene wild type while deletion from all mutant clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and have replaced the wt gene locus. Asterisk (*) indicate DNA lanes of mutants which were used for pathogenicity assays. **D** Loss of virulence *Δabftf5* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with four different concentrations of conidia of the wild type and *Δabftf5* mutant on a detached leaf (table 3.5). Abbreviations: X = *Xba*1 enzyme digestion site, WT= wild type.

Table 3.5: Decreased virulence of two strains of *Δabftf5* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf5-1	3000	11	16.5±3.0	9.7±4.0	41%	0.01
Δabftf5-1	1500	11	15.2±2.7	10.5±6.0	30%	0.01
Δabftf5-6	2000	5	18.0±4.4	10.5±1.0	42%	5.00E-03
Δabftf5-6	1000	5	16.0±8.4	10.8±8.0	32%	6.00E-02

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

Gene *Abftf5* encodes for 450 amino acid residues. The 3' flanking region is shorter than 1 kb length as there is a gap of 720bps. Thus to make deletion construct we included 129 bps from 3'end of the coding region. 6 mutant colonies were selected and southern hybridization shows all six to be single copy replacement mutants. Pathogenicity assays were done with two different clones with different spore concentrations. In all assays it shows reduction in virulence with statistical significance. Future studies using complementation of mutant and more pathogenicity assays can be done to confirm this gene to be a virulence factor.

33.3.2.5 *Abftf6*

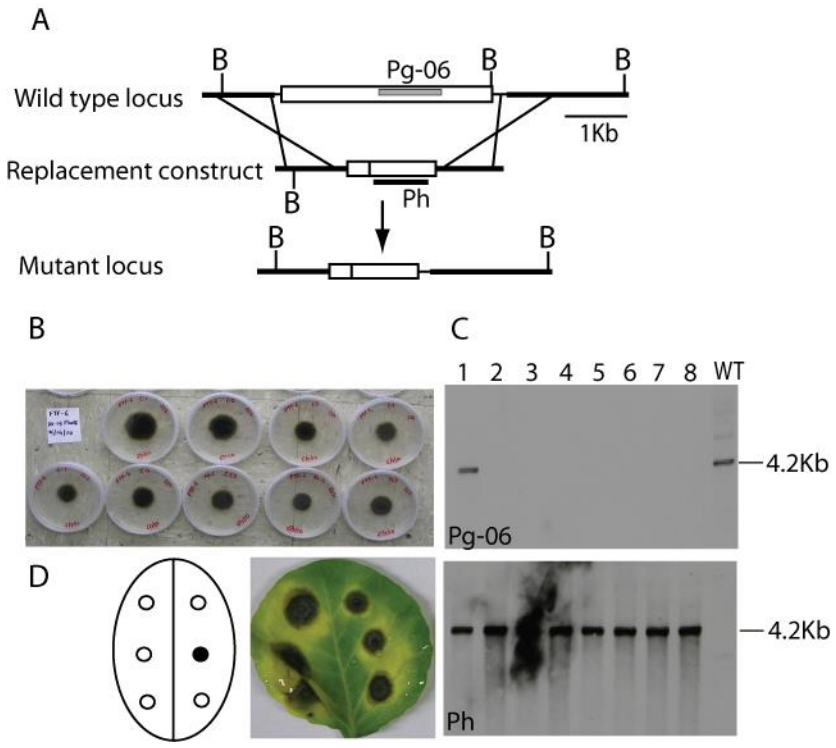

Fig.3.8: Targeted deletion and pathogenicity assay of *Abftf6* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 9 transformants that were verified. Probes used for hybridization are indicated as Pg-06 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 9 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** 8of 9 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe only band of appropriate size appeared in wild type lane showing the presence of gene wild type while deletion in all except in lane E1 where it shows presence of 5' insertion. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and have replaced the wt gene locus. **D** status of virulence *Δabftf6* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf6* mutant on a detached leaf (table 3.6). Asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: B= BamHI enzyme digestion site.

Table 3.6: Decreased virulence of two strains of *Δabftf6* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-6L1	3000	10	18.3±3.5	18.2±4.1	0%	0.78
Δabftf-6L1	1500	7	17.3±4.5	16.8±4.2	3%	0.17
Δabftf-6L1	3000	12	12.5±2.3	13.0±2.9	0%	3.80E-01
Δabftf-6L1	1500	12	12.7±3.0	13.0±3.2	0%	4.60E-01

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

Abftf6 encodes for 915 amino acid residues and have Zn_2Cys_6 fungal-specific DNA binding domain (IPR007219, PF04082) predicted by Interpro (Bateman *et al.*, 2004). This gene is present in a single copy in the *A.brassicicola* genome. A BLAST search annotates it as a hypothetical protein. The pathogenicity assays suggests that this transcription factor is not participating in regulation of virulence trait.

3.3.2.6 *Abftf7*

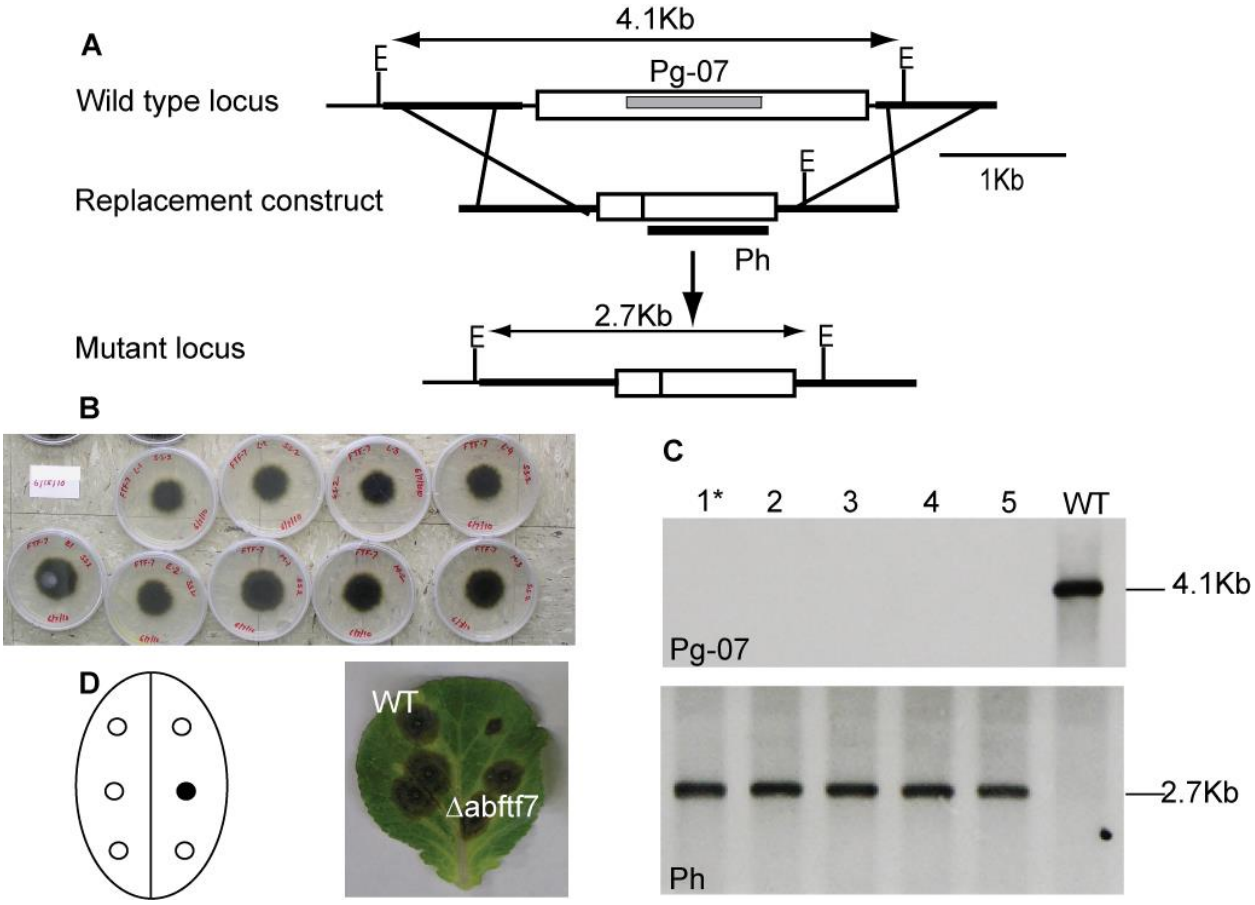

Fig.3.9: Targeted deletion and pathogenicity assay of *Abftf7* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus,showing the double recombination event that occurred to produce the 9 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-07 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 9 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** 5of 9 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe only band of appropriate size appeared in wild type lane showing the presence of gene wild type while deletion in all clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and have replaced the wt gene locus. **D** status of virulence *Δabftf7* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with three different concentrations of conidia of the wild type and *Δabftf7* mutant on a detached leaf (table 3.7). Asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: E=*ECoR*I enzyme digestion site, WT= wild type.

Table 3.7: Decreased virulence of two strains of *Δabftf7* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf7-1	3000	11	16.9±3.4	15.5±3.3	8%	0.01
Δabftf7-1	1500	11	15.7±3.2	14.2±4.2	9%	0.15
Δabftf7-1	2000	5	13.1±4.9	12.6±3.1	4%	6.40E-01

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Abftf7 encodes for 525 amino acid residues and is present in single copy in genome of *A.brassicicola*. The pathogenicity assays shows a decrease of 4-9% in virulence which can be considered as marginal, hence this gene is not a virulence factor in *A.brassicicola*. Their homologs in other fungi are annotated as putative transcription factors and hypothetical proteins.

3.3.2.7 *Abftf8*

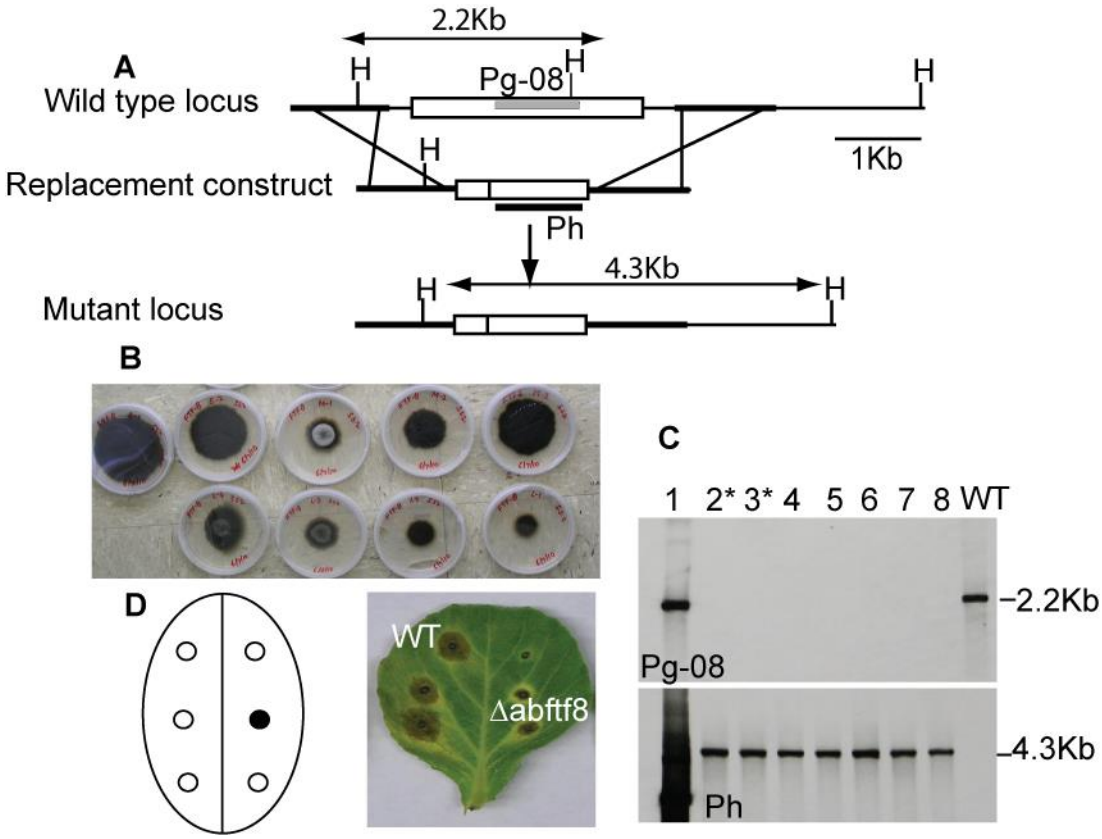

Fig.3.10: Targeted deletion and pathogenicity assay of *Abftf8* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 9 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-08 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 9 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** 8of 9 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe only band of appropriate size appeared in wild type lane and lane 1 showing the presence of gene wild type while deletion in all clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and have replaced the wt gene locus. The presence of bands with both probes in lane 1 suggests of insertion event had occurred and hyg containing gene cassette is ectopically inserted in the genome. **D** status of virulence *Δabftf8* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with three different concentrations of conidia of the wild type and *Δabftf8* mutant on a detached leaf (table 3.8). Asterisks (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: H= HindIII enzyme digestion site, WT=wild type.

Table 3.8: Decreased virulence of two strains of *Δabftf8* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf8-3	3000	5	12.6±2.5	5.3±5.2	58%	0
Δabftf8-3	1000	5	11.7±7.7	3.3±3.7	71%	0.038
Δabftf8-3	3000	5	15.1±6.3	3.3±4.5	79%	2.80E-03
Δabftf8-3	1500	5	16.8±8.8	3.3±5.4	80%	4.10E-03
Δabftf8-2	3000	10	14.3±2.8	12±2.8	15%	7.20E-03
Δabftf8-2	1500	12	13.3.±8.11	8.7±4.7	34%	0.0001
Δabftf8-2	3000	10	14.4.±2.16	6.2±4.2	57%	2.84E-06

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

The gene *Abftf8* is a putative transcription factor that encodes for 682 amino acids. It is present in single copy in genome of *A.brassicicola* and is annotated as novel or hypothetical protein. The pathogenicity assays shows reduction in lesion size produced by mutants in the range of 15% to 80%. But the combined average of reduction in lesion size of all pathogenicity assays done with two clones is 56% that suggests as a strong virulence factor.

3.3.2.8 *Abftf9*

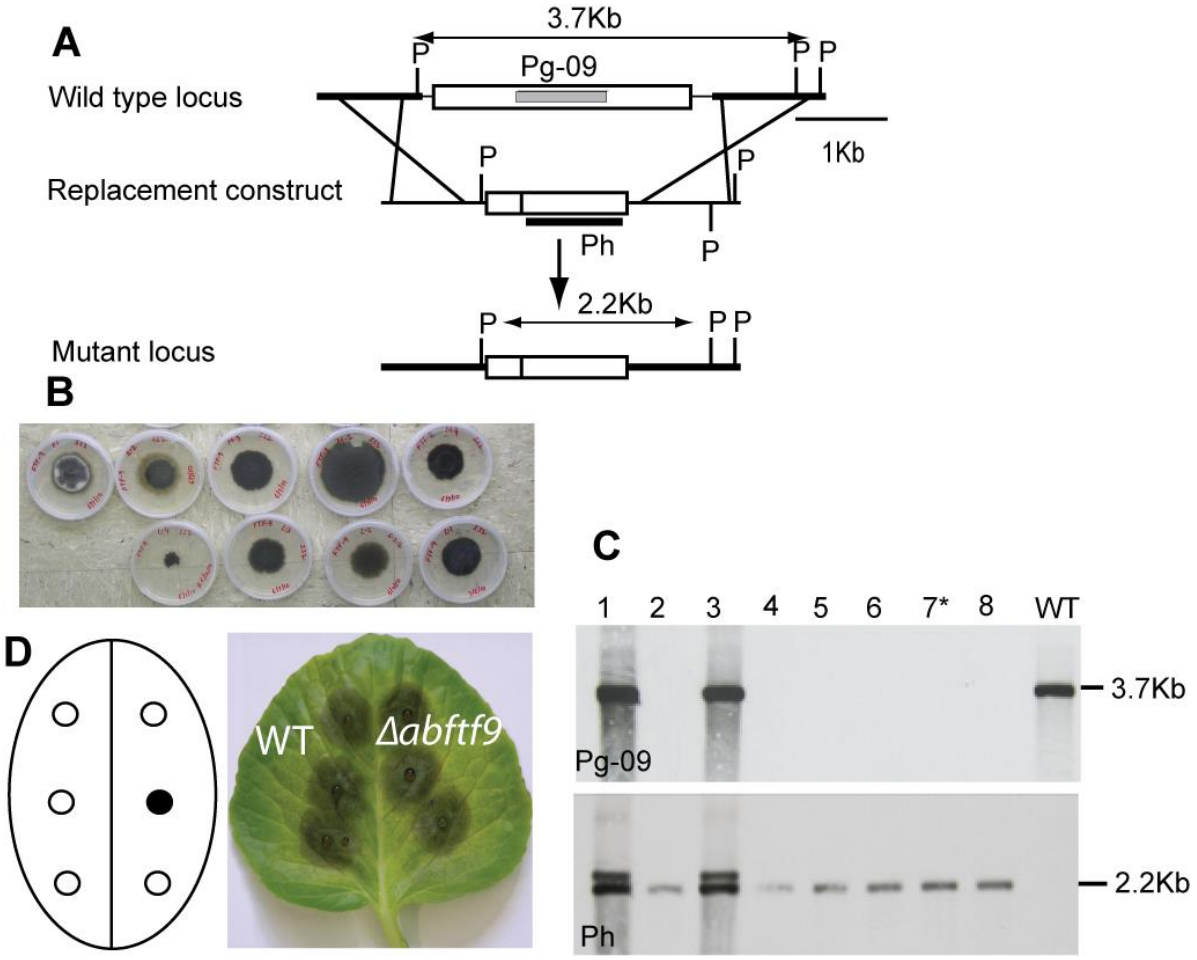

Fig.3.11: Targeted deletion and pathogenicity assay of *Abftf9* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 9 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-09 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 9 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** 8of 9 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe only band of appropriate size appeared in wild type lane and in lane 1 and lane 3 showing the presence of gene wild type while deletion in other clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and have replaced the wt gene locus. The presence of bands with both probes in lane 1 and 3suggests of insertion event had occurred and hyg containing gene cassette is ectopically inserted in the genome. **D** status of virulence *Δabftf9* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with four different concentrations of conidia of the wild type and *Δabftf9* mutant on a detached leaf (table 3.9). Asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: P= *Pst* I enzyme digestion site WT=wild type.

Table 3.9: Decreased virulence of two strains of *Δabftf9* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-9-7	3000	5	14.3±6.0	4.0±5.8	72%	0.000112
Δabftf-9-7	1000	5	7.5±5.3	6.3±5.7	15%	0.481
Δabftf-9-7	3000	5	16.5±7.4	8.6±4.6	48%	8.24E-03
Δabftf-9-7	1500	5	17.0±9.6	6.5±7.4	60%	3.46E-02
**Δabftf-9-7*	2000	10	11..3±2.8	12±2.8	15%	7.20E-03

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Gene Abftf9 is a putative transcription factor that codes for 740 amino acid residues. The pathogenicity assays were done four different concentrations of conidia from one clone. The assays though show internal variability in lesion size. Hence to ascertain that gene *Abftf9* can be considered as one of candidate for virulence factor. We performed controlled pathogenicity assays with two different concentrations of conidia (1000 and 3000 spores in 10µl). In this pathogenicity assay the deletion mutant Δ*abftf9* showed same level of vigor in pathogenesis as wild type. (result shown as *) and therefore we conclude that *Abftf9* has no apparent role in regulation of pathogenesis and is not a virulence factor in *A.brassicicola*.

3.3.2.9 *Abftf11*

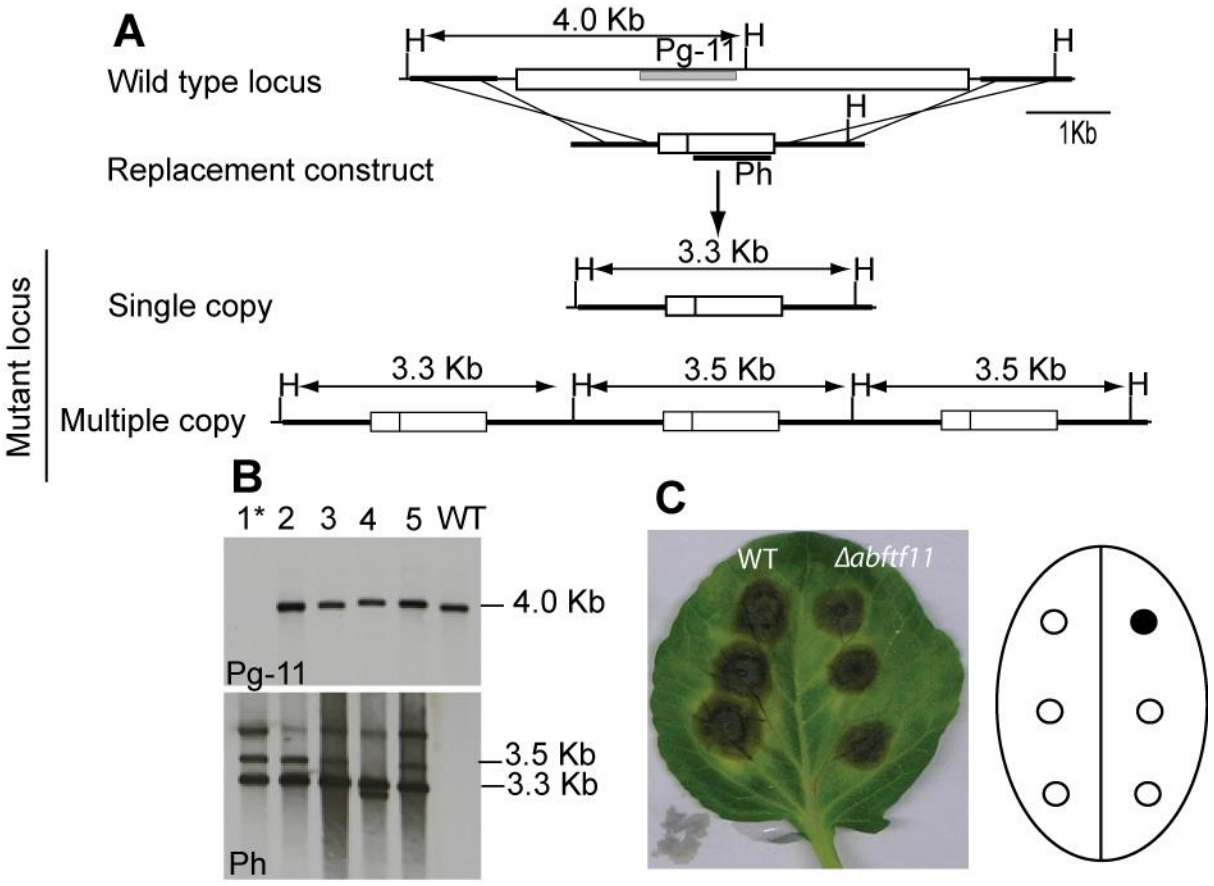

Fig.3.12: Targeted deletion and pathogenicity assay of *Abftf11* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-11 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B** 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in all lanes except lane 1 showing the presence of wild type locus in all lanes except in lane 1 where deletion has occurred in clone 1. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted but have replaced the wt gene locus only in clone 1. The presence of bands with both probes in all lanes except in lane 1suggests of hyg containing gene cassette is ectopically inserted in the genome. **C** status of virulence *Δabftf11* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf11* mutant on a detached leaf (table 3.10). Abbreviations: H=*Hind* IIII enzyme digestion site, WT=wild type

Table 3.10: Decreased virulence of two strains of *Δabftf11*deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf11-1	3000	5	16.3±4.5	14.3±5.7	12%	0.04
Δabftf11-1	1500	5	13.5±2.8	7.5±6.6	44%	0.06
**Δabftf11-1*	2000	17	16.24±1.3	16.6±1.3	-2.54	0.07

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Gene *Abftf11* encodes for 1,173 amino acid putative transcription factor protein. The pathogenicity assays show decrease in virulence, but this decrease are not statistically well supported and shows presence of internal variations. Most of the internal variation arose because of use of old aged leaves which are traditionally more robust and resistant to infection and hence contributed to skewing the data. A controlled pathogenicity assay using leaves from 50 days old plants was done (results are shown by * in the table 3.10) clearly suggest that *Abftf11* gene is not a virulence factor in *A.brassicicola*.

3.3.2.10 *Abftf12*

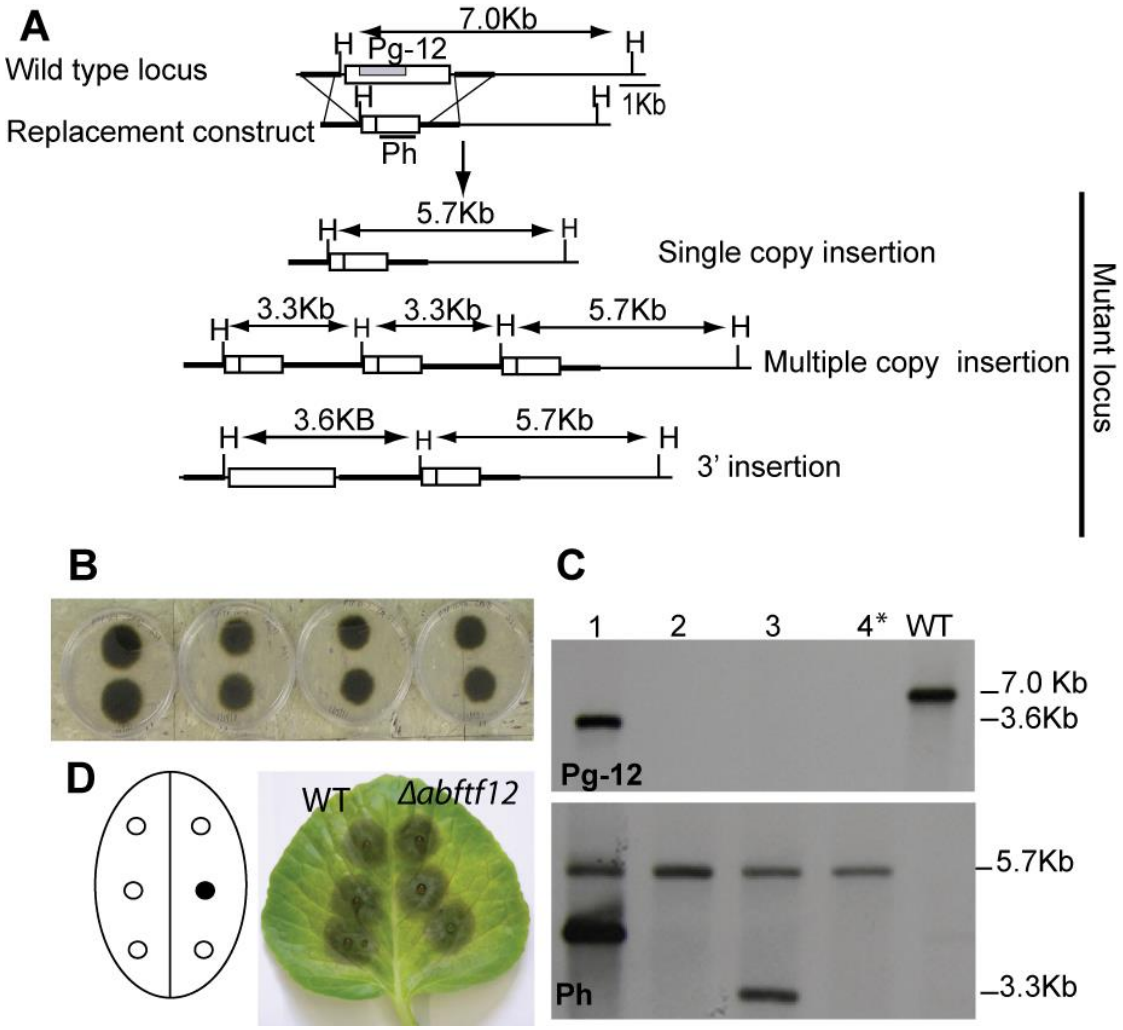

Fig.3.13: Targeted deletion and pathogenicity assay of *Abftf12* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 4 transformants that were verified. The details of this process is already discussed in the method section, upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-12 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**.4 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** Clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane and lane 1 showing the presence of gene in wild type and in clone1 while deletion from all remaining mutant clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. In clone 1 (lane 1) the insertion has occurred as a result of insertion of hyg cassette at 3' end of target gene and the clone 3 (lane 3) suggests incorporation of multiple copies of hyg cassette after replacing target wt gene. Asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: H = *Hind*III enzyme digestion site, WT= wild type.

Table 3.11: Decreased virulence of two strains of *Δabftf12*deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf12-4	2000	7	14±10.1	10.2±9.4	28%	0.1
**Δabftf12-4*	2000	17	15.6±4.7	12.7±5.4	0.18%	0.05

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

The gene *Abftf12* encodes for 728 amino acid protein which is annotated as hypothetical protein in *Pyrenophora teres* (E value=0) and as fungal specific transcription factor domain containing protein on *Ajellomyces dermatitidis* (ATCC18188) (E- value= 4e-144). Though it shows 28% decrease in lesion in one path assay, the high standard deviation does not support decrease in lesion size. A controlled pathogenicity assay was done (results are shown by * in the table 3.11) clearly suggest that *Abftf12* gene is not a virulence factor in *A.brassicicola*

3.3.2.11 *Abftf13*

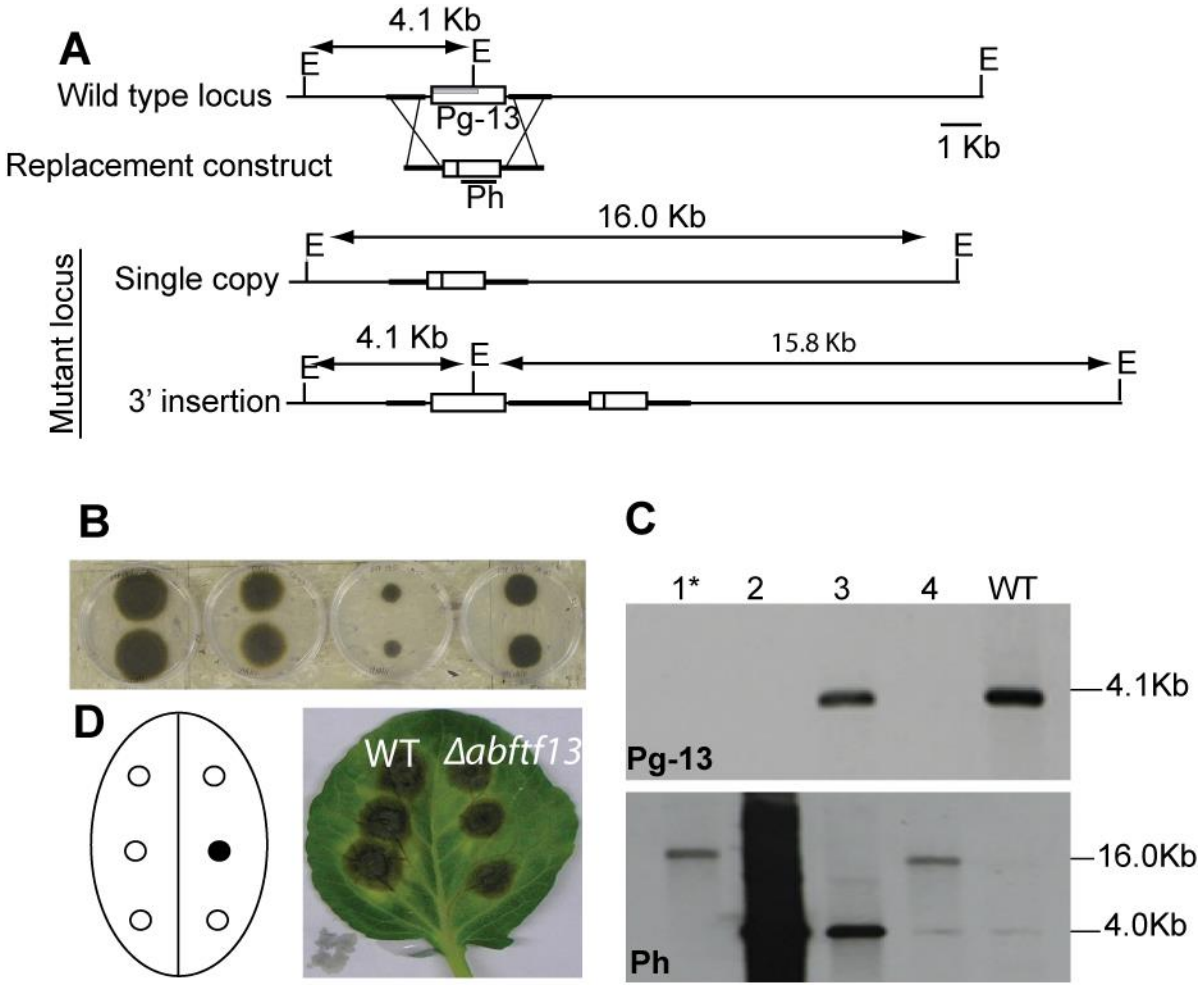

Fig.3.14: **Targeted deletion and pathogenicity assay of *Abftf13* gene**. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 4 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-13 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 4 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** 4 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe band of appropriate size appeared in wild type lane and in lane 3 having clone 3 showing the presence of gene wild type while deletion from all mutant clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. The wt gene was successfully replaced by single copy of Hyg gene cassette in clone 1 (lane 1) while clone 3 (lane 3) shows that hyg gene cassette was inserted at the 3' region as a result of single recombination event. An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. **D** virulence status of *Δabftf13* mutants on host plants (*Brassica oleracea*) as a result of pathogenicity assays. Abbreviations: E = *Eco*RI enzyme digestion site, WT= wild type.

Table 3.12: Decreased virulence of two strains of *Δabftf13* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-13-1	3000	5	18.1±4.5	16.5±6.2	9%	0.15
Δabftf-13-1	1500	5	14.6±3.9	10.3±6.2	24%	0.06
**Δabftf13-1*	2000	17	16.59±1.7	16.8±2.0	-1.77	0.19

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

The gene *Abftf13* encodes for 546 amino acid residue protein and is present in single copy. The pathogenicity assays done with two different concentrations of conidia show decrease in 9% and 24% reduction in lesion. To further confirm that Δ*abftf13* reduces virulence we performed pathogenicity assay with more number of replicates. The result of pathogenicity assay shows an insignificant increase (1.77%) in lesion size produced by the mutants in comparison to the wild type. This clearly suggests that *Abftf13* is not a virulence factor (results are shown as * in table).

3.3.2.12 *Abftf14*

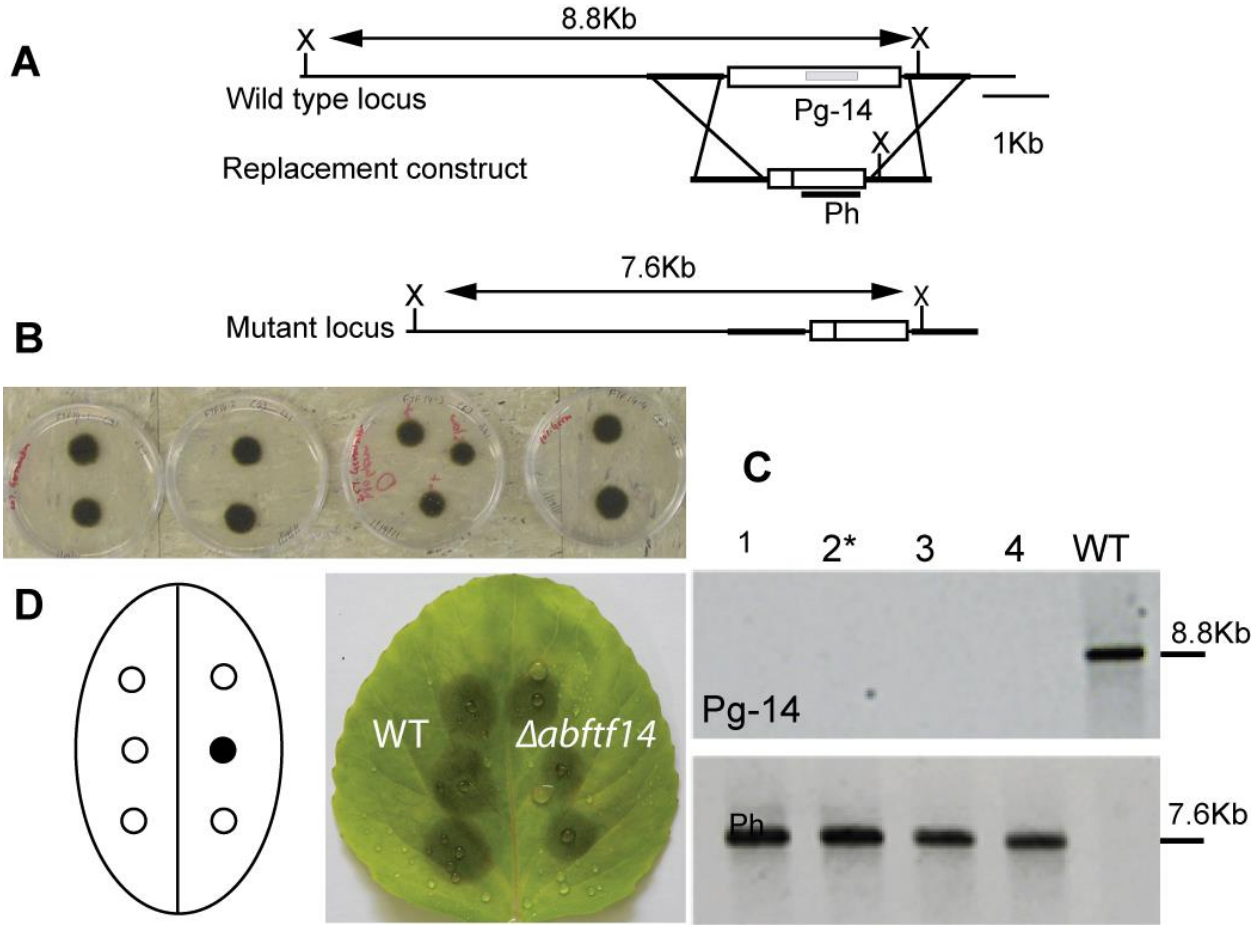

Fig.3.15: Targeted deletion and pathogenicity assay of *Abftf14* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 4 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-14 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 4 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 4 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe only band of appropriate size appeared in wild type lane showing the presence of gene wild type while deletion in all clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted and replaced the wt gene locus. **D** status of virulence *Δabftf14* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with three different concentrations of conidia of the wild type and *Δabftf7* mutant on a detached leaf (table 3.7). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: X=*Xba*I enzyme digestion site, WT=wild type.

Table 3.13: Decreased virulence of two strains of *Δabftf14*deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-14-2	3000	5	19±2.2	13±8.02	31%	0.09
Δabftf-14-2	1500	8	14.8±4.6	11.3±2.9	23%	0.01
Δabftf-14-2	1000	10	20±6.9	14.0±9.4	30%	0.02

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Abftf14 gene encodes for 563 amino acid protein and is annotated as cutinase transcription factor 1 alpha in *Pyrenophora repentis* (E=0.00). Cutinases are the most studied hydrolases are expected to be involved during initial stages of pathogenesis. In *A.brassicicola* genome about 7 cutinase genes are present and were initially considered as pathogenicity or virulence factors. Several studies have been made in this direction but could not identify any single cutinase which exclusively controls the pathogenesis. The studies concluded that most of cutinase genes which are usually present in multiple numbers are redundant in function. We expected *Abftf14* to be a regulator of at least some if not all cutinase and it should have influence on pathogenesis. On bio assays with deletion mutant clones though shows a decrease of 16%-31% reduction in virulence (table 3.13)

3.3.2.13 *Abftf16*

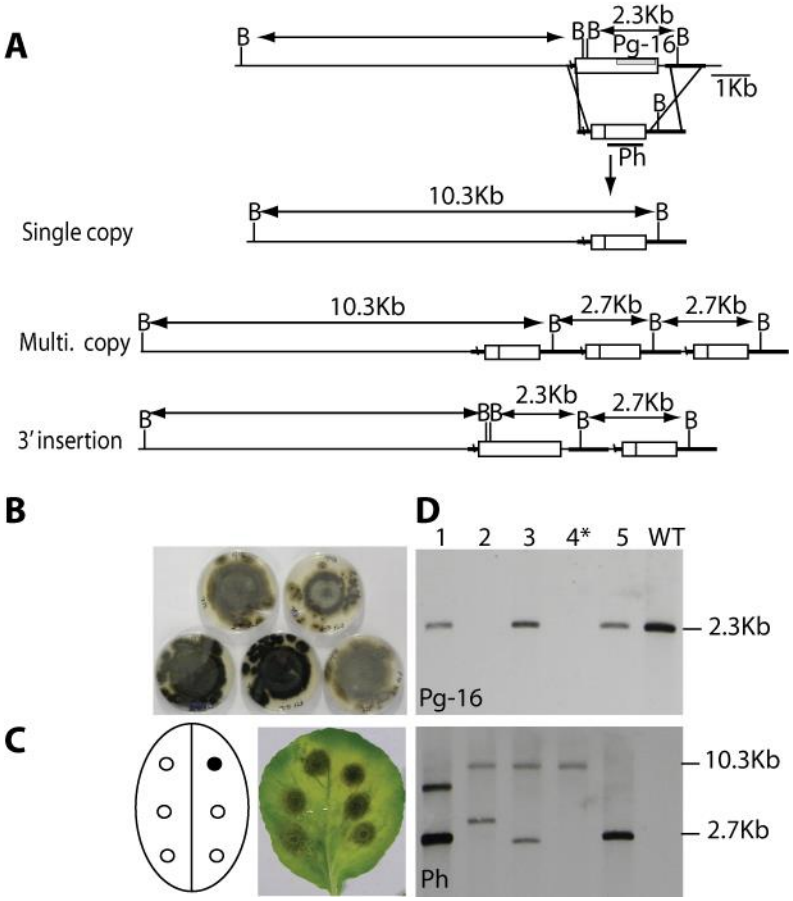

Fig.3.16: Targeted deletion and pathogenicity assay of *Abftf16* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus,showing the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-16 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in lanes 1 and 5 showing the presence of gene wild type while deletion in all remaining clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. In clone 2 (lane 2) multiple copy of the Hyg gene cassette were inserted while, in clone 4 (lane 4) only single copy of hyg gene replaced the wt gene. Lanes 1,3 and 5 shows incorporation of hyg gene at the 3' region of the gene without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf16* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with three different concentrations of conidia of the wild type and *Δabftf16* mutant on a detached leaf (table 3.14). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: X=*Xba*I enzyme digestion site.

Table 3.14: Decreased virulence of two strains of *Δabftf16*deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-16-4	3000	5	1.59±5.3	11.3±4.32	19%	0.79
Δabftf-16-4	1500	5	10.1±8.9	9.6±6.9	4%	0.6

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Abftf16 encodes for a putative C6 domain containing transcription factor. The length of amino acid residues is 675. It is not a pathogenicity or virulence associated gene as the average decease in lesion size is small (9%). The 20% decrease is also not considered as it is poorly supported by statistics and shows high internal variations.

3.3.2.14 *Abftf18*

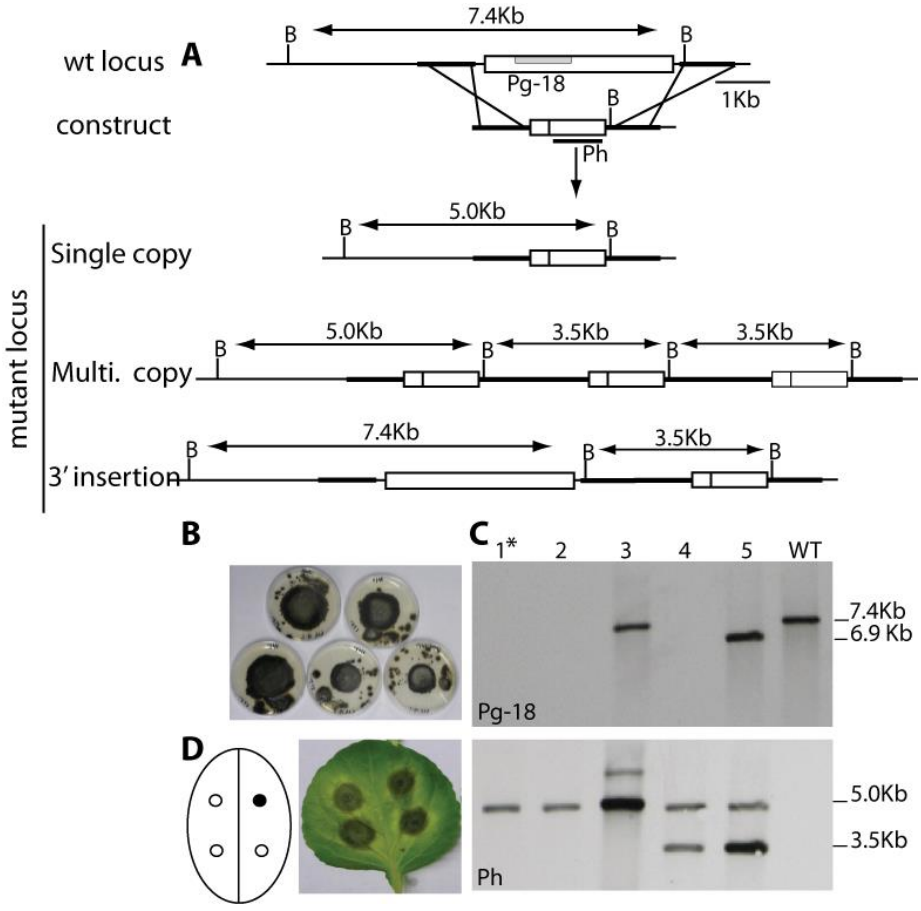

Fig.3.17: Targeted deletion and pathogenicity assay of *Abftf18* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-16 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in lanes 3 and 5 showing the presence of gene wild type while deletion in all remaining clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. In clone 4 (lane 4) multiple copy of the Hyg gene cassette were inserted while, in clone 1 and 2 (lane 1 and 2) only single copy of hyg gene replaced the wt gene. Lanes 3 and 5 show incorporation of hyg gene at the 3' region of the gene without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf16* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with three different concentrations of conidia of the wild type and *Δabftf16* mutant on a detached leaf (table 3.15). Abbreviations: B=*BamH*I enzyme digestion site.

Table 3.15: Decreased virulence of two strains of *Δabftf18*deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-18-1	3000	5	16.7±3.8	16±2.5	4%	0.5
Δabftf-18-1	1500	5	13±1.8	11.5±2.8	12%	0.1

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

Gene *Abftf18* encodes for 1039 amino acid residues and is a putative transcription factor. The pathogenicity assays show very small reduction of virulence (table 3.15) hence it is not associated with regulation of pathogenesis.

3.3.2.15 *Abftf19*

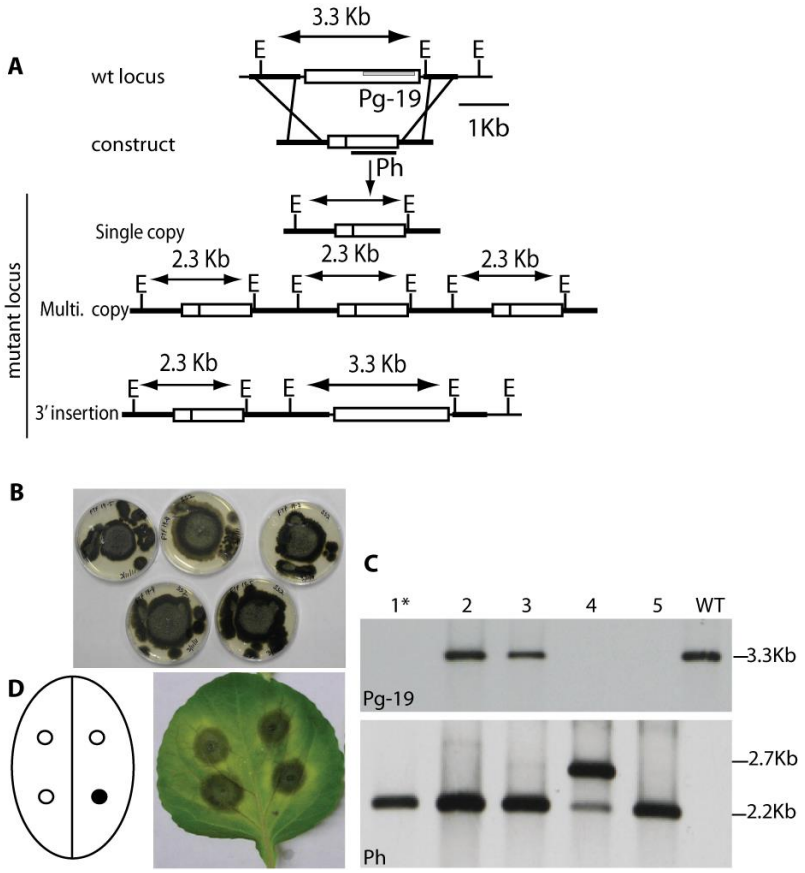

Fig.3.18: Targeted deletion and pathogenicity assay of *Abftf19* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-16 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in lanes 2 and 3 showing the presence of gene wild type while deletion in all remaining clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. In clone 4 (lane 4) multiple copy of the Hyg gene cassette were inserted while, in clone 1 and 5 (lane 1 and 5) only single copy of hyg gene replaced the wt gene. Lanes 2 and 3 show incorporation of hyg gene at the 3' region of the gene without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf19* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf19* mutant on a detached leaf (table 3.16). An assterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays Abbreviations: E=*EcoR*I enzyme digestion site.

Table 3.16: Decreased virulence of two strains of *Δabftf19*deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-19-1	3000	5	18.5±2.08	17.7±2.5	4%	0.5
Δabftf-19-1	1500	5	15.7±5.0	17±4.08	-8%	0.3

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

Gene *Abftf19* encodes for 743 amino acid containing protein as annotated as hypothetical protein in *Pyrenophora repentis* (E=0.00). The pathogenicity assays with two different concentrations of conidia shows no change in the lesion size produced by mutant and wt (table 3.16). Hence the assay suggests that *Abftf19* does not regulate pathogenesis in *A. brassicicola*.

3.3.2.16 *Abftf20*

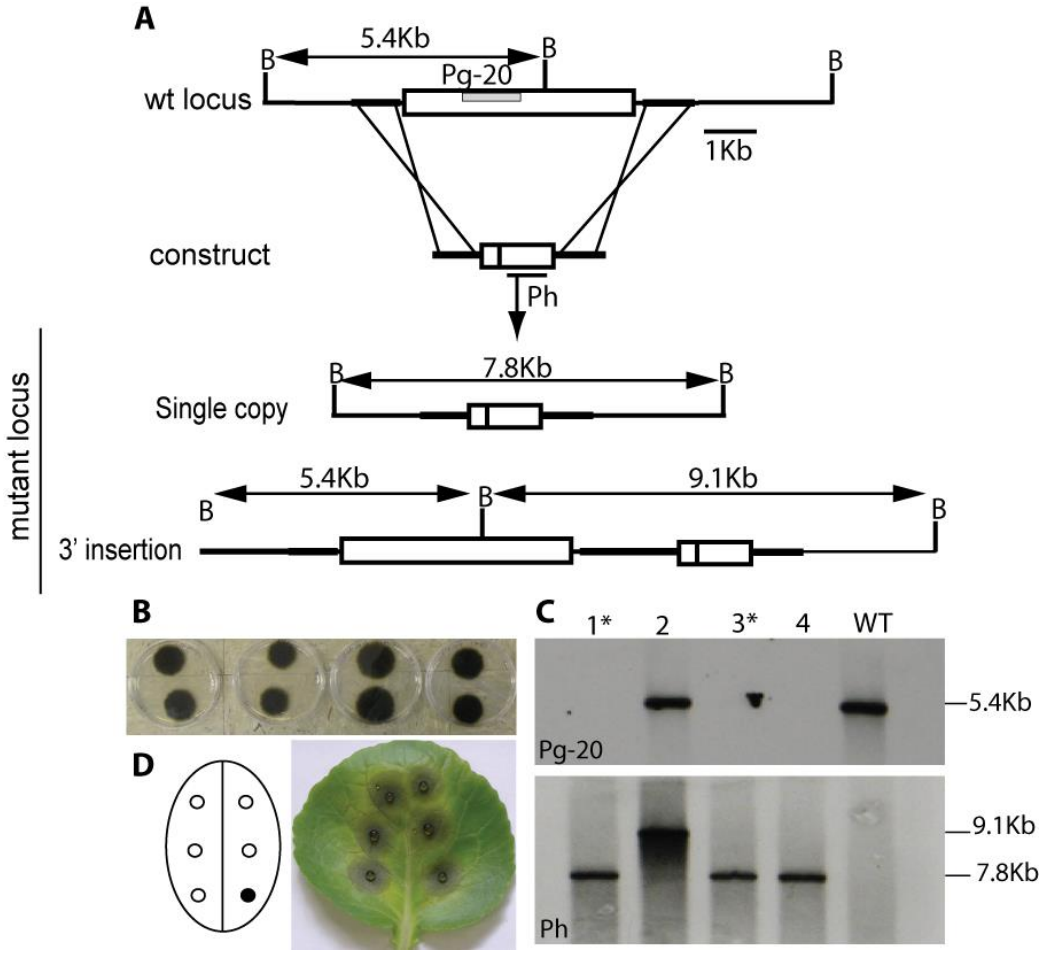

Fig.3.19: Targeted deletion and pathogenicity assay of *Abftf20* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus, showing the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-20 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 4 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 4 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in lane 2 showing the presence of gene wild type, while deletion in all remaining clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. Lane 1,3 and 4 shows successful replacement by a single copy of hyg cassette while the lane 2 shows incorporation of hyg gene at the 3' region of the gene without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf20* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf20* mutant on a detached leaf (table 3.17). an asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: B=*BamH*I enzyme digestion site.

Table 3.17: Decreased virulence of two strains of *Δabftf20* deletion mutants compared to wild-type *Alternaria.brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-20-1	2000	5	12.5±3.3	9.6±5.7	23%	0.1
Δabftf-20-3	1000	5	9.3±5.0	4.6±5.9	37%	0.07
**Δabftf-20-1*	2000	17	16.4±2.8	16.2±3.0	1%	0.4

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Gene *Abftf20* encodes for 1091 amino acid. The pathogenicity assays done for initial screening suggests this gene to be putative virulence factor. Since they assays were performed had fewer data points we thought of doing a comprehensive pathogenicity. The data obtained from the controlled pathogenicity assay produced only1% reduction of virulence (results shown by * in the assay table) suggesting that *Abftf20* is not a virulence factor.

3.3.2.17*Abftf21*

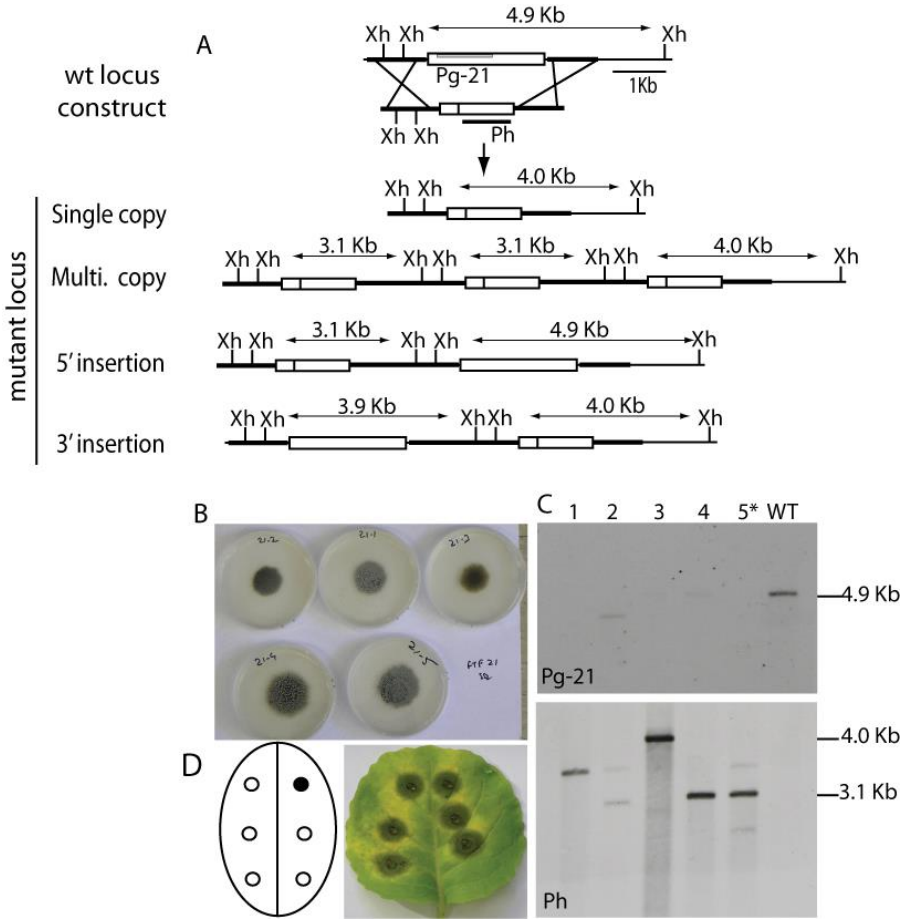

Fig.3.20: Targeted deletion and pathogenicity assay of *Abftf21* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-21 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in lane 4 showing the presence of gene wild type, while deletion in all remaining clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. Lane3 shows successful replacement by a single copy of hyg cassette while the lane 1,2 and 4 shows incorporation of hyg gene at the 3'or 5' region of the gene without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf21* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf21* mutant on a detached leaf (table 3.18). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: Xh=*Xho*I enzyme digestion site.

Table 3.18: Decreased virulence of two strains of *Δabftf21* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-21-5	3000	5	10.5±4.5	9.3±4.6	10%	0.4
Δabftf-21-5	3000	8	20.5±7.4	20.3±4.4	1%	1
Δabftf-21-5	1500	8	19.3±5.8	18.5±6.9	4%	5.00E-01

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Gene *Abftf21* encodes for 628 amino acid polypeptide that is present in single copy. it is present in single copy in *A. brassicicola*. pBLAST annotates as hypothetical protein in *Pyrenophora teres* f. teres (E-value= 0.00) and shows presence of C6 fungal specific transcription factor in *Aspergillus fumigatus* (E-value 0.0). The pathogenicity assays shows very marginal difference in the lesion size produced by mutant and wild type therefore *Abftf21* is not involved in regulation of pathogenesis.

3.3.2.18 *Abftf22*

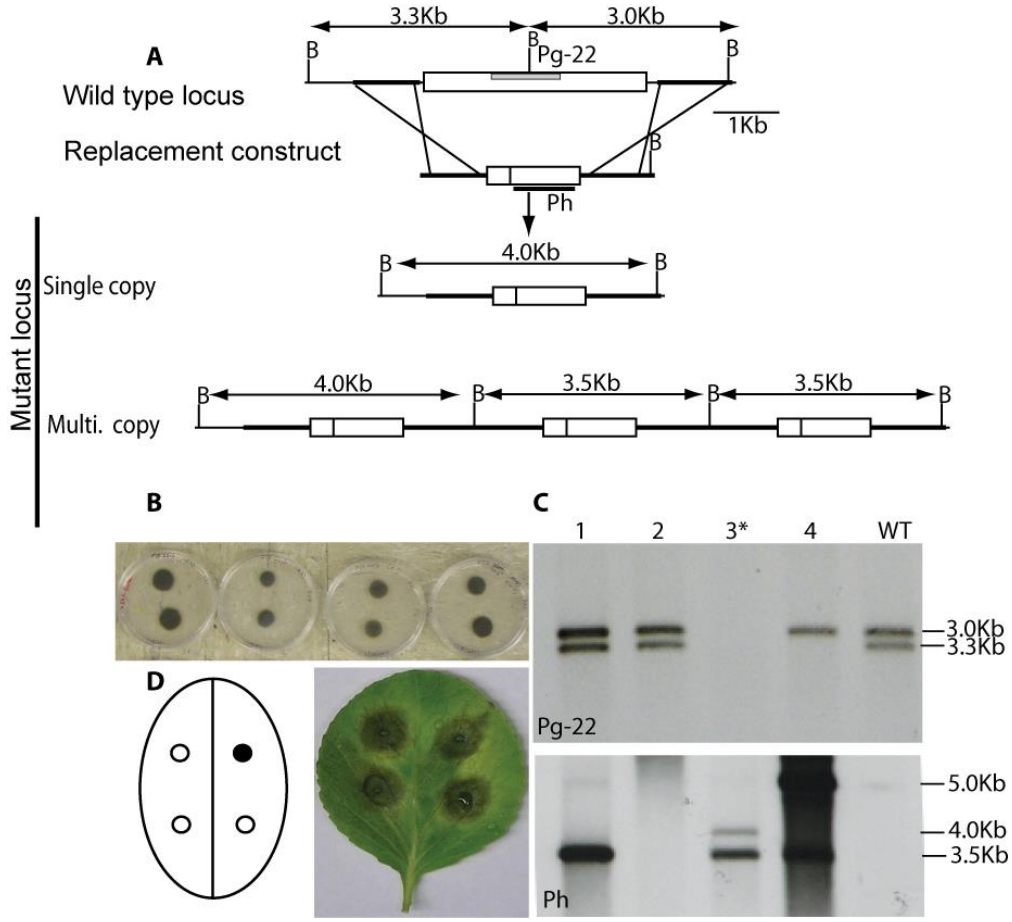

Fig.3.21: Targeted deletion and pathogenicity assay of *Abftf22* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 4 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-22 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 4 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 4 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in lanes 1, and 4 showing the presence of gene wild type, while deletion in all remaining clones. The double band in upper panel is due to the position of probe which includes the restriction site. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. Lane3 shows successful replacement by multiple copy of hyg cassette while the lane 1 and 4 shows incorporation of hyg gene at the 3'or 5' region of the gene without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf22* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf22* mutant on a detached leaf (table 3.19). Abbreviations: B=*BamH*I enzyme digestion site.

Table 3.19: Decreased virulence of two strains of *Δabftf22* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-22-3	3000	5	17.5±0.9	15.2±4.3	14%	0.2
Δabftf-22-3	1500	5	13.5±6.1	14±4.6	-4%	0.7

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Gene abftf22 encodes a 919 amino acid containing putative transcription factor present in single copy in genome of *A.brassicicola*. It is annotated as hypothetical protein with fungal specific TF and chitin binding domain in *Pyrenophora teres* (E- value=0) and *Magnaporthe oryzae* (E value= 3e-144). The pathogenicity assays however does not suggest this gene as a putative virulence factor (Table 3.19).

3.3.2.19 *Abftf24*

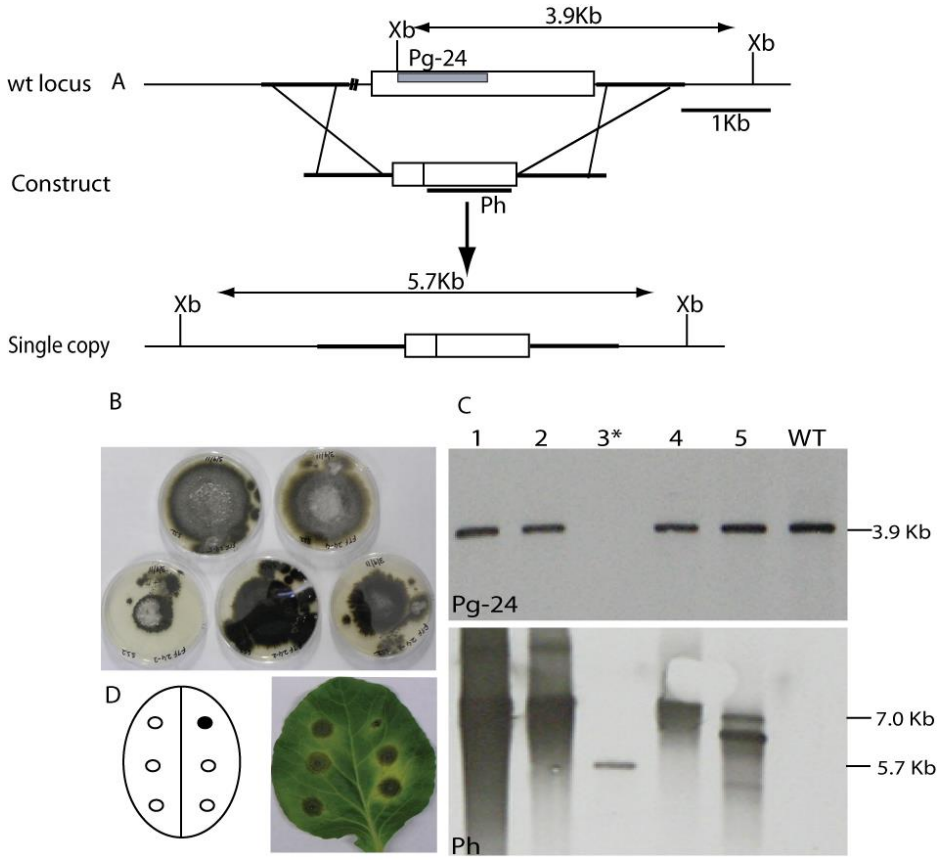

Fig.3.22: Targeted deletion and pathogenicity assay of *Abftf24* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 4 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-24 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in all lanes except in lane 1 showing the presence of gene wild type, while deletion clone 1. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted though the insertion is largely ectopic except in case of clone 3 (lane3) which shows successful replacement of wt gene by single copy of hyg cassette while the remaining lanes show incorporation of hyg gene at the 3'or 5' region of the gene without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf24* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf24* mutant on a detached leaf (table 3.20). An asterisk (*) indicate DNA lanes of mutants which were used for pathogenicity assays Abbreviations: Xb=*Xba*I enzyme digestion site.

Table 3.20: Decreased virulence of two strains of *Δabftf24* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-24-3	3000	5	10.8±2.9	1.1±1.8	89%	7.0E-05
Δabftf-24-3	1500	5	13.5±7.4	4±6.9	70.3%	0.7
**Δabftf24-3*	2000	17	17.4±4.6	6.2±4.9	64.1%	0

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Gene *Abftf24* is 744 amino acid containing protein and is annotated as hypothetical protein in *Pyrenophora teres* (E- value=0). The initial pathogenicity assays suggested *Abftf24* to be a virulence factor. To ascertain results we performed a very well controlled pathogenicity assays with 50 days old green cabbage plants, using Δ*abftf24* colonies grown on PDA for 5 days. The assays were in sync with the previous results (Shown by * in the table above). Based on these results we conclude that *Abftf24* is a strong virulence factor. In the present study we did not performed any other experiments but for future work *Abftf24* can serve as a very good candidate studying and characterizing its role in pathogenesis.

3.3.2.20 *Abftf25*

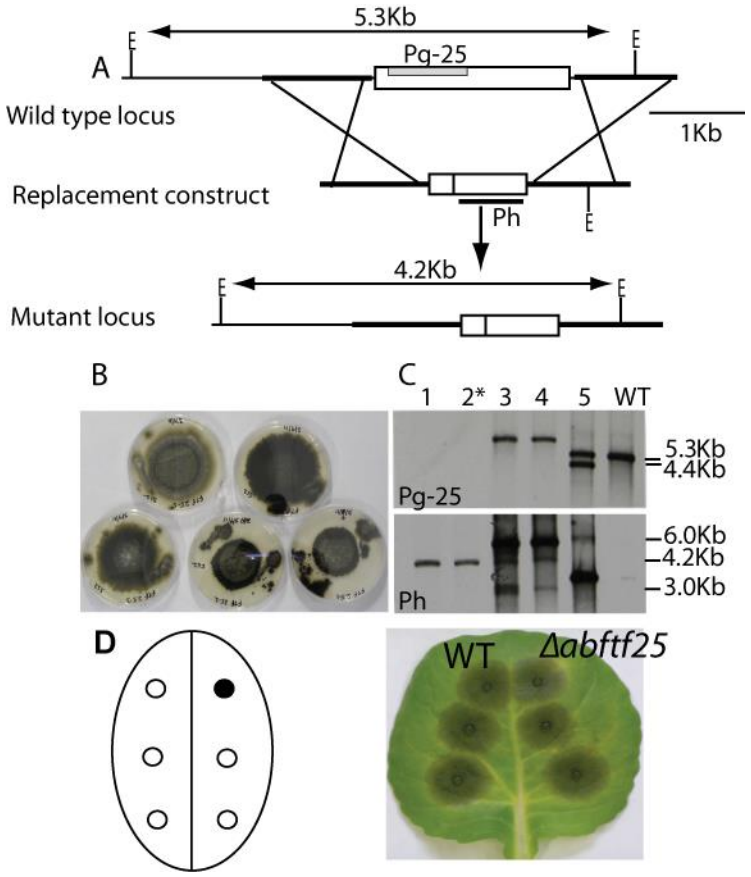

Fig.3.23: Targeted deletion and pathogenicity assay of *Abftf25* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus. It also shows the double recombination event that occurred to produce the 4 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-25 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in lanes 3,4 and 5 showing the presence of wild type gene, while deletion in clones 1 and 2. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted though the insertion is largely ectopic except in case of clone 1 and 2 (lanes 1 and 2) which shows successful replacement of wt gene by single copy of hyg cassette while the remaining lanes show incorporation of hyg gene at the 3'or 5' region of the gene without replacing the wt gene by virtue of single homologous recombination event. An asterisk (*) indicate DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: E=*EcoRI* enzyme digestion site.

Table 3.21: Decreased virulence of two strains of *Δabftf24* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-25-2	3000	5	10.6±2.8	7.1±6.1	33%	1.8E-01
Δabftf-25-2	1500	5	13.0±9	11±8.6	10%	0.1
Δabftf-25-2	3000	11	13.5±3.4	12.8±3.0	5%	0.6
Δabftf-25-2	1500	11	12.4±2.9	13.4±1.3	0%	2.00E-01
**Δabftf25-2*	2000	17	15.6±3.6	13.9±5.8	7.4%	0.14

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Gene *Abftf25* encodes 657 amino acid containing protein. The pathogenicity assays were done for four times and shows very small reduction in virulence by mutant in three of them (0-10%). In one assay there was reduction of 33% which may be because of use of relatively susceptible plants in pathogenicity assays. The trend illustrates that *Abftf25* is not a virulence factor.

3.3.2.21 *Abftf26*

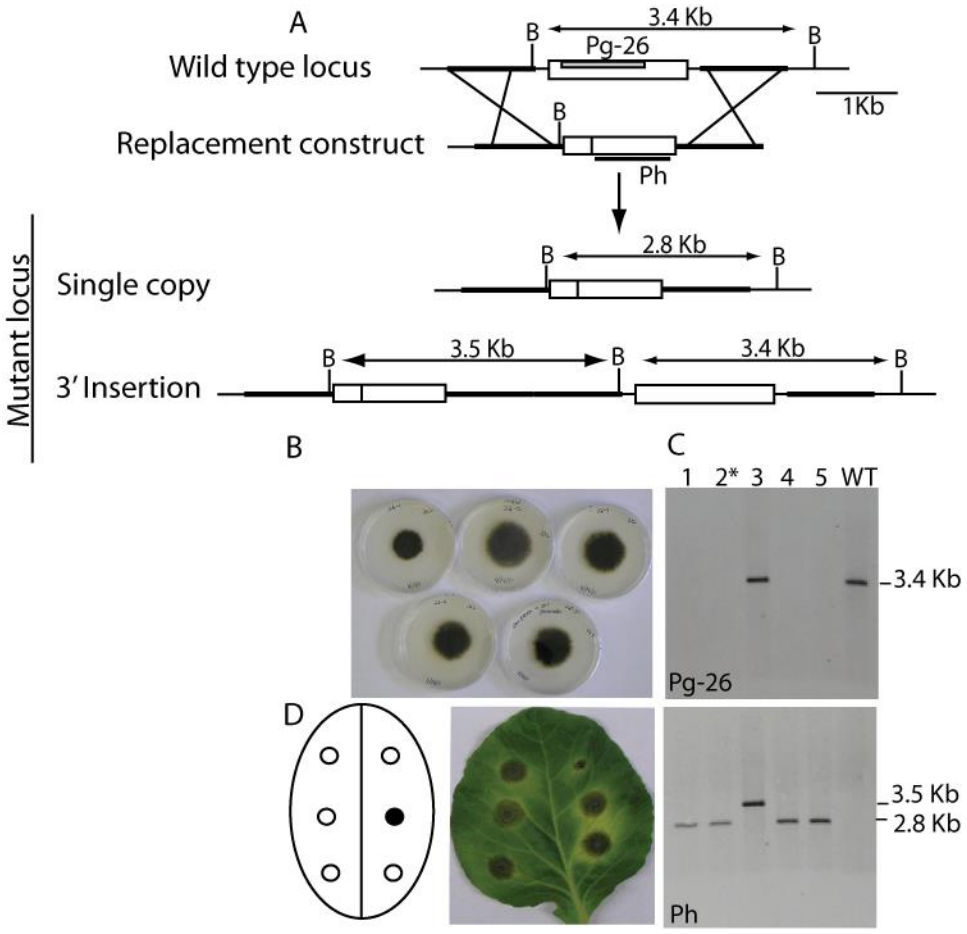

Fig.3.24: Targeted deletion and pathogenicity assay of *Abftf26* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-26 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in lane 3 showing the presence of gene wild type, while deletion in all remaining clones. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. Lane3 shows incorporation of hyg gene at the 3'or 5' region of the gene without replacing the wt gene by virtue of single homologous recombination event, remaining clones shows replacement of wild type allele by single copy of hyg. Gene cassette. **D** status of virulence *Δabftf26* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf26* mutant on a detached leaf (table 3.22). An asterisk (*) indicate DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: B=*BamH*I enzyme digestion site.

Table 3.22: Decreased virulence of two strains of *Δabftf26* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-26-2	3000	5	13.6±4.9	2.6±6.5	80%	1.0E-02
Δabftf-26-2	1500	5	10.0±3.8	0±0	100%	0.0015
Δabftf-26-2	3000	8	23.5±6.2	20.75±7.5	16%	0.14
Δabftf-26-2	1500	8	17±9.8	18.6±8.0	-10%	1.80E-01
Δabftf-26-2	3000	11	14±3.9	14±2.5	0%	0.21
Δabftf-26-2	1500	11	13±3.5	12.8±1.6	0%	0.001
Δabftf-26-2	3000	11	13.4±4.4	12.4±2.9	7%	0.56
Δabftf-26-2	1500	11	12.7±3.6	14.5±1.2	14%	0.1

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Abftf26 is a putative fungal specific transcription factor gene encoding for 543 amino acid residue. The initial pathogenicity assay showed dramatic decrease in virulence (80-100%) inkling it to be a putative virulence factor. To confirm it as a virulence factor we further performed 6 more pathogenicity assays and discovered there was very small difference between virulence caused by *Abftf26* mutant and wt. the initial two assays show dramatic reduction because of use of very susceptible plants for assays.

3.3.2.22 *Abftf28*

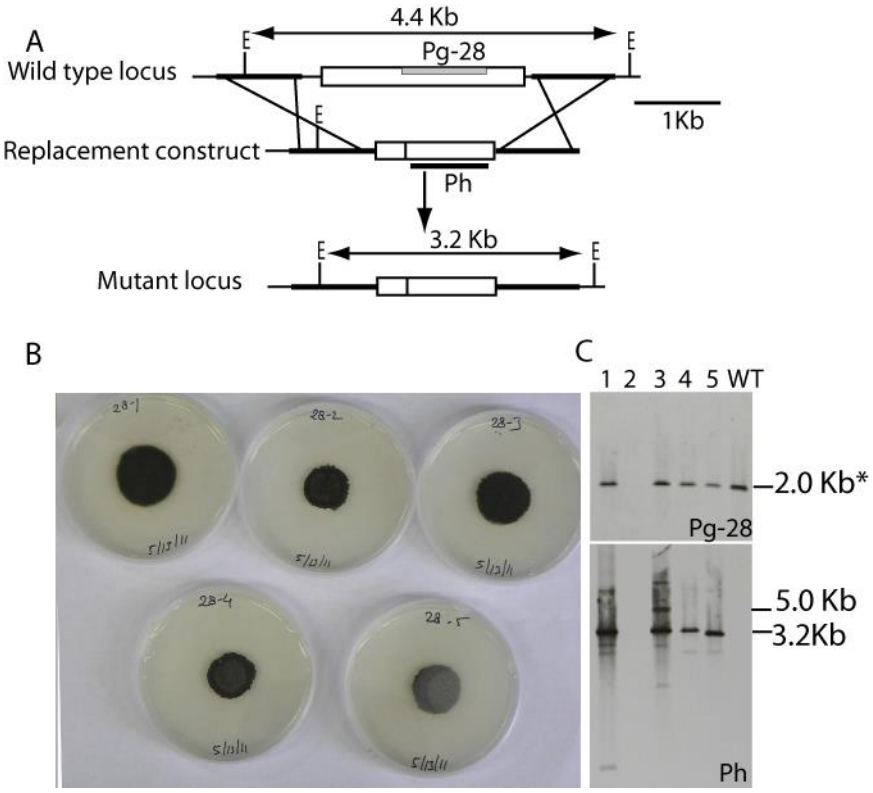

Fig.3.25: Targeted deletion and pathogenicity assay of *Abftf28* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-28and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in all of th remaining lanes except lane 2, showing the presence of wild type gene, in all clones which indicates that the gene was not deleted. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt and clone 2 indicating that Hyg cassette was successfully incorporated but at the 3'or 5' region of the gene without replacing the wt gene by virtue of single homologous recombination event. Abbreviation E= *Eco*RI ,.

Since, the southern blots suggest presence of no true mutant among all transformants; we did not perform any pathogenicity assays for this gene.

3.3.2.23 *Abftf29*

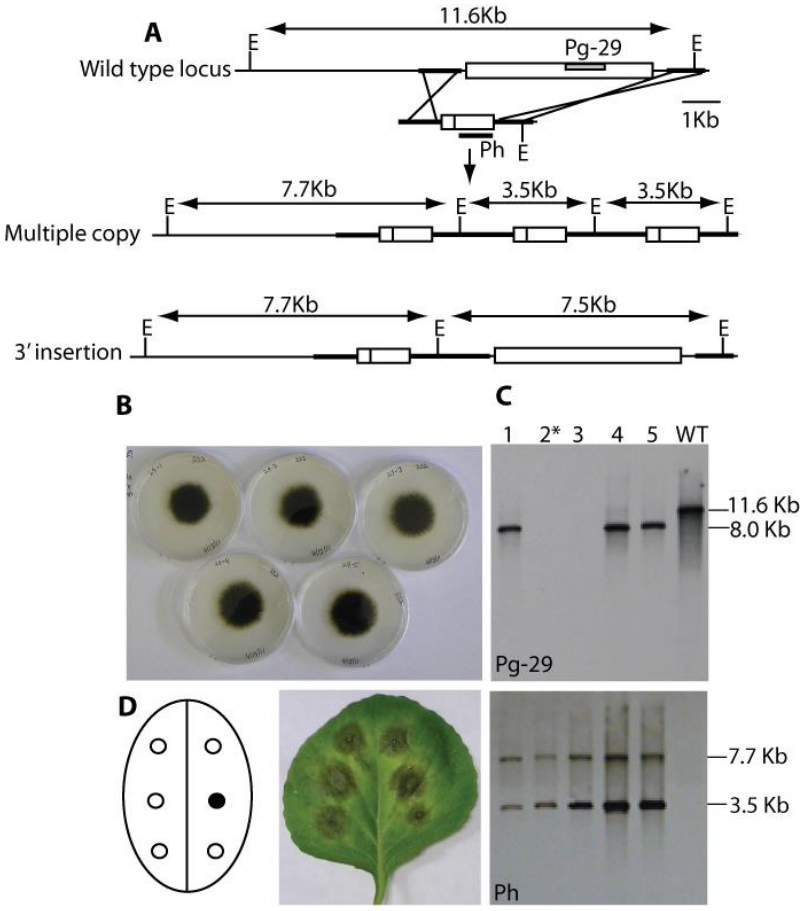

Fig.3.26: Targeted deletion and pathogenicity assay of *Abftf29* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-29 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well lanes 1,4 and 5 showing the presence of wild type gene, while deletion clone 2 and 3 (lane 2 and 3). In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette was successfully inserted. However the banding pattern indicates that in clone 2 and 3 the wild type gene was replaced by multiple copy of Hyg resistance gene cassette, while in lane 1, 4 and 5 the insertion occured at the 3' end by multiple copy of Hyg cassette without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf29* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf29* mutant on a detached leaf (table 3.23). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: E=*EcoR*I enzyme digestion site.

Table 3.23: Decreased virulence of two strains of *Δabftf29* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-29-2	3000	5	12±4.1	13.8±2.9	-15%	2.0E-02
Δabftf-29-2	1000	5	10.6±6.5	11.8±6.2	-10%	0.2

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

The *Abftf29* gene shows no reduction; rather increase in lesion size produced by the mutant in comparison to the wt lesion. The increase however is marginal hence we concluded that this putative transcription factor is not a virulence factor and is not involved in pathogenesis of *A.brassicicola*.

3.3.2.24 *Abftf30*

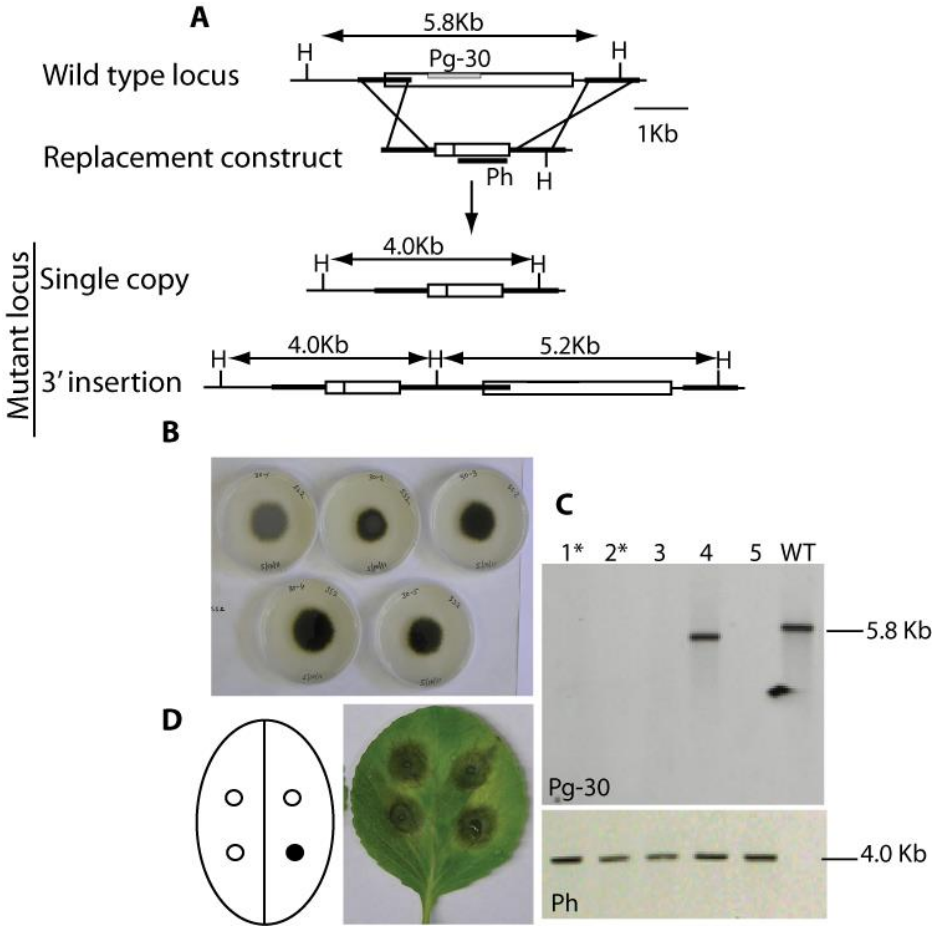

Fig.3.27: Targeted deletion and pathogenicity assay of *Abftf30* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-30 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in lane 4 showing the presence of wild type gene, while deletion clone in lanes 1,2,3 and 5. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that Hyg cassette successfully replaced wt gene. However the banding pattern indicates that in clone 4 insertion occured at the 3' end by Hyg cassette without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf30* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf30* mutant on a detached leaf (table 3.24). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays Abbreviations: H=*Hind*III enzyme digestion site, wt = wild type.

Table 3.24: Decreased virulence of two strains of *Δabftf30* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-30-1	3000	5	19±1.4	17±2.5	11%	2.0E-02
Δabftf-30-2	1000	5	15.6±3.8	14.7±4.6	3%	0.3
Δabftf30-2	2000	17	16.6±4.6	16.5±4.2	0%	0.7

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

Gene *Abftf30* is a 1030 amino acid encoding gene is annotated as hypothetical protein in *Pyrenophora teres* (E- value=0) having C6 transcription factor domain of *Aspergillus oryzae* (E-value= 3e-95). The pathogenicity assays with two different clones of the *Abftf30* mutant illustrates no significant reduction in lesion size as compared to made by wt. hence we conclude that this gene cannot be categorized as virulence factor in *A.brassicicola*..

3.3.2.25 *Abftf31*

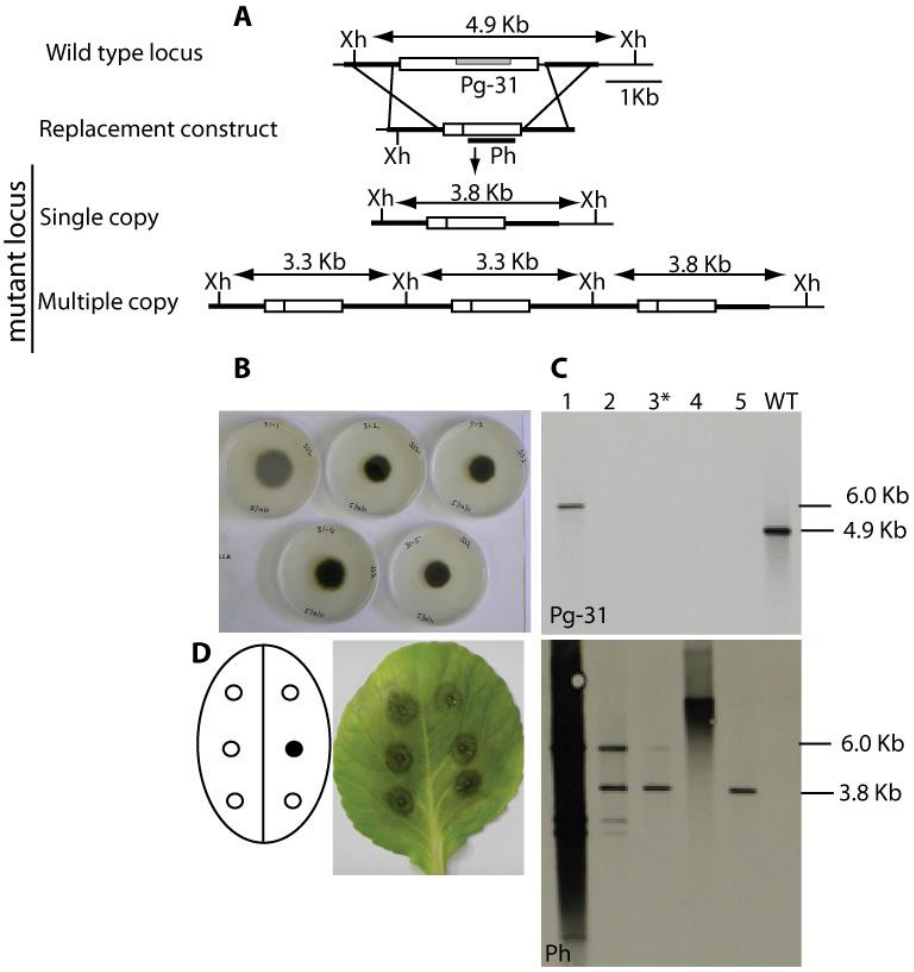

Fig.3.28: Targeted deletion and pathogenicity assay of *Abftf31* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-31 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in lane 1 showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt. In clone 3 and 5 (lane 3 and 5) the wt gene was successfully replaced by the single copy of hyg cassette, while in clone 2 wt gene is replaced by the multiple copy of hyg gene. In clone 1 insertion occurred at the 3' end by Hyg cassette without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf31* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf1* mutant on a detached leaf (table 3.25). An asterisk (*) indicate DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: Xh=*Xho*I enzyme digestion site.

Table 3.25 Decreased virulence of two strains of *Δabftf31* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-31-3	3000	5	19±1.3	14.5±2.3	26%	7.0E-04
Δabftf-31-3	1000	5	18.8±1.7	8.83±6.9	53%	0.01
**Δabftf31-3*	2000	17	12.06±3.1	11.5±3.5	4%	0.03

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

Abftf31 encodes for 708 amino acid protein. The pathogenicity assays showed reduction in virulence. Since very small numbers of data points were taken where one or two inconsistent infection site skews data, therefore we decided to do a comprehensive and well controlled pathogenicity assay. The results obtained from this pathogenicity assay suggest that there is no appreciable difference between lesion size made by *Δabftf-31* and wild type (result shown by *), hence we conclude that this gene is not involved in regulation of pathogenesis.

3.3.2.26 *Abftf32*

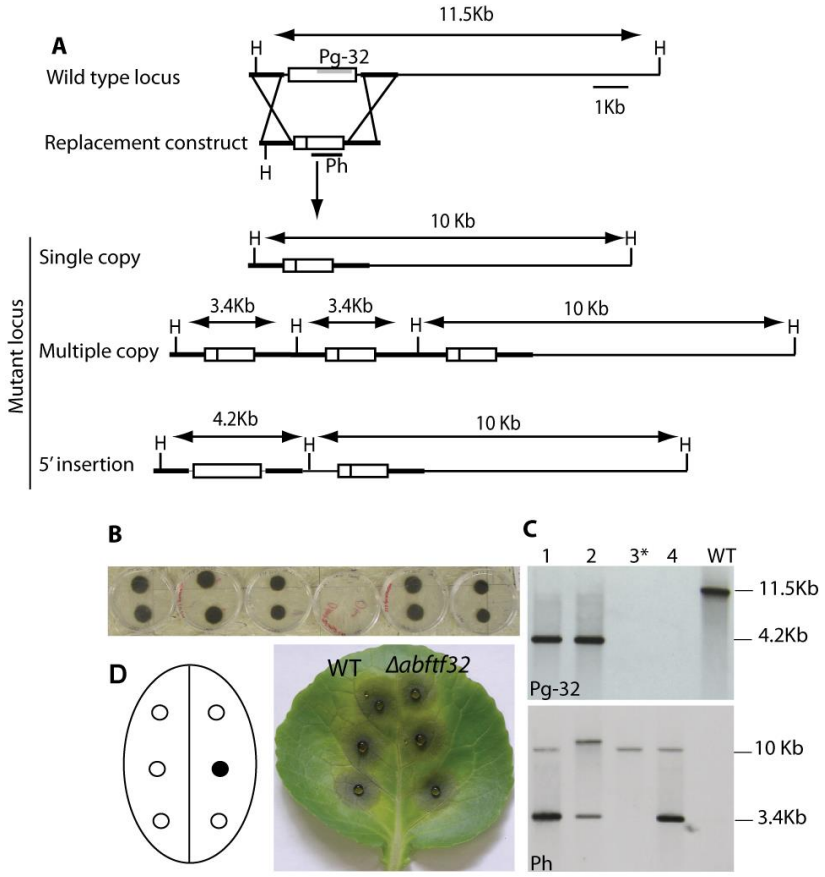

Fig.3.29: Targeted deletion and pathogenicity assay of *Abftf32* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 6 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-32 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 4 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 4 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in lanes 1and 2 showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt. In clone 3 and 4 (lane 3 and 4) the wt gene was successfully replaced by the single and multiple copy of hyg cassette respectively. Clones 1 and 2 illustrate incorporation of hyg gene at the 3' end of wt gene by a single homologous recombination event. **D** status of virulence *Δabftf32* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf31*mutant on a detached leaf (table 3.28). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: H=*Hind III* enzyme digestion site, wt = wild type.

Table 3.26: Decreased virulence of two strains of *Δabftf32* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-32-3	3000	8	14.4±3.7	12±3.7	17%	0.01
Δabftf-32-3	1500	8	11.1±2.0	11.1±2.1	0%	1

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

The pathogenicity assays suggest that the lesion size produce by mutant *Abftf32* were similar to lesions produced by wt hence this genes is not a virulence factor.

3.3.2.27 *Abftf33*

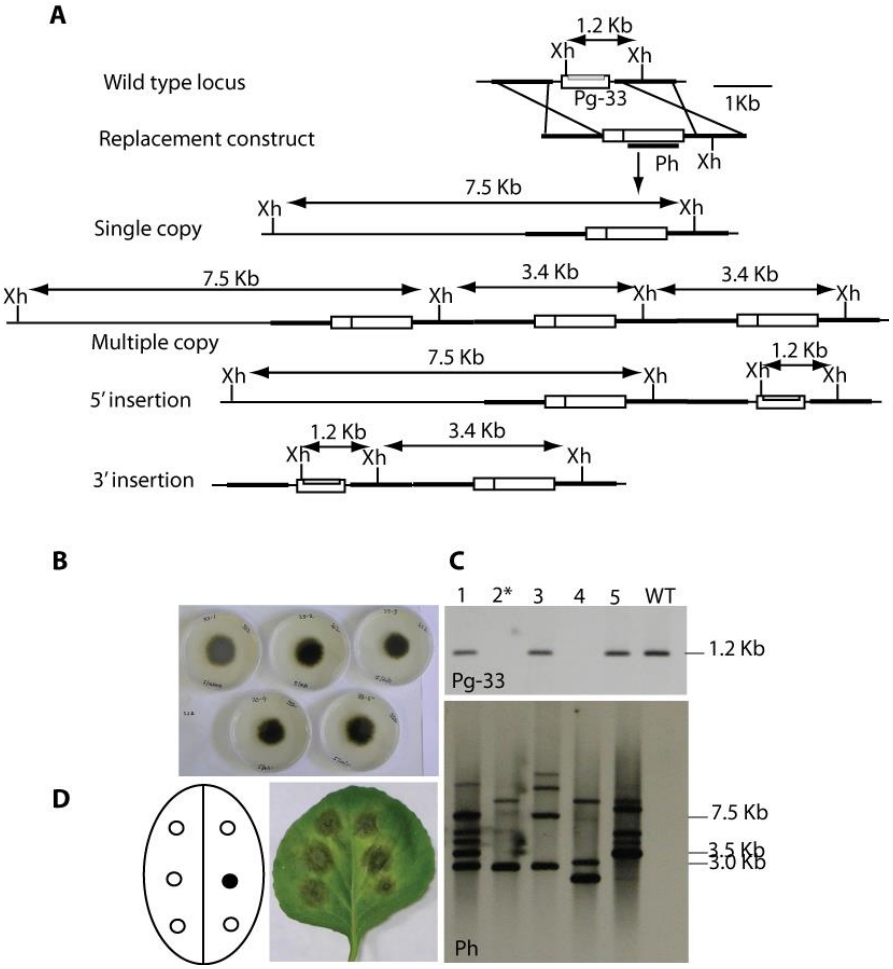

Fig.3.30: Targeted deletion and pathogenicity assay of *Abftf33*gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-33 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in lanes 1,3and 5 showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt. In clone 2 and 4 (lane 2 and 4) the wt gene was successfully replaced by multiple copy of hyg cassette. Clones 1, 3 and 5 illustrate incorporation of multiple copy of hyg gene at the 3' end of wt gene by a single homologous recombination event. **D** status of virulence *Δabftf33* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf33*mutant on a detached leaf (table 3.27). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: Xh=*XhoI* enzyme digestion site.

Table 3.27: Decreased virulence of two strains of *Δabftf33* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-33-2	3000	5	12.3±4.2	11.1±6.5	10%	4.0E-01
Δabftf-33-2	1000	5	10.5±5.7	9.8±5.4	7%	0.6

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter

Abftf33 is a small protein made up of 249 amino acid residues and annotated as putative The $Zn(II)_2Cys_6$ fungal specific transcription factor in *Aspergillus flavus* (e-value=5e-85). The pathogenicity assay results (table 3.27) show very small difference between lesion size of mutant and wt. *A brassicicola* therefore this gene cannot be a virulence factor.

3.3.2.28 *Abftf35*

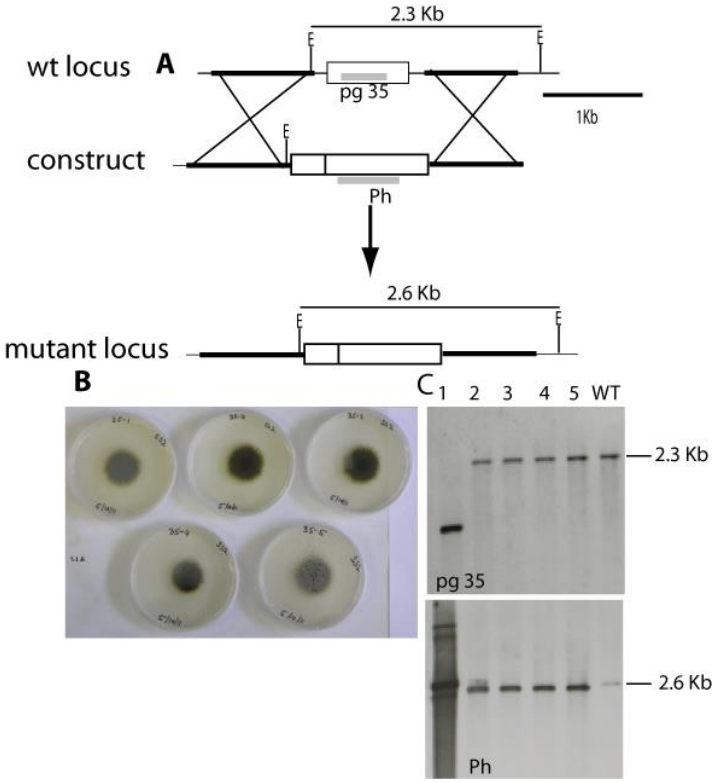

Fig.3.31: Targeted deletion and pathogenicity assay of *Abftf35* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as pg-35and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe bands of appropriate size appeared in wild type lane as well as in all of the remaining lanes showing the presence of wild type gene, in all clones which indicates that the gene was not deleted. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt and clone 2 indicating that Hyg cassette was successfully incorporated but at the 3'or 5' region of the gene without replacing the wt gene by virtue of single homologous recombination event. Abbreviation E= *Eco*RI.

Since, the southern blots suggest presence of no true mutant among all transformants; we did not perform any pathogenicity assays for this gene.

3.3.2.29 *Abftf36*

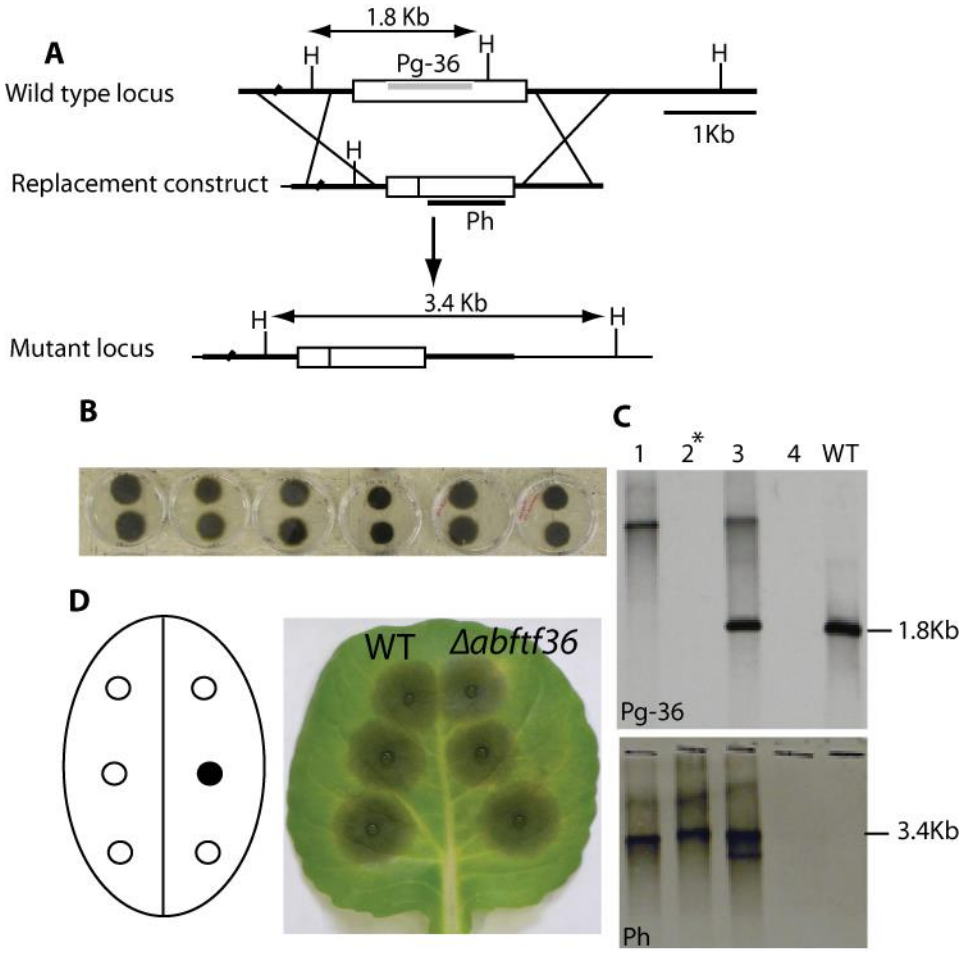

Fig.3.32: Targeted deletion and pathogenicity assay of *Abftf36*gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 6 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-36 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**.4 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 4 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in lanes 1 and 3 showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt. and lane 4. Single copy of hyg gene replaced wt gene in clone 2 while 1 and 3 represents insertion mutants resulted from single recombination event. **D** status of virulence *Δabftf36* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf36* mutant on a detached leaf (table 3.30). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: Xh=*XhoI* enzyme digestion site, WT= wild type.

Table 3.28: Decreased virulence of two strains of *Δabftf36* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf-36-2	3000	11	16.7±3.0	13.3±4.6	20%	1.0E-03
Δabftf-36-2	1500	11	16.1±2.9	14.2±4.3	12%	0.01
Δabftf36-2	2000	17	15.4±3.8	14.3±5.4	7.2	0.18

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

The pathogenicity assays suggest that the lesion size produce by mutant Abftf36 were not very different from lesions produced by wt. Therefore, this gene cannot be referred as virulence factor.

3.3.2.30 *Abftf37*

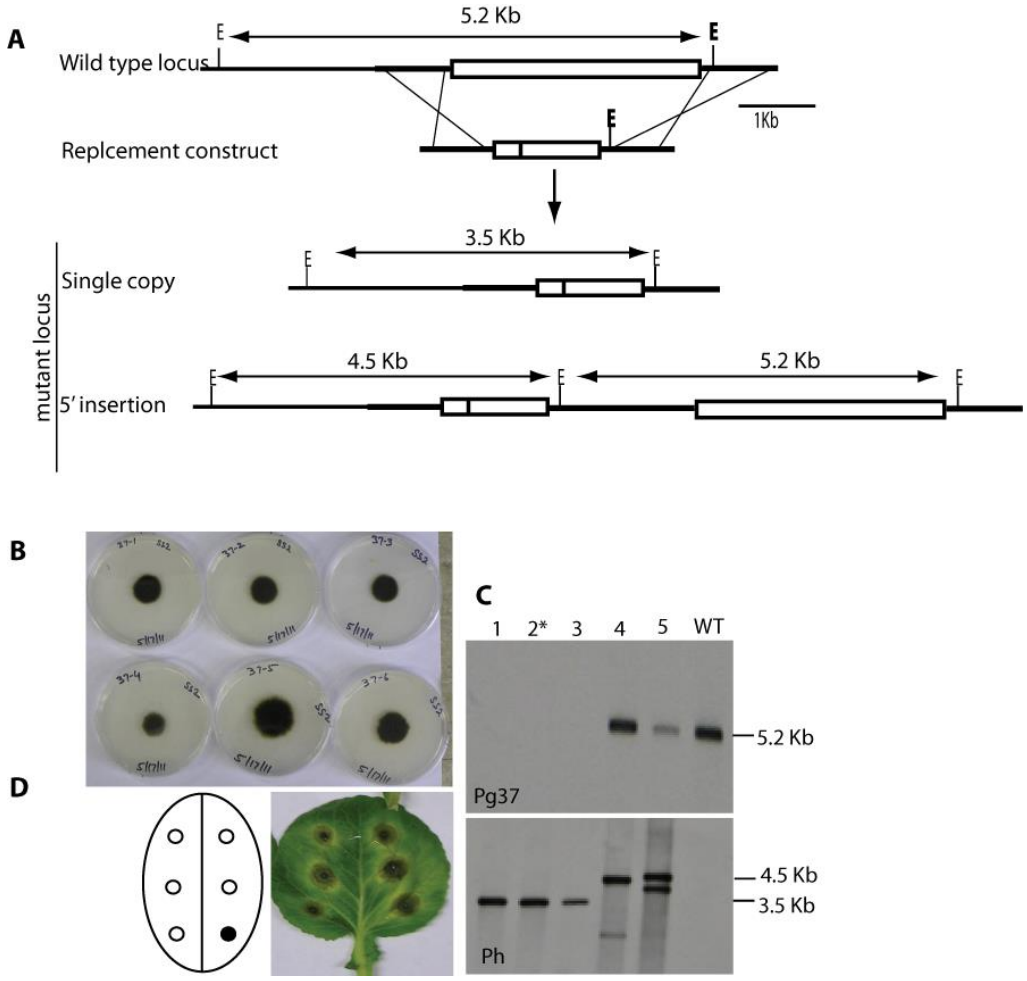

Fig.3.33: Targeted deletion and pathogenicity assay of *Abftf37*gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 6 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-37 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in lanes 4 and 5 showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt. In clone 1, 2 and 3 (lane1,2 and3) the wt gene was successfully replaced by single copy of hyg cassette. Clones 4 and 5 illustrate incorporation of multiple copy of hyg gene at the 3' end of wt gene by a single homologous recombination event. **D** status of virulence *Δabftf37* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf37*mutant on a detached leaf (table 3.31). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: E=Ec*oRI* enzyme digestion site.

Table 3.29: Decreased virulence of two strains of *Δabftf37* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf37-2	3000	6	12.6±3.3	14.5±1.3	-15%	1.4E-01
Δabftf37-2	1500	6	11.6±2.8	8.6±3.4	26%	0.01
Δabftf37-2	3000	8	23.0±5.3	22.1±6.4	3.1%	0.4
Δabftf37-2	1500	8	16.0±9.7	19.6±6.7	-10.0%	0.09
**Δabftf37-2*	2000	17	12.76±2.5	12.4±2.4	2%	1

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

The gene *Abftf37* encodes 657 amino acids. It shows annotation with very low similarity in *A. brassicicola* genome. The general trend of pathogenicity assay indicates that it does not influence virulence. To ascertain its role in pathogenicity we performed a separate pathogenicity assay with 50 days old green cabbage. The results show very marginal difference between the lesion size produced by the *Δabftf37* and wild type *A. brassicicola*. Hence, we conclude that this gene is associated with regulation of pathogenesis in *A. brassicicola*.

3.3.2.31 *Abftf38*

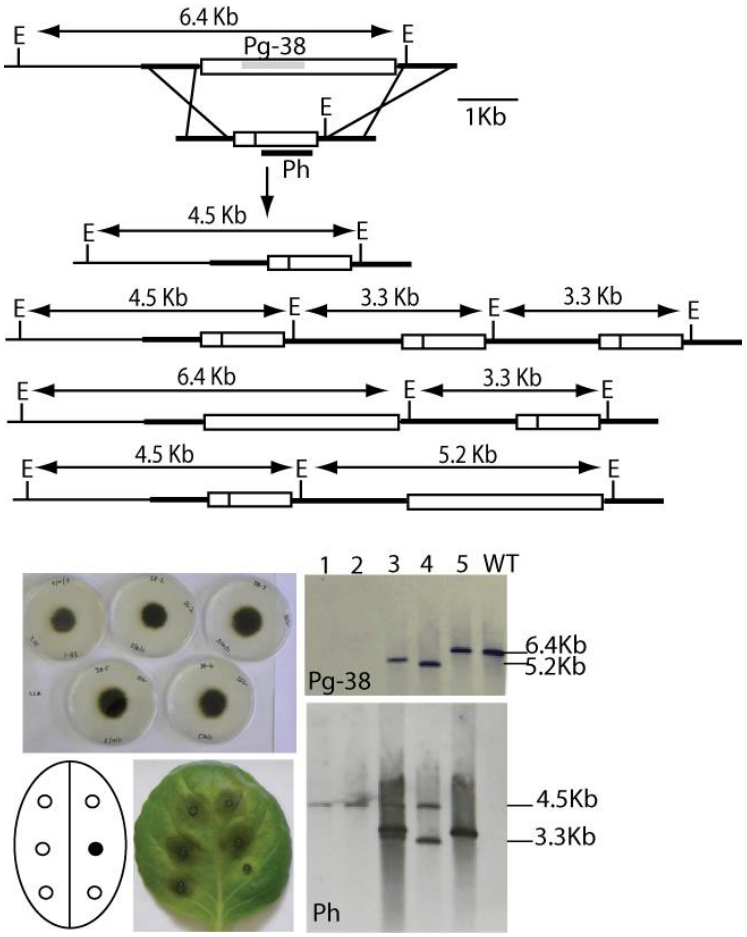

Fig.3.34: Targeted deletion and pathogenicity assay of *Abftf38* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-38 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in lanes 3,4 and 5 showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt. In clone 1 and 2 (lane1 and 2) the wt gene was successfully replaced by single copy of hyg cassette. Clones 3,4 and 5 illustrate incorporation of multiple copy of hyg gene at the 3' end of wt gene by a single homologous recombination event. **D** status of virulence *Δabftf38* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf38* mutant on a detached leaf (table 3.30). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: E=Ec*oRI* enzyme digestion site.

Table 3.30: Decreased virulence of two strains of *Δabftf38* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf38-1	3000	6	19.5±1.3	15.1±2.04	23%	5.0E-04
Δabftf38-1	1500	6	18.1±1.6	11.6±4.8	36%	0.01
**Δabftf38-1*	2000	17	15.8±4.1	15.0±5.1	5%	0.09

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

The gene *Abftf38* encodes for 810 amino acid residue. The average reduction in lesion size from wt is 30%. The initial pathogenicity assay suggests that this gene is a weak virulence factor. We performed a controlled pathogenicity with 50 days old green cabbage. The results of this assay showed only reduction of 5% in virulence in comparison to wild type *A. brassicicola* (results shown as * in table above). Thus, we finally conclude that *Abftf38* is not associated with regulation of pathogenesis and is not a virulence factor in *A. brassicicola*.

3.3.2.32 *Abftf50*

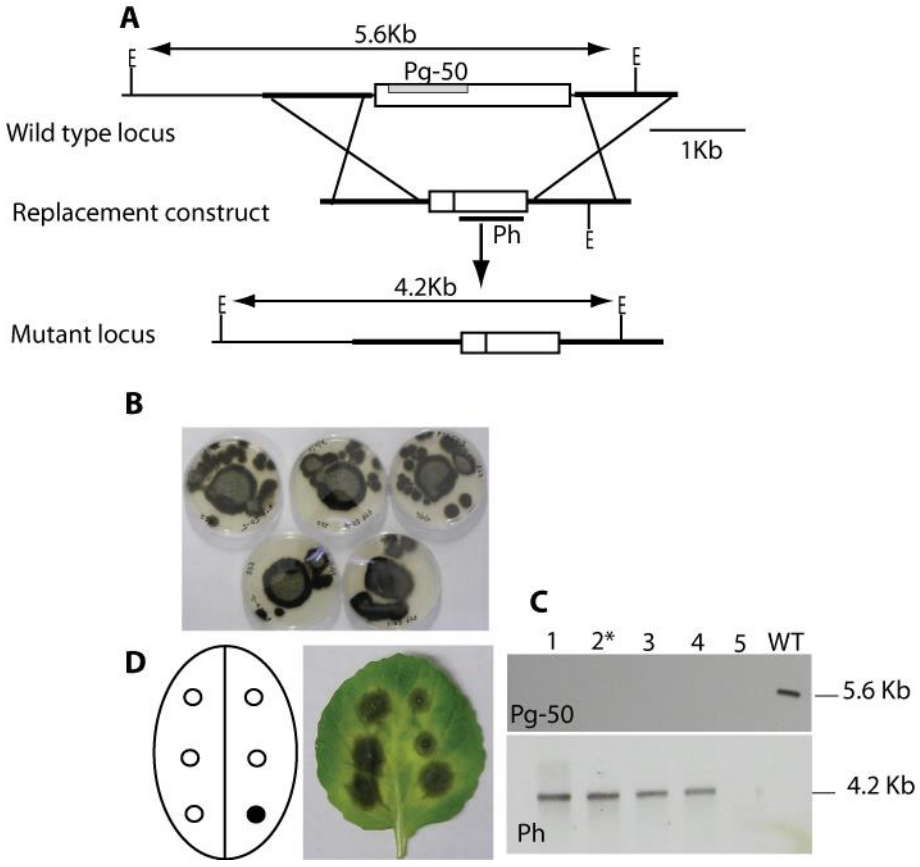

Fig.3.35: Targeted deletion and pathogenicity assay of *Abftf50* gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-38 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt indicating that the wt gene was successfully replaced by single copy of hyg cassette. **D** status of virulence *Δabftf 50* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf50* mutant on a detached leaf (table 3.31). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: E=Ec*oRI* enzyme digestion site.

Table 3.31: Decreased virulence of two strains of *Δabftf50* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf50-1	3000	6	19.8±1.3	16.8±2.6	15%	5.0E-02
Δabftf50-1	1500	6	18.6±1.8	12±3.3	35%	0.01
**Δabftf50-1*	2000	17	15.6±4.5	16.6±4.2	-6.3%	0

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Abftf50 is 499 amino acid encoding gene. The initial pathogenicity assays with *Δabftf50* shows a small reduction in virulence. We performed another pathogenicity assay with in a more controlled condition and with more number of replicated (18 replicates) to confirm the reduction in virulence. The results of the assay show that the average lesion size produced by the mutant was 6.3% more than the lesions size of wild type. Thus we concluded that the deletion of *Abftf50* gene from *A. brassicicola* does affect the vigor of producing disease hence *Abftf 50* is not a virulence factor.

3.3.2.33 *Abftf53*

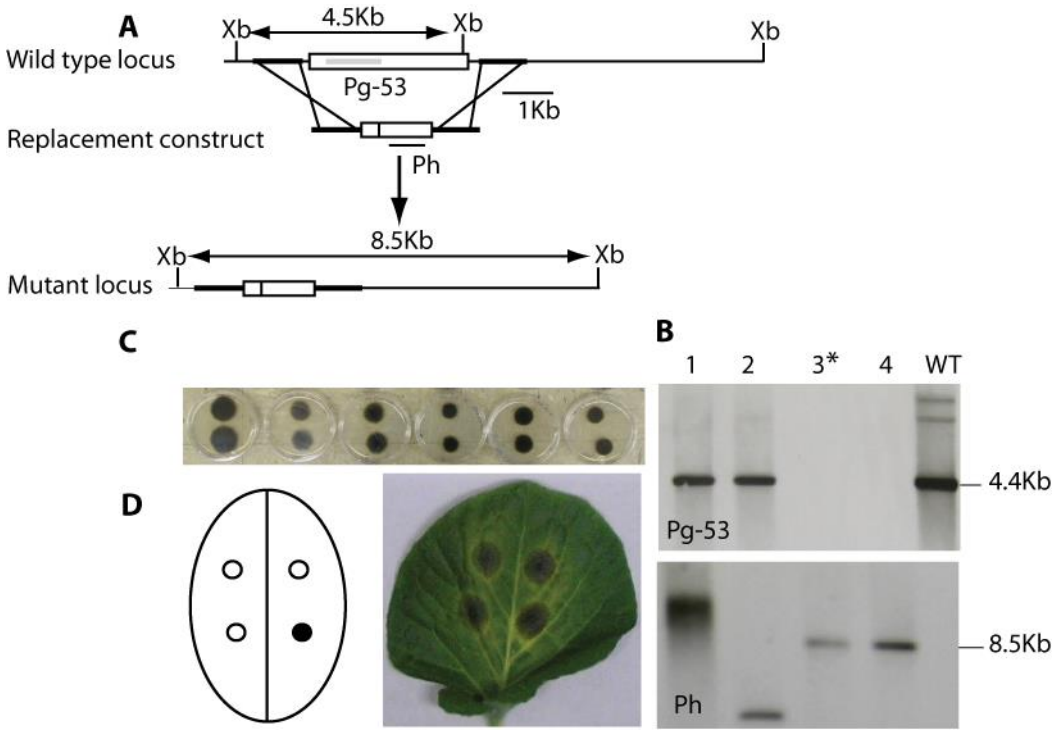

Fig.3.36: Targeted deletion and pathogenicity assay of *Abftf53*gene. A. Schematic diagram of wild-type locus, replacement construct, and mutant locus. It also shows the double recombination event that occurred to produce the 6 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-53 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**.4 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 4 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in lane 1 and 2 showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt. In clone 3 and 5 (lane 3 and 5) the wt gene was successfully replaced by the single copy of hyg cassette, while in lane 1 and 2 insertion occurred at the 3' end by Hyg cassette without replacing the wt gene by virtue of single homologous recombination event. **D** status of virulence *Δabftf53* mutants on host plants (*Brassica oleracea*). Lesions produced 5 days after inoculation with two different concentrations of conidia of the wild type and *Δabftf53*mutant on a detached leaf (table 3.32). An asterisk (*) indicates DNA lanes of mutants which were used for pathogenicity assays. Abbreviations: Xb=*Xba*I enzyme digestion site.

Table 3.32: Decreased virulence of two strains of *Δabftf53* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf53-2	3000	11	17.0±4.6	16.5±4.6	3%	5.1E-01
Δabftf53-2	1500	11	17.1±4.4	17.7±4.9	0%	0.1
Δabftf53-3	3000	11	18.5±3.7	17.6±4.9	5%	0.1
Δabftf53-3	1500	11	18.2±5.2	17.0±5.07	6.5%	0.07

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

Gene *Abftf53* encodes for 842 amino acid containing protein which is annotated as cutinase transcription factor 1 alpha (*Pyrenophora tritici-repentis* e=0.00) and also having C6 fungal specific transcription factor domain. Cutinase are one of the enzymes that harbingers infection and their regulatory genes are expected to be a potential virulence or pathogenicity factor. The pathogenicity assays however suggests converse and the mutants of *Abftf53* gene shows very marginal in lesion than wt. we performed the pathogenicity assays with two different single copy replacement clone and got similar results (fig 3.35 and table 3.32), hence based on bioassays we conclude that *Abftf53* is not a virulence factor.

3.3.2.34 *Abftf55*

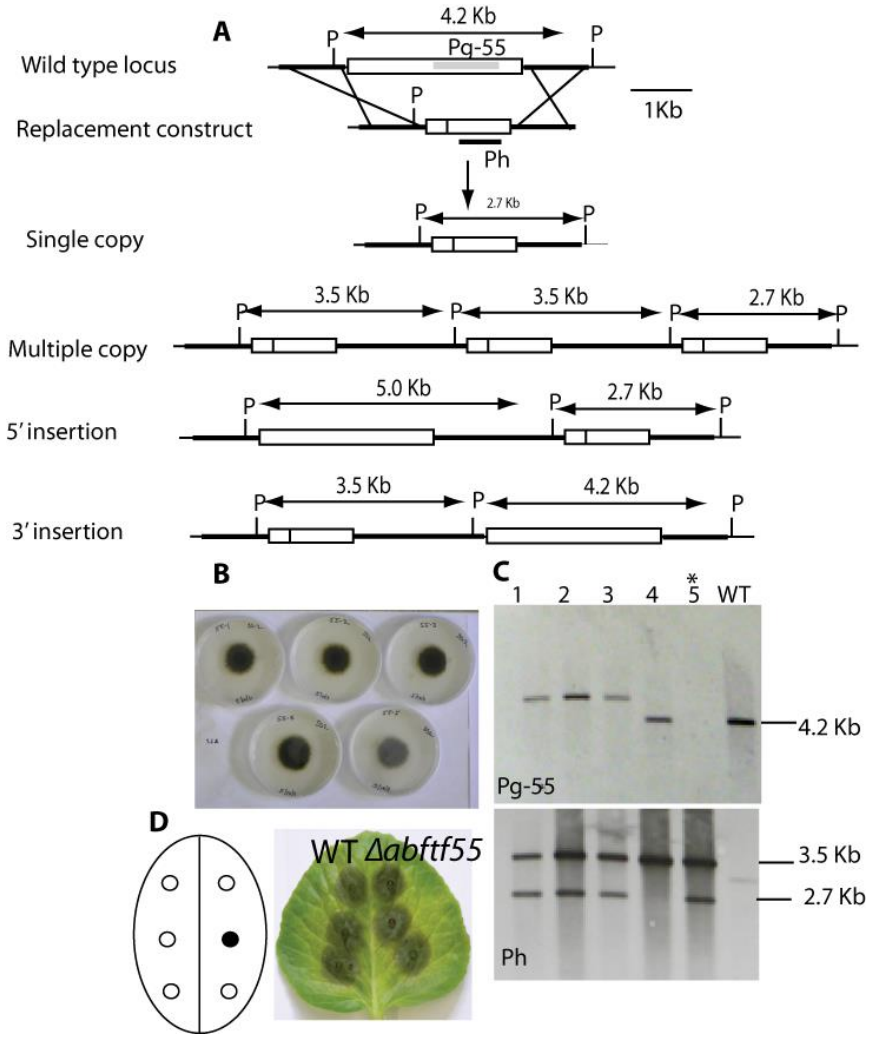

Fig.3.37: Targeted deletion and pathogenicity assay of *Abftf55*gene. **A**. Schematic diagram of wild-type locus, replacement construct, and mutant locus and three possible scenarios of insertion. It also shows the double recombination event that occurred to produce the 5 transformants that were verified. Upper arrows shows the expected band size in the southern blot when hybridized with appropriate probes which are indicated as Pg-55 and Ph in the same diagram for gene specific and Hyg cassette specific probes respectively. **B**. 5 clones were randomly selected from the transformation plate and single spore purified for two rounds before used DNA extraction for southern verification. **C** all 5 clones were used in southern hybridization with two probes as described above. When hybridized with gene specific probe, bands of appropriate size appeared in wild type lane as well in all lanes except lane 1, showing the presence of wild type gene. In next hybridization with Hyg specific probe (bottom panel) bands appeared in all except wt. The banding pattern of both blots suggests that only in clone 5 the wt gene was replaced my multiple copy of hyg gene and the remaining all clones shows either 5' or 3' insertion as a consequence of single homologous recombination. An asterisk (*) indicate DNA lanes of mutants which were used for pathogenicity assays Abbreviations: P=*Pst I* enzyme digestion site, WT = wild type.

Table 3.33: Decreased virulence of two strains of *Δabftf55* deletion mutants compared to wild-type *Alternaria brassicicola*

Mutants	Spores/10µl	d.f.	Lesion diameter		Decrease in lesion diameter	p-value
			Wild type	Mutant		
Δabftf55-5	3000	9	13.2±2.8	11.8±3.0	11%	3.0E-02
Δabftf55-5	3000	9	17.8±3.2	15.7±6.0	11%	0.1
Δabftf55-5	1500	9	17.0±4.0	15.8±4.7	7.0%	0.06

Legend for table: d.f. = degrees of freedom, p = probability, lesion size indicates the average lesion diameter.

The gene Abftf55 encodes for 845 amino acid residue and is annotated as hypothetical protein in *Pyrenophora tritici-repentis* e=0.00 with a fungal specific transcription factor domain. The pathogenicity assays shows very small difference between the lesions made by *Δabftf55-5* and wt (table 3.31). Gene *abftf55* is therefore not a virulence factor in *A .brassiciola*.

3.4 Discussion

Presence of taxon specific transcription factors is not unusual. There are examples of transcription factors that are specifically found only in bacteria or in plants or in animals. The $Zn(II)_2Cys_6$ motif containing transcription factor family is exclusively present in the fungal kingdom (MacPherson *et al.*, 2006). In the present work we attempted to make thirty five deletion mutants of the $Zn(II)_2Cys_6$ transcription factors. Of the 32 viable knock out mutants only 6 (16.6%) were shown to be associated with virulence. Deletion of members of the $Zn(II)_2Cys_6$ transcription factor family in *Neurospora. crassa*, resulted in aberrant mutant phenotypes in 42% (30/72) of the knock out mutants (Colot *et al.*, 2006; Son *et al.*, 2011). However, in *Fusarium.graminearum* only 16% (46/296) aberrant mutant phenotypes were observed when all 296 $Zn(II)_2Cys_6$ transcription factors were knocked out (Son *et al.,* 2011). In these studies phenotypes such as sexual development, structure of spores, vegetative growths were also studied apart from virulence. However in the present study virulence phenotype was the major focus. Of the six genes one gene *AbVf01* showed a similar phenotype as similar to the wild type in terms of vegetative growth and colony morphology but shoed reduction in virulence during pathogenicity assays on green cabbage and other brassicas it also showed reduced growth in presence of phytoalexin- brassinin (Srivastava *et al*, unpublished; chapter 4). This transcription factor is important for the detoxification of phytoalexins which are secreted by the plants in order to protect themselves from plant pathogens during early stages of infection.

Deletion mutant of *Abpf2* produced no lesions on the leaves of the green cabbage (*Brassica oleracia*). Like Δ*abvf01*, Δ*abpf2* is indistinguishable from the wild type in all morphological and physiological properties. This mutant also showed no signs of colonization

even when inoculated on wounded leaf surfaces. The *Abftf8* deletion mutants are also like wild type in phenotype, but have shown compromised growth in presence of oxidative stress inducing H_2O_2. This gene is could be related to reactive oxidation species (ROS) which play a very important role during the initial stages of pathogenesis. *Abftf14* have been annotated as a cutinase transcription factor gene, this could be the first cutinase related gene that is found to be involved in pathogenesis. Previous attempt have failed to identify any cutinase gene related to pathogenesis phenomenon. Further studies are required to characterize the function of these and *Abftf5* and *Abftf24* genes and will be done in future.

In summary, we found more numbers of pathogenesis related gene in Zn(II)2Cys6 transcription factor family in comparison to previous study done on C2H2 transcription factors, where only 8 genes were identified associated with virulence out of more than knock out 200 genes. The ratio of pathogenesis associated genes identified in the present study with the fungal specific transcription factor is much higher. This fact also changes the old notion that suggested that Zn(II)2Cys6 are mostly associated only with metabolic processes.

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CHAPTER 4

AbVf01 is a Zinc-binuclear Fungal Specific Transcription Factor Involved in Detoxification of Brassinin.

Abstract

Phytoalexins are low molecular weight antimicrobial compounds biosynthesized *de novo* by plants in response to abiotic as well as biotic cues including attack from different plant pathogens. Brassinin is one such phytoalexin synthesized exclusively by members of *Brassica* species. In the present study, characterization of a $Zn(II)_2Cys_6$ fungal specific transcription factor is described, which is involved in regulation of virulence and detoxification of the phytoalexin, brassinin in the plant pathogenic fungus *Alternaria brassiciola*. Pathogenicity assays on leaves of green cabbage plant with deletion mutant Δ*abvf01* showed significant (75%) reduction in virulence. Consistent reduction of virulence was observed on plants of different species of Br*assica*. Pathogenicity assays with *Arabidopsis thaliana* however, produced no significant changes in virulence between Δ*abvf01* and wild type *A. brassicicola*. The bioinformatics data suggests that this protein is a transcription factor. The vegetative growth of Δ*abvf01* was extremely compromised during *in vitro* growth assays in the presence of 0.1mM and 0.2mM brassinin. Thin layer chromatography also showed that Δ*abvf01* was unable to degrade brassinin added to the culture media, while wild type *Alternaria brassicicola* does this very efficiently within 6-8 hours of its addition. Discovery that *AbVf01* gene is a virulence factor involved in brassinin detoxification is very important and could lead to designing of novel environment friendly antifungal control agents.

4.1Introduction

Plants are predisposed to pathogen attacks and for protection plants have well developed multi layered defense mechanisms that include cell wall reinforcement, formation of reactive oxygen species (ROS), production of pathogenesis related (PR) proteins, the induction of defense genes, and production of antimicrobial compounds such as phytoanticipins and phytoalexins. Phytoanticipins are compounds that are constitutively expressed (VanEtten *et al.*, 1994; Morrissey and Osbourn, 1999) and phytoalexins are compounds which are synthesized *de novo* by plants in response to pathogen invasion or other cues (Bailey and Mansfield, 1982; Brooks and Watson, 1985; Smith, 1996). The term phytoalexin was introduced to describe induced antibiotic plant metabolites and is derived from the Greek words phyton = plant and alexein = protecting substance (Müller and Börger, 1940; Islam, 2008). By definition phytoalexins are low molecular weight secondary metabolites that are secreted in small quantities to display plant resistance towards biological or environmental cues. The first phytoalexin to be isolated and chemically characterized was 'pisatin' from pea (*Pisum sativum*) (Cruickshank and Perrin, 1960). Phytoalexins from crucifers were first reported in 1986 by Takasugi *et. al*, brassinin, 1-methoxybrassinin, and cyclobrassinin that were isolated from Chinese cabbage (*B. campestris* L. ssp. pekinensis) infected with *Pseudomonas cichorii* (Takasugi *et al.,* 1988). Currently, close to 44 phytoalexins have been identified in *Brassicaceae* (Pedras *et al.*, 2011c).

Signals due to pathogenic microbial attack, heavy metal salts, UV radiation and other environmental insults stimulate plants to secrete phytoalexins. To overcome the resistance generated by phytoalexins phytopathogens can detoxify phytoalexins to a significant level and

this can be correlated with virulence. Tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*) and alfalfa (*Medicago sativa*) were individually transformed with stilbene synthase that enabled the plants to secrete higher loads of grapevine (*Vitis vinifera*) phytoalexin, resveratrol. These transformed plants showed increased resistance to *Botrytis cinerea*, a fungal pathogen of tobacco (Hain *et al.*, 1993) *Phytophthora infestans* (Thomzik *et al.*, 1997) a pathogen of tomato and to *Phoma medicaginis* a pathogen of alfalfa. (Hipskind and Paiva, 2000, Pedras and Ahiahonu, 2005)

Brassinin is one of the most important phytoalexins synthesized by members of *Brassica spp*. of Brassicaceae family. It is involved in antimicrobial activity against *A. brassicicola* and is also a biosynthetic precursor of many other phytoalexins, such as cyclobrassinin, brassilexin and spirobrassinin as well. To this point it is known that *A. brassicicola* can detoxify brassinin during its infection on brassica plants. The degradation product indole-3-methanamine enzyme brassinin hydrolase involved in this process is also known (Pedras *et al.*, 2000; Pedras and Ahiahonu, 2005; Pedras *et al.*, 2009b; Sexton *et al.*, 2009; Pedras *et al.*, 2011a; Pedras and Minic, 2012). But whether their degradation *in planta* is associated with the virulence has not been established.

During the screening of $Zn(II)_2Cys_6$ fungal specific transcription factors associated with pathogenesis a mutant of gene *AbVf01* was discovered that showed approximately 70% reduction in virulence and severe retardation in fungal growth in two concentration of brassinin in liquid medium. The gene deletion mutants showed no phenotypic change in colony morphology, vegetative growth and conidia structure. The growth was equal to the wild type *A. brassicicola* on potato dextrose agar (PDA) and on 1% glucose with 0.5% yeast extract (GYEB),

however on green cabbage and other brassica plants and on PDA and GYEB media supplemented with brassinin it showed drastic reduction in growth. The aim of this study is to investigate the importance of brassinin detoxification in successful pathogenesis.

4.2. Materials and Methods

4.2.1 Fungal strain, growth conditions, transformation and southern hybridization

Alternaria brassicicola isolate ATCC 93866 96866 (American Type Culture Collection, Manassas, VA) isolated from green cabbage (*Brassica oleracia*) was used in this study. Fungus was grown on Potato dextrose agar (PDA) (Difco^{TM} , BD Co. Sparks, MD, USA) under dark at 25ºC as solid medium and on 1% glucose and 0.5% yeast extract (GYEB) in liquid medium by continuous shaking at 100 rpm at 25ºC in dark in a temperature controlled incubator shaker (C-25 incubator shaker, New Brunswick Scientific, Edison, NJ, USA). Protoplasts prepared from growing mycelial balls were transformed as previously described in chapter 2. Purification of transformed colonies through two rounds of single spore isolation, followed by nucleic acid preparation and southern hybridizations were done to identify true mutants as described previously in chapter 2 and 3. Four probes were used for Southern hybridization (Fig. 4.3). Sequences of primers used to produce deletion mutants and DIG labeled probes are shown on table S-1.

4.2.2 Bioinformatic Analysis

Predicted 808 amino acid sequences of *AbVf01* were analyzed using BLASTp against Refseq database, using Blastp algorithm and BLOSUM 62 matrix (Altschul *et al.*, 1990). Interpro (Bateman *et al.*, 2004). WoLF PSORT was used to determine the DNA binding domain

and identify the nucleus localization signal (NLS) in the amino acid sequence (Horton *et al.*, 2007).

4.2.3 Generation of two strains of deletion mutants for *AbVf01*

All transformation constructs described were produced as described previously in chapter 2 and 3. *AbVf01* deletion mutants were made by replacing the gene with a *Hyg.B* cassette (*Δabvf01:TrpP-HygB)*. The replacement construct was produced with the following three sets of primers. The primer sets P1 and P2 and P5 and P6 were used respectively to amplify ~1 Kb region at the 5' and 3' flanking regions of the targeted locus. Another set of primers, P3 and P4, was used to amplify the *Hyg.B*-selectable marker gene cassette (1,436 bp) from pCB1636. Sequences of primers are available in S-1. The final transformation constructs were produced by PCR amplification using the mix of products from three reactions as template DNA and two primers, P1 and P6 (table S-1).The schematic diagram in figure 3.2 in chapter 3 describes the production of the deletion construct.

AbVf01 deletion mutants were also produced that express green fluorescence under the control of the *AbVf01* gene promoter (*Δabvf01*:*AbVf1*P-GFP:HygB). To make the construct, the *AbVf01* promoter region and 3' flanking region were amplified with primer pairs P1 and PGFP2 and P5 and P6, respectively. Another set of primers, PGFP3 and P4, were used to amplify the 2,423 bp that covered the coding regions of the GFP and the *Hyg. B* cassette from plasmid pCB16G6 (Cho *et al.*, 2006). The amplicons from three reactions were then mixed and amplified with primer pairs P1 and P6 to make the final construct that was used for transformation of wild type strain (fig. 4.1). Sequences of primers are available in table S-1.

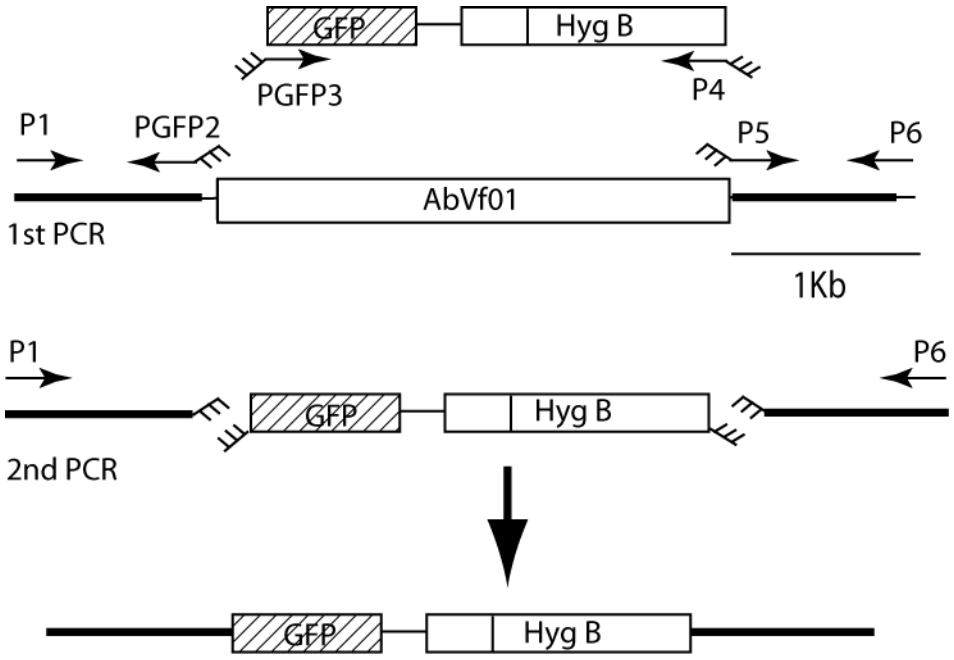

Fig 4.1 schematic diagram describing the two PCR reactions used to make gene replacement construct, where the target gene is replaced by a cassette of GFP and hygromycinB resistance gene.

4.2.4 Generation of mutants expressing *AbVf01-GFP* fusion proteins.

In order to create mutants expressing green fluorescence protein fused to the C-terminal of AbVf01, the *AbVf01* coding region (1,156 bp) and 3' flanking region (221 bp) were amplified with 1FTF1GFP-GA and 2'FTF1GFP, and P5 and 6'FTF1-DP6, respectively. Another set of primers, 3'FTF1GFP and P4, was used to amplify the 2,384 bp that covered the coding regions of the GFP and the HygB cassette from pCB16G6. The final transformation constructs were produced by PCR amplification from the mixture of the PCR products using 1FTF1GFP-GA and 6'FTF1-DP6 (Fig.4.2).

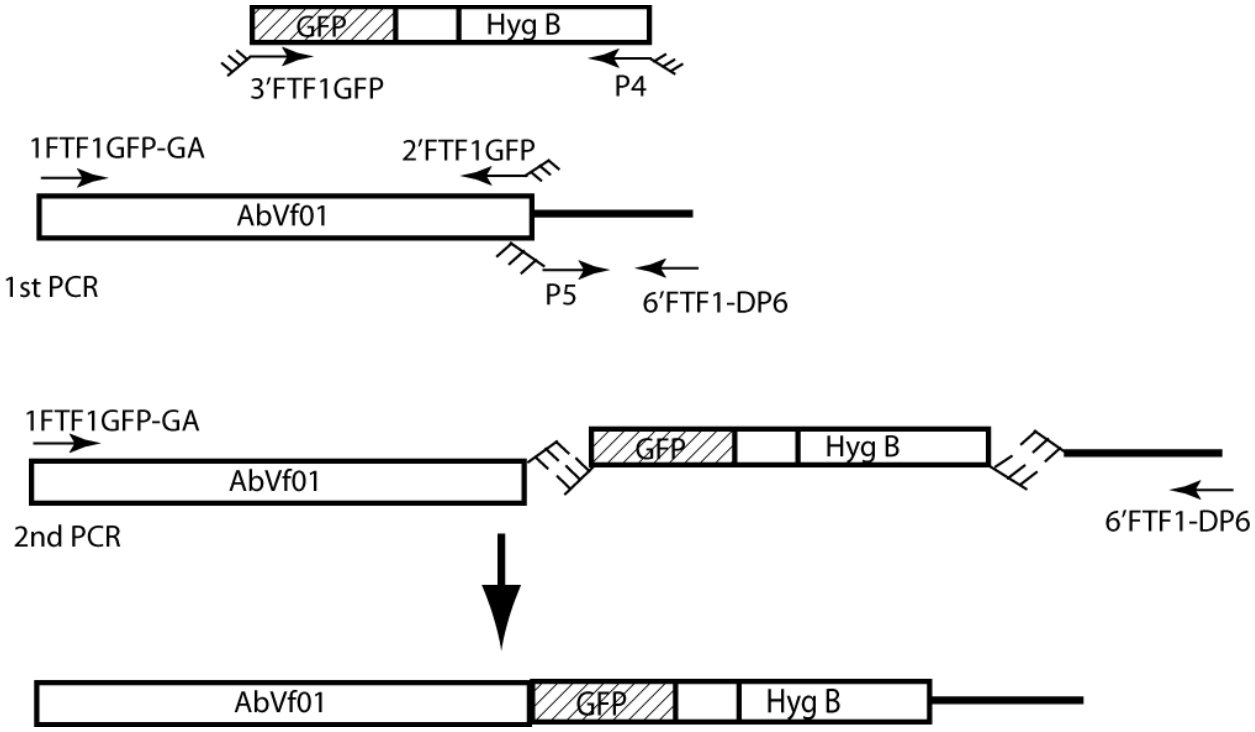

Fig 4.2 Schematic diagram describing the two PCR reactions used to make fusion protein construct.

4.2.5 Complementation of the *Δabvf01* mutants

The *Δabvf01-5* mutant was complemented with the wild-type *AbVf01* allele with its native promoter. Two primers P1 and P6 were used to amplify the 4,722 bp wild-type allele, of the *AbVf01* gene using *A. brassicicola* genomic DNA as a template. The amplified product is used to reintroduce wild-type *AbVf01* into the *Δabvf01-5* mutant (*Δabvf01: AbVf01*). The PCR product included a 1,047 bp 5' flanking region, 2,692 bp complete coding region, and a 983 bp 3' flanking region. Separately, pNR-20F and pNR-775R were used to amplify a 2,226 bp-long nourseothricin-resistant cassette, as a selectable marker gene, using a pNR vector as the template (Malonek *et al.*, 2004). The sequences of primers are in table S-1. These two products (7 µg of *AbVf01* and 3 µg of *NTC* cassette) were mixed to transform the *Δabvf01-5* mutant.

Complemented mutant clones were purified by two rounds of single-spore isolation and Southern hybridizations were done to confirm the introduction of the wild type allele into the *Δabvf01-5* mutant (Fig.4.5).

4.2.6 Pathogenicity assays

Conidia from 5-7 days old PDA plates were harvested in sterilized water and washed twice before inoculation. Pathogenicity assays were performed on detached leaves from 5-to-8-week-old green cabbage (*Brassica oleracia var.capitata)*) plants. For detached leaf assays, leaves were removed, placed in mini-moist chambers, and located on a laboratory bench. Pathogenicity assays were also performed on whole plants of wild type *Arabidopsis thaliana* (Col-0) or the *pad3* mutant (Col-0 background) (Zhou *et al.*, 1999). Pathogenicity assays with oil seed mustard (*B. juncea*), cauliflower (*B. oleracea var. bortrytis*), broccoli (*B. oleraceae var.Italica*) and Chinese cabbage (*B. rapa var. pekinensis*) were performed on 5 weeks old whole plants at the same time under the same condition using 2,000 conidia in 10 µl of water. For the pathogenicity assays on whole plants, potted plants were placed in a semi-transparent plastic trough with adequate water. The troughs with plants inside were sealed with saran wrap after inoculation to keep the relative humidity close to 100%. The decreased virulence of each mutant was calculated using the formula $(\sum(Dwi\text{-}Dmi)/ \sum(Dwi)) \times 100$, where Dw*i* was the lesion diameter created by the wild-type for the *i*th sample and Dm*i* was the lesion diameter produced by the mutant for the *i*th sample.

4.2.7 Germination assays in the presence of Brassinin

Lyophilized powder of 100 mg brassinin (Sata Cruz Biotechnolgy Inc., Santa Cruz CA, USA) was dissolved in 1 ml dimethyl sulfoxide (DMSO). 250 µl of 100 mg/ml Brassinin solution was kept in -20° C for long term storage. One tube was thawed just before use to minimize freeze and thaw cycles. To achieve 0.1 and 0.2 mM brassinin concentration in PDA, we respectively added 12.5 µl and 25 µl solution in 50 ml of melted PDA when it reached 50° C. It was thoroughly mixed and immediately poured at 80 or 150 mm disposable petri dish. Conidia of either *Δabvf01* mutant or wild type *A. brassicicola* harvested from 5 days old PDA plates were inoculated using 10 µl sterile disposable pipette tips. Similarly, PDA containing Brassinin was poured on microscope glass slides and conidia were added and slides were maintained in the moist petri plates. Germination was recorded under a phase contrast microscope (Olympus, BX4, Olympus Corp. Tokyo, Japan) and images were captured by the CCD camera mounted on the microscope (Infinity 2C, Lumenera Corp. Ottawa, ON, Canada). Vegetative growth on plates was evaluated by measuring the colony diameter using side ruler and images of plates were recorded using digital camera (Olympus, SZX16, Olympus Corp. Tokyo, Japan).

4.2.8 Mycelia growth in GYEB medium with brassinin

Fungal mycelia were grown for 3 days in 1% glucose and 0.5% yeast extract broth (GYEB) with an exchange of the medium 16 hours ahead of harvest. We harvested the mycelia in 50 ml conical tubes, centrifuged and transferred mycelia (equivalent to 0.25 g in dry weight) to a new GYEB medium containing 0.0 mM, 0.1 mM, or 0.2 mM Brassinin. They were cultured at 25° C in a shaker incubator for 24 hours, harvested, and their dry weights were measured.

4.2.9 Brassinin digestion assay

During the fungal mycelial growth assays in the presence of brassinin, 2 ml GYEB was recovered from each culture. After removal of mycelia by centrifugation at 13,000 rpm for 5 minutes, 0.8 ml of media were transferred to a clean tube and extracted with 0.8 ml chloroform twice. The combined chloroform extracts were dried in Savant Speedvac Concentrator (Savant scientific Inc., Farmingdale, NY, USA). The pellet was dissolved in 16 µl DMSO and spotted on thin layer of silica gel coated aluminum plates (Whatman Ltd. Maidstone, Kent, England). The relative concentration and integrity of brassinin were evaluated under ultraviolet light (254 nm) before or after separation of compounds by thin layer chromatography with ethylacetate (EtOAc) as a mobile phase. (Fig 4.6, 4.7)

4.2.10 Tryphan blue staining

Tryphan blue stain (MP Biomedicals, LLC, Solon, OH, USA) was used to study the various stages of conidia germination, germ tube elongation, formation of specialized structure for pathogenesis. Green cabbage leaves were inoculated with wild type and *Δabvf01*conidial spores and stained with tryphan blue stain at different times. After 48 hours of staining the infection locations were destained and washed with 70% ethanol. The stained area is carefully excised from the leaf using a razor blade and mounted on a slide for making observations with a compound microscope and microphotographs were taken using CCD camera mounted on microscope.

4.2.11 Epiflouresence microscopy

Plant tissues at different stages of infection were trimmed with a razor blade, placed on microscope slides, and covered with gold seal cover glass, images were acquired using a epiflouresent microscope (Olympus BX-5 fitted with epifluorescence, DIC optics, and an Optronics MacroFire digital camera) using e-GFP objective with excitation at 365 nm and emission at 420 nm. Spectra for fungal tissues expressing standard green fluorescence and for plant cells emitting autofluorescence were collected and image was recorded using a digital camera mounted on the microscope.

4.2.12 Salt stress and pharmacological tests

Each fungal strain from glycerol stocks was grown on PDA containing an appropriate selectable agent and cultured in the dark for 5 days at 25˚C. In order to test the fungal cultures for sensitivity to osmotic stress, cell wall stressors and antifungal agents, wild-type and mutant conidia were pipetted onto PDA containing 2% (w/v) NaCl, 1.2 M sorbitol (Sigma-Aldrich St. Louis, MO, USA). Calcoflour white was used to evaluate cell wall stress. (Sigma-Aldrich Chemie GmbH, Switzerland). Colony diameters were measured after 4 days of addition. Pharmocological tests were done by adding anti-fungal agents: fluconazole 0.25µg/ml, amphotericin B 0.15µg/ml and ketonazole 0.5µg/ml (Sigma-Aldrich, St. Louis, MO, USA) in 50 ml of PDA and poured into petri plates. Equal number conidia were pipetted into each plate. Colony size was measured after six days of addition of conidia and photographs were taken. Before conducting this experiment, each of the antifungal agents was dissolved in appropriate

solvents as instructed by the manufacture. We also estimated the EC_{50} values for each of the anti-fungal agents prior to conducting experiment.

4.3. Results

4.3.1 Initial screening of mutants for the *Abvf01* gene

Gene knockout mutants of fungal-specific transcription factors were created using gene deletion methods previously described in chapter 3 (Fig.3.2). The 2796 nucleotides (nt) spanning the partial promoter (91 nt), full protein-coding region (658 nt), and a short 3' untranslated region (13 nt) was replaced with a Hygromycin B transferase (*Hyg.B*) gene cassette. Southern hybridization verified that the *Abvf01* coding region was replaced by a single copy of the *Hyg. B* resistance cassette in seven mutants (Fig.4.3, A, lane 1-6 and 7). During pathogenicity assays, a gene was observed whose mutants similar to wild-type *A. brassicicola* in its phenotype, but showed severe reduction in its virulence. The gene was predicted to encode 808 amino acids. It included a $Zn(II)_2Cys_6$ fungal-specific DNA binding domain (IPR001138, PF00172) predicted by Interpro (Bateman *et al.*, 2004) and WoLF PSORT (Horton *et al.*, 2007) . Single copy of this gene is present in the *A.brassicicola* genome and homolog of this gene has been found in the genome of all filamentous fungi whose genome sequence is available in the public domain.

4.3.2 Replacement of the *Abvf01*gene with a HygB –GFP cassette

To confirm the association of *Abvf01* in virulence, additional mutants of the gene were created. a second set of six targeted gene deletion mutants by replacing the coding region by another replacement construct were also produced. The construct consists of a coding gene of green fluorescent protein (GFP) coding gene and *Hyg B* cassette (fig 4.1). The southern

hybridization with gene specific and hygromycin probe suggests *Abvf01* coding region was replaced by a single copy of the replacement construct (fig 4.3B). The GFP gene is under the control of the *Abvf01* promoter. A nonpathogenic mutant (*Δabvf01-5*) was complemented with the wild-type allele of the *Abvf01*-coding gene.

4.3.3 Reduction of virulence in the *Δabvf01* mutants on *Brassica* plants

Pathogenicity assays were performed using three mutants (*Δabvf01-5, -9,* and *-12*) on compatible host plants, green cabbage (*Brassica oleracea var. capitata*) (Cho *et al.*, 2007b; Cho *et al.*, 2009a). All three *Δabvf01* mutants (*Δabvf01-5, 9,* and *12)* caused disease spots that were 67-68% smaller in diameter than those produced by the wild type *A. brassicicola* when inoculated with 1,000-2,000 conidia on green cabbage leaves (Fig4. 4, A-D and table 4.1). The *Δabvf01-5* complemented with the wild-type allele of *Abvf01* produced lesions comparable to wild-type *A. brassicicola,* indicating that their pathogenicity and virulence on cabbage had been restored (Fig. 4.4, A). We further tested the mutant's ability to cause lesions to five additional *Brassicia* species, two cultivars of oil seed mustard (*B. juncea*), cauliflower (*B. oleracea var. bortrytis*), broccoli (*B. oleracia var. italica*) and Chinese cabbage (*B. rapa var. pekinensis*). The *Δabvf01-5* mutant consistently produced smaller lesions than the wild type in these *Brassica* plants (Fig.4 4, E-H and Table 4.3). We also inoculated the cabbage leaves which were mechanically injured and found that though the wild type showed more vigor in pathogenicity or production of lesion but the mutants maintained slow progression of lesion size in comparison to wild type. In other words, we could not find any differences among results obtained from injured and non-injured plants. The *Δabvf01* mutants showed reduced virulence in all tested *Brassica* plants but not in *A. thaliana*. One of major difference between the *Brassica* plants and *A.*

thaliana is in the different kind of phytoalexins they produce and we speculate from this observation that this gene might be involved in detoxification of phytoalexin brassinin.

4.3.4 No reduction of virulence in the *Δabvf01* mutants on *Arabidopsis thaliana*

Also virulence of the *Δabvf01-5* mutant was tested on the non-B*rassica* host plant *Arabidopsis thaliana*, ecotype Col-0 and the *pad3* mutants that lack the phytoalexin, camalexin (Zhou *et al.*, 1999). The mutant colonized both with the wild type *A. thaliana* and the *pad3* mutant. The lesion size produced by *A. brassicicola* was bigger on the *pad3* mutants than the wild type *A. thaliana*. However, an average lesion size cause by the *Δabvf01-5* mutant and wild type *A. brassicicola* was not statistically different in both *pad3* mutants and wild type *A. thaliana* (Fig.4.4, I,J and K). The *Δabvf01* mutants showed reduced virulence in all tested *Brassica spp* plants but not in *A. thaliana*. One of major difference between the *Brassica spp.* plants and *A. thaliana* is in the different kind of phytoalexins they produce and we speculate from this observation that this gene might be involved in detoxification of phytoalexin brassinin.

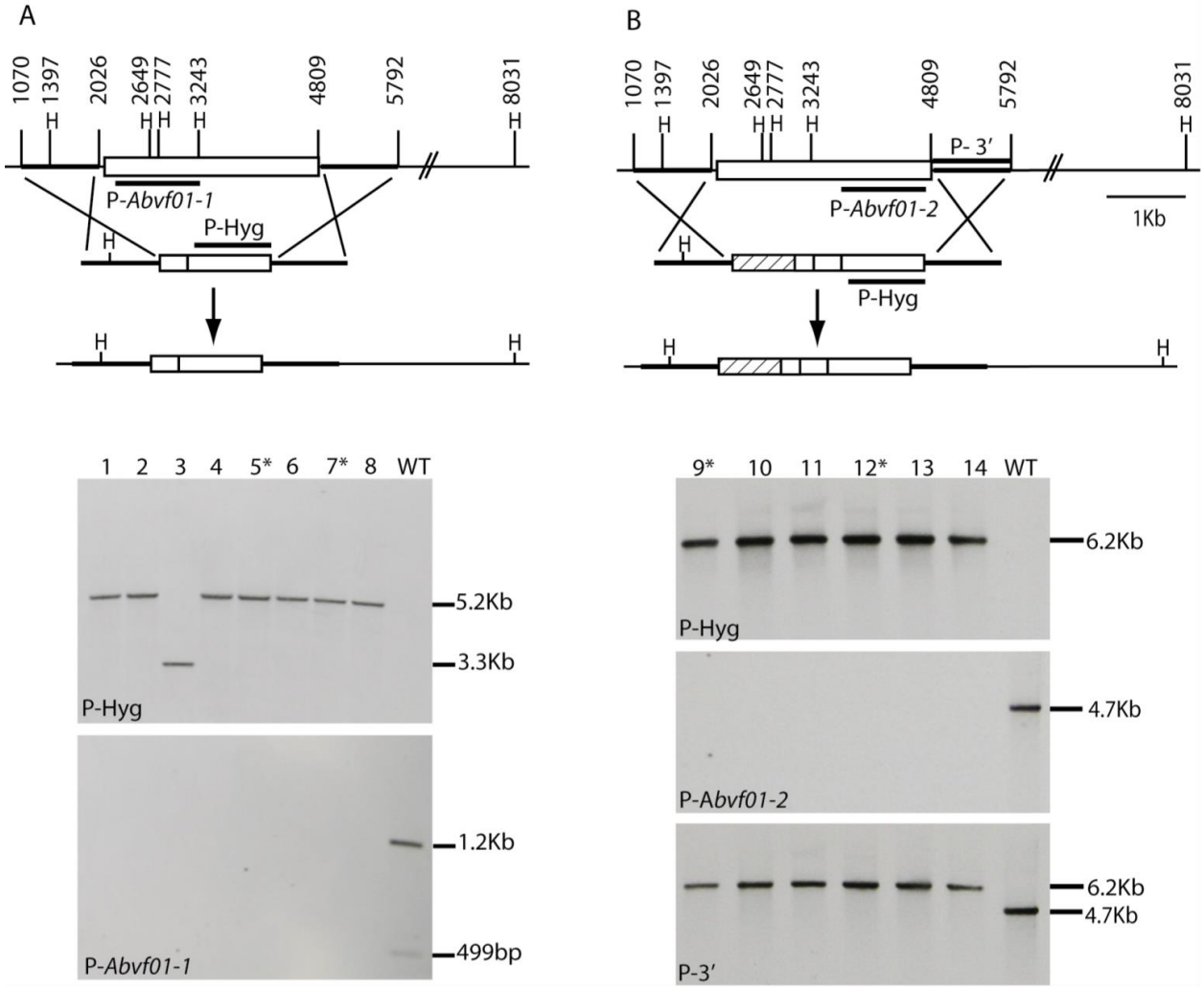

Fig. 4.3. Targeted deletion of *AbVf01* gene. **A**. Replacement of the AbVf01 coding region with a selectable marker Hygromycin B transferase (Hyg B) cassette. **B**. Replacement of the AbVf01 coding region with a green fluorescent protein coding region (hatched box) plus a HygB cassette. In both A and B, the upper panel is a schematic diagram of wild-type locus, replacement construct, and mutant locus in order and the lower panel is a collection of Southern blots displaying loss of the AbVf01 gene in selected mutants. Asterisks (*) indicate DNA lanes of mutants whose pathogenicity assay results presented in this study. Probe regions are marked by P-AbVf01, P-3', and P-Hyg. Abbreviations: H = HindIII enzyme digestion site.

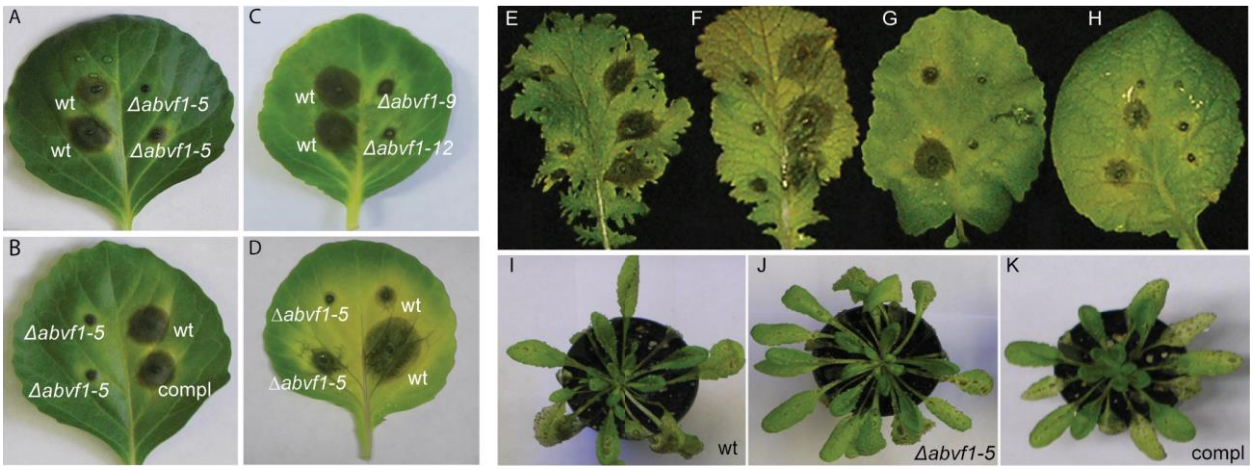

Fig.4.4. Reduced virulence of *ΔAbvf01* mutants on host plants (*Brassica oleracea*) and normal virulence on *Arabidopsis thaliana*. **A** Lesions on *B. oleracea* 5 days after inoculation with 2,000 conidia of the wild type and Δabvf01-5 on detached leaves.**B**. Reduced lesion size by mutant and restoration size produced by a complemented mutant and the wild type compared to the small lesions produced by the *Δabvf01-5* mutant. **C**. Lesions produced by 1,000 conidia of the wild type and 1,000 conidia of a *Δabvf01-9* and *Δabvf01-12* mutants. **D** Mechanical injury, top 2 spots un injured inoculation site, bottom 2 shows injured ones data was collected 4 dpi. inoculated with 1,500 spores **E-H**. Lesions caused by ~1,500 conidia of the wild type and the *Δabvf01-5* mutant *Alternaria brassicicola* on whole plants of different species of brassica. **E** Green Mustard Homiz, **F**. Green Mustard Pizzo, **G**. Cauliflower, **H**. Chinese cabbage. **I - K** *Arabidopsis thaliana* (Col-0) inoculated with 3000 spores of wt, mutant and complementaion mutant showing appearence of lesion. Abbreviations: wt = wild-type *A. brassicicola*; Δabvf01 = AbVf01 deletion mutant; compl = Δabvf01-5 mutant complemented with a native allele of the AbVf01 gene (Δabvf01:AbVf01).

Table 4.1. Decreased lesion size caused by three *Δabvf1* deletion mutants compared to wild-type *Alternaria brassicicola*.

Mutants	d.f.	Lesion diameter (mm)		% Decrease in lesion diameter	p-value
		Wild type	Mutant		
Δabvf01-5	11	9.3±4.3	3.0±2.7	68	1.2E-05
Δabvf01-9	11	11.5±4.1	3.7±2.5	67	7.8E-07
Δabvf01-12	11	11.3±4.0	3.7±2.6	67	5.6E-06

d.f. = degrees of freedom, p-value = probability, lesion size indicates the average lesion diameter.

Table 4.2. Decrease in lesion size of deletion mutant and wild type when inoculated by causing mechanical injury to the leaf.

Mutants	Spores/10µl	d.f.	Lesion diameter		% Decrease in lesion diameter	p-value
			Wild type	Mutant		
*Δabvf01-5*0-1*	1500	5	26.1±2.4	9.8±2.9	62	1.41E-06
Δabvf01-5	1500	5	6.6±1.6	1.6±1.5	75	0.0007

wt = wild type, d.f. = degrees of freedom, p-value = probability, lesion size indicates the average lesion diameter.

Table 4.3. Decreased lesion size caused by *Δabvf1-5* mutant compared to wild-type *Alternaria brassicicola* on five Brassica species.

Plant	d.f.	Lesion diameter (mm)		%Decrease in lesion diameter	p-value
		wt	*Δabvf01-5*		
Green Mustard Homiz *(Brassica juncea)*	6	16.1±1.5	3.2±1.2	80	3.6E-06
Green Mustard Pizzo *(Brassica juncea)*	6	16.2±1.7	2.2±0.7	86	3.5E-07
Cauliflower *(Brassica oleracea* var.*botrytis)*	4	6.4±4.3	1.2±1.0	81	0.065
Chinese cabbage *(Brassica rapa* var.*pekinensis)*	4	5.8±2.2	1.6±0.8	72	0.001
Broccli (*Brassica oleraceae var. italiana*)	4	11.8±0.5	1.3±0.9	89	2.4E-04

wt = wild type ,d.f. = degrees of freedom, p-value = probability, lesion size indicates the average lesion diameter measured after 8 days of inoculation, 1,500 conidia/10µl of sterile water were used for inoculations. All host plants were 5 weeks old and pathogenicity assays were performed at the same time.

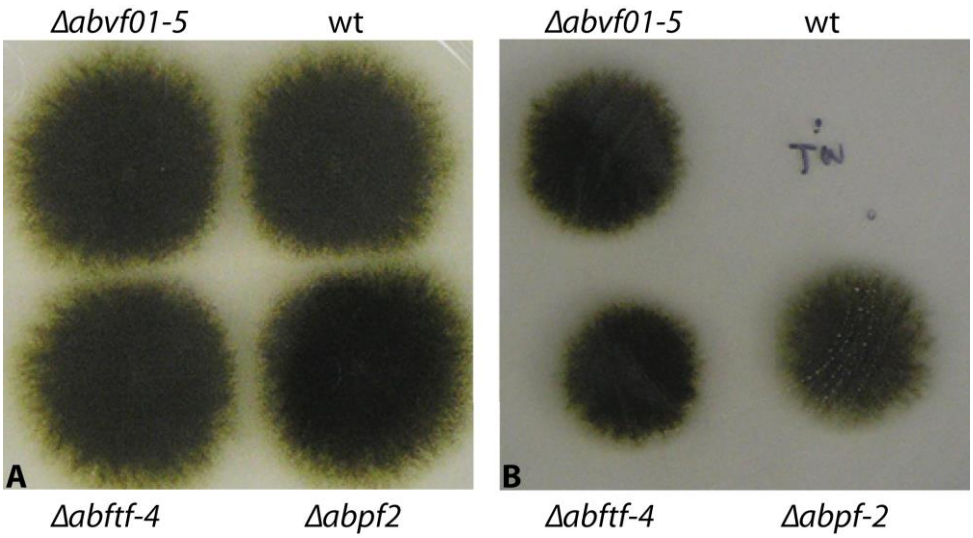

Fig.4.5 Growth assays of Δabvf01. Deletion mutants Δabvf01, Δabftf-4 and Δabpf-2 and wt were used to assay the changes in growing pattern and morphology of mutants in comparison to wild type. The assay was carried out on Potato dextrose agar (PDA) (fig4.3,A.) and on PDA containing 30ng/ml Hygromycin B. No phenotypic changes were observed between wt and Δabvf01 however a general reduction in growth was observed in case of all mutants. wt did not grow in PDA+Hyg. plate because it does not have the resistance gene in its genome.

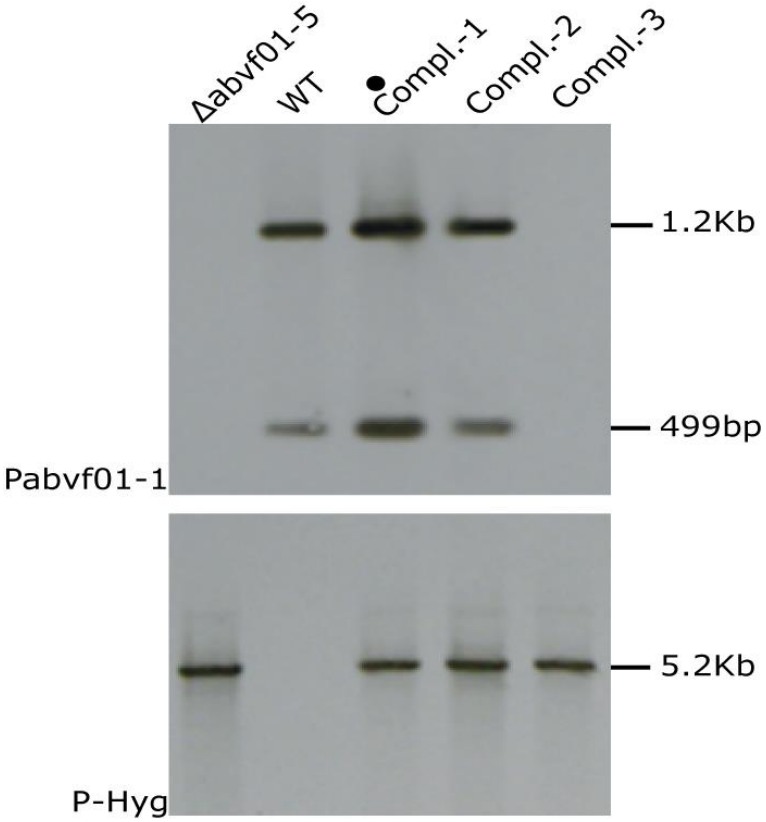

Fig. 4.6: **Southern showing complementation**: Δ*abvf01-5* was used for generating the complementation strain. Three colonies were obtained and analyzed through southern hybridization. Compl.-1 and Compl.-2 shows reinsertion of wild type allele as present in lane WT. While compl-3 does not show any reinsertion event and is like Δ*abvf01*. The complementation clone denoted by '●' indicates the clone used for pathogenicity assays and produced lesion equal to wt Fig4.3, C.

4.3.5 No difference in germination and appressorium-like structure formation

Germination and formation of an appressorium-like structure were compared between the *Δabvf01* mutants and wild type on host plant *B. oleracea* (Fig. 4.8). The mutant conidia germinated and earlier than wt and the length of germ tubes were highly variable within each group (wild type and mutant). The difference in average length between mutant and wild type germ tubes was not statistically significant (table 4.4). Germination was slower on *Brassica oleracea* (green cabbage) than on nutrient rich media for both the wild type and the *Δabvf01* mutant. A small swollen structure (appressorium-like structure thereafter) was formed by 24 hpi in a similar frequency at the tips of germ tubes produced by both the mutant and wild type fig 4.8. In addition, both the mutant and the wild type caused host plant cell death during the pathogenesis.

There were no differences in germination and colony growth between the wild type and mutants during growth on potato dextrose agar (PDA), or medium containing PDA+ different stress inducing agents like 0.7 M Nacl,1.0 M sorbitol 10 µg/ml Calcoflour white and antifungal agents fluconazole 0.25 µg/ml, amphotericin B 0.15 µg/ml and ketonazole 0.5 µg/ml (fig. 4.7 A-H). We observed no difference between mutant and wt colonies with any of the reagents.

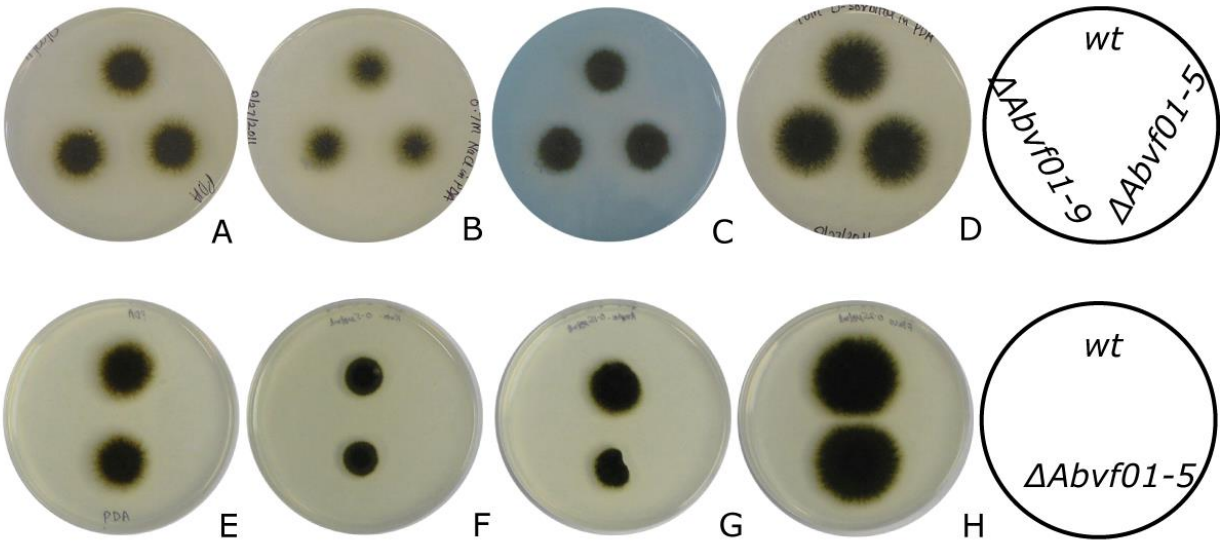

Fig.4.7: Growth assays of mutant and wild type: Two mutants developed by replacement of AbVf01 gene by hygromycinB resistance gene *(Δabvf01-5)* and by GFP-hygromycin B resistance gene cassette. *(Δabvf01-9*). **A-D** The growth assays were done in presence of plain PDA 0.7M NaCl 10µg/ml calcoflour white and 1.0 M sorbitol. The bottom row shows pharmacological assays of mutant and wild type. Abvf01 and wt were cultured in the presence of PDA and antifungal drugs **E-H** amphotericin B 0.15µg/ml, ketonazole 0.5µg/ml and fluconazole 0.25µg/ml. Equal numbers of conidia were collected from 6 days old PDA plates. Photographs and measurements were taken after 6 days of growth.

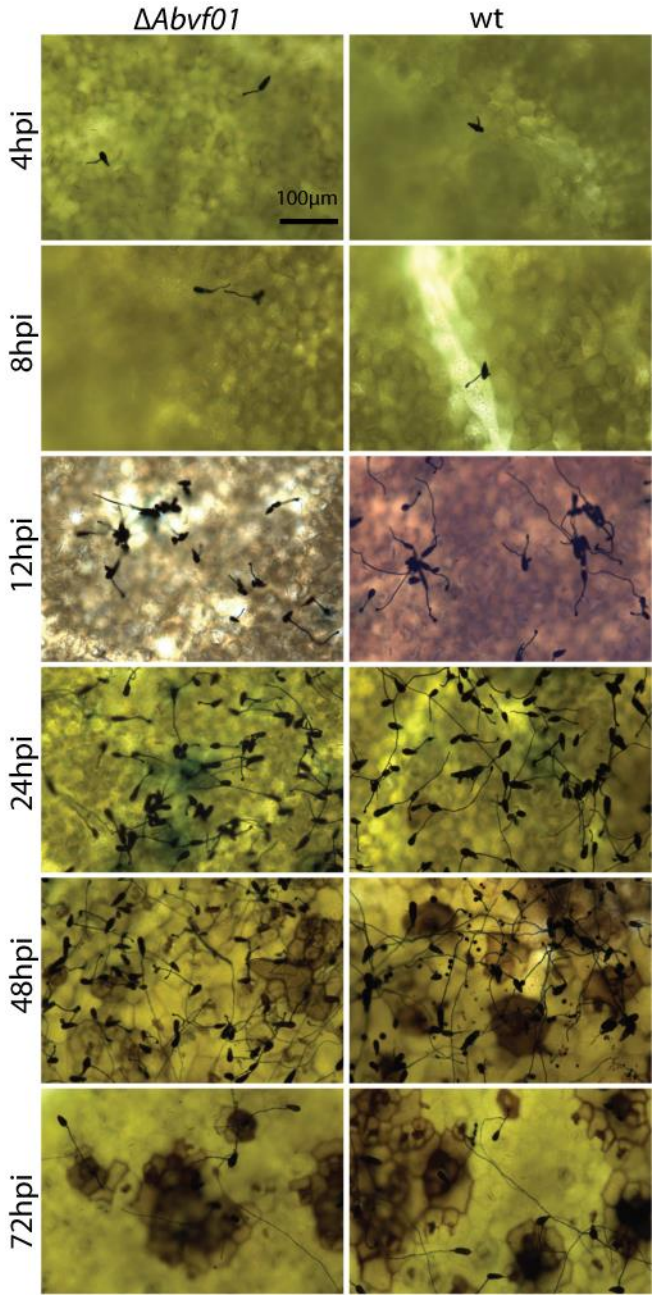

Fig.4.8: Germination and initial stages of pathogenesis on host plant. Comparison between *Δabvf01-5* mutant and wild type *Alternaria brassiciciola* on host plant during initial stages of infection. Individual images displays tissues of green cabbage leaf infected with *A. brassicicola.* Fungal tissues were stained with tryphan blue. Dead plant cells appeared darker than adjacent tissues in four images representing 48 and 72 hpi.

Table 4.4. Vegetative growth of *Δabvf1-5* deletion mutant and wild-type *Alternaria brassicicola*.

	d.f.	Length of hyphae (mm)		p-value
		Wild type	Mutant	
12 hpi	16	64±43	78±36	0.31
24 hpi	150	118±91	105±69	0.16
48 hpi	161	139±145	109±63	0.14
72 hpi	18	481±288	361±166	0.06

Abbreviations: d.f. = degrees of freedom, p-value = probability, lesion size indicates the average lesion diameter.

4.3.6 Brassinin effects on growth and germination of *Δabvf01*

The *Δabvf01* mutants (*Δabvf01*-5 and *Δabvf01*-9) and wild type *A. brassicicola* were grown on PDA containing brassinin in various concentrations to evaluate the effect of the *abvf01* gene in detoxification of brassinin. Both the *Δabvf01* mutants and the wild type *A. brassicicola* grew with equal rates on plain PDA or PDA containing 1.0% dimethyl sulfoxide (DMSO) (Fig. 4.9). In the presence of 0.1 mM Brassinin, colony size was approximately 50% smaller for the mutants than the wild type. In the presence of 0.2mM Brassinin, the *Δabvf01*mutants failed to form colonies while the wild type and complemented mutants showed reduction in colony size. The complemented mutant, however, showed almost similar colony size as wild type in presence of 0.1 mM and 0.2 mM Brassinin. The stereoscope image shows that delayed germination in presence of 0.1 mM and 0.2 mM Brassinin in mutants after 12 hours of conidia inoculation on plates. Wild type though showed germination in case of 0.1 mM Brassinin but it showed very marginal germination at concentration of 0.2 mM Brassinin. The reduction in growth and germination of mutant in presence of Brassinin was evaluated and the tests were repeated with three different colonies representing simple replacement of *Abvf01* gene by hygromycin B gene and by replacement with GFP-hygromycin cassette *(*Δ*abvf01-9*) as well as with complementation mutant. Similar trend of reduction germination and growth in deletion mutant while normal and equivalent to wild type growth occurred in case of complemented mutant in the presence of 0.1 mM and 0.2 mM Brassinin was observed. The detailed result of this experiment is described in Table 4.5.

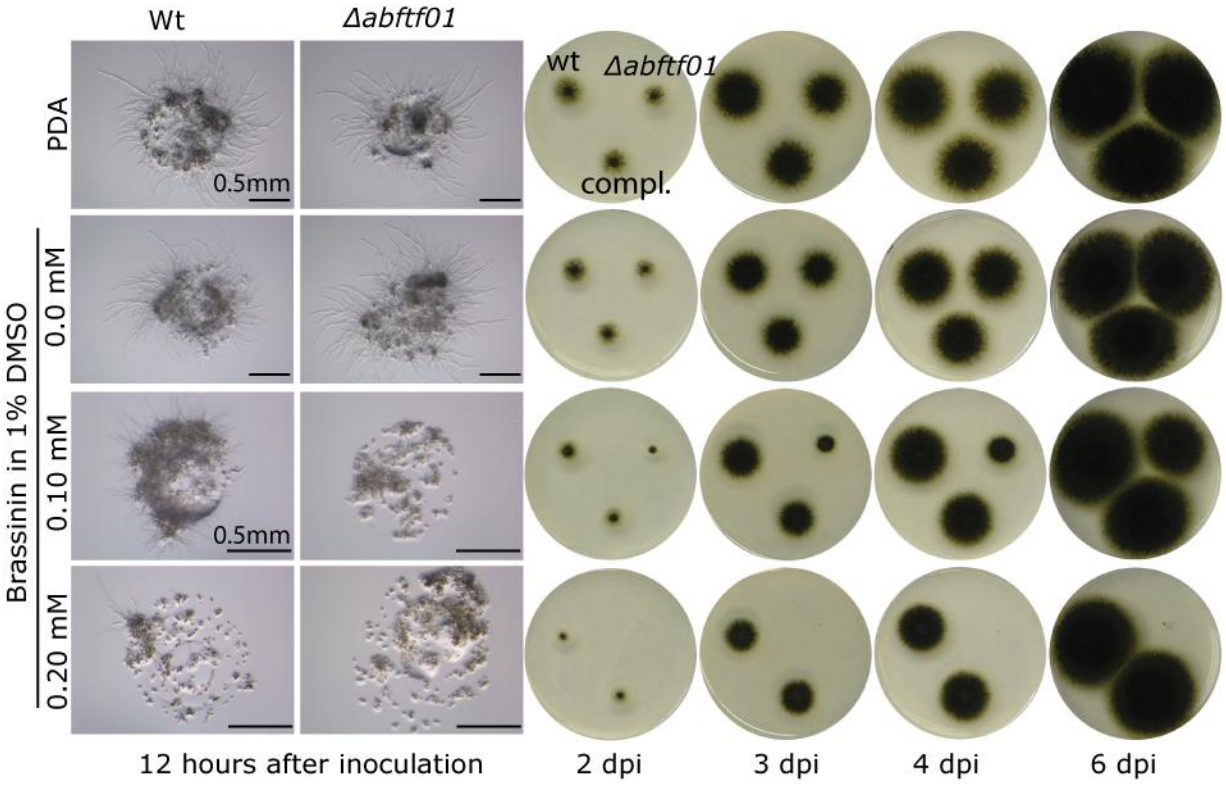

Fig. 4.9 . **Adverse effect of Brassinin to colony expansion of Δ*abvf01* mutant**. Fungal colonies were grown on PDA plates with two different concentrations of brassinin for six days.To evaluate the status of germination and very initial stages of growth all plates were observed under stereomicroscope after 12 hours of inoculation. Abbreviations dpi = days post inoculation, wt = wild type *Alternaria brassicicola*, Δ*abvf1* = deletion mutant of *AbVf1*. comp = Δ*abvf1-5* complemented with wild type allele of *AbVf1 (*Δ*abvf1:AbVf1*), DMSO = dimethyl sulfoxide, PDA = Potato Dextrose Agrose.

Table 4.5: The colony size of wt, mutants and complemented mutant in presence of brassinin

Conc. Of brassinin	wt	Δ*abvf01-5*	Δ*abvf01-7*	Δ*abvf01-9*	Compl.
0mM	32.5±2.5	34.3±0.5	33±2.0	34±1.00	27.7±1.5
0.1mM	28.5±1.5	16±3.0	14±1.0	20±1.0	26.7±0.5
0.2mM	27.5±0.5	0±0.00	0±0.00	0.6±1.1	26.7±0.6

The colony size was measured after 6 days of inoculation. All measurements are in milli meters (mm). Abbreviation: wt= wild type, Δ*abvf01-5* and Δ*abvf01-7* are deletion mutant by replacing the gene with hygromycin gene, Δ*abvf01-9* is deletion mutant by replacing target gene by hygromycin and GFP gene cassette. Compl= complementation mutant.

4.3.7 Effect of Brassinin on germination and vegetative growth

Germination and hyphal growth with a compound microscope was compared at 48 hours after spores were placed on PDA with Brassinin on a glass slide to know if the germination only or both germination and growth are effected by the presence of brassinin. Both wild type and mutants germinated 100% by 4 hours and their vegetative growth was comparable on plain PDA or 1.0% dimethyl sulfoxide (DMSO) containing PDA. In the presence of 0.1 mM Brassinin, both the *Δabvf01* mutant and wild type germinated at 8 hours, which was delayed approximately 4 hours. Hyphal growth was dense and short for both *Δabvf01* mutant and the wild type, but the mutants were more severely affected. In the presence of 0.2 mM brassinin, the wild type germinated at 12 hpi, which was delayed about 8 hours. The *Δabvf01*mutatns failed to germinate until 48 hpi (Fig4.10).

To clarify the reasons why the mutants formed smaller colonies than the wild type, we also compared hyphal growth in the liquid medium, 1% glucose and 0.5% yeast extracts broth (GYEB). Mycelia of the wild type or mutants were transferred to GYEB with Brassinin of 0.1 mM or 0.2 mM concentration. The wild type and mutants (*Δabvf1*-5 and *Δabvf1*-9) grew similarly in GYEB with 0.05% DMSO, the solvent for the brassinin. Mycelia of the wild type grew equally well in the presence and absence of 0.1 mM brassinin but its growth was reduced ~50% in the presence of 0.2mM brassinin (Fig. 4.8). Mycelial growth of mutants (*Δabvf1*-5 and *Δabvf1*-9) were over 50% and over 95% reduced in the presence of 0.1 mM and 0.2 mM brassinin, respectively (Fig. 4.11).

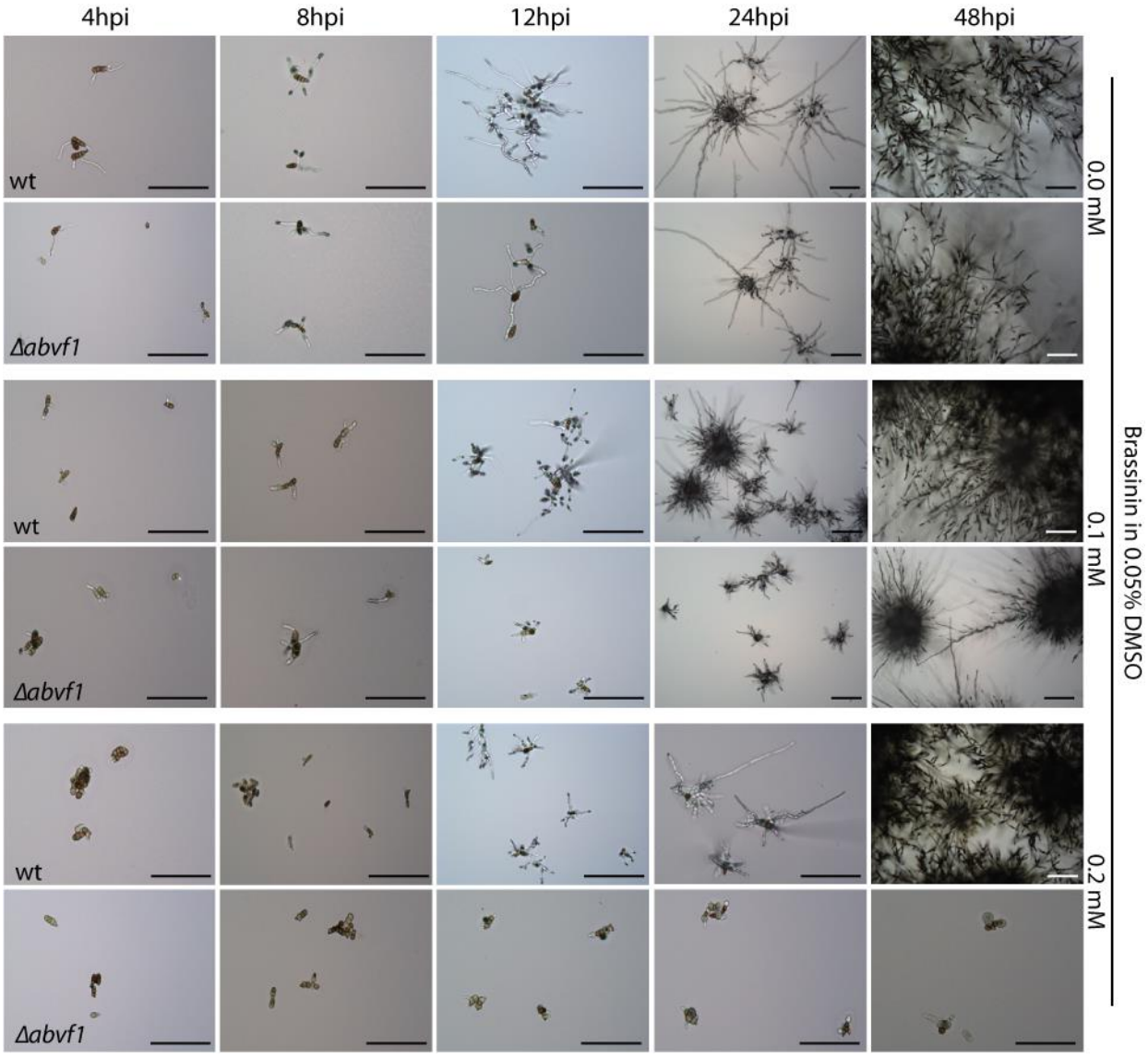

Fig. 4.10 Germination ofmMutant and wild in presence of two concentration of brassinin. hpi = hours postinoculation, wt = wild type *Alternaria brassicicola*, Δ*abvf1* = deletion mutant of *AbVf1*. comp = Δ*abvf1* complemented with wild type allele of *AbVf1 (*Δ*abvf1:AbVf1*), DMSO = dimethyl sulfoxide, PDA = Potato Dextrose Agarose.

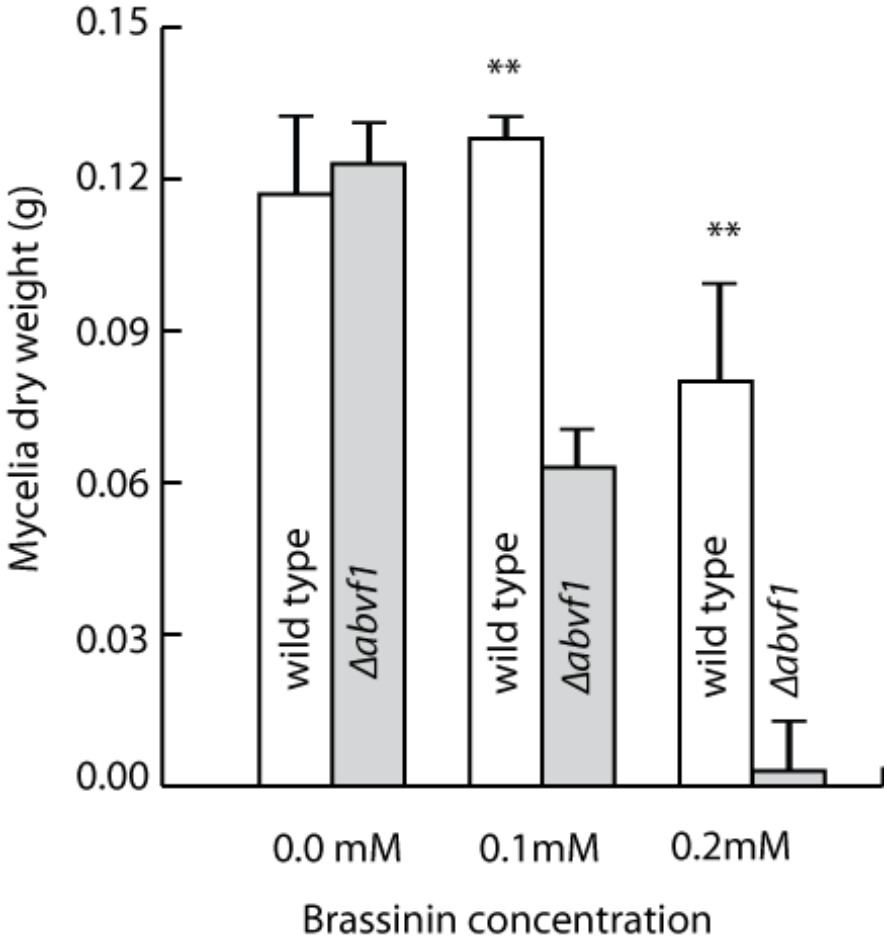

Fig 4.11 Effect of brassinin on vegetative growth of the *Δabvf1* mutants and wild type. Data show mean dry weight in grams. Bars represent standard deviation. ** = (p<0.01).

4.3.8 Mutants failed to degrade brassinin

During the growth of mycelium in the presence of brassinin, 2ml of culture medium was collected and evaluated amounts and integrity of brassinin. After 24 hours culture of wild type, brassinin was not detectable from the medium. In contrast, the brassinin was still detectable from the medium where mutants (*Δabvf1*-5 and *Δabvf1*-9) were grown (Fig.4.12,A). Brassinin was extracted from cultures of mutant and wild type collected at different time points and spotted on a TLC plate. I observed that the fungi starts degrading brassinin as soon as 3 hours of their addition and by 6 hours the visual signals suggests that more than 50% of brassinin was degraded (Fig. 4.12,B), however brassinin remained intact in mutant cultures. This observation supports our hypothesis that this gene is involved in brassinin degradation and is active during very early stages of infection. When the extract was separated with thin-layer chromatography, size of the standard brassinin was similar to the compound extracted from culture media grown with the mutant and (Fig 4.13). In contrast, there was very little compound left in the extract of culture medium where wild type was grown.

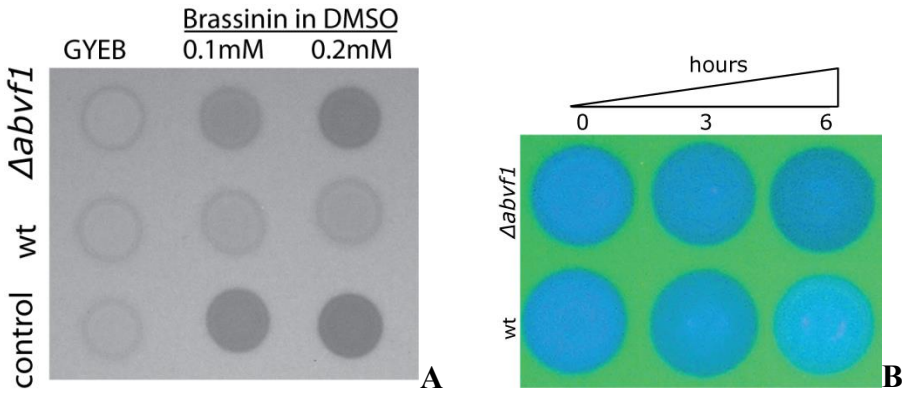

Fig. 4.12. Brassinin degradation *in vitro* culture. A. Brassinin was extracted from the GYEB alone liquid culture and cultures having GYEB supplemented with 0.1mM and 0.2mM brassinin dissolved in 1% DMSO inoculated with *Δabvf1,* wt and without inoculation with any fungi (used as control). **B**. shows the degradation of brassinin over course of time in wt, while

with *Δabvf01*very slow degradation was observed largly the brassinin remained intact. Spot intensity correlates brassinin concentration.

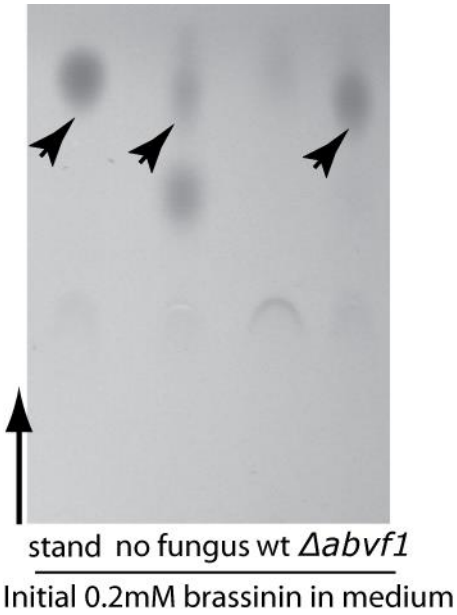

Fig.4.13: Reduced degradation of brassinin by *Δabvf1* during mycelial growth. Thin-layer chromatography (TLC) showing brassinin preservation. Lane 1 has brassinin added to flask containing water, lane to has brassinin added to GYEB medium without any fungal inoculum, lane three had brassinin added to GYEB and inoculated with wt and lane 4 had brassinin added to GYEB and mutant. An arrow indicates TLC run direction

4.3.9 Expression and localization of AbVf01 protein

To survey the expression and localization of the AbVf01 protein, we tagged its gene at the C-terminus right before the stop codon with a *GFP* coding sequence (Lorang *et al.*, 2001) The tagging construct was designed for the GFP protein to be expressed as a fusion protein with an AbVf01 gene that was regulated by its native enhancer and promoter elements (fig 4.2). As a negative control, we also made mutants that expressed GFP regulated by an *Abvf01*promoter (fig 4.1). While most of the GFP signals remained in the cytoplasm we observed that in some conidia, the GFP signals were located in the nuclei of the some mutant conidia expressing AbVf01-GFP fusion proteins 12 hours after inoculation on cabbage leaf(Fig 4.14).The localization of AbVf01proteins into nuclei seems to be specific to initial stage of infection, we

failed to see localization of GFP signals in nuclei of hypha during any stage of infection however we do observed accumulation of signals at the tip of germination tube during early stages of plant infection after 8 hours of inoculation, indicating that this protein was being actively expressed at the early stage of infection probably during detoxification of phytoalexins. (Data not shown).

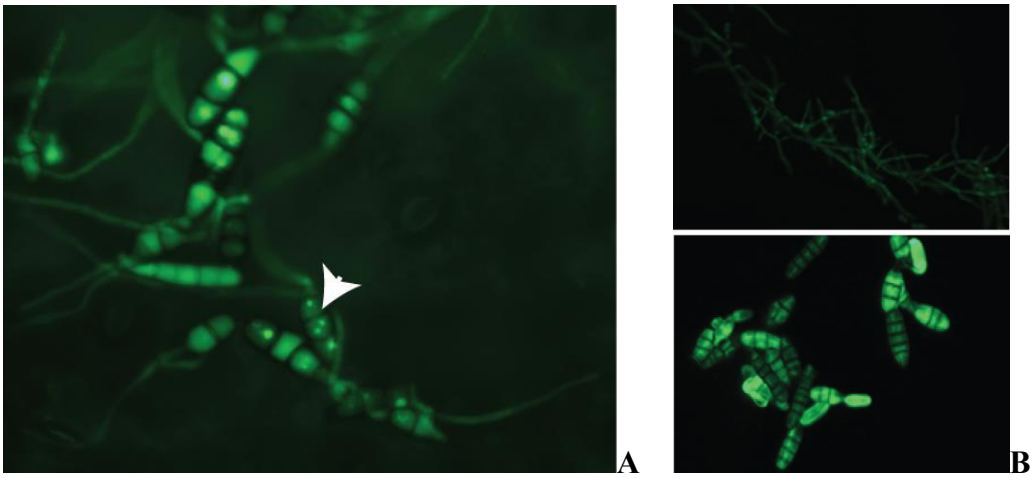

Fig 4.14.Localization of GFP signals in nuclei. **A.** The GFP signals were location in the conidia to show that the Δ*abvf01* is location marked by arrow. **B** GFP tagged on promoter and is used as a control to show that all GFP signal is in cytoplasm in mycelium (above) and in conidia(bottom).

4.4 Discussion

Deletion mutants of AbVf01 gene showed approximately 70% reduction in virulence compared to wild type *A. brassicicola*. Other strains where the coding sequence was replaced by the *GFP-Hyg* cassette also showed similar level of reduction in virulence. The reduced virulence was restored by complementing a *Δabvf01* mutant with the wild-type allele of its *AbVf01* gene. Growth of *ΔabVf01* was also severely effected in the presence of phytoalexin-brassinin. The pathogenicity and growth assay results with *ΔabVf01* show that the reduction of virulence as well as reduction of growth in the presence of brassinin, was due to the loss of the *AbVf01* function. The GFP localization on nuclei, the presence of nuclear localization signal (NLS) on the protein and presence of $Zn(II)_2Cys_6$ domains on protein sequence suggests that the AbVf01 protein is a transcription factor and belongs to zinc binuclear fungal specific class of transcription factors (pfam ID: PF00172) (Bateman *et al.*, 2004).

4.4.1 Reduction in virulence is only specific to Brassica species

The pathogenicity assays with different strains of deletion mutants of AbVf01 on whole plants and on detached leaves of green cabbage showed aproximately70% reduction in virulence. Similar reduction in virulence in the mutant was observed when pathogenicity assays were conducted with wounded and non-wounded cabbage leaves. Although, wild type *A. brassicicola* produced large lesion size at the mechanically injured infection site in comparison to the uninjured leaf mainly because the pathogen had an easy access to the plant cells. Plants belonging to different Brassica species namely: Chinese cabbage, broccoli, mustard and

cauliflower also showed reduction in virulence in the range of 70-90%. The variability in the virulence can be explained as the variable amount of resistance offered by each species of plant to the invading pathogen. But unexpectedly the pathogenicity assays with two ecotypes of *Arabidopsis thaliana* namely 'pad3' and 'Columbia' were totally different. *Δabvf01* mutant produced similar symptoms and same sized necrotic lesions as the wild type *A. brassicicola*. The mutant *pad3 A. thaliana* cannot produce the phytoalexin, camalexin, and is more susceptible to wild type *A. brassicicola* (Zhou *et al.*, 1999; Narusaka *et al.*, 2003) produced more severe symptoms with the *Δabvf01*mutant than on the Columbia ecotype, though these results were not statistically significant. Another strong virulence factor AbVf19 discovered in a previous study showed similar trend of decrease in lesion size in both of the *A. thaliana* ecotypes as well as on green cabbage leaves. The differential gene expression study of AbVf19 deletion mutant showed that it is involved in regulation of cell wall degradation enzymes (CWDEs) (Srivastava *et al.*, 2011). Arabidopsis and brassicas differs in the type of phytoalexin they produce, the former one secretes camalexin while later one secretes brassinin in response to a microbial attack. This fact drew attention to the possible function of AbVf01 in detoxification of brassica specific phytoalexins brassinin during the initial stages of infection.

4.4.2 AbVf01 is involved in phytoalexin biotransformation:

Brassinin is an indolic phytoalexin toxic to invading, microbial pathogens. The toxicity of brassinin is due to presence of dithiocarbamate a toxophore responsible for antifungal activity of brassinin (Szolar, 2007; Joubert *et al.*, 2011). During microbial infection brassinin not only limits the invasions of pathogens but also induces the biosynthesis of several brassinin derived compounds like cyclobrassinin, brassilexin, spirobrassinin to strengthen the plant defense.

(Pedras and Minic, 2012). To carryout successful infection and colonization *A. brassicicola* should have a mechanism to detoxify brassinin at early stages of infection. Brassinin hydrolases BHAb detoxifies brassinin to 3-indolylmathanamine in *A. brassicicola* (Pedras *et al.*, 2009a). Similarly two other enzymes brassinin oxidase and brassinin glucosyl transferase have been reported to detoxify brassinin in *L. maculans* and *S. sclerotiorum* respectively (Pedras and Ahiahonu, 2005; Sexton *et al.*, 2009). In *A. brassicicola* 96 to 18 proteins having different functional role in the cell and are found to differentially expressed when fungal mycelia were treated with 0.5 mM and 0.1 mM brassinin (Pedras and Minic, 2012), however there direct involvement in detoxification and pathogenesity is yet to be established. None of the previously known brassinin detoxifying enzymes has shown any influence on the virulence. The expression pattern of SsBGT1 gene encoding for *S. sclerotiorum* brassinin glucosyl transferase, suggests a redundant role in pathogenicity. The induced secretion of brassinin in response to inoculation of *S. sclerotiorum* on *B. napus* leaves is coherent with the transcript levels of SsBGT1 gene transcripts. However, lack of deletion or disruption mutants could not substantiate the role of SsBGT1 as pathogenicity factor (Sexton *et al.*, 2009). AbVf01 is the first gene whose null mutants simultaneously showed decrease in virulence and severe reduction in vegetative growth in the presence of brassinin.

During the infection process, the host plant secretes phytoalexins at the initial stages of infection to display resistance imposed by the plant. Pathogens in order to establish themselves and colonize host must overcome this resistance by detoxifying the phytoalexins. Closely followed observation of brassinin level during infection of *B. juncea* by *A. brassicicola* (Pedras *et al.*, 2009a) and levels of brassinin as well as transcripts of genes encoding for brassinin

detoxification enzyme, SsBGT1, in *S. sclerotiorum* infection of *B. napus* (Sexton *et al.*, 2009), strongly suggests that the phytoalexins like brassinin are induced initial stages of infection around 48-96hpi and thereafter it decreases. During the present study, a similar trend, which can be considered as indirect evidence in support of above mentioned phenomenon since, the brassinin was not quantified. During the very initial stages of inoculation of *A. brassicicola* on green cabbage leaves at 4-8 hpi the mutants showed similar growth pattern as wild type. Rather it was observed that mutants grew better than wild type *A. brassicicola* as they germinate early with large germ tubes length. Though this observation is not statistically supported because during staining and destaining large number of unattached conidia were washed off resulting in reduction in the number of data points. But the initial growth vigor by the mutant can be explained by the fact that just after few hours of inoculation since the plants have not yet started the secretion of phytoalexins gives the mutant an opportunity to grow faster or with equal rate to wild type. But as soon as the concentration of brassinin is increased around 8 hour onwards the growth of mutant strain on plant surface start retarding. By 12 hours though the mutant forms appressoria with same frequency as the wild type growth is slow in comparison to its wild type. After 24 hours the plant maintains a steady level of brassinin secretion thus the mutant continues to grow and form lesions albeit at a very slow rate.

4.4.3 *In vitro* degradation of brassinin

The growth assays on liquid and solid media in presence of different concentrations of brassinin clearly show that the growth of the mutant is dramatically reduced when compared to the wild type. The germination process was also affected in the presence of brassinin showing

that that brassinin effects both vegetative growth as well as germination. Extraction of brassinin from media inoculated with wild type and mutant also show that brassinin was degraded in wild type culture where *AbVf01* gene was active. While, brassinin remained intact (not degraded) in culture where *AbVf01* deletion mutants were used. These findings also provide direct evidences that *AbVf01* gene is involved in detoxification of phytoalexin – brassinin and contributes in pathogenesis.

Results from this study is important because other studies on brassinin biotransformation although have described about enzymes related to detoxification of brassinin, but none of the have shown its effect on virulence. This study reports the identification of a transcription factor which in involved in brassinin detoxification and simultaneous reduction in virulence on many brassica plants. The gene identified in the present study can serve as ideal candidate for management of black spot disease cause by *A. brassicicola* and a target gene for the green pesticides called 'paldoxins' that will block the fungal destruction by destroying phytoalexin detoxification inhibitors (Islam, 2008; Pedras *et al.*, 2010; Pedras *et al.*, 2011a; Pedras *et al.*, 2011b).

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CHAPTER 5

Conclusion and Impact of study

Fungi are considered as the major cause of crop losses occurring due to any biotic factors, both as pre-harvest and post-harvest pathogens. Among fungal phytopathogens necrotrophs represent the largest group of plant pathogens. Although they account for just 4% of fungal diversity, they cause as much as 80% of the losses from fungal diseases in some parts of the world. In necrotrophic pathogenesis a multi-step process, begins as soon as the spore comes in contact of the surface of host plant and continues till the pathogen converts all the biomass of the host into its own. Various physiological and morphological characteristics are believed to be involved in fungal pathogenesis. These characteristics include: specialized morphology; molecular sensing of hosts; germination of spore; induction of cell wall degrading enzymes (CWDE), other digestive proteases, lipases and secondary metabolites (Fig. 5.1). Although knowledge of various phenomena associated with pathogenesis is very crucial for developing detailed understanding of pathogenesis mechanism and for designing of efficient management practices, our understanding of host-pathogen interactions at present is subtle.

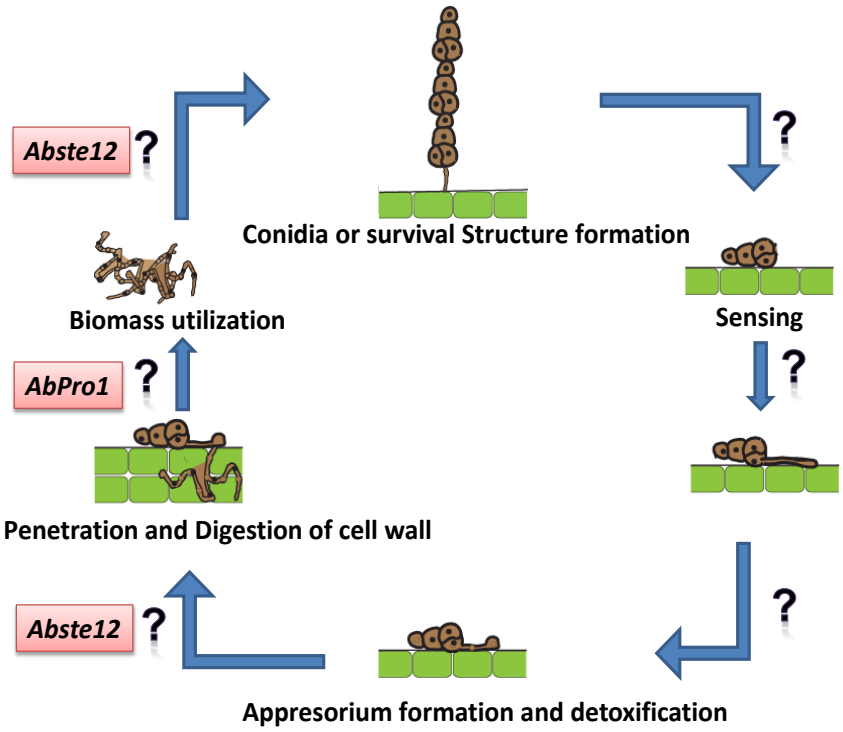

Fig 5.1. Schematic diagram of the disease cycle of *Alternaria brassicicola*. At the time of initiation of present study very little information was available regarding the regulatory genes associated with different stages of pathogenesis (marked by '?') in *A. brassicicola*. Putative functions of only two transcription factors Abste12 and AbPro1 was available.

The study of the host pathogen interaction was done by systematically knocking out transcription factors encoding genes and studying the changes in the gene minus mutants in the necrotroph *Alternaria brassicicola*. Throughout our study *A. brassicicola* and Green cabbage (*Brassica oleracia*) were used as a model system, while at some instance we also used *Arabidopsis thaliana* as another hosts. *A. brassicicola* is a devastating foliar and ubiquitous post-harvest pathogen and causes black spot disease among members of family Brassicaceae which includes many vegetable and oil crops e.g. cabbage, cauliflower, mustard and canola.

The whole genome of *A. brassicicola* (ATCC 93866) has been sequenced and is available in the public domain and the same strain was used in the present study. Out of the 13 different families of transcription factors we selected zinc finger domain containing transcription factors and systematically made knockout mutants by gene disruption. About 6 genes were identified, the mutants of which showed reduction in pathogenesis. All except one gene were novel and were discovered for the first time in *A. brassicicola* and fungal necrotrophs. The only known gene *AbPaCc*, homologs of which have been found in other fungi is known to regulate the pH response. It has shown almost 100% reduction in virulence. Two novel transcription factor genes AbVf19 and Amr1 are involved in the late stages of infection and works antagonistically to regulate the expression of hydrolytic enzymes. The mutants of *AbVf19* showed reduction in virulence while disruption and deletion mutants of Amr1 showed increase in virulence. *Amr1* is the first gene reported in *A. brassicicola* and to best of our knowledge the only known transcription factor that negatively regulates its downstream gene. The gene expression profiling of *AbVf19* and *Amr1* suggests that they commonly regulate 77 genes encoding for different hydrolases. Also, gene *Ab9637* was identified that is although not involved in pathogenesis but plays important role during conidiogenesis and believed to be active during the late stages of infection.

In the later part of study genes knock out mutants were made by deletion of the entire gene encoding sequence and replaced it by selectable marker hygromycin B genes. The change in methodology has negated any chance of reversal in mutants. 35 Zinc(II)2Cys6, or zinc binuclear transcription factor encoding gene were knocked out. This family of transcription factor is exclusively present in fungi. Six genes were associated with virulence. We report identification of two pathogenicity factors in this study previously only one such gene was

reported in *A. brassicicola* till present date. The *abPacC* disruption and Δ*abpf2* deletion mutants were unable to produce any lesion on the host without showing any difference in its morphological or physiological characteristics. *AbPf2* is found to regulate infection switch that allows *A. brassicicola* to become to pathogenic from saprophyte. We found another gene that has shown to be involved in regulation of detoxification of brassinin a brassica specific phytoalexin. These two genes have crucial role during the early stages of infection. The remaining four genes are yet to be studied for their function.

The major outcome of this study is the identification of 12 transcription factors that are involved in the different stages of pathogenesis (Fig5.2). This study will help in enhancing knowledge about pathogenesis mechanism, tailoring of efficient management practices and even envisioning newer application. For example the fungal specific transcription factors associated with pathogenesis can serve as very attractive molecular targets for designing control of pathogenic fungi with no effect on other animals or plants. Also, newer strains of *A. brassicicola* can be designed for industrial purpose by attaching high expression inducible promoters to the transcription factors involved in the regulation of suits of hydrolytic enzymes. Hence, the creation of pathogenesis associated transcription factor mutant library will serve as foundation for carrying out future researches devoted to deepen our understanding of molecular mechanisms involved behind pathogenesis not only in *A. brassicicola* but also by studying their homologs in other fungal species.

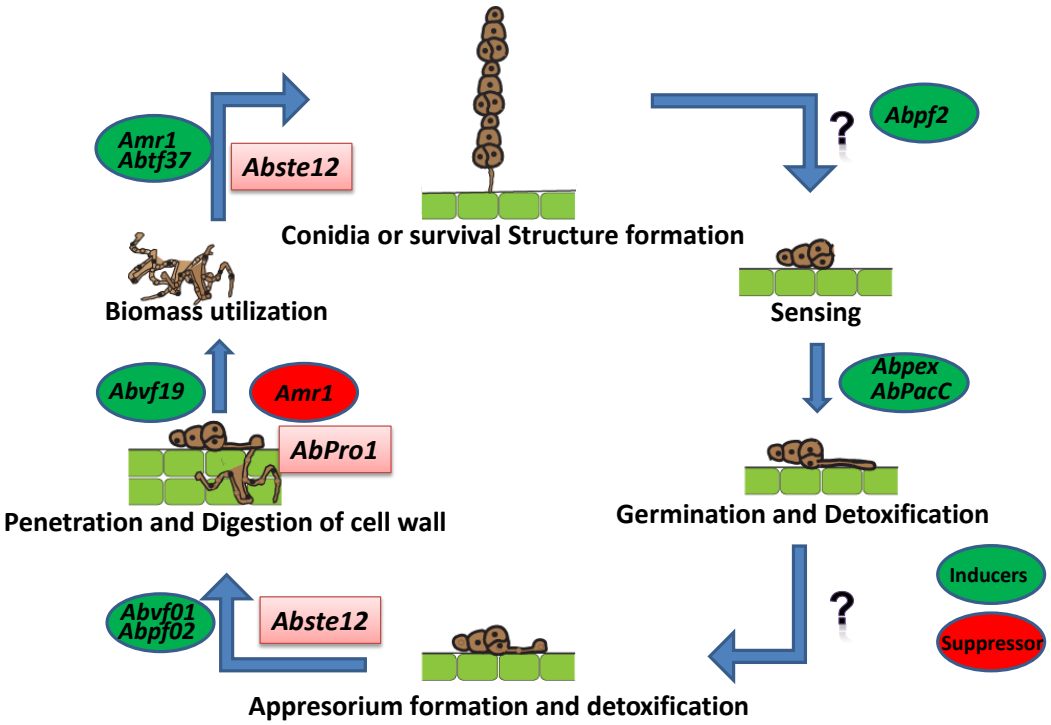

Fig 5.2. Schematic diagram of disease cycle of *Alternaria brassicicola* with newly discovered genes. By the end of study 13 transcription factor encoding genes were discovered. The figure above shows the genes (in ovals) whose putative functional role was identified during this study. The major impact of this study can be assessed by observing the replacement of '?' mark (in Fig 5.1) with the names of several newly discovered genes.

Appendix

List of primers for Zn(II)2Cys6 transcription factor gene deletion constructs.

ABFTF 1

ABFTF1-DP1	ACGAGGAAGAGGGAGAGGAG
ABFTF1-DP2	ATCAGTTAACGTCGACCTCGGATCGCGGAAGTTTGTTGAT
ABFTF1-DP3	ATCAACAAACTTCCGCGATCCGAGGTCGACGTTAACTGAT
ABFTF1-DP4	ATCCAGTTAAACGCCAGCACCGTCGACGTTAACTGGTTCC
ABFTF1-DP5	GGAACCAGTTAACGTCGACGGTGCTGGCGTTTAACTGGAT
ABFTF1-DP6	ACAACTGGTGGGTCTCCAAG

ABFTF 2

ABFTF2-DP2	CTCGGAACCTTGACTGCTTC
ABFTF2-DP2	ATCAGTTAACGTCGACCTCGTAGCATCACTACGCCAGTCG
ABFTF2-DP3	CGACTGGCGTAGTGATGCTACGAGGTCGACGTTAACTGAT
ABFTF2-DP4	ACGATGTGAGCACGCTGTAGCGTCGACGTTAACTGGTTCC
ABFTF2-DP5	GGAACCAGTTAACGTCGACGCTACAGCGTGCTCACATCGT
ABFTF2-DP6	GATCTGCAAGCCCTGTTAGC

ABFTF 4

ABFTF4-DP1	GACCTTCGGGATCGTCTACA
ABFTF4-DP2	ATCAGTTAACGTCGACCTCGCCGATCTTTCCATTGTTGCT
ABFTF4-DP3	AGCAACAATGGAAAGATCGGCGAGGTCGACGTTAACTGAT
ABFTF4-DP4	GTGTGGTCAGTTGCCCTCTTCGTCGACGTTAACTGGTTCC
ABFTF4-DP5	GGAACCAGTTAACGTCGACGAAGAGGGCAACTGACCACAC
ABFTF4-DP6	GCGGCTAATCTAACGCAGAC

ABFTF 5

ABFTF5-DP1	CTTTCCGGATGGTGGTATGT
ABFTF5-DP2	ATCAGTTAACGTCGACCTCGAAGAAGGCGATAACCGGAAT
ABFTF5-DP3	ATTCCGGTTATCGCCTTCTTCGAGGTCGACGTTAACTGAT
ABFTF5-DP4	CAGTGATCGAAGGACCCATTCGTCGACGTTAACTGGTTCC
ABFTF5-DP5	GGAACCAGTTAACGTCGACGAATGGGTCCTTCGATCACTG
ABFTF5-DP6	TGAGATGAGATCAGCCATGC

ABFTF 6

ABFTF6-DP1	ACCATCATGTTCGAATGCAA
ABFTF6-DP2	ATCAGTTAACGTCGACCTCGTGTGGCTGTGCTGAGCTATC
ABFTF6-DP3	GATAGCTCAGCACAGCCACACGAGGTCGACGTTAACTGAT
ABFTF6-DP4	ATTCATCCTTCCAAAACCCCCGTCGACGTTAACTGGTTCC
ABFTF6-DP5	GGAACCAGTTAACGTCGACGGGGGTTTTGGAAGGATGAAT
ABFTF6-DP6	AAGGAGACGAGGGATCAGGT

ABFTF 7

ABFTF7-DP1	TGGTGGTATTCTCCGAGTCC
ABFTF7-DP2	ATCAGTTAACGTCGACCTCGTACGTGGAGACGCTAGCTGA
ABFTF7-DP3	TCAGCTAGCGTCTCCACGTACGAGGTCGACGTTAACTGAT
ABFTF7-DP4	CAGTAAGCCTCGTTTCGGAGCGTCGACGTTAACTGGTTCC
ABFTF7-DP5	GGAACCAGTTAACGTCGACGCTCCGAAACGAGGCTTACTG
ABFTF7-DP6	CCATCGACGGTAACGAGAAT

ABFTF 8

ABFTF8-DP1	TAGAAGGCATGGAAGGATGG
ABFTF8-DP2	ATCAGTTAACGTCGACCTCGTCTCAGCAAGCAGGTCTCAA
ABFTF8-DP3	TTGAGACCTGCTTGCTGAGACGAGGTCGACGTTAACTGAT
ABFTF8-DP4	ATCTTTCGGTTCCTTTCGGTCGTCGACGTTAACTGGTTCC
ABFTF8-DP5	GGAACCAGTTAACGTCGACGACCGAAAGGAACCGAAAGAT
ABFTF8-DP6	CACTTTTGCCCGCTGATTAT

ABFTF 9

ABFTF9-DP1 GGCCATGACAAGGGTAAAGA
ABFTF9-DP2 ATCAGTTAACGTCGACCTCGTGACCGATACCGAGGCTAAC
ABFTF9-DP3 GTTAGCCTCGGTATCGGTCACGAGGTCGACGTTAACTGAT
ABFTF9-DP4 CCAATGTGTGTATTCAGCCGCGTCGACGTTAACTGGTTCC
ABFTF9-DP5 GGAACCAGTTAACGTCGACGCGGCTGAATACACACATTGG
ABFTF9-DP6 CTAGTGGGCGTTGTCGGTAT

ABFTF 10

ABFTF10-DP1 AGGGTGTTTTGCCTGTCAAC
ABFTF10-DP2 ATCAGTTAACGTCGACCTCGCGGCAAGATACAAAAGCACA
ABFTF10-DP3 TGTGCTTTTGTATCTTGCCGCGAGGTCGACGTTAACTGAT
ABFTF10-DP4 ACCTCCACTGAACGATGTCCCGTCGACGTTAACTGGTTCC
ABFTF10-DP5 GGAACCAGTTAACGTCGACGGGACATCGTTCAGTGGAGGT
ABFTF10-DP6 TGACTTCGACGCAGATTGAC

ABFTF 11

ABFTF11-DP1 GTCCTTGGACGGAAAGCATA
ABFTF11-DP2 ATCAGTTAACGTCGACCTCGACTCGCCGACTACTGAATCG
ABFTF11-DP3 CGATTCAGTAGTCGGCGAGTCGAGGTCGACGTTAACTGAT
ABFTF11-DP4 CTCCAAGCCATCGATTTTGTCGTCGACGTTAACTGGTTCC
ABFTF11-DP5 GGAACCAGTTAACGTCGACGACAAAATCGATGGCTTGGAG
ABFTF11-DP6 GGTTGGTCAGGGTGACAAGT

ABFTF 12

ABFTF12-DP1 AACTCTTCCTCTTGCGACCA
ABFTF12-DP2 ATCAGTTAACGTCGACCTCGTCCAATTGACCCCAGCTAGT
ABFTF12-DP3 ACTAGCTGGGGTCAATTGGACGAGGTCGACGTTAACTGAT
ABFTF12-DP4 TTTTCGAGCCGCTAAACTGTCGTCGACGTTAACTGGTTCC
ABFTF12-DP5 GGAACCAGTTAACGTCGACGACAGTTTAGCGGCTCGAAAA
ABFTF12-DP6 TTCGTGGCTCCTTCTGAACT

ABFTF13

ABFTF13-DP1 ATACTCACCGACGACGAAGG
ABFTF13-DP2 ATCAGTTAACGTCGACCTCGAGGTGCGAGTGCTACGGTAT
ABFTF13-DP3 **ATACCGTAGCACTCGCACCT**CGAGGTCGACGTTAACTGAT
ABFTF13-DP4 GATAGTGTGAGGGGCGAGAGCGTCGACGTTAACTGGTTCC
ABFTF13-DP5 GGAACCAGTTAACGTCGACG**CTCTCGCCCCTCACACTATC**
ABFTF13-DP6 ATGGCAGTTTGCTGGATACC

ABFTF 14

ABFTF14-DP1 CTCCTGTGTTTGCTCGATGA
ABFTF14-DP2 ATCAGTTAACGTCGACCTCGATTCAATCCAGTGCGTCACA
ABFTF14-DP3 TGTGACGCACTGGATTGAATCGAGGTCGACGTTAACTGAT
ABFTF14-DP4 CACAATGAAGGCGGGAGTATCGTCGACGTTAACTGGTTCC
ABFTF14-DP5 GGAACCAGTTAACGTCGACGATACTCCCGCCTTCATTGTG
ABFTF14-DP6 GTCGTCAACGTTCAAGCTCA

ABFTF 16

ABFTF16-DP1 GGTTGGGTCTGTGCTAGGAG
ABFTF16-DP2 ATCAGTTAACGTCGACCTCGACTATTCGATGTCGGCGTCT
ABFTF16-DP3 AGACGCCGACATCGAATAGTCGAGGTCGACGTTAACTGAT
ABFTF16-DP4 TTCTCATCTTCCACCCGATCCGTCGACGTTAACTGGTTCC
ABFTF16-DP5 GGAACCAGTTAACGTCGACGGATCGGGTGGAAGATGAGAA
ABFTF16-DP6 GAAAAGCAATGCACAGCAAA

ABFTF 18

ABFTF18-DP1 GATATTTTCGCGGGATGAGA
ABFTF18-DP2 ATCAGTTAACGTCGACCTCGGATTGCACGAGAAAGGGTGT
ABFTF18-DP3 ACACCCTTTCTCGTGCAATCCGAGGTCGACGTTAACTGAT
ABFTF18-DP4 ACTCGCAAGCGTGGATAGAGCGTCGACGTTAACTGGTTCC
ABFTF18-DP5 GGAACCAGTTAACGTCGACGCTCTATCCACGCTTGCGAGT
ABFTF18-DP6 ACCTTGCTCCAATCATCCTG

ABFTF 19

ABFTF19-DP1	ACAGCACCATGATTCTGCAA
ABFTF19-DP2	ATCAGTTAACGTCGACCTCGTGGAGGATGCAACAACTCTG
ABFTF19-DP3	CAGAGTTGTTGCATCCTCCACGAGGTCGACGTTAACTGAT
ABFTF19-DP4	GAGTAGGCATGGTGCGAATTCGTCGACGTTAACTGGTTCC
ABFTF19-DP5	GGAACCAGTTAACGTCGACGAATTCGCACCATGCCTACTC
ABFTF19-DP6	GGAAGAATGGCAACGGTCTA

ABFTF 20

ABFTF20-DP1	ACATCTTCCATACGCCGTTC
ABFTF20-DP2	ATCAGTTAACGTCGACCTCGGCGTAGGTTATCTCCGTTGG
ABFTF20-DP3	CCAACGGAGATAACCTACGCCGAGGTCGACGTTAACTGAT
ABFTF20-DP4	TCATCTGCTGGTTCATGCTCCGTCGACGTTAACTGGTTCC
ABFTF20-DP5	GGAACCAGTTAACGTCGACGGAGCATGAACCAGCAGATGA
ABFTF20-DP6	CCTCGACGAGTCACGTACAA

ABFTF 21

ABFTF21-DP1	TGGGTCAACTTGATCATGGA
ABFTF21-DP2	ATCAGTTAACGTCGACCTCGCGACATGCAGGCTTAGAACA
ABFTF21-DP3	TGTTCTAAGCCTGCATGTCGCGAGGTCGACGTTAACTGAT
ABFTF21-DP4	GAGAGTATTGACGGGAGGGGCGTCGACGTTAACTGGTTCC
ABFTF21-DP5	GGAACCAGTTAACGTCGACGCCCCTCCCGTCAATACTCTC
ABFTF21-DP6	CTCACCTTCGTTCCCAAAAC

ABFTF 22

ABFTF22-DP1	ATTGCTTGATGCGAGGCTAT
ABFTF22-DP2	ATCAGTTAACGTCGACCTCGTGTGGAAAACGAGTGTGGAA
ABFTF22-DP3	TTCCACACTCGTTTTCCACACGAGGTCGACGTTAACTGAT
ABFTF22-DP4	CTTCGACAACTCGCGTGATACGTCGACGTTAACTGGTTCC
ABFTF22-DP5	GGAACCAGTTAACGTCGACGTATCACGCGAGTTGTCGAAG
ABFTF22-DP6	TTGCCTACATCACCACCGTA

ABFTF 24

ABFTF24-DP1	GGCGAACAGGTCATGGTACT
ABFTF24-DP2	ATCAGTTAACGTCGACCTCGAGTCAGTGTTGCCCGGATAC
ABFTF24-DP3	GTATCCGGGCAACACTGACTCGAGGTCGACGTTAACTGAT
ABFTF24-DP4	TTGATCTCGGTGAAAAAGGCCGTCGACGTTAACTGGTTCC
ABFTF24-DP5	GGAACCAGTTAACGTCGACGGCCTTTTTCACCGAGATCAA
ABFTF24-DP6	CGGATTCGCTCTCTGTCTCT

ABFTF 25

ABFTF25-DP1	AAAGAGAGCGTTGGACTGGA
ABFTF25-DP2	ATCAGTTAACGTCGACCTCGTTGCCATGACTCTTCGTGAG
ABFTF25-DP3	CTCACGAAGAGTCATGGCAACGAGGTCGACGTTAACTGAT
ABFTF25-DP4	TCTCAGCGCAGCTAGCAGTACGTCGACGTTAACTGGTTCC
ABFTF25-DP5	GGAACCAGTTAACGTCGACGTACTGCTAGCTGCGCTGAGA
ABFTF25-DP6	ACGGTGGAAATTCTGACTGG

ABFTF 26

ABFTF26-DP1	CGTGGTTTCTGGATCTGGTT
ABFTF26-DP2	ATCAGTTAACGTCGACCTCGTTTTCGTCTTTGGGACAAGG
ABFTF26-DP3	CCTTGTCCCAAAGACGAAAACGAGGTCGACGTTAACTGAT
ABFTF26-DP4	CGTAGGCGGGATGTTAGTGTCGTCGACGTTAACTGGTTCC
ABFTF26-DP5	GGAACCAGTTAACGTCGACGACACTAACATCCCGCCTACG
ABFTF26-DP6	TATGCCTTTCAACCCTCCAC

ABFTF 28

ABFTF28-DP1	TGAGTTCACGAATCCCACAA
ABFTF28-DP2	ATCAGTTAACGTCGACCTCGGGCAGCATCATCATCATCAC
ABFTF28-DP3	GTGATGATGATGATGCTGCCCGAGGTCGACGTTAACTGAT
ABFTF28-DP4	CCTACGAACCTCGAGAGACGCGTCGACGTTAACTGGTTCC
ABFTF28-DP5	GGAACCAGTTAACGTCGACGCGTCTCTCGAGGTTCGTAGG
ABFTF28-DP6	GTACTGCAAAGGCCTGAAGC

ABFTF 29

ABFTF29-DP1	ATGTGAGCGCGCCTAATACT
ABFTF29-DP2	ATCAGTTAACGTCGACCTCGACAACATTTCAGGCGAATCC
ABFTF29-DP3	GGATTCGCCTGAAATGTTGTCGAGGTCGACGTTAACTGAT
ABFTF29-DP4	CAAGCCTACGATCGCATGTACGTCGACGTTAACTGGTTCC
ABFTF29-DP5	GGAACCAGTTAACGTCGACGTACATGCGATCGTAGGCTTG
ABFTF29-DP6	TCCAACCCCTCACATCTCTC

ABFTF 30

ABFTF30-DP1	CCAGCCCAATATCGTTGTTT
ABFTF30-DP2	ATCAGTTAACGTCGACCTCGGACTACCAGTCACCGCGATT
ABFTF30-DP3	AATCGCGGTGACTGGTAGTCCGAGGTCGACGTTAACTGAT
ABFTF30-DP4	GTTCCGTTGCTATTGGAAGCCGTCGACGTTAACTGGTTCC
ABFTF30-DP5	GGAACCAGTTAACGTCGACGGCTTCCAATAGCAACGGAAC
ABFTF30-DP6	TGGACGTAACCACAGGTCAA

ABFTF 31

ABFTF31-DP1	AAGCATGGGTGCAGTAAAGG
ABFTF31-DP1	ATCAGTTAACGTCGACCTCGCTCATCACGACCATCATTCG
ABFTF31-DP3	CGAATGATGGTCGTGATGAGCGAGGTCGACGTTAACTGAT
ABFTF31-DP4	TCTAGGCGTAACAAATCCGGCGTCGACGTTAACTGGTTCC
ABFTF31-DP5	GGAACCAGTTAACGTCGACGCCGGATTTGTTACGCCTAGA
ABFTF31-DP6	CCAAGCCCGAAACATGTACT

ABFTF 32

ABFTF32-DP1	AAGACCAATTCGTTGCCTTG
ABFTF32-DP2	ATCAGTTAACGTCGACCTCGCTTTCACGCCCTACTTCTCG
ABFTF32-DP3	CGAGAAGTAGGGCGTGAAAGCGAGGTCGACGTTAACTGAT
ABFTF32-DP4	AAGTGGTTCTAGCCAAGCGACGTCGACGTTAACTGGTTCC
ABFTF32-DP5	GGAACCAGTTAACGTCGACGTCGCTTGGCTAGAACCACTT
ABFTF32-DP6	GGCAGACGAATCTAGCAAGG

ABFTF 33

ABFTF33-DP1	AACCATCACCGAGGTGTCTC
ABFTF33-DP2	ATCAGTTAACGTCGACCTCGGCCTTCCAAACAAGGATTGA
ABFTF33-DP3	TCAATCCTTGTTTGGAAGGCCGAGGTCGACGTTAACTGAT
ABFTF33-DP4	GCTTAAAGTACGACGCCAGCCGTCGACGTTAACTGGTTCC
ABFTF33-DP5	GGAACCAGTTAACGTCGACGGCTGGCGTCGTACTTTAAGC
ABFTF33-DP6	GCAGATGCGTACACCTGAGA

ABFTF 35

ABFTF35-DP1	CCTTCACCTTCCACAACGAT
ABFTF35-DP2	ATCAGTTAACGTCGACCTCGAATTCGCCAGATTGCCATAC
ABFTF35-DP3	GTATGGCAATCTGGCGAATTCGAGGTCGACGTTAACTGAT
ABFTF35-DP4	GTGTCTCAGCCAAATGAGCACGTCGACGTTAACTGGTTCC
ABFTF35-DP5	GGAACCAGTTAACGTCGACGTGCTCATTTGGCTGAGACAC
ABFTF35-DP6	CCCAACGGTCTGATCACTCT

ABFTF 36

ABFTF36-DP1	CAGTATCACGGCAGCATGAG
ABFTF36-DP2	ATCAGTTAACGTCGACCTCGTAACAATCGCTCGTGGTTCA
ABFTF36-DP3	TGAACCACGAGCGATTGTTACGAGGTCGACGTTAACTGAT
ABFTF36-DP4	TTTGAGTTTGGAGCATGCAGCGTCGACGTTAACTGGTTCC
ABFTF36-DP5	GGAACCAGTTAACGTCGACGCTGCATGCTCCAAACTCAAA
ABFTF36-DP6	TCCTCTTCCACCATTTCGAC

ABFTF 37

ABFTF37-DP1	TAAAGTTTTACGCTCTTAAGGTCTATT
ABFTF37-DP2	ATCAGTTAACGTCGACCTCGAAGCAAGCAGCTACCCTAAGT
ABFTF37-DP3	ACTTAGGGTAGCTGCTTGCTTCGAGGTCGACGTTAACTGAT
ABFTF37-DP4	TTCAGTCAACGAGTCAAGCGCGTCGACGTTAACTGGTTCC
ABFTF37-DP5	GGAACCAGTTAACGTCGACGCGCTTGACTCGTTGACTGAA

ABFTF37-DP6 GAACAGCCGACTCTTTGCTC

ABFTF 38

ABFTF38-DP1 GCACTGACAAGAAACGGTGA
ABFTF38-DP2 ATCAGTTAACGTCGACCTCGGAGGAAGGAGCCCTGGACTA
ABFTF38-DP3 TAGTCCAGGGCTCCTTCCTCCGAGGTCGACGTTAACTGAT
ABFTF38-DP4 GCTTGAAATGCCTGTCATCACGTCGACGTTAACTGGTTCC
ABFTF38-DP5 GGAACCAGTTAACGTCGACGTGATGACAGGCATTTCAAGC
ABFTF38-DP6 GACATGTGCTGCCTACTCCA

ABFTF 50

ABFTF50-DP1 GCTTAACGCGAAGACCAAAG
ABFTF50-DP2 ATCAGTTAACGTCGACCTCGCGGTCTAGTTCCGTCGTGTT
ABFTF50-DP3 AACACGACGGAACTAGACCGCGAGGTCGACGTTAACTGAT
ABFTF50-DP4 CAACTCCTTGCTTCGCTAGCCGTCGACGTTAACTGGTTCC
ABFTF50-DP5 GGAACCAGTTAACGTCGACGGCTAGCGAAGCAAGGAGTTG
ABFTF50-DP6 AATACGCGGGATACCCTGAT

ABFTF 53

ABFTF53-DP1 GTCCGAGACTTCACCGAGAG
ABFTF53-DP2 ATCAGTTAACGTCGACCTCGGTGCTAGGACACGCAGACAA
ABFTF53-DP3 TTGTCTGCGTGTCCTAGCACCGAGGTCGACGTTAACTGAT
ABFTF53-DP4 TTTCCATCACATCTTTGCCACGTCGACGTTAACTGGTTCC
ABFTF53-DP5 GGAACCAGTTAACGTCGACGTGGCAAAGATGTGATGGAAA
ABFTF53-DP6 CTCCTCCTCACAAACGAAGC

ABFTF 55

ABFTF55-DP1 AACACCAACTCGGAATGAGG
ABFTF55-DP2 ATCAGTTAACGTCGACCTCGGCAAGTAAACCCAGCAAAGG
ABFTF55-DP3 CCTTTGCTGGGTTTACTTGCCGAGGTCGACGTTAACTGAT
ABFTF55-DP4 ATTACGCGGAGGTACACAGCCGTCGACGTTAACTGGTTCC
ABFTF55-DP5 GGAACCAGTTAACGTCGACGGCTGTGTACCTCCGCGTAAT
ABFTF55-DP6 GGGGAAAGTATGCGGTATGA

List of primers for southern blotting.

ABFTF 1

ABFTF1-HybF	TCTCGCCTGTGCATCACTAC
ABFTF1-HybR	CCTCTAAGCTTGCGGCATAC

ABFTF 2

ABFTF2-HybF	GCTGTCACCGAAGAAAGGTC
ABFTF2-HybR	ATTTGACATGTGGGCCATTT

ABFTF 4

ABFTF4-HybF	GCAAACGTAGACCCAATGGT
ABFTF4-HybR	GTACGAACGGCGAATTTCAT

ABFTF 5

ABFTF5-HybF	TGCCTAGCAGAAAGTGAGCA
ABFTF5-HybR	TCTCTGGCGAAATCGAGTCT

ABFTF 6

ABFTF6-HybF	TCACGCATAGTCGAGACAGG
ABFTF6-HybR	ACTCGTGTTGCCGAGAGACT

ABFTF 7

ABFTF7-HybF	GACTGTCGGCTTCTCCAAAG
ABFTF7-HybR	CAACACTCACCACGATCACC

ABFTF 8

ABFTF8-HybF	GAAGGATCAGTCGCTCAAGG
ABFTF8-HybR	TGTTTTCAAGTGTCGCTTCG

ABFTF 9

ABFTF9-HybF	ACACGACCATTCGAAACTCC
ABFTF9-HybR	CCACCCTGGAGAACACAGTT

ABFTF 10

ABFTF10-HybF	GTTTGTATACGCGGCCTGAT
ABFTF10-HybR	GCCATTGCTTTCTCTTCCTG

ABFTF 11

ABFTF11-HybF	TCTCAAGAACGCCATGAGTG
ABFTF11-HybR	AAGAGCGTGACCAAAGCAGT

ABFTF 12

ABFTF12-HybF	AACATCCCGAGCTGTGTACC
ABFTF12-HybR	CCCAGCATTCATGTTGTTTG

ABFTF13

ABFTF13-HybF	CGAGTATCGAAAGCGTGTGA
ABFTF13-HybR	CGTGTCGAGCATACGAAAGA

ABFTF 14

ABFTF14-HybF	ACGCGAGGAAAGTGAGTCAT
ABFTF14-HybR	CCACTCGGGTCCAGTAAAAA

ABFTF 16

ABFTF16-HybF	GCCTGCAGTACGACCTCTTC
ABFTF16-HybR	CTTGCCCAAAAGTCCTGTGT

ABFTF 18

ABFTF18-HybF	CCTTTGCACGGGTAGTGTTT
ABFTF18-HybR	GACTTTCCGATCCTCACCAA

ABFTF 19

ABFTF19-HybF	AGTGCGCAAGATTCTCTGGT
ABFTF19-HybR	TGCATCTGCGTCGTTCTATC

ABFTF 20

ABFTF20-HybF	CAACTATGTCGGCACCAATG
ABFTF20-HybR	TGCTGTCAAAGACGGTCAAG

ABFTF 21

ABFTF21-HybF	TCGACAATGAAGCAAAGTGC
ABFTF21-HybR	TGTCCAGGAAGTGCTCAATG

ABFTF 22

ABFTF22-HybF	ACAGGACGCTTTCCAAGCTA
ABFTF22-HybR	TGCGATCTGTGCTAACCTTG

ABFTF 24

ABFTF24-HybF	GACACGTATTTCGCCAGGAT
ABFTF24-HybR	GAAAGCTCAGCCAAGTCCAC

ABFTF 25

ABFTF25-HybF	CAGTTCTTCGCCAAAAGAGG
ABFTF25-HybR	ATGACGGTCCTGTGCTTACC

ABFTF 26

ABFTF26-HybF	TTCTTCCCTCTCGCATGACT
ABFTF26-HybR	GACTATCGATGCGTGCTTCA

ABFTF 28

ABFTF28-HybF	GACAACATACGAAGCGCTGA
ABFTF28-HybR	GTGATCCATGCAAACAATCG

ABFTF 29

ABFTF29-HybF	CAGCAAATGAGCCAACTCAA
ABFTF29-HybF	AGCGAGAGTCTGGACATCGT

ABFTF 30

ABFTF30-HybF	TGATAGCACTGCCGACTTTG
ABFTF30-HybR	GTTCTGCATAGGCAGCATCA

ABFTF 31

ABFTF31-HybF	CGCCAGATAGTTGGAAGCTC
ABFTF31-HybR	CTGCATATTCGCGACGTCTA

ABFTF 32

ABFTF32-HybF	ATCAGTCTGTCGCTCGACCT
ABFTF32-HybR	TCCAAAATCTCCACCAAAGC

ABFTF 33

ABFTF33-HybF	ACTTTTCGATAACCGCATGG
ABFTF33-HybR	CACAACCCAACGGAAGTTTT

ABFTF 35

ABFTF35-HybF	ATCAGCCAGCAGCTTGAAGT
ABFTF35-HybR	ACGGAAATTTCGCTGTAGGA

ABFTF 36

ABFTF36-HybF	GGTGCAGCATCTGATTAGCA
ABFTF36-HybR	GAACGGTCGGAAAAGATGAA

ABFTF 37

ABFTF37-HybF	CCGTCAATCAAGCACTAGCA
ABFTF37-HybR	GCAGGAACAGAGAAGCTTG

ABFTF 38

ABFTF38-HybF	AAAGGGTCTGTCCACGTCAC
ABFTF38-HybR	GGCCTAGACCAGCATCAGAG

ABFTF 50

ABFTF50-HybF	CCTGGTGCCACGTATTTTCT
ABFTF50-HybR	TCCTGTTGTTTGAGCGAGTG

ABFTF 53

ABFTF53-HybF	AAAGCGTGTCAGTGTTGTCG
ABFTF53-HybR	CTGACTTTGCCTTTGCTTCC

ABFTF 55

ABFTF55-HybF	TCGCATGCCCATTATCTGTA
ABFTF55-HybR	TCCACTGGATGATGTCGTGT

Table S-1. List of primers used chapter 4

P1	ACGAGGAAGAGGGAGAGGAG
P2	ATCAGTTAACGTCGACCTCGGATCGCGGAAGTTTGTTGAT
P3	ATCAACAAACTTCCGCGATCCGAGGTCGACGTTAACTGAT
P4	ATCCAGTTAAACGCCAGCACCGTCGACGTTAACTGGTTCC
P5	GGAACCAGTTAACGTCGACGGTGCTGGCGTTTAACTGGAT
P6	ACAACTGGTGGGTCTCCAAG
HYB-1F	TCTCGCCTGTGCATCACTAC
HYB-1R	CCTCTAAGCTTGCGGCATAC
HYB-2F	TGTTGCTGAGCAGTCCTTTG
HYB-2R	AAGTTGTTGGTCGGAAGTGG
PGFP-2	CTCGCCCTTGCTCACCATCTCGATTCGCTGTGTTC
PGFP-3	GAACACAGCGAATCGAGATGGTGAGCAAGGGCGAG
PNR-20F	AAAGGGAACAAAAGCTGGAG
PNR-775R	CCTCGAGGTCGACGGTATC
1FTF1GFP-GA	TGTTGCTGAGCAGTCCTTTG
2'FTF1GFP	CAGCTCCTCACCCTTACTCACGAAACCTTGCGCATTGAAATC
3'FTF1GFP	GATTTCAATGCGCAAGGTTTCGTGAGTAAGGGTGAGGAGCTG
6'FTF1-DP6	TCAATTCTCTCGCACACTGC