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PATHOGENICITY DETERMINANTS OF *FUSARIUM GRAMINEARUM* ON WHEAT EARS

Submitted by Andrew Mark Beacham to the University of Exeter as a thesis for the degree of *Doctor of Philosophy by Research in Biological Sciences*, September 2010

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Signed

A M Beacham

Abstract

Some specialist microbes can deploy a range of mechanisms to cause disease on one or more host plant species. To identify entirely new classes of pathogenicity and virulence factors, a bioinformatics-reverse genetics approach has been applied to a plant pathogen where near complete genomic sequence information was available. A genomic region was identified on chromosome 1 of the important cereal pathogen *Fusarium graminearum* that contains a significant grouping of homologues of known virulence genes. Targeted deletion of these genes revealed a role for the neutral trehalase (*NTH1*) and protein kinase A regulatory subunit (*PKAR*) genes in the rate of disease symptom spread by *F. graminearum*, in addition to the previously reported *SNF1* Ser/Thr protein kinase and *STE7* MAP kinase kinase genes. Subsequent investigation of further genes at this locality revealed the presence of a gene, here named *Fusarium graminearum Contributor to Virulence 1* (*FCV1*), which represent a novel class of gene required for a full rate of symptom spread. Targeted deletion of *FCV1* led to a reduced rate of disease development by *F. graminearum* on wheat ears and *Arabidopsis* floral tissue, but did not affect trichothecene mycotoxin production. The *fcv1* deletion mutant also exhibits altered hyphal growth, reduced asexual sporulation and altered sensitivity to oxidative and osmotic stress. In the complemented strain, wild-type traits were completely or partially restored. This micro-region of < 40 kb containing these five important genes represents a novel type of gene cluster containing genes required for a full rate of disease development. This micro-region is located in a genomic region of low recombination, is highly conserved in three other *Fusarium* species, but is less conserved in other plant pathogenic species. The micro-region is not defined by a distinct GC content or coordinated gene expression patterns, nor is it flanked by highly repetitive sequences. This micro-region is distinct from the previously identified fungal and bacterial virulence gene clusters and the clustered biosynthesis-associated genes required to synthesis metabolites which contribute to pathogenicity. This method for novel disease development-contributing gene identification is applicable to any sequenced pathogen species.

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Chapter 1. Introduction

1.1 Aims, Objectives and Results

The aim of this study was to locate novel classes of gene that contribute to disease development by the plant pathogen *F. graminearum* (teleomorph *Gibberella zeae*) while demonstrating the feasibility of an approach designed to highlight genomic micro-regions for targeted gene deletion to investigate their roles in disease development. The objectives were as follows:

- To locate a region of the *F. graminearum* genome that may show enrichment for genes contributing to disease symptom development.
- To investigate one such region by bioinformatic and reverse genetic approaches, including:
 - Determining the distribution and degree of conservation of the region across different fungal species
 - Searching the region for features commonly found in previously described virulence gene clusters
 - Analysing published expression datasets to determine the *in planta* expression patterns of the genes in the region
 - Generating targeted single-gene strains that lack one of the genes in the region under study
 - Investigating the effect of gene deletion on disease development
 - Characterising further any strains exhibiting a reduced rate of disease symptom development for the production of mycotoxin, *in vitro* growth and other possible phenotypes

Bioinformatic analysis of the cluster indicated a series of features that distinguish this region from previously characterised virulence gene clusters, such as the lack of either flanking repetitive sequences, a distinct GC content or genes for secreted proteins. Of three homologues of known virulence genes in other species initially deleted, two showed a reduced rate of disease progression phenotype. Deletion of a further six genes, not previously known to be associated with virulence, revealed an additional strain with a reduced disease progression rate. The further examination of these three affected strains revealed additional defects that included reduced asexual sporulation, germination viability, mycotoxin production, virulence on *Arabidopsis* and altered stress sensitivity.

1.2 Contributions Made to the Field

This study has revealed three previously uncharacterised genes in *F. graminearum* that are required for a full rate of disease progression. One of these genes represents a class of gene not previously linked to disease symptom development. Two of the three genes also contribute to sporulation and one to mycotoxin production, which are important features of the *Fusarium*-wheat pathosystem. These genes therefore constitute possible new targets for chemical intervention. The investigations described here have highlighted a novel genomic feature in fungi. The genes described above were found to reside in a new type of virulence gene cluster unlike any previously reported.

This study has also demonstrated a new application of a technique for the rapid location of novel disease-contributing factors in sequenced pathogen species. Previous methods used to locate such factors have had to rely on either a trial and error or a large-scale mutant-screening approach. By highlighting genomic hotspots that are more likely to contain genes contributing to disease development, this technique increases the success rate and speed of locating novel classes of such genes, while greatly reducing labour and resource requirements.

This approach is applicable to all pathogens or any organism type or host range with a largely completed genome sequence. For pathogens with smaller genomes, and, due to the advent of next-generation sequencing technology, increasingly those with somewhat larger genomes, genome sequencing is becoming more rapid, simpler and cheaper, so increasing the potential for this technique further.

By applying the technique to a key plant pathogen species, the study has been able to locate not only a new class of disease development-contributing gene in pathogenic fungi, but also a novel type of disease development-contributing gene micro-region. Genes of related sequence or function to *FCV1* have not previously been shown to play a role in disease progression by pathogenic fungal species. *FCV1* resides within a genomic locus that is distinctly different to the previously characterised pathogenicity islands of bacteria or the secreted protein and secondary metabolite biosynthesis clusters of fungi.

1.3 Contribution to Co-authored Papers

The results described in this thesis are in preparation for submission:

Andrew M. Beacham, Martin Urban, John Antoniw, Amy Freeman, Sue Welham and Kim E. Hammond-Kosack (2011). Identification of a novel virulence contributor class located in a conserved *Fusarium* gene cluster.

I curated data for the PHI-base database (www.PHI-base.org) that allowed a publication outlining the release of an updated version of the database and describing its new contents and features. I reviewed and amended the manuscript which was published as:

***Winnenburg, R., *Urban, M., *Beacham, A., Baldwin, T.K., Holland, S., Lindeberg, M., Hansen, H., Rawlings, C., Hammond-Kosack, K.E., and Köhler, J. (2008).** PHI-base update: Additions to the Pathogen-Host Interaction Database. *Nucleic Acids Research* **36** (Database issue): D572-6.

*Joint first authors

I wrote a paper for the Institute of Biology *Biologist* Journal discussing the growing problem of plant pathogens, the recently published *F. graminearum* genome, and new methods for investigating pathogens:

Beacham, A.M., Antoniw, J., and Hammond-Kosack, K.E. (2009). A Genomic Fungal Foray. *The Biologist* **56** (2): 98-105.

I contributed to the beta testing and further refinement of the functionality of a novel whole-genome display software called OmniMapFree developed by Dr John Antoniw at Rothamsted Research. A manuscript has recently been submitted to BMC Bioinformatics:

John Antoniw, Andrew Beacham, Thomas Baldwin, Martin Urban, Jason J. Rudd and Kim E. Hammond-Kosack

OmniMapFree: A new tool to visualise and explore sequenced genomes (submitted Sept 2010)

1.4 Literature Review

1.4.1 The Problem of Plant Pathogens

With a rapidly increasing global population it is essential to improve the characteristics of food crops. An increase in the yield, drought tolerance or hardiness of a crop, for example, could vastly increase food supplies. However, crop diseases constitute a serious constraint on yield. These diseases cause billions of dollars worth of crop losses worldwide every year and, in the worst cases, lead to devastating food shortages.

The problem of disease is not new. Perhaps the most familiar historical case is the Irish potato famine of the 1840s, caused by the potato late blight pathogen *Phytophthora infestans* (Agrios, 1997), but many other examples of dramatic crop losses are known. The black stem rust pathogen *Puccinia graminis tritici* caused the loss of large percentages of the American wheat crop in the 1950s. Resistant and high-yielding wheat varieties were soon developed to alleviate the problem, but the disease has since returned to prominence in Africa with the discovery of Ug99, a new and highly virulent strain, in Uganda in 1999. This strain is not controlled by the wheat varieties developed previously and so is currently spreading across Africa and could enter the Middle East (MacKenzie, 2009). As well as mass food shortages, plant diseases can even lead to cultural alterations such as in the case of coffee rust (*Hemileia vastatrix*) in the 1870s. This rust disease decimated coffee production of Sri Lanka and led to the adoption of tea as the caffeinated drink of choice in Britain. Global banana production is currently under threat from black sigatoka (*Mycosphaerella fijiensis*), or black leaf streak, which can cause yield losses over 50% and result in prematurely ripening fruit which are difficult to export (Ploetz, 2001).

Pathogens are not static, new species and strains can arise and spread rapidly across large areas. Emerging pathogens constitute a serious threat to regions that have to cope with the arrival of new pathogens which may in addition carry altered resistance to current antifungal treatments. To take one example: in Florida, enormous economic losses are predicted if newly acquired pathogens are able to spread and infect crops such as citrus, tomato and strawberry

(Emerging Pathogens Institute, Autumn 2010). The wide range of different ecosystems and diverse agriculture in this state may be particularly vulnerable to new pathogens, with wind-borne pathogens arriving or re-appearing from the Caribbean and Latin America via the action of hurricanes (Emerging Pathogens Institute, Autumn 2010). The University of Florida has highlighted a list of 17 important new and emerging plant diseases in the state including bacterial wilt, karnal bunt, soybean rust and citrus greening (Harmon *et al.*, 2002). This last disease, also known as Huanglongbing (HLB), affects all citrus cultivars and can rapidly destroy entire citrus groves. Infected trees can become non-productive and can be killed by this bacterial disease. The insect psyllid vector of HLB and the disease itself have been detected in Florida and prevailing climate conditions would enable a rapid spread of HLB through the state (Chung and Briansky, 2005).

Crop disease is now of increasing concern as world food supplies must continue to nourish a rapidly growing population. As the problem of disease grows, so new ways to protect crops are needed. As disease results from an interaction between a host plant and a pathogen, the fight against disease can be approached from two sides: that of the host and that of the pathogen itself. One approach is to understand how pathogens cause disease. This information may then allow the successful modification of either host defences, the virulence determinants of the pathogen, or both, in order to help reduce disease. One key aim of this approach is to understand the genetic basis of pathogenicity/virulence i.e. which of the genes in the genome of the pathogen allow successful colonisation of a plant and disease symptom development. Sequencing the entire genomes of plant pathogens can help in identification of such genes. It is hoped that, in addition to providing a large amount of information about a particular species and opening the door for many experimental techniques, further insights into pathogenicity/virulence can be gained through the comparison of genomes of different species. This can lead to the discovery of chemical control targets to help combat the pathogen, so reducing disease incidence and severity, both in the crop and post-harvest.

Fungi constitute a large proportion of the plant pathogenic microorganisms present in the temperate and sub-tropical regions of the world. Plant pathogenic fungi exhibit a wide range of lifestyles and infection mechanisms and frequently produce harmful toxins. In 2002, the first fungal plant pathogen genome was sequenced and the results published in 2005 (Dean *et al.*, 2005). *Magnaporthe oryzae* was selected because this organism is responsible for the globally devastating rice blast disease (Agrios, 1997). Since then, the genome sequences of a number of pathogenic and related non-pathogenic fungal species have been made available. The sequenced pathogenic species represent a wide range of infection biology types that can successfully cause disease on many different plant species. The non-pathogenic species provide a useful comparison and can be investigated in parallel to highlight factors required for disease.

The globally important pathogenic species *Fusarium graminearum*, a causative agent of Fusarium ear blight of wheat and barley and other plant diseases is one such species whose genome has been sequenced and recently published (Cuomo *et al.*, 2007). This study has investigated the genetic basis of *F. graminearum* pathogenicity/virulence at a discrete chromosomal location and is described in the following chapters.

1.4.2 Fusarium Ear Blight

Fusarium ear blight (FEB) is a highly destructive disease that affects wheat, barley, maize, sorghum, oat and rye crops (Schroeder and Christensen, 1963; Steffenson, 2003). FEB now has been reported in most wheat-growing areas of the world (Parry *et al.*, 1995). The main causal agents of FEB are the filamentous ascomycetes *Fusarium graminearum*, *F. culmorum* and *F. avenaceum*, together with *F. poae* in parts of Europe (Snijders, 1994; Parry *et al.*, 1995; Bai *et al.*, 2003; Gale, 2003; Mesterhazy, 2003). *F. graminearum* and *F. culmorum* are regarded as some of the most pathogenic Fusarium species (Stack and McMullen, 1985; Mesterhazy, 2003). To date, *F. graminearum* is the more intensely studied species of the two and the biology of these two closely

related species has been determined predominantly from studies of *F. graminearum*.

Infection by *Fusarium* leads to a reduction in kernel/grain set and weight, ear bleaching and a loss of starch granules and cell wall material that reduces yield and grain quality (Snijders, 2004). In addition, many *Fusarium* species produce trichothecene mycotoxins (such as deoxynivalenol (DON), its acetylated derivatives (3A-DON and 15A-DON) and nivalenol (NIV)) and other toxins, such as zearalenone, that contaminate grain, presenting a serious health hazard to humans and animals as well as exhibiting phytotoxicity (Stob *et al.*, 1962; Ueno and Ishii, 1985; Wang and Miller, 1988; Wakulinski, 1989) (**Figure 1.1**). It has been estimated that around 25% of the world's food crops are contaminated to some degree by mycotoxins (Charmley *et al.*, 1995), but this figure may be even higher for some toxins, such as DON (Bottalico, 1998). Due to the risk posed by such toxins, strict guidelines have been implemented such that grain with DON levels higher than around 1 ppm (even less in some cases) is rejected (Food Standards Agency, 2006). Current EU limits, in effect from 1 July 2006, are as follows:

Table 1.1. Current EU mycotoxin limits (Food Standards Agency, 2006).

	Legal limit (ppb)	
	Deoxynivalenol (DON)	Zearalenone (ZEA)
Unprocessed wheat	1250	100
Flour	750	75
Finished products	500	50
Infant food	200	20

Infected grain is therefore harder to market and is more difficult to process, further increasing the impact on the wheat and barley industry and in addition, *Fusarium* fungal material can remain in soil on debris from a previous season's crop, aiding disease perseverance and making eradication more difficult (discussed in McMullen *et al.*, 1997).

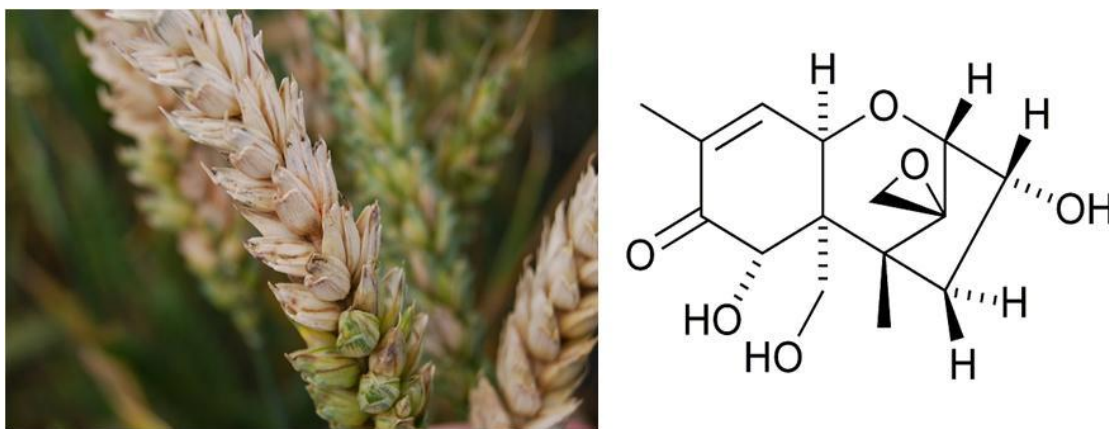


Figure 1.1. Fusarium ear blight (FEB) disease symptoms in the field (left) and the structure of the mycotoxin deoxynivalenol (right).

1.4.3 Importance and Economic Effects

The consequences of FEB infections are drastic. In the USA and China alone, this amounts to losses of millions of tons of grain, and hence billions of dollars. Over 7 million hectares of wheat in China have been affected by FEB, with severe epidemics causing losses of over 1 million tons (Wang and Miller, 1987; Lu *et al.*, 2001; Bai *et al.*, 2003; Leonard and Bushnell, 2003). Recent numerous epidemics in American wheat and barley crops, especially in the Northern Great Plains, and also Canada from 1991-1997 resulted in around \$1.3 billion of direct losses and \$4.8 billion of accumulated economic impact (Johnson *et al.*, 2003), and have led to FEB being considered by the US Department of Agriculture (USDA) as the worst plant disease in the USA since stem rust in the 1950s (Wood *et al.*, 1999). Between 1998 and 2000, yield reduction and price discount effects (due to poor quality and toxin-infected grain) of FEB in wheat and barley in the USA were estimated at \$870 million, which is more than the annual value of all barley and oats produced in the USA in 1999 and 2000 (Nganje *et al.*, 2001). On average, 6.9 percent of the total value of all U.S. winter wheat production was lost due to FEB (Nganje *et al.*, 2001). Such large losses force many farmers to switch to the production of different crop species or supplement their income with other work. Increasing debt (an average of over \$400,000 in the Red River Valley in 1998) has led many to abandon farming (Windels, 1999).

It is not only the producers who suffer from the results of FEB. Businesses that depend on revenue from crop sales are also affected. For every \$1 dollar of losses incurred by the producer, \$2 in losses are incurred in other areas of rural and state economies (Nganje *et al.*, 2001). In the USA, FEB occurs in many parts of the Great Plains that are predominately dependent upon small grain production. In addition, FEB is occurring during periods of depressed farm prices and low net farm income (Nganje *et al.*, 2001).

Europe and the UK

Fusarium is found in all European cereal-growing areas, causing root, stem, and ear diseases and subsequent reductions in yield of 10-40% (Bottalico and Perrone, 2002). *F. culmorum* appears to dominate in cooler areas such as northern Europe, with *F. graminearum* predominating in the warmer south, but spreading northwards (Parry *et al.*, 1995; Bottalico and Perrone, 2002).

Wheat and barley comprise nearly 80% of European small grain production (Bottalico and Perrone, 2002). It is thought that almost all wheat and barley grown in northern Europe is contaminated by Fusarium mycotoxins to some extent. In Europe DON and its derivatives are the most common mycotoxins, which are produced by *F. graminearum* and *F. culmorum* (Bottalico and Perrone, 2002). The situation in Europe appears to have worsened over recent years, with increased levels of Fusarium colonisation and infection (Magan *et al.*, 2002). An increase in the cultivation of bread wheat in Europe in the 1980s had led to an increase in FEB similar to that seen in Canada (Sutton, 1982; Parry *et al.*, 1995) and the recent adoption of higher yielding but more susceptible winter wheat varieties in Western Europe has also heightened the problem of FEB in this area (Snijders, 2004).

Reports from many countries have indicated the extent of the Fusarium problem in Europe and the effect of weather on disease severity. For example, in Italy, disease incidence is worst in the central regions of the country due to the larger influence of warmer maritime weather here (Bottalico and Perrone, 2002). In the Netherlands, FEB infection levels have been known to vary greatly – in 1991 34% of cereal samples were infected but this rose to 83% in 1993. Such

differences are possibly due to weather conditions at anthesis (Bottalico and Perrone, 2002). Severe infections are also known in countries including Bulgaria (37.2% incidence) (Vrabcheva and Vrabchev, 1997), Poland (epidemics in the north in 1998 and west and south in 1999) (Tomczak *et al.*, 2002), Romania and Hungary (discussed in Parry *et al.*, 1995). More precise data on disease impact in the field is available from experiments using inoculated crops, for example the finding that *F. culmorum* and the non-mycotoxin producing species *Microdochium nivale* decreased wheat yield by 60% and 15%, respectively, in Switzerland (Hani, 1981).

In the UK, FEB was thought to be a potentially serious but sporadically occurring disease (Nicholson *et al.*, 2003). Surveys in the years 1989-1990 determined that *F. culmorum*, *F. avenaceum*, *F. poae* and *Microdochium nivale* (a *Fusarium* relative) were the main cause of FEB (Polley and Turner, 1995). However, the proportion of disease due to *F. graminearum* is increasing year on year and first exceeded that due to *F. culmorum* in 2002 (**Figure 1.2**) (HGCA Crop Monitor, 2010). By 2009, *F. graminearum* was responsible for 13% of FEB symptoms compared to just 5% for *F. culmorum* and is spreading northwards through the UK (HGCA Crop Monitor, 2010). *F. graminearum* now comprises a serious threat due to the high level of damage it can inflict (Jennings *et al.*, 2004). *F. graminearum* and *F. culmorum* are now the main cause of DON mycotoxin contamination of wheat grain in the UK, with *F. graminearum* generally being regarded as more aggressive and causing more contamination than *F. culmorum* (HGCA Crop Monitor, 2010).

Results from the HGCA Crop Monitor Winter Wheat Survey indicate that FEB incidence levels fluctuate annually, being highly dependent upon weather conditions during crop flowering (**Figure 1.3**) (HGCA Crop Monitor, 2010). In years of particularly high FEB incidence, such as in 2007, over 80% of wheat samples were affected. Such surveys have highlighted the south west and East Anglia as the worst affected areas (**Figure 1.4**), however in 2009, the north east and south east were worst affected (HGCA Crop Monitor, 2010).

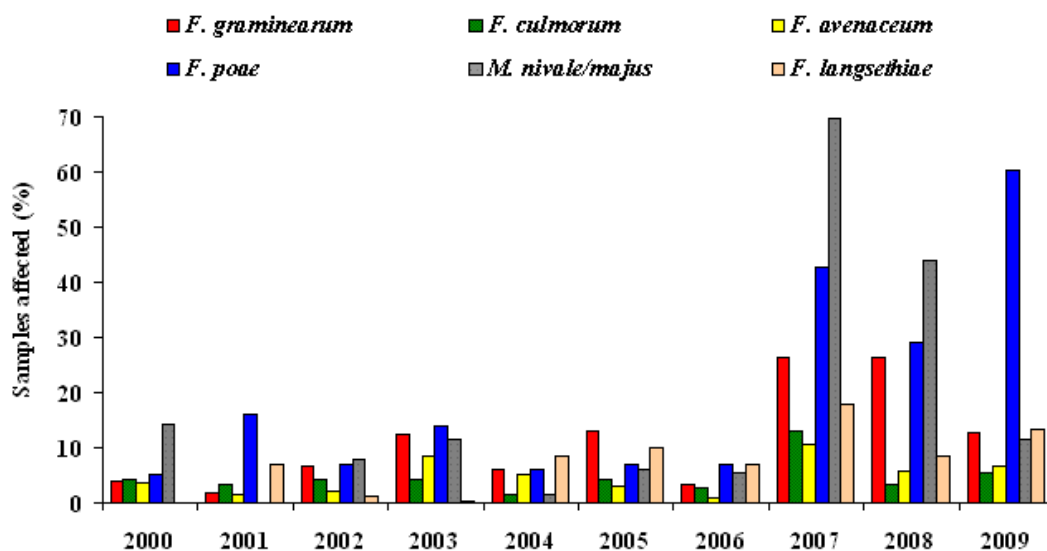


Figure 1.2. Species responsible for causing Fusarium ear blight symptoms in the UK (HGCA Cropmonitor, 2010).

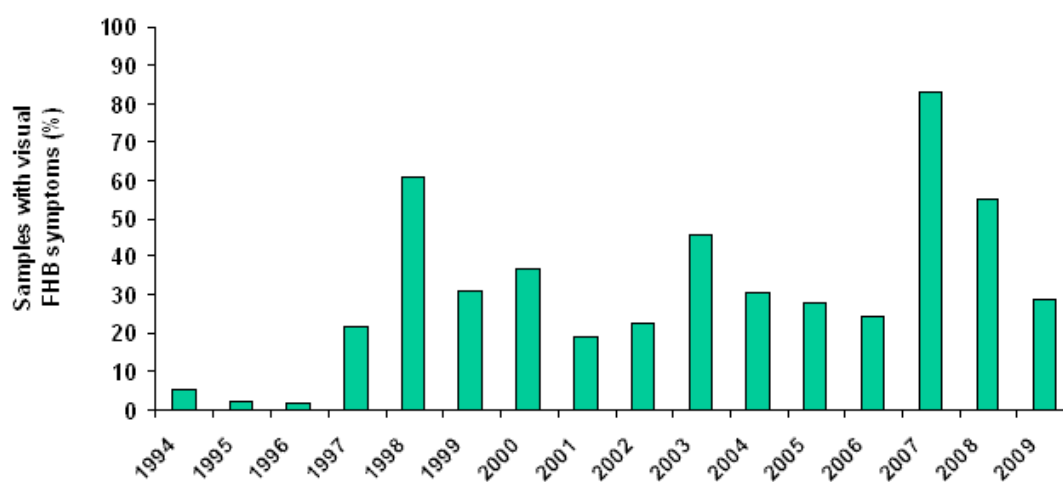


Figure 1.3. National incidence of Fusarium ear blight symptoms from 1994 to 2009. From HGCA Crop Monitor (2010).

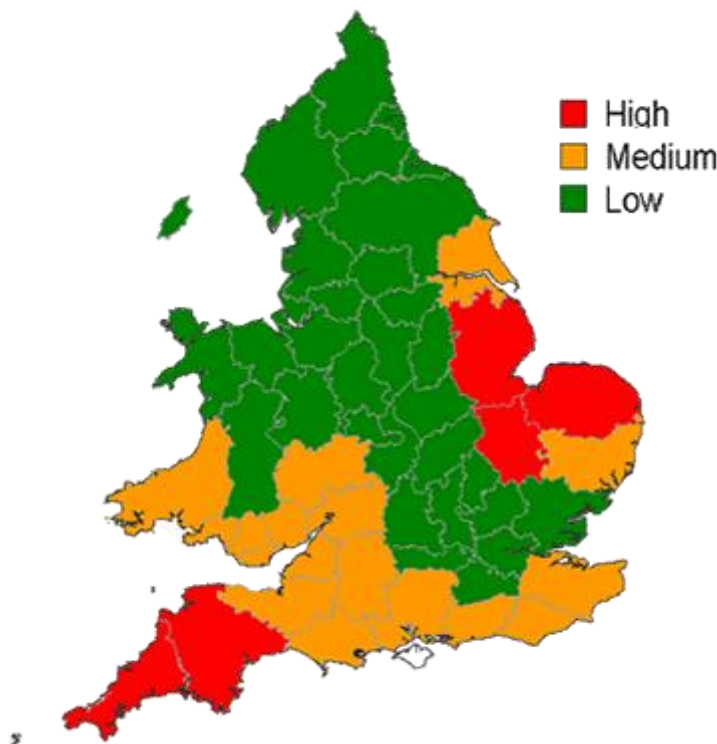


Figure 1.4. Risk map based on incidence of FEB symptoms 1992-2006. From HGCA Crop Monitor (2010)

Globally it is thought that unusually wet weather at anthesis, an increase in conservation tillage, which allows the fungus to survive on crop residues, and the use of maize in the rotation have helped increase the abundance of the disease in wheat crops (Windels, 1999; Dill-Macky and Jones, 2000; HGCA Crop Monitor, 2010).

1.4.4 Disease Development

F. graminearum produces both sexual (ascospores) and asexual (macro and microconidia) spores, while *F. culmorum* produces asexual spores only. *Fusarium* ascospores and conidia are thought to be the main infectious propagules, with the sequentially flowering wheat ears on each tiller comprising the route of infection (Stack and McMullen, 1985; Bai and Shaner, 1994; Miller, 1994; Parry *et al.*, 1995; McMullen *et al.*, 1997; Shaner, 2003). Spores are generally thought to primarily infect spikelets during anthesis, a time when they are most susceptible (Pugh *et al.*, 1933; Parry *et al.*, 1995). Spore dispersal from fungus existing saprophytically on crop debris could be aided by rain

splash, wind and even arthropod vectors (discussed in Parry *et al.*, 1995). Growth, reproduction and spore germination of *Fusarium* are all affected by water potential (Sung and Cook, 1981), which may help to explain why wet weather increases the occurrence of the disease (Andersen, 1948; Dill-Macky and Jones, 2000).

Infection begins with the formation of brown spots on spikelets and eventually spreads up and down the rachis (Guenther and Trail, 2005), commonly leading to bleaching of the ear prior to natural senescence (Atanasoff, 1920; Pugh *et al.*, 1933; Parry *et al.*, 1995; Bushnell *et al.*, 2003). The extent of disease development depends on host developmental stage, inoculum amount, exposure duration, air temperature and requires a period of high moisture level (Andersen, 1948). The fungus appears to form a hyphal network in the spikelet cavity and colonise the glume, ovary, palea and lemma and other tissues of the wheat ear (Pritsch *et al.*, 2000; Wanyoike *et al.*, 2002; Jansen *et al.*, 2005; Brown *et al.*, 2010). Subcuticular growth, including that along glume stomatal rows has also been observed (Pritsch *et al.*, 2000) and may lead to direct penetration of epidermal cells (Kang and Buchenauer, 2000). Within 24-36 hours of infection (hai), infection hyphae have formed (Wanyoike *et al.*, 2002) and penetrate through stomata to the interior of the ear tissues (Pritsch *et al.*, 2000). Entry via anthers may also be an important infection route for hyphae (discussed in Strange and Smith, 1978). Mass spectrometry of wheat extracts that stimulate *F. graminearum* growth *in vitro* and *in vivo* determined the active compounds as choline and betaine (Strange, 1972, 1974), which were present at the highest concentration in anther extracts (Pearce *et al.*, 1976). A range of similar quaternary ammonium compounds (esters of choline) were later found to also stimulate the early stages of *F. graminearum* hyphal growth *in vitro*. Choline and betaine had no effect on macroconidial germination or resulting germling growth, however, their effects appeared to be restricted to an early stage of hyphal extension (Strange and Smith, 1978).

In susceptible wheat genotypes, *Fusarium* hyphae grow through the cortex and vascular tissue and spread apically along the rachis, infecting neighbouring florets, and continue down the stem (Brown *et al.*, 2010). The fungus grows both inter- and intracellularly, penetrating cell walls and leading to the collapse

of such cells (Wanyoike *et al.*, 2002; Jansen *et al.*, 2005; Brown *et al.*, 2010). The infection destroys starch granules and damages cell walls, reducing detectable cellulose, xylan and pectin levels (Wanyoike *et al.*, 2002) and also affects endosperm storage proteins (Snijders, 2004). At later infection stages, the fungus switches from vertical to predominantly lateral growth and accumulates below the rachis surface, degrading cells and rupturing the rachis surface (Brown *et al.*, 2010), where, within 48 to 76 hours after inoculation (hai), the fungus sporulates, allowing further spread of infection via the release of spores (Pritsch *et al.*, 2000; Guenther and Trail, 2005). Macroconidia can be produced on florets and glumes, and (in the case of *F. graminearum*) perithecia can eventually form and propel ascospores into the air to be dispersed by wind and rain (discussed in Bushnell *et al.*, 2003). Trichothecene mycotoxins produced by *Fusarium* species have the ability to inhibit plant growth and cause lesions, and may spread through the host ahead of the developing fungus (Kang and Buchenauer 1999). Toxins are transported through the xylem and phloem of the rachis to uninfected florets and are widely distributed in infected spikelets (Kang and Buchenauer, 1999). However in a recent study, death of cells ahead of the infection front was only noted in the chlorenchyma (Brown *et al.*, 2010). The toxins may therefore serve to combat host defence responses rather than, or in addition to, causing host cell death *per se* (Jansen *et al.*, 2005).

Fusarium genes expressed during initial disease development include those encoding a two-component sensing response regulator homologue (*Rrr1*; whose mutation slightly delays spread of the disease on wheat and reduces sporulation), an ABC transporter (*Abc2*) and lysine permease (*Lyp1*) (Goswami *et al.*, 2006). From observation of gene expression changes made by the fungus during infection, it has been suggested that mitochondria may play an important role in pathogenesis, perhaps for increased energy provision or catabolism of plant material (Goswami *et al.*, 2006). Other genes whose expression is altered upon infection include those that could be used to overcome a plant oxidative attack and various metabolic enzymes, including some involved in glycolysis, possibly for utilisation of carbon sourced from the plant (Zhou *et al.*, 2006). The fungus also produces a range of polysaccharide-degrading enzymes to aid infection of the plant via the destruction of host cell

wall material. When the fungus is grown on hop cell walls, its exoproteome includes many proteins thought to be involved in the degradation of xylan, the main constituent of hemicellulose (Phalip *et al.*, 2005; Hatsch *et al.*, 2006). The complexity and variety of hemicellulose polymers would require a range of enzymes to break them down. The large number of xylan-degrading enzymes, coupled with differential expression patterns of their cognate genes is thought to allow greater flexibility in the response of the pathogen to its environment (Hatsch *et al.*, 2006). The microscopy study by Brown *et al.* (2010) has, however, revealed that substantial plant cell wall degradation is only a feature of the later stages of the infection of wheat ears by *F. graminearum*. The fungus also secretes lipases during infection, some of which, such as FGL1, are required for successful ear colonisation (Voigt *et al.*, 2005).

Using an Affymetrix Gene Chip microarray, Güldener *et al.* (2006) analysed the expression of *F. graminearum* genes on infected barley plants at 24 hr intervals over a 144 hour time course and compared this to *in vitro* gene expression. In total, transcripts hybridising to 431 probe sets were detected exclusively *in planta*. Many corresponded to genes of unknown function, but also included were genes encoding proteins involved in carbohydrate metabolism, fatty acid metabolism, secondary metabolism, ion homeostasis, disease, virulence and defence functions. These included genes encoding plant cell wall-degrading enzymes, such as xylanases, mannanases, pectinases, glucanases, galactosidases and cutinases, and also trichothecene biosynthesis genes. Various patterns of gene expression were noted *in planta*, some genes appearing to be constitutively expressed (138 probe sets), while expression of others was only detectable after a period of time (48, 72, 96, or 144 hr). Other genes exhibited transient detectable expression for a 24 or 48 hr period at different sections of the time course. A similar time course for wheat ear infection has been generated but has not yet been published (E. Lysoe and H-C Kistler, pers comm.)

Studies have also been made of the changes in host plant gene expression and protein levels upon infection. Defence response genes, including those encoding PR (pathogenesis-related) proteins have been noted to be up-regulated in response to Fusarium infection (discussed in Pritsch *et al.*, 2000).

For example, using the Barley1 Affymetrix GeneChip probe array, Boddu *et al.* (2006) observed changes in barley gene transcript levels upon infection by *F. graminearum*. 467 transcripts were differentially regulated in *F. graminearum*-infected versus control plants. Up-regulated genes included those encoding defence response proteins, oxidative burst-associated enzymes and potential trichothecene catabolism/transport proteins.

Proteomic analysis of infected wheat ears has revealed the up-regulation of genes encoding proteins involved in oxidative burst (several antioxidant genes for a potential defence response) and jasmonic acid signalling pathways, pathogenesis-related response (such as antifungal chitinases and β -glucanases), amino acid biosynthesis and nitrogen metabolism and down-regulation of some photosynthetic protein-encoding genes (Zhou *et al.*, 2005, 2006). A comparison of wild-type and a trichothecene non-producing *tri5* loss of function strain, showed that a subset of barley genes were up-regulated specifically in response to trichothecenes and were distinct from the basal defence response of this species (Boddu *et al.*, 2007). These genes included some encoding proteins that may function in trichothecene export or detoxification. Other defence response genes whose expression is enhanced included those associated with cell death, a process which, for this necrotrophic pathogen, may actually help to promote disease. Proteomic analysis of the resistant wheat cultivar Wangshuibai after infection with *F. graminearum* revealed an increase in expression of genes specifying stress response and plant defence proteins (Wang *et al.*, 2005). Pritsch *et al.* (2000) noted an earlier and larger accumulation of certain PR gene transcripts in a resistant cultivar compared to a susceptible one. A second study of a resistant wheat genotype indicated a series of genes whose expression was altered by the presence of *F. graminearum*, including several involved in defence responses or similar to disease resistance genes, whose expression appeared to be resistance-specific (Kong *et al.*, 2007). A study of barley proteins that respond to the presence of *F. graminearum* in several genotypes of varying resistance indicated an increased abundance of PR proteins in resistant and intermediate barley genotypes but a decreased abundance in a susceptible genotype. Oxidative burst and oxidative stress response proteins increased in abundance in susceptible, intermediate and one resistant genotype. As noted in the earlier barley gene expression

study (Boddu *et al.*, 2007), this response may contribute to susceptibility by increasing host cell death which could aid disease symptom progression by this pathogen (Boddu *et al.*, 2007; Geddes *et al.*, 2008).

F. graminearum also appears to influence ethylene signalling during infection of both monocotyledonous (wheat and barley) and dicotyledonous (Arabidopsis) plants (Chen *et al.*, 2009). Arabidopsis mutants with reduced ethylene signalling exhibited increased resistance to *F. graminearum* in leaf tissue while those with increased ethylene signalling levels were more susceptible than normal. However, in floral tissue, the various ethylene signalling mutations had no effect on infection (Cuzick *et al.*, 2008b). In wheat, down-regulation of ethylene signalling increased resistance to *F. graminearum*, However, in this study, a full characterisation of the anti-transgenic wheat plants was not conducted and so the influence of unknown non-target effects cannot be discounted. The *NPR1* and *EDS11* genes have been shown to contribute to resistance against Fusarium in Arabidopsis (Cuzick *et al.*, 2008b). The *npr1* and *eds11* mutants of Arabidopsis exhibited enhanced susceptibility and increased mycotoxin contamination. In wheat, over-expression of the Arabidopsis NPR1 protein leads to enhanced resistance (Makandar *et al.*, 2006). Finally, the Arabidopsis signalling mutant *sgt1b* shows increased resistance to Fusarium infection but no reduction in mycotoxin accumulation (Cuzick *et al.*, 2009).

1.4.5 Mycotoxins

The mycotoxins produced by *Fusarium* depend on both species and strain, with a range of trichothecenes, polyketides and other toxins known. The b-type trichothecenes (containing a keto group at carbon eight), the best-studied of the *Fusarium* toxins, are potent inhibitors of translation, interacting with the peptidyl transferase site of eukaryotic ribosomes (Ueno *et al.*, 1973; Ehrlich and Daigle, 1987). These trichothecenes, such as deoxynivalenol (DON), can also activate MAP kinases after ribosome binding via the ribotoxic stress response which mediates their effects on immune system cells: the upregulation of cytokines and other genes at low dose via modulation of transcription factor binding, leading to immunostimulation and promotion of leukocyte apoptosis and so

immunosuppression at high dose (Shifrin and Anderson, 1999, discussed in Pestka *et al.*, 2004). In the host plant, DON appears to activate hydrogen peroxide production, programmed cell death and defence responses (Nishiuchi *et al.*, 2006; Desmond *et al.*, 2008). DON may induce reactive oxygen species (ROS) production *in planta* in order to stimulate host cell death that would aid the spread of the pathogen, however this signal would also appear to stimulate host defence responses (Desmond *et al.*, 2008). However, DON, when directly injected into the apoplast of plant tissue is only able to induce host cell death at high concentrations (millimolar range) compared to other mycotoxins produced by *Fusarium* species (Nishiuchi *et al.*, 2006).

Most genes required for the biosynthesis of the trichothecene mycotoxins of *Fusarium* are grouped together as the 'TRI5 cluster', which in *F. graminearum* consists of 10 genes: *TRI8*, *TRI3*, *TRI4*, *TRI6*, *TRI5*, *TRI10*, *TRI9*, *TRI11*, *TRI12* and *TRI14* (Brown *et al.*, 2001, 2004), and is located on chromosome 2 in a region of moderate genetic recombination. Expression of this cluster of genes is under control of the transcriptional regulators *TRI6* and *TRI10* (Proctor *et al.*, 1995a, Seong *et al.*, 2009). *TRI6* has been shown by electrophoretic mobility shift assay to bind the promoters of various *TRI* biosynthesis genes in *F. sporotrichioides*, probably using Zn finger domains located at its C-terminus (Hohn *et al.*, 1999). Mutation of the *TRI6* gene lowers transcript production and site-directed mutagenesis of the Zn finger or mutation of the DNA-binding motif abolishes binding to *TRI* cluster promoters (Proctor *et al.*, 1995a; Hohn *et al.*, 1999). Targeted deletion, meanwhile, of either *TRI6* or *TRI10* reduces the expression of most *TRI* genes and also the expression of isoprenoid biosynthesis genes involved in the metabolic pathway preceding trichothecene biosynthesis (Tag *et al.*, 2001; Peplow *et al.*, 2003a; Seong *et al.*, 2009). Deletion of *TRI6* also increases the expression of *TRI10*, suggesting a level of negative regulation of this gene by *TRI6*. Conversely, deletion of *TRI10* has little effect on the expression of *TRI6* (Seong *et al.*, 2009). *TRI6* and *TRI10* appear to regulate overlapping but distinct sets of genes that also include genes involved in virulence, defence, transport and secondary metabolism (Seong *et al.*, 2009). A number of additional genes have been found to exhibit expression that is correlated with that of trichothecene biosynthesis genes *in planta* and are highly induced by agmatine, a stimulant of DON production (Gardiner *et al.*, 2009a).

Many of these genes were found to possess predicted *TRI6* binding sites in their promoter regions. Interestingly, two of these genes, *FGSG_00007* and *FGSG_10397* (which possess one and three predicted *TRI6* binding sites, respectively) were found to highly negatively regulate DON production, their targeted deletion leading to large increases in DON production in both non-inducing and inducing conditions (Gardiner *et al.*, 2009).

The expression of *TRI* genes appears to be influenced, at least *in vitro*, by ambient pH (Merhej *et al.*, 2010, Gardiner *et al.*, 2009b). Water activity and temperature have also been reported to affect the expression of *TRI* genes (Marin, *et al.*, 2010; Schmidt-Heydt *et al.*, 2010) as has the application of oxidative stress (Ponts *et al.*, 2006, 2007, 2009), a situation which could, in the field, result from sub-lethal doses of fungicide (Audenaert *et al.*, 2010). Amine compounds such as agmatine (described above) and putrescine also increase trichothecene production by *F. graminearum* (Gardiner *et al.*, 2009a, c), while magnesium has been reported to decrease production of these compounds (Pinson-Gadais *et al.*, 2008).

The role of many *TRI* genes has been elucidated in the related species *F. sporotrichioides*, which produces the trichothecene T-2 toxin. However, many of the *TRI* genes are conserved across *Fusarium* species and are also found in *F. graminearum* (Brown *et al.*, 2001; Kimura *et al.*, 2003). Several such genes have been experimentally disrupted and the resulting accumulation of trichothecene intermediates analysed to determine the function of the particular gene. *TRI5* encodes trichodiene synthase, which catalyses the first committed step in trichothecene biosynthesis and is required for full virulence on wheat, rye seedlings and maize (Proctor *et al.*, 1995b; Desjardins *et al.*, 1996; Harris *et al.*, 1999) although this effect is dependent on wheat variety (Proctor *et al.*, 1995b). *TRI4* encodes a multifunctional P-450 monooxygenase, disruption of which in either *F. graminearum* or *F. sporotrichioides* halts mycotoxin production. Precursor feeding experiments suggest that this enzyme appears to perform four steps in trichothecene biosynthesis (McCormick *et al.*, 2006). *TRI14*, meanwhile is required for full virulence of *F. graminearum* and for detectable DON production *in planta* but not *in vitro* (Dyer *et al.*, 2005).

Different *Fusarium* species and strains produce different trichothecenes and some differences in their *TRI* biosynthetic genes complement have been noted. For example, *TRI13* is required for C-4 oxygen addition in T-2 toxin and NIV biosynthesis. In DON-producing *F. graminearum* strains this gene appears to be non-functional (possessing numerous stop codons and no start codon due to nucleotide deletions) but is functional in *F. sporotrichioides* and *F. graminearum* NIV strains, consistent with the requirement for a C-4 hydroxylase in T-2 toxin and NIV biosynthesis, but not for DON (Brown *et al.*, 2002). Similarly, *TRI7* is required by *F. sporotrichioides* for acetylation of the C-4 oxygen of T-2 toxin and is non-functional in DON-producing *F. graminearum* (Brown *et al.*, 2001). Disrupting *TRI13* in a NIV-producing *F. graminearum* strain leads it to produce DON instead, while expression of a *TRI13* gene from a NIV-producing *F. graminearum* in a DON-producing strain caused it to produce NIV (Lee *et al.*, 2002). Very recently, the sequence of the *TRI8* enzyme was shown through yeast over-expression experiments to determine whether 3A-DON or 15A-DON arises from the intermediate diacetylated 3A,15A-DON precursor in the pathway (Alexander *et al.*, unpublished).

A small number of *TRI* genes have been discovered at loci unlinked to the main *TRI* cluster. *TRI1* of *F. sporotrichioides* encodes a P-450 monooxygenase required for C-8 oxygenation not found in the main cluster (Meek *et al.*, 2003). *F. graminearum* cDNA libraries from cultures grown in trichothecene production-inducing conditions were used to identify additional *TRI* genes in this species and found a new P-450-encoding gene on chromosome 1 required for C-7 and C-8 oxygenation that is probably a homologue of *TRI1* (McCormick *et al.*, 2004). *TRI16*, required for C-8 OH group esterification, and located close to *TRI1*, appears to be non-functional in DON-producing *F. graminearum*, consistent with the presence of a C-8 keto rather than ester group in DON (Brown *et al.*, 2003; Peplow *et al.*, 2003b; McCormick *et al.*, 2004). *TRI101*, a trichothecene 3-O-acetyltransferase-encoding gene, is another *TRI* gene not found in the main cluster (Kimura *et al.*, 1998a-c) and is located on chromosome 4 in *F. graminearum*. The precise role of *TRI101* is currently under debate. A self-defence role for *TRI101* was proposed after trichothecene 3-O-acetyl derivatives showed significantly reduced toxic activity in an *in vitro* rabbit reticulocyte translation system (Kimura *et al.*, 1998a). However, disruption of

this gene in *F. sporotrichioides* indicates it is not essential for self-protection in this species and may instead play a role in T-2 toxin biosynthesis. It has been suggested that *TRI8* encodes a toxicity factor with *TRI101* again acting in self-protection as a free C-3 OH group is important for toxicity (McCormick and Alexander 2002). But the expression patterns of the two genes were not investigated, so no explanation could be made as to how the organism prevents cycling between acetylated and deacetylated forms of trichothecenes and how self-protection could actually be achieved when a C-3 deacetylase is present. Further evidence regarding the role of *TRI101* is presented later. *TRI15*, which may encode a negative regulator of other *TRI* genes, is also unlinked to the main *TRI* cluster and located on chromosome 3 (Alexander *et al.*, 2004). Comparison of the *TRI* cluster in a number of *Fusarium* species has revealed that in some species, the *TRI1* and *TRI101* genes appear to have moved into the main *TRI* cluster. The cluster also demonstrates gene loss as well as non-functionalisation by rearrangement between different species (Proctor *et al.*, 2009).

In addition to trichothecenes, *Fusarium* species also produce a range of other toxins. These include the polyketide zearalenone (ZEA), which shows estrogenic effects in farm animals (Stob *et al.*, 1962). Polyketide synthase genes, such as *ZEA1* and *ZEA2*, which appear to be transcribed divergently from a common promoter region, are required for ZEA synthesis (Gaffoor and Trail., 2006). Disruption of the polyketide synthase genes that are involved in the biosynthesis of ZEA and other polyketides such as aurofusarin and fusarin C, however, has little effect on the pathogenicity of the fungus (Gaffoor *et al.*, 2005; Lysoe *et al.*, 2006).

The precise role mycotoxins play in plant infection by *Fusarium* species is not entirely clear. While disruption of the *TRI5* gene decreases *Fusarium* virulence to wheat (Proctor *et al.*, 1995b; Desjardins *et al.*, 1996) and DON has been shown to inhibit plant and seedling growth and regeneration (discussed in Rocha *et al.*, 2005), infection is not completely prevented by *TRI5* disruption and this effect is also dependent on cereal variety, suggesting trichothecenes play a role in virulence but are not essential (Proctor *et al.*, 1995b). Further studies of *F. graminearum* *TRI5* disruption mutants, which do not produce DON,

indicated that reduced infection on particular cereal varieties was limited to the inoculated spikelets and could not spread to uninoculated spikelets (Proctor *et al.*, 1995b; Bai *et al.*, 2002), due to the presence of plant cell wall thickenings blocking spread of the fungus in wheat (Jansen *et al.*, 2005). The role of DON and similar trichothecenes may therefore be to suppress such a defence response by wheat or to combat other fungal species (Jansen *et al.*, 2005), although the elicitation of hydrogen peroxide production by DON may also serve to activate host defences (Desmond *et al.*, 2008). *TRI5* expression *in planta* does not appear to be uniform, with increased expression in the developing kernel and rachis node (Ilgen *et al.*, 2009) suggesting increased DON production at sites of increased host resistance or defence responses such as cell wall thickening. On barley ears, a loss of function *tri5* strain appeared to show reduced virulence and fungal biomass in planta (Boddu *et al.*, 2007). The loss of *TRI5* does not, however, affect the infection of Arabidopsis by *F. graminearum*, indicating that DON does not play a role in disease symptom progression on this species (Cuzick *et al.*, 2008a). This later result indicated that despite its ubiquitous target site, the ribosome, DON belongs to the class of host-selective toxin (Agrios, 1997).

1.4.6 Pathogenicity/Virulence Factors

A number of *F. graminearum* genes have been disrupted/deleted by either forward or reverse genetics experiments in the search for pathogenicity/virulence factors required by the fungus for full disease-causing ability. These genes and the effect of their disruption on *F. graminearum* virulence are summarised in **Table 1.2**. Interestingly, the *CHS5* and *CHS7* genes are predicted to bind chitin and may act as chitin synthases (Kim *et al.*, 2009). Alternatively, the proteins encoded by these genes could serve to prevent host detection of chitin released from invading hyphae in a manner similar to that of the Ecp6 protein of *Cladosporium fulvum* (de Jonge *et al.*, 2010).

Table 1.2. Characterised genes in *F. graminearum*. Accession numbers, where not otherwise available, are provided based on the gene locus ID number as defined in the first version of the *F. graminearum* genome gene call (FG1, Broad Institute, MIPS).

Gene locus ID	Gene name	Accession	Putative function encoded	Phenotype of deletion/disruption	Host tested	Reference
<i>FGSG_02506</i>	<i>ADE5</i>	XP_382682.1	Phosphoribosylamine-glycine ligase	Reduced virulence	Barley	Kim et al., 2007
<i>FGSG_01939</i>	<i>ARG2</i>	XP_382115.1	Acetylglutamate synthase	Reduced virulence	Barley	Kim et al., 2007
<i>FGSG_02324</i>	<i>AUR1</i>	XP_382500.1	Aurofusarin synthesis	Unaffected pathogenicity	Wheat	Urban et al., 2003
<i>FGSG_01932</i>	<i>CBL1</i>	EAA68828	Cystathionine beta-lyase	Reduced virulence	Wheat	Seong et al., 2005
<i>FGSG_01364</i>	<i>Cch1</i>	XP_381540.1	Voltage-gated calcium ion channel	Reduced virulence	Maize	Seong et al., 2005
<i>FGSG_01964</i>	<i>CHS5</i>	XP_382140.1	Chitin synthase	Unaffected pathogenicity	Wheat	Hallen and Trail, 2007
<i>FGSG_12039</i>	<i>CHS7</i>	EAA69453	Chitin synthase	Reduced virulence	Barley	Kim et al., 2009
<i>FGSG_04355</i>	<i>CID1</i>	XP_384531.1	Cyclin-C like	Reduced virulence	Barley	Kim et al., 2009
<i>FGSG_06631</i>	<i>CPS1</i>	XM_386807	Adenylate forming enzymes	Reduced virulence	Wheat	Zhou et al., 2009
<i>FGSG_02095</i>	<i>FBP1</i>	XP_382271.1	F-box protein	Reduced virulence	Wheat	Zhou et al., 2009
<i>FGSG_05159</i>	<i>FET3</i>	XP_385335.1	Ferroxidase	Unaffected pathogenicity	Maize	Lu et al., 2003
					Wheat	Han et al., 2007
						Greenshields et al., 2007

<i>FGSG_05906</i>	<i>FGL1</i>	AAQ23181	Secreted Lipase	Reduced virulence	Maize	Voigt et al., 2005
				Reduced virulence	Wheat	Voigt et al., 2005
<i>FGSG_01665</i>	<i>FSR1</i>	EAA68366	Signalling scaffold protein	Reduced virulence	Barley	Shim et al., 2006
<i>FGSG_00332</i>	<i>FTL1</i>	XP_380508.1	Transducin beta-like	Reduced virulence	Wheat	Ding et al., 2009
	<i>FTR1</i>	Not available	Iron permease	Unaffected pathogenicity	Barley	Park et al., 2006
	<i>FTR2</i>	Not available	Iron permease	Unaffected pathogenicity	Barley	Park et al., 2006
<i>FGSG_05955</i>	<i>GCS1</i>	XP_386131.1	Glycosylceramide synthase	Reduced virulence	Wheat	Ramamoorthy et al., 2007b
				Reduced virulence	Maize	Ramamoorthy et al., 2007b
				Unaffected pathogenicity	Arabidopsis thaliana	Ramamoorthy et al., 2007b
<i>FGSG_02328</i>	<i>GIP1</i>	XP_382504.1	Putative laccase	Unaffected pathogenicity	Tomato	Ramamoorthy et al., 2007b
				Unaffected pathogenicity	Barley	Kim, JE et al., 2005
				Unaffected pathogenicity	Barley	Yu et al., 2008
<i>FGSG_05535</i>	<i>GPA1</i>	XP_385711.1	G protein alpha subunit	Unaffected pathogenicity	Barley	Yu et al., 2008
<i>FGSG_09614</i>	<i>GPA2</i>	XP_389790.1	G protein alpha subunit	Reduced virulence	Barley	Yu et al., 2008
<i>FGSG_04104</i>	<i>GPB1</i>	XP_384280.1	G protein beta subunit	Reduced virulence	Barley	Yu et al., 2008
<i>FGSG_06385</i>	<i>GPMK1</i>	AAL73403	MAPK	Loss of pathogenicity	Wheat	Jenczmionka et al., 2003

FGSG_06385	GPMK1	AAL73403	MAPK	Loss of pathogenicity	Wheat	Urban et al., 2003
				Loss of pathogenicity	Arabidopsis thaliana	Urban et al., 2003
				Loss of pathogenicity	Tomato	Urban et al., 2003
FGSG_03964	GRS1	XP_384140.1	Polyketide synthase	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005
FGSG_09988	GPA3	XP_390164.1	G protein alpha subunit	Unaffected pathogenicity	Barley	Yu et al., 2008
	HIS7	Not available	Glutamine aminotransferase	Loss of pathogenicity	Barley	Seo et al., 2007
FGSG_09197	HMR1	XP_389373.1	HMG-CoA Reductase	Reduced virulence	Wheat	Seong et al., 2006
FGSG_09612	HOG1	XP_389788.1	MAPK	Reduced virulence	Wheat	Ramamoorthy et al., 2007a
				Unaffected pathogenicity	Tomato	Ramamoorthy et al., 2007a
	LIP1	EAA67628	Secreted lipase	Unaffected pathogenicity	Wheat	Feng et al., 2005
				Unaffected pathogenicity	Barley	Feng et al., 2005
FGSG_06680	Mes1	XP_386856.1	Homologue of <i>Aspergillus nidulans</i> MesA	Reduced virulence	Wheat	Rittenour and Harris, 2008
FGSG_05658	metE	EAA75229	Homoserine O-acetyltransferase	Reduced virulence	Wheat	Han et al., 2004
FGSG_10313	MGV1	AAM13670	MAPK	Loss of pathogenicity	Wheat	Hou et al., 2002
				Loss of pathogenicity	Tomato	Hou et al., 2002

FGSG_10825	MSY1	EAA75179	Methionine synthase	Reduced virulence	Maize	Seong et al., 2005
				Reduced virulence	Wheat	Seong et al., 2005
FGSG_05593	MT2	XP_385769.1	Sphingolipid C-9 methyltransferase	Reduced virulence	Wheat	Ramamoorthy et al., 2009
				Reduced virulence	Arabidopsis thaliana	Ramamoorthy et al., 2009
				Reduced virulence	Tomato	Ramamoorthy et al., 2009
				Reduced virulence	Maize	Ramamoorthy et al., 2009
FGSG_00376	NOS1	XP_380552.1	Ubiquinone oxidoreductase	Reduced virulence	Wheat	Seong et al., 2005
				Reduced virulence	Maize	Seong et al., 2005
FGSG_03747	NPS6	XP_383923.1	Non-ribosomal peptide synthetase	Reduced virulence	Wheat	Oide et al., 2006
FGSG_07118	OS1	XM_387294	Histidine kinase	Not known		Ochiai et al., 2007
FGSG_00408	OS4	XM_380584	MAP KKK	Not known		Ochiai et al., 2007
FGSG_08691	OS5	XM_388867	MAP K	Not known		Ochiai et al., 2007
FGSG_09182	PGL1	XP_389358.1	Black perithecial pigment synthesis	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005
FGSG_10548	PKS1	AAS57885	Polyketide synthase	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005
FGSG_01790	PKS11	AAS57295	Polyketide synthase	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005

<i>FGSG_12040</i>	<i>PKS12</i>	Not available	Polyketide synthase	Not known		Kim et al., 2007
<i>FGSG_02395</i>	<i>PKS13 (ZEA2)</i>	ABB90282	Zearalenone synthesis	Unaffected pathogenicity	Barley	Gaffoor et al., 2005; Kim, YT et al., 2005
<i>FGSG_03340</i>	<i>PKS17</i>	XP_383516.1	Polyketide synthase	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005
<i>FGSG_04694</i>	<i>PKS2</i>	AAS57287	Polyketide synthase	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005
<i>FGSG_12126</i>	<i>PKS4 (ZEA1)</i>	ABB90283	Zearalenone synthesis	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005; Lysoe et al., 2006
<i>FGSG_05794</i>	<i>PKS5</i>	AAS57290	Polyketide synthase	Unaffected pathogenicity	Barley	Gaffoor et al., 2005; Lysoe et al., 2006
<i>FGSG_08208</i>	<i>PKS6</i>	AAS57291	Polyketide synthase	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005
<i>FGSG_08795</i>	<i>PKS7</i>	AAS57292	Polyketide synthase	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005
<i>FGSG_10464</i>	<i>PKS9</i>	AAS57293	Polyketide synthase	Unaffected pathogenicity	Wheat	Gaffoor et al., 2005
<i>FGSG_08695</i>	<i>PLS1</i>	XP_388871.1	Tetraspanin	Unaffected pathogenicity	Wheat	Rittenour and Harris, 2008
<i>FGSG_05061</i>	<i>PPG1</i>	XP_385237.1	Pheromone precursor	Not known		Kim et al., 2008

<i>FGSG_02655</i>	<i>PRE2</i>	XP_382831.1	Peromone receptor	Not known		Lee et al., 2008
<i>FGSG_04111</i>	<i>PTC1</i>	XP_384287.1	Type 2C protein phosphatase	Reduced virulence Not known, appears to be essential	Wheat	Jiang et al., 2010
<i>FGSG_09778</i>	<i>RAS1</i>	XP_389954.1	Ras GTPase			Bluhm et al., 2007
<i>FGSG_10114</i>	<i>RAS2</i>	XP_390290.1	Ras GTPase	Reduced virulence	Maize	Bluhm et al., 2007
				Reduced virulence	Wheat	Bluhm et al., 2007
<i>FGSG_05371</i>	<i>SID1</i>	XM_385547	Siderophore biosynthetic gene	Reduced virulence	Wheat	Greenshields et al., 2007
<i>FGSG_05484</i>	<i>STE11</i>	XP_385660.1	MAPKKK	Loss of pathogenicity	Wheat	Ramamoorthy et al., 2007a
				Loss of pathogenicity	Tomato	Ramamoorthy et al., 2007a
<i>FGSG_09903</i>	<i>STE7</i>	XP_390079.1	MAPKK	Loss of pathogenicity	Wheat	Ramamoorthy et al., 2007a
				Loss of pathogenicity	Tomato	Ramamoorthy et al., 2007a
<i>FGSG_00950</i>	<i>SYN1</i>	XP_381126.1	Syntaxin-like SNARE	Reduced virulence	Barley	Hong et al., 2010
<i>FGSG_09928</i>	<i>SYN2</i>	XP_390104.1	Syntaxin-like SNARE	Reduced virulence	Barley	Hong et al., 2010
<i>FGSG_00332</i>	<i>TBL1</i>	EAA69638	Transducin beta-subunit	Reduced virulence	Maize	Seong et al., 2005
				Reduced virulence	Wheat	Seong et al., 2005
<i>FGSG_06874</i>	<i>TOP1</i>	XP_387050.1	Topoisomerase	Reduced virulence	Wheat	Baldwin et al.,

						2010
	<i>TRI10</i>	AF365969	Transcription factor	Reduced virulence	Wheat	Seong et al., 2009
<i>FGSG_03543</i>	<i>TRI14</i>	EAA72509	DON toxin biosynthesis	Reduced virulence	Wheat	Dyer et al., 2005
<i>FGSG_03537</i>	<i>TRI5</i>	AAM90953	Trichodiene synthase	Unaffected pathogenicity	Oat	Proctor et al., 1995b
				Reduced virulence	Rye	Proctor et al., 1995b
				Unaffected pathogenicity	Maize	Proctor et al., 1995b
				Reduced virulence	Wheat	Proctor et al., 1995b
				Unaffected pathogenicity	Arabidopsis thaliana	Cuzick et al., 2008
<i>FGSG_03536</i>	<i>TRI6</i>	BAA83722	Transcription factor	Reduced virulence	Wheat	Seong et al., 2009
	<i>ZEB1</i>	ABB90284	Zearalenone synthesis	Unaffected pathogenicity	Barley	Kim, YT et al., 2005
<i>FGSG_02398</i>	<i>ZEB2</i>	EAA67238	Zearalenone synthesis	Unaffected pathogenicity	Barley	Kim, YT et al., 2005
<i>FGSG_01555</i>	<i>ZIF1</i>	EAA68510	b-ZIP transcription factor	Reduced virulence	Maize	Seong et al., 2005
				Reduced virulence	Wheat	Seong et al., 2005
<i>FGSG_00007</i>		XP_380183.1	Unknown Major Facilitator Superfamily	Increased virulence	Wheat	Gardiner et al., 2009
<i>FGSG_00416</i>		XP_380592.1	Transporter	Reduced virulence	Wheat	Dufresne et al., 2008

<i>FGSG_01974</i>	XP_382150.1	Similar to HET-C2 glycolipid transfer protein	Reduced virulence	Wheat	Dufresne et al., 2008
<i>FGSG_02077</i>	XP_382253.1	Unknown	Reduced virulence	Wheat	Dufresne et al., 2008
<i>FGSG_02549</i>	XP_382725.1	Phosphoglycerate mutase family	Reduced virulence	Wheat	Dufresne et al., 2008
<i>FGSG_04510</i>	XP_384686.1	Unknown	Unaffected pathogenicity	Wheat	Dufresne et al., 2008
<i>FGSG_04610</i>	XP_384786.1	Similar to maltose permease	Unaffected pathogenicity	Wheat	Dufresne et al., 2008
<i>FGSG_07062</i>	XP_387238.1	Unknown	Unaffected pathogenicity	Wheat	Dufresne et al., 2008
<i>FGSG_08737</i>	XP_388913.1	Unknown	Unaffected pathogenicity	Wheat	Dufresne et al., 2008
<i>FGSG_09759</i>	XP_389935.1	Cation efflux family	Unaffected pathogenicity	Wheat	Dufresne et al., 2008
<i>FGSG_10057</i>	XP_390233.1	Unknown	Reduced virulence	Wheat	Dufresne et al., 2008
<i>FGSG_10397</i>	XP_390573.1	Unknown	Increased virulence	Wheat	Gardiner et al., 2009
<i>FGSG_12019</i>	Not available	Unknown	Reduced virulence	Wheat	Dufresne et al., 2008
<i>FGSG_12753</i>	Not available	Unknown	Reduced virulence	Wheat	Dufresne et al., 2008

1.4.7 Resistance to *Fusarium* Infection

The resistance of wheat to infection by *Fusarium* species was last reviewed by Snijders (2004). Resistance in wheat is at best incomplete and varies between genotype, with no complete resistance having been discovered. Often, the most resistant genotypes possess the worst agronomic characteristics. For example, the highly resistant genotype Sumai 3 (origin China) lacks resistance to most other diseases and also has poor grain quality traits (Snijders, 2004). As such, both transgenic and marker-assisted breeding approaches aiming to increase resistance are under investigation.

Resistance to FEB can be regarded as comprising of two components. Type I resistance against initial penetration and type II resistance against spread of the pathogen within the host (Schroeder and Christensen, 1963). Genotypes of wheat that exhibit resistance show a slower development and spread of disease symptoms (Snijders and Perkowski, 1990; Ribichich *et al.*, 2000). Resistance mechanisms affecting kernel/grain DON content via degradation or tolerance may also be present (Miller *et al.*, 1985; Mesterhazy *et al.*, 2002). Resistance to FEB does not show specificity to either *F. graminearum* or *F. culmorum* (Snijders, 2004).

In wheat, *Fusarium* resistance is a quantitative trait with relatively high heritability and is controlled by a few genes with major effect (Snijders, 1990; Singh *et al.*, 1995; Van Ginkel *et al.*, 1996). Identification of quantitative trait loci (QTLs) for resistance to FEB is a main focus of the international resistance research community, with the possibility of accumulating such loci in wheat lines to increase resistance. QTLs may in some cases be coincident for genes controlling morphology (e.g. lax ears and a lack of awns tend to show lower disease occurrence: Mesterhazy, 1987; Snijders, 2004). The Sumai 3 genotype, which originated as a transgressive segregant from a cross involving two parents with only moderate resistance to FEB, is the best-studied source of resistance. Breeding programmes rely heavily on this resistance source, and recently released genotypes with improved resistance such as Pioneer 25R18 in the USA and Wonder in Canada are two such examples (Snijders, 2004). The recent detailed study of the infection of the entire ear of a susceptible

genotype has revealed an extensive symptomless phase to the infection process (Brown *et al.*, 2010). It is currently not known whether resistance genotypes prevent or permit this symptomless phase of infection.

It has recently emerged that a QTL affecting resistance to FEB appears to co-localise with the green revolution semi-dwarfing *Rht-D1* locus. In the resistant winter wheat cultivar Arina, it is thought that the linkage of other genes that confer increased susceptibility to the *Rht-D1b* (*Rht2*) locus is responsible, rather than resistance variation due to height (Draeger *et al.*, 2007). In a mapping population from a cross between the resistant cultivar Spark and the susceptible cultivar Rialto, lines possessing the *Rht-D1b* allele showed reduced type I resistance but were unaffected in type II resistance (Srinivasachary *et al.*, 2008). Again, the variation in resistance appeared not to be due to height differences per se. A later study further supported the role of the *Rht-D1b* allele in FEB susceptibility in European winter wheat (Holzapfel *et al.*, 2008). Recently, it has been found that both the *Rht-D1b* and the second semi-dwarfing *Rht-B1b* allele also used extensively in global wheat breeding affect FEB resistance but in different manners. An investigation using the resistant variety Soissons showed a major FEB QTL linked to the *Rht-D1* locus but no effect of the *Rht-B1b* allele on FEB resistance. However, using Mercia and Maris Huntsman, containing both both *Rht-D1b* and *Rht-B1b*, reduced type I resistance under high disease pressure. *Rht-D1b* did not affect type II resistance but *Rht-B1b* actually increased type II resistance (Srinivasachary *et al.*, (2009).

1.4.8 Infection Post-Harvest

Production of mycotoxins during infection by *Fusarium* leads to contaminated grain which poses a serious health risk to the consumer. As well as aiming to reduce crop infection by *Fusarium*, control of grain contamination after harvest is also important in improving grain quality and safety.

A large number of factors, both biotic and abiotic, affect the quality of grain after harvest. Such factors include grain and mould respiration, water availability, temperature and insect and fungal presence, and can influence contaminant

toxigenic fungi such as *F. graminearum* and *F. culmorum* and their production of mycotoxins (Magan *et al.*, 2003; Aldred and Magan, 2004; Schrodter *et al.*, 2004).

The pre-harvest condition of the grain is important in determining its quality and contamination level post-harvest (Schrodter *et al.*, 2004). Factors affecting the pre-harvest level of infection by *Fusarium* include climate conditions, crop cultivar, cutting height at harvest, the use of crop rotation, ploughing methods and fertiliser and fungicide use (Aldred and Magan, 2004; Schrodter *et al.*, 2004). Due to the lack of fully resistant wheat cultivars, a combination of fungicide use, partially resistant cultivars and specific farming practices (such as deep ploughing to remove surface contamination and crop rotation to break the infection cycle) have to be employed to try and reduce infection (Mesterhazy, 2002; Aldred and Magan, 2004).

At the post-harvest stage, maintaining grain at a low moisture content ($a_w \leq 0.65-0.70$) should prevent fungal spoilage (Magan *et al.*, 2003; Aldred and Magan, 2004; Schrodter *et al.*, 2004). As such, immediate drying after harvest should be implemented (Schrodter *et al.*, 2004). It is thought that the chemical stability of trichothecenes is such that no significant decrease will occur during a drying operation itself (Schrodter *et al.*, 2004). Mechanical damage to grain and an incorrect storage temperature (toxin production is much higher at 25°C than 15°C) can also aid fungal infection (Magan *et al.*, 2003; Schrodter *et al.*, 2004).

Competition with other fungal and bacterial species will also influence contamination by fungi and toxin production. Magan and Lacey (1984) developed an Index of Dominance to assist in analysis of fungal communities in grain, which was found to vary depending on temperature and water activity (a_w). *F. culmorum* competed well with storage moulds at $a_w > 0.93-0.95$. Upon testing the interaction of this species with others, it was found that *F. graminearum* was more competitive in all water activity and temperature conditions, suggesting why these two species are such important pathogens in post-harvest cereal grain (Magan *et al.*, 2003). More recently, Niche Overlap Indices calculated from carbon source utilisation patterns have been used to assess fungal competition in such environments, and have shown dynamic behaviour and dependence on temperature, water availability and nutrient

status (Magan *et al.*, 2003). Fungal species such as *Microdochium nivale* have been found to enhance *F. culmorum* growth and DON production on grain, but at lower water activity, other species, such as *Penicillium verrucosum* can reduce DON production, while enhancing growth. Insects can also encourage the spread of fungi (Aldred and Magan, 2004). Due to the physiological and biochemical similarities between fungi and early insect development stages, combined insecticidal/fungicidal control may be possible (Magan *et al.*, 2003).

Good post-harvest management, such as maintenance of suitable low moisture conditions, detection and separation of contaminated from healthy grain and minimisation of pre-drying storage time will therefore help reduce mycotoxin contamination of grain (Aldred and Magan, 2004).

1.4.9 Combating Fusarium Ear Blight (FEB)

A range of techniques have been employed in attempts to combat FEB and reduce disease occurrence, mycotoxin accumulation and symptom severity. As yet, a complete cure or preventative measure for FEB has not been found. As described earlier, resistance in wheat is at best partial, often occurring in cultivars lacking traits favourable for commercial use (Snijders, 2004), such as high yield and tolerance of poor weather conditions.

Other potential means to protect crops against FEB include the introduction of genes from heterologous organisms or over-expression of specific host genes. For example, the *TRI101* trichothecene 3-acetyltransferase gene from *F. sporotrichioides* has been introduced to wheat plants where it provides partial resistance to *F. graminearum* spread in inoculated spikelets (Okubara *et al.*, 2002), but as yet provided no overall resistance in field trials. A similar result has been noted in tobacco, where the *TRI101* gene product increases plant tolerance to the trichothecene diacetoxyscirpenol (DAS) in a seed germination assay (Muhitch *et al.*, 2000). In rice, *TRI101* expression prevented the inhibitory effects of DON on root growth, however, the activity of the transgene was only assayed in leaves rather than the roots themselves (Ohsato *et al.*, 2006).

A series of pathogenesis-related (PR) genes isolated from a cDNA library of *F. graminearum*-infected resistant wheat cv. Sumai-3 have been introduced into susceptible wheat cv. Bobwhite in various combinations. By producing wheat expressing a rice thaumatin-like protein and a specific combination of a wheat chitinase and a wheat glucanase, partial resistance to FEB, indicated by a lower average number of infected spikelets, was achieved in greenhouse conditions. However, such resistance was not evident in field trials with heavy inoculum loads (Chen *et al.*, 1999; Anand *et al.*, 2003). A study using the over-expression of wheat defence-response genes alpha-1-purothionin, thaumatin-like protein 1 and beta-1, 3-glucanase has also reported the production of lines exhibiting reduced ear blight severity and DON contamination (Mackintosh *et al.*, 2007). Transgenic wheat cv. Bobwhite expressing the *Arabidopsis thaliana* *AtNPR1* gene, which regulates the activation of systemic acquired resistance, shows increased resistance to FEB caused by *F. graminearum*, associated with a faster defence response to infection with rapid, high-level expression of *PR1* (Makandar *et al.*, 2006). However, under field conditions, resistance was not evident (Shah, pers comm.). Introduction of a barley chitinase into wheat also showed limited success against *F. graminearum* infection (Shin *et al.*, 2008). The identification of a DON UDP-glycosyltransferase from *Arabidopsis thaliana* that is able to detoxify DON and 15-ADON may provide another route to follow, although no protective activity was noted against NIV (Poppenberger *et al.*, 2003). In *Arabidopsis* itself, over-expression of the Golden2-like (*GLK1*) transcription factor increased the expression of a series of defence-related proteins and increased resistance to *F. graminearum* despite a noted decrease in the expression of the defence gene *PR-1* (Savitch *et al.*, 2007). In maize, a transgenic line possessing a zearalenone-degrading enzyme has been reported to reduce levels of the toxin in infected kernels (Igawa *et al.*, 2007).

Compounds or proteins antagonistic to FEB have been obtained from a number of species. Alfalfa (*Medicago sativa*) seed defensin MsDef1 inhibits the *in vitro* growth of *F. graminearum*, disrupting hyphal elongation and leading to a hyper-branching phenotype (Spelbrink *et al.*, 2004). This activity requires the Arg 38 residue and is diminished by Ca^{2+} presence. The corn defensin PDC1 has also be reported to show antifungal activity towards *F. graminearum* (Kant *et al.*, 2009). A *Trichoderma* isolate producing 6-pentyl-alpha-pyrone (6PAP) reduced

DON production by *F. graminearum* when grown in a competition assay system, as could *F. subglutinans*, but some strains of this latter species are also toxin producers and so would not represent suitable combat agents (Cooney *et al.*, 2001).

Plant extracts and essential oils have been investigated in the search for means to reduce FEB incidence. Extracts of medicinal plants in ethyl acetate or methanol are claimed to exhibit growth-inhibiting properties towards *F. culmorum*, such as ethyl acetate extracts of *Cineraria grandiflora*, *Coccinia adoensis* and *Pavonis urens*, and methanol extracts of *Pavonia urens* and *Marattia fraxinea* (de Boer *et al.*, 2005). A series of essential oils were also claimed to inhibit *F. graminearum* growth and DON production, depending on environmental conditions and *F. graminearum* strain used (Velluti *et al.*, 2004). The isoprenoid farnesol, interestingly, has also been reported to exhibit antifungal effects against *F. graminearum* (Semighini *et al.*, 2008).

Biological control methods using bacteria have also been examined for the possibility of reducing FEB symptoms and mycotoxin accumulation. Several *Pseudomonas* species have been claimed to reduce the severity of FEB symptoms caused by *F. culmorum*, reducing expression of the fungal trichodiene synthase (*TRI5*) gene that is required for mycotoxin synthesis, and possibly aiding plant resistance induction (Khan *et al.*, 2006). Other bacteria that may antagonise FEB include *Cryptococcus* and *Bacillus* species for FEB caused by *F. graminearum* (Khan *et al.*, 2001, 2004) and *Pseudomonas* and *Bacillus* species for FEB caused by *F. culmorum* (Czaban *et al.*, 2004).

1.4.10 Fungal Pathogen Signalling

The number of sequenced fungal pathogens available for study is increasing at an ever faster rate as sequencing technology progresses and next-generation procedures are adopted. This is allowing rapid progress in the comparison of conserved biological processes between species. Such processes include core signalling pathways common to many species and which in many cases are integral in disease development.

In order to convey changes in the environment to transcriptional, behavioural and morphological alterations that allow the fungus to adapt to such changes, a signal is required to pass from the surface of the organism to the internal machinery. Animal and plant pathogenic fungi require such signalling pathways to control virulence and response to the host. Some signalling pathways appear to be widespread and show a high degree of conservation between plant- and animal-pathogenic fungi, for example, MAP kinase and cAMP signalling cascades.

MAP kinase signalling in fungal pathogens shows homology to MAP kinase cascades identified in the yeast *Saccharomyces cerevisiae* (**Figure 1.5**). Homologues of individual *S. cerevisiae* kinases and sometimes entire kinase cascades have been identified in both plant- and animal-pathogenic species. In *S. cerevisiae*, MAP kinase pathways containing the PAK kinase (Ste20)-MAPKKK (Ste11)-MAPKK (Ste7)-MAPK (Fus3/Kss1) cascade regulate pheromone response and filamentous growth. In the case of pheromone response, an upstream G-protein coupled receptor (GPCR) and heterotrimeric G-protein system is used, but in the case of filamentous growth, activation of the pathway is by Ras2, Cdc42 and 14-3-3 proteins (Mosch *et al.*, 1996; Roberts *et al.*, 1997; Xu, 2000). Other MAP kinase pathways include those regulating cell integrity (Bck1-Mkk1/2-Slt2 pathway) and high osmolarity response (Ssk2/Ssk22/Ste11-Pbs2- Hog1 pathway) (Xu, 2000, Hohmann 2002, Levin 2005). Some components of the pathways may act in more than one cascade (Hohmann 2002).

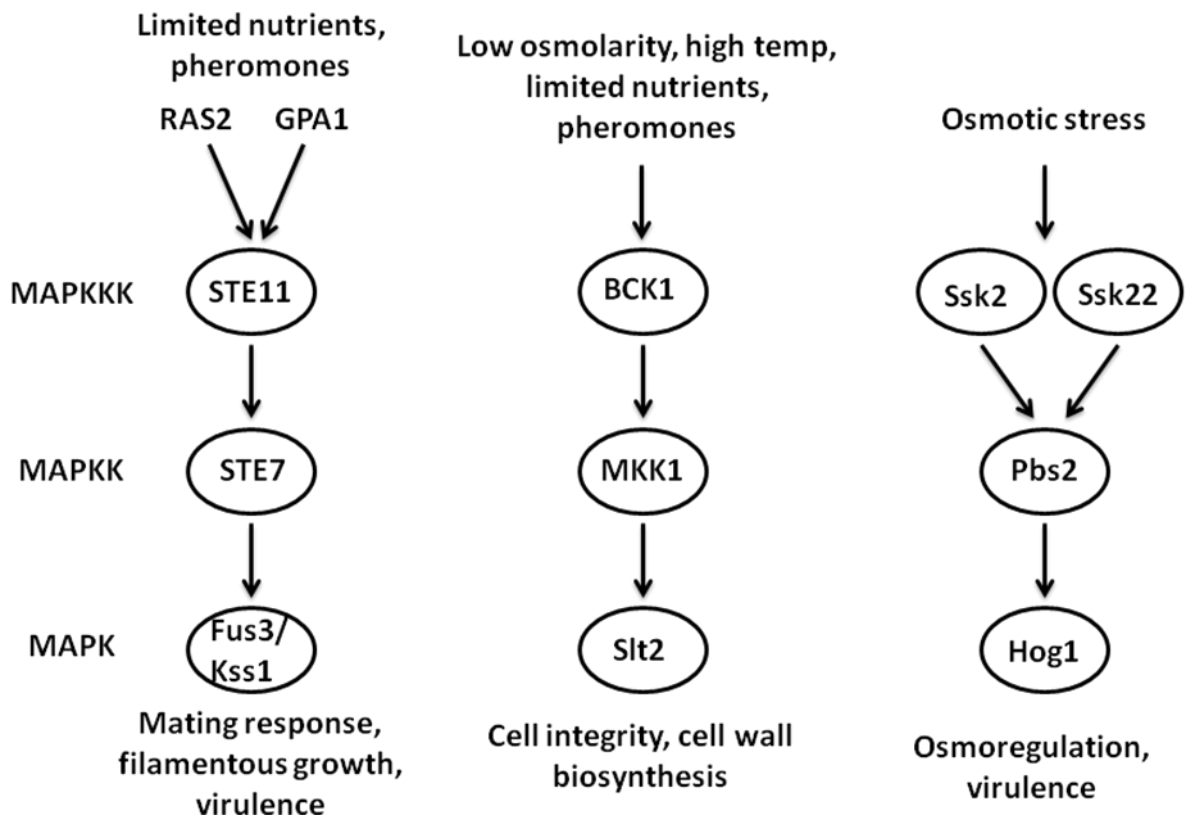


Figure 1.5. The core MAP kinase signalling pathways of fungi. Names shown are for the *S. cerevisiae* proteins but corresponding pathways are found in *M. oryzae*, *C. albicans* and other fungal species. These pathways regulate a variety of processes in response to a range of stimuli (as shown).

The core signalling cascades of animal and plant pathogenic fungi are highly homologous to those of *S. cerevisiae* and outlined here using the examples of the animal pathogen *Candida albicans* and plant pathogen *Magnaporthe oryzae*. The causal agent of Candidiasis, *C. albicans* possesses a signalling pathway homologous to the *S. cerevisiae* pheromone response pathway with the CST20 PAK kinase and HST7-CEK1 MAPK cascade, but in this species the pathway instead regulates hyphal transition and pathogenicity (Kohler and Fink, 1996; Leberer *et al.*, 1997; Csank *et al.*, 1998). Similarly, the rice blast fungus *M. oryzae* possesses the homologous MST11-MST7-PMK1 MAPK cascade which regulates pathogenicity and may interact with upstream components Ras1, Ras2 and Cdc42 via the Mst50 adaptor protein (Xu and Hamer, 1996; Zhao *et al.*, 2005; Park *et al.*, 2006). Homologues of both *Slit2* (*MKC1* in *C. albicans* and *MPS1* in *M. oryzae*) and *Hog1* (*CaHOG1* in *C. albicans* and *OSM1* in *M. oryzae*) have also been identified in both animal and plant pathogenic fungi and regulate a number of processes such as cell wall biogenesis,

virulence and osmoregulation (Xu, 2000; Idnurm *et al.*, 2005). In *M. oryzae*, *OSM1* regulates turgor during hyperosmotic stress but not virulence (Dixon *et al.*, 1999), while *MPS1* is involved in host penetration (Xu *et al.*, 1998). In *C. albicans*, *HOG1* is essential for virulence (Alonso-Monge *et al.*, 1999; Arana *et al.*, 2007) and deletion of this gene increases osmotic and oxidative stress sensitivity (Alonso-Monge *et al.*, 1999, 2003; Smith *et al.*, 2004), while *MKC1* is involved in cell integrity and cell wall formation (Navarro-Garcia *et al.*, 1995, 1998), stress response (Navarro-Garcia *et al.*, 2005) and virulence (Diez-Orejas *et al.*, 1997).

Other components of these MAPK signalling cascades have also been investigated in *M. oryzae* and *C. albicans*, for example, *MCK1* of *M. oryzae*, homologous to the *S. cerevisiae* MAPKKK *BCK1* of the Slt2 pathway regulates cell integrity and pathogenicity in this species (Jeon *et al.*, 2008), while further elements of the *Hog1* pathway homologous to those in yeast have been identified in *C. albicans*, including the two-component kinases *YPD1* and *SSK1*, MAPKKK *SSK2* and MAPKK *PBS2* (Calera and Calderone, 1999; Calera *et al.*, 2000; Arana *et al.*, 2005; Cheetham *et al.*, 2007).

cAMP-dependent signalling is frequently initiated by receptors, such as G-protein-coupled receptors, (GPCRs) which serve to activate G proteins, that in turn activate adenylate cyclase. Adenylate cyclase catalyses the formation of cAMP, activating cAMP-dependent protein kinase (protein kinase A, PKA), which proceeds to phosphorylate a range of target proteins. *M. oryzae* possesses three G α subunits, MagA, MagB and MagC (Liu and Dean, 1997), two G β , Mgb1 and Mgb2 (Nishimura *et al.*, 2003) and one G γ subunit (Dean *et al.*, 2005). Disruption of *MGB1* reduces conidiation and causes defective appressorium formation and consequent loss of pathogenicity (Nishimura *et al.*, 2003). Deletion of *MagA* or *MagC* does not affect growth, appressorium formation or virulence, although deletion of *MagC* reduces conidiation. However, disruption of *MagB* significantly reduces growth, conidiation, appressorium formation and virulence. In addition, *magB* mutants do not form perithecia, while both *magA* and *magC* mutants are unable to produce mature asci (Liu and Dean, 1997). MoRic8 interacts with MagB and appears to regulate cAMP-dependent signalling in pathogenicity and cell differentiation in

sporulation, sexual development and plant infection in *M. oryzae* (Li *et al.*, 2010). Deletion of the adenylate cyclase gene *Mac1* reduces growth, conidiation, and conidial germination of *M. oryzae* and renders the fungus unable to form perithecia or appressoria, nor to penetrate host leaves. The application of exogenous cAMP derivatives is able to restore appressorium formation (Choi and Dean, 1997). In *M. oryzae* deletion of the catalytic subunit of PKA, *CPKA*, causes delayed appressoria formation and reduced virulence (Mitchell and Dean, 1995, Xu *et al.*, 1997). The *cpkA* mutant forms smaller appressoria that are defective in penetrating plant cells, but inoculation of wounds still allows lesion formation indicating that post-penetration disease development may be unaffected (Xu *et al.*, 1997, 1996).

C. albicans possesses two G α , one G β and one G γ subunit. The G α subunits appear to be involved in the response to pheromone signals and in mating (Sadhu *et al.*, 1992; Bennett and Johnson, 2006; Dignard *et al.*, 2008). The adenylate cyclase *CDC35* is also required for wild-type growth rates, the yeast-hyphal transition and pathogenicity in *C. albicans*. cAMP is able to restore the yeast-hyphal transition in *cdc35* mutants. *CDC35* may act downstream of *Ras1* and upstream of MAP kinase pathway components (Rocha *et al.*, 2001). The small G proteins *Ras1* and *Ras2* also appear to regulate cAMP-dependent signalling in this species. Deletion of *Ras2* alone does not affect hyphal growth but loss of this gene in a *ras1* strain increases the growth defect of this strain. Deletion of *Ras2* also restores the decrease in cellular cAMP found in the *ras1* strain. *Ras1* deletion increases resistance to H₂O₂ and sensitivity to Co²⁺, however, *ras2* strains have opposing phenotypes (Zhu *et al.*, 2009). *Ras1* is also required for the yeast-hyphal transition in *C. albicans* and consequently affects virulence (Feng *et al.*, 1999; Leberer *et al.*, 2001). Deletion of the *TPK2* gene, encoding a PKA catalytic subunit, results in reduced virulence (Sonneborn *et al.*, 2000) while deletion of the PKA regulatory subunit-encoding gene appears to be lethal (Cassola *et al.*, 2004). Disruption of *TPK2* or a second catalytic subunit, *TPK1*, causes defects in hyphal morphogenesis, although under different conditions for each of the two genes (Bockmuhl *et al.*, 2001).

The MAPK and cAMP signalling pathways also appear to be linked to each other in many species. Examples include *S. cerevisiae*, where Ras2 activates both types of pathway (Mösch *et al.*, 1996; Lorenz and Heitman, 1997; Mösch and Fink, 1997; Mösch *et al.*, 1999) the two pathways converging to regulate the *FLO11* gene (Pan and Heitman, 1999; Rupp *et al.*, 1999), and cAMP inhibits the expression of MAPK-regulated reporter genes (Lorenz and Heitman, 1997). It has recently been found that activation of MKC1 of *C. albicans* is controlled by both protein kinase C (PKC1) (which is placed upstream in the same pathway) and HOG1, providing a link between two different MAPK signalling pathways (Navarro-Garcia *et al.*, 2005). Furthermore, both pathways are activated under conditions of oxidative stress (Navarro-Garcia *et al.*, 2005). In addition, Ras activates both MAPK and cAMP signalling for regulation of filamentation and virulence in this species (Leberer *et al.*, 2001). In *M. oryzae*, the G β subunit, MGB1, is suggested to be linked to cAMP signalling and may lie upstream of the Pmk1 MAPK in regulating appressorium formation (Nishimura *et al.*, 2003).

Upstream regulation of signalling pathways is complex, sometimes involving different subunits of heterotrimeric G proteins to activate different pathways in the same organism. For example, in *C. neoformans*, the heterotrimeric G protein β subunit GPB1 activates MAPK signalling in mating and haploid differentiation (Alspaugh *et al.*, 1997, D'Souza *et al.*, 2001; Wang *et al.*, 2001; Alspaugh *et al.*, 2002), while the G α GPA1 activates cAMP-dependent regulation of virulence (Alspaugh *et al.*, 1997). Heterotrimeric G-protein α subunits have been studied in many plant pathogenic fungi, examples including *M. oryzae* MagB, *F. oxysporum* Fga1 and *Stagonospora nodorum* Gna1, disruption of all of which reduces pathogenicity (Liu and Dean, 1997; Jain *et al.*, 2002; Solomon *et al.*, 2004). The Mst50 adaptor protein of the PMK1 cascade in *M. oryzae* may be regulated by multiple upstream proteins including Ras1, Ras2, Cdc42 and Mgb1 (Park *et al.*, 2006).

In some fungi, a two-component signalling system has been identified, which involves an autophosphorylating membrane-bound sensor histidine kinase and a response regulator for phosphotransfer. For example, in the *S. cerevisiae* Hog1 MAPK pathway, a complex four-step phosphotransfer system is situated upstream of the MAPK cascade, comprising the sensor hybrid-histidine kinase

Sln1 (containing both a conserved His and Asp residue) (Ota and Varshavsky, 1993; Posas *et al.*, 1996), intermediate His kinase Ypd1, and response regulators Ssk1 (for osmoadaptation) and Skn7 (for oxidant adaptation and cell wall biosynthesis) ((Li *et al.*, 1998, 2002). A Sln1 homologue has been partially characterised in *C. albicans* (Nagahashi *et al.*, 1998; Yamada-Okabe *et al.*, 1999). Other *C. albicans* histidine kinases include Nik1 (Alex *et al.*, 1998; Selitrennikoff *et al.*, 2001), and Chk1 (Calera *et al.*, 1998; Calera and Calderone, 1999b), which appear to be cytoplasmic. A His kinase, Fos1, has also been identified in *Aspergillus fumigatus* (Pott *et al.*, 2000). A response regulator homologue and histidine kinase have recently been characterised in *F. graminearum*, (Goswami *et al.*, 2006; Ochiai *et al.*, 2007).

In addition to the two highly conserved signalling pathways above, signalling involving lipid molecules has also recently been shown to be important in animal pathogenic fungi development. For example, in *C. neoformans*, diacylglycerol derived from sphingolipids induces expression of App1 (Mare *et al.*, 2005), a virulence factor inhibiting phagocytosis by macrophages, and also activates protein kinase C, whereby cell wall stability is increased (Heung *et al.*, 2004, 2005). The oxylipin quorum-sensing molecule farnesol regulates genes involved in yeast-hyphal transition and biofilm formation in *C. albicans*, although the exact mechanism used is unknown (Hornby *et al.*, 2001; Oh *et al.*, 2001; Cao *et al.*, 2005; Mosel *et al.*, 2005; Navarathana *et al.*, 2005). However, the very same molecule has been found to induce cell death in *A. nidulans* (Semighini *et al.*, 2006). Secreted eicosanoids may also have a role in signalling in such fungi (Noverr *et al.*, 2001).

1.4.11 Signalling in *F. graminearum*

Three G α (*GPA1-3*) and one G β (*GPB1*) subunit have been identified and characterised in *F. graminearum* (Yu *et al.*, 2008). In addition, *F. graminearum* appears to possess a G γ subunit and adenylate cyclase in addition to catalytic and regulatory subunits of cAMP-dependent protein kinase (protein kinase A, PKA) (Yu *et al.*, 2008). Loss of *GPA2* and *GPB1* leads to a large reduction in virulence of *F. graminearum*. Deletion of any of the three *GPA* genes,

meanwhile, slightly reduces growth on minimal and complete media, but deletion of *GPB1* by comparison, reduces hyphal growth on these media types by 25% compared to wild type. *GPA1*, but not the three other G protein subunits investigated, is required for perithecia production, while *gpa2* deletion strains appeared to exhibit increased cell wall chitin content. *GPA1* and *GPB1* also appear to negatively regulate toxin production.

F. graminearum appears to possess two Ras GTPase genes (*RAS1* and *RAS2*) (Bluhm *et al.*, 2007). Deletion of *RAS2* led to a reduced growth rate on solid media, failure to produce perithecia, delayed spore germination and reduced virulence on wheat and maize. No difference in conidiation or DON production was noted between *ras2* and wild-type strains, however. A *RAS1* deletion strain was not obtained suggesting this gene may be essential for viability. Expression of *RAS1* and *RAS2* is increased by the disruption of *Fst7* or *Fst11*, (*STE7* and *STE11*) while *RAS2* expression is increased to a greater extent than that of *RAS1* in a *tbl1* (transducin β -subunit-like gene) or *CpkA* (catalytic subunit of cAMP-dependent protein kinase) mutant background. Intracellular cAMP levels were not affected by the loss of *RAS2*. This may suggest little or no role of this gene in cAMP signalling, however *RAS2* appears to be negatively regulated by *CpkA* (Bluhm *et al.*, 2007) and so if cAMP acts upstream of *RAS2* in cAMP signalling, then no effect on cAMP levels or of cAMP application would be expected for *ras2* strains. *RAS2* deletion did, however, reduce phosphorylation of *Gpmk1* and secretion of *Fgl1*. The data suggest a link of *RAS2* and MAPK signalling pathways in *F. graminearum* that may participate in the regulation of secreted enzymes important to virulence.

The MAPKKK *STE11*, MAPKK *STE7* and MAPK *Gpmk1* have been proposed to act as a MAP kinase cascade in *F. graminearum* based on their homology to the Fus3/Kss1 cascade of other fungal species and their similar sensitivity to the *Medicago sativa* seed defensins MsDef1 and MsDef4 (Ramamoorthy *et al.*, 2007). *STE11*, *STE7* and *Gpmk1* mutants show reduced virulence on tomato fruit, while the MAPK *MGV1* is required for pathogenicity (Urban *et al.*, 2003; Ramamoorthy *et al.*, 2007). On wheat ears, *ste11*, *ste7*, *gpmk1* and *mgv1* are severely reduced in virulence (Hou *et al.*, 2002; Jenczmionka *et al.*, 2003; Urban *et al.*, 2003; Ramamoorthy *et al.*, 2007). *MGV1* is required for perithecia

production but not for the production or normal germination of conidia, although formation of thick-walled cells within conidia was reduced in *mgv1* mutants and a small fraction of conidia fragmented after several days (Hou *et al.*, 2002). While in liquid media, growth of *mgv1* is unaffected, on solid media, however, growth is reduced compared to wild type. The *mgv1* mutant also appears to possess weakened cell walls, increased temperature sensitivity and a severe reduction in DON production (Hou *et al.*, 2002). The homologous gene in yeast (*S. cerevisiae*), to MG1 is the *Sl2* gene, which controls cell wall integrity (Xu, 2000).

Two independent studies have characterised the role of *Gpmk1* in two different strains of *F. graminearum*. In one study by Jenczmionka *et al.* (2003) of strain 8/1, it was noted that *Gpmk1* disruption does not affect growth on complete media, However, on minimal media, the production of aerial mycelium is drastically reduced (Jenczmionka *et al.*, 2003). Growth of *gpmk1* in liquid media, however, is comparable to wild type, with approximately the same biomass production (Jenczmionka *et al.*, 2003). A study of *Gpmk1* in strain 16A by Urban *et al.* (2003) found that disruption of this gene leads to a reduced growth rate on solid minimal, V8 and oatmeal media in addition to reduced aerial hyphae production (Urban *et al.*, 2003); *gpmk1* colonies are unable to produce perithecia (Jenczmionka *et al.*, 2003; Urban *et al.*, 2003). *Gpmk1* also appears to positively regulate the induction of DON production (Urban *et al.*, 2003) and of extracellular endoglucanolytic, proteolytic, xylanolytic and lipolytic activities (Jenczmionka and Schäfer, 2005). *Gpmk1* is also required for full virulence and DON production on Arabidopsis (Cuzick *et al.*, 2008a).

Another identified pathogenicity/virulence factor is the *FSR1* gene, which may encode a signalling scaffold protein, and is required for full *F. graminearum* virulence on barley and maize and perithecia production, while also contributing to *in vitro* growth rate on solid complete media (Shim *et al.*, 2006).

The Hog1 pathway regulates the response to osmotic and oxidative stress in fungi (Brewster *et al.*, 1993; Xu, 2000). Disruption of the *F. graminearum* *HOG1* MAP kinase gene prevented growth on 1M NaCl but did not affect growth on standard complete media (Ramamoorthy *et al.*, 2007). The *hog1* disruption

strain also showed reduced virulence on wheat ears, rarely spreading from the inoculated spikelets to adjacent spikelets (Ramamoorthy *et al.*, 2007).

Additional components of the *HOG1* pathway have also been studied in *F. graminearum* (Ochiai *et al.*, 2007a). The histidine kinase (*Os1*), MAP kinase kinase kinase (*Os4*), MAP kinase kinase (*Os5*) and *Hog1* MAP kinase (also termed *Os2*) genes were disrupted and the resulting effects on stress sensitivity and secondary metabolite production observed. All four *Os* mutants were more resistant to the fungicides iprodione and fludioxonil. *Os1* was also less sensitive to osmotic stress than the other *Os* mutants yet still more sensitive to this stress than the wild-type strain. Sensitivity of *Os1* to H₂O₂ and tBOOH (*t*-butyl hydroperoxide) was comparable to wild type and less than the other *Os* mutants. Sensitivity to diamide however, was less for *Os1* than wild type. *Os5* and *Os2* were comparable to wild type, while *Os4* has increased sensitivity to diamide compared to wild type. *Os1* showed reduced aurofusarin production but no change in trichothecene production while the other *Os* mutants showed increased aurofusarin production and reduced trichothecene production. The opposing effects of *Os1* and the other *Os* mutants on aurofusarin and trichothecene production are suggested to result from divergent downstream components that are linked to the same *Os1* phosphorelay system (Ochiai *et al.*, 2007a). NaCl reduces the production of trichothecenes (Ochiai *et al.*, 2007b). This effect must overcome the positive regulatory effect of *Os4*, *Os5* and *Os2* on trichothecene production (Ochiai *et al.*, 2007b). Again, additional downstream components may play a role.

1.4.12 The Role of Reactive Oxygen Species Production in Plant and Animal Pathogenesis

A role for reactive oxygen species (ROS) in eukaryotic signalling, encompassing both pathogenic fungi and their hosts is currently emerging. The exact nature of ROS use in pathogen-host interactions is often unclear but both organisms appear to be able to use such molecules in some of the roles of attack, defence or other signalling. ROS are well-suited for a signalling role, being small, diffusible and with rapid production and short lifetimes that allow

localised signalling. ROS have been implicated in cell proliferation, induction or inhibition, apoptosis activation and inhibition and necrosis induction at high concentrations (Gamaley *et al.*, 1999).

A main source of ROS is the enzyme NADPH oxidase. NADPH oxidases are plasma (or phagosome) membrane proteins that generate extracellular (or phagosomal) superoxide anions (Meier, 2001). Xanthine oxidoreductase and peroxidases are also thought to be important sources of ROS (Bolwell *et al.*, 1995; Harrison, 2000). Hydrogen peroxide, which possesses no charge and is therefore able to freely diffuse through the membrane to inside the cell, is formed from the external superoxide anions produced by NADPH oxidase complex. Hydrogen peroxide has been shown to regulate a large number of different genes (Allen and Tresini, 2000). MAP kinases may also be activated by ROS, so linking these two signalling types (Fialkow *et al.*, 1994; Guyton *et al.*, 1996; Desikan *et al.*, 1999; Kovtun *et al.*, 2000). Hydrogen peroxide may also be linked to signalling phosphatases, phospholipases, ion channels and other cellular components (Goldman *et al.*, 1992; Goldman and Zor, 1994; Sullivan *et al.*, 1994; Dai *et al.*, 1995; Taglialatela *et al.*, 1997; Wu *et al.*, 1998; Pei *et al.*, 2000).

NADPH oxidases are found in organisms that differentiate multicellular structures during their life cycle but are not present in unicellular organisms. NADPH oxidase homologues are widespread in filamentous fungi including *F. graminearum* (Lalucque and Silar, 2003). It is therefore suggested that NADPH oxidases play a role in differentiation during the formation of multicellular structures. Signalling of a cell's metabolic status is required to allow nutrient flow from nutrient-scavenging cells to specialised cells. NADPH oxidases may play such a role as, by using NADPH as a substrate, they are linked to the cell's metabolic activity and in addition they produce extracellular products that can diffuse between cells. Alternatively, NADPH oxidases could act as oxygen sensors that allow the direction of multicellular structure organisation via detection of an oxygen gradient (Blackstone, 2000; Lalucque and Silar, 2003).

NADPH oxidases are required for sexual reproduction and ascospore germination in *Podospora anserina*. *PaNox1* is required for the differentiation of

fruiting bodies and *PaNox2* for ascospore germination. *PaNox1* is thought to act upstream of a MAPK signalling system (Malagnac *et al.*, 2004). NoxA in *A. nidulans* represents a novel NADPH oxidase subfamily present in lower eukaryotes. It is induced during sexual development and required for differentiation of sexual fruiting bodies (cleistothecia). This signalling appears to be connected to MAP kinase signalling. Deletion of the gene does not affect hyphal growth or asexual development. The ubiquity of this class of NADPH oxidase in lower eukaryotes suggest similar roles may be performed by this enzyme in animal pathogenic *Aspergillus* species (*A. fumigatus* contains a single *noxA* gene) and perhaps in plant pathogenic fungi also (*F. graminearum* and *M. oryzae* both contain two *noxA* homologues) (Lara-Ortiz *et al.*, 2003). In *M. oryzae*, both the *Nox1* and *Nox2* NADPH oxidase genes are required for penetration of the host cuticle and so for pathogenicity, indicating a requirement for ROS production in disease establishment (Egan *et al.*, 2007). In the endophyte *Epichloë festucae*, NADPH oxidase is required for the maintenance of a symbiotic relationship with the host. Disruption of this gene leads to pathogenic behaviour of this species (Scott *et al.*, 2007).

In both cereal and non-cereal species of plants, ROS are important during defence – in activation of the highly localised hypersensitive response (HR), in cell wall thickening and defence gene expression (Bradley *et al.*, 1992; Levine *et al.*, 1994; Jabs *et al.*, 1997; Thordal-Christensen *et al.*, 1997). However, HR does not protect, for example, against the necrotrophic phytopathogen *Botrytis cinerea*. This species has been suggested to in fact trigger the hypersensitive response to aid its colonisation of the host (Govrin and Levine, 2000).

ROS scavengers may be important to invading pathogens for protection or successful attack. *B. cinerea* Cu-Zn superoxide dismutase (encoded by *bcSOD1*) is required for virulence on bean leaves. The role of this enzyme may be dismutation of superoxide produced by the host plant defence system or by the fungus itself, or indeed the production of hydrogen peroxide by *bcSOD1* may be important in pathogenesis, perhaps via activation of the hypersensitive response (Rolke *et al.*, 2004). In *F. graminearum*, meanwhile, accumulation of DON and 15-ADON has been shown to be modulated by oxidative stress *in*

vitro and was found to be increased following daily additions of hydrogen peroxide to the liquid cultures (Ponts *et al.*, 2006, 2007, 2009).

ROS also have a direct attacking role in many pathogenic fungi, which release toxins capable of producing ROS to damage host cells. Several plant pathogenic fungi genera produce photoactivated perylenequinone toxins (reviewed in Daub *et al.*, 2005), for example cercosporin produced by species of the plant pathogenic *Cercospora* genus, which absorbs light energy and generates ROS (Dobrowolski and Foote, 1983; Daub *et al.*, 2000). Another class of ROS-producing fungal toxins is the Epipolythiodioxopiperazine (ETP) toxins. The best known ETP is gliotoxin, an *Aspergillus fumigatus* compound which may be important in pathogenesis (Sutton *et al.*, 1994; Reeves *et al.*, 2004).

In comparing the signalling pathways employed by plant and animal pathogenic fungi, some can be seen to possess a high level of conservation. MAP kinase and cAMP signalling appear widespread with homologues of multiple components present in both types of pathogen. Differences in the number of components and points of crosstalk in a particular pathway may differ in some cases, but as yet, knowledge is often incomplete. However, such highly conserved signalling systems often regulate different processes in different species. Both plant and animal pathogens appear to use heterotrimeric and small G-proteins, and two-component systems as upstream signalling elements. Fundamental differences in signalling between the two pathogen types may have been demonstrated by the observation of lipid signalling in human pathogens.

1.4.13 The *F. graminearum* genome

In 2003, the genome sequence of *F. graminearum* was made available at the Broad Institute of MIT and Harvard, and a report on the genome was published in *Science* (Cuomo *et al*, 2007). This new resource has greatly enhanced the possibilities for investigation of this important pathogen. The genome sequence of *F. graminearum* strain PH-1 (a North American isolate) revealed a number of important features. *F. graminearum* possesses very little repetitive DNA sequence, at least a factor of 15 less than that of other related fungi. Few recently duplicated genes are found in the genome and transposons are minimal and non-functional. The lack of repetitive sequence is thought to be due to a combination of the low number of transposons, the self-fertility of *F. graminearum* (repetitive DNA is rarely gained from crosses) and the presence of a specific genetic mechanism, already known to be present in several other species, called repeat-induced point mutation (RIP), which efficiently eliminates repetitive DNA. RIP is thought to act as a defence mechanism for the genome in which duplicated sequences are selectively mutated before meiosis in the sexual cycle. The *F. graminearum* genome also contains a larger number of genes predicted to encode proteins for transcription factors, hydrolytic enzymes and transmembrane transporters than the related Ascomycete species *N. crassa* *M. oryzae* and *A. nidulans*.

Unlike other plant pathogenic fungi, *F. graminearum* contains only four large chromosomes. The very low level of repetitive DNA facilitated the alignment of the genome and genetic maps of *F. graminearum*. This had not been possible for the previously published fungal plant pathogen genomes because of the lower quality of sequence information obtained as well as the abundance of repetitive sequences. These features also limited the ability of these other fungal genomes to be completely assembled from the overlapping fragments of sequence obtained in the sequencing project.

Comparison of the sequence of strain PH-1 to that of a second strain of *F. graminearum*, GZ3639 (another North American isolate) revealed the presence of 10,495 single nucleotide polymorphisms (SNPs). These SNPs tended to be

clustered together, with many present near the ends of the four chromosomes at the telomeres. However, a few regions in the middle of three of the four chromosomes also showed high SNP density. This led the authors to suggest that the large chromosomes are the result of fusion of previous, smaller chromosomes in the progenitor species. This may help to explain the low number of chromosomes in *F. graminearum*. The regions of highest SNP density also correlated with high recombination frequency and low GC content, further suggesting that these regions represent former telomeres. Genes that were found to be specific to *F. graminearum* also tended to occur more frequently in the SNP-dense regions, as did genes specifically expressed during the first six days of barley ear colonisation. Such genes included those encoding predicted plant cell-wall degrading enzymes and several with similarity to known disease-causing factors. The conserved core genes, such as those involved in basal transcription and protein translation, tended to occur away from these regions. Overall, these genomic features make the landscape of the *F. graminearum* chromosomes very different from that of previously- sequenced fungi. A summary of the features of the *F. graminearum* PH-1 genome sequence is shown in **Table 1.3**.

Table 1.3. Summary of the *F. graminearum* genome (36.1 Mb) (compiled from Cuomo *et al.*, 2007). Genes were called by both the Broad Institute of MIT and Harvard and the Munich Information Centre for Protein Sequences (MIPS). The Broad call used BlastX with a threshold value of $E < 10^{-5}$ to query public protein databases with the genome sequence. A combination of FGENESH, FGENESH+ and Genewise was used to predict genes. Predicted genes and the genome itself were then compared to known publically available EST sequences. For the MIPS annotation, FGENESH with a matrix trained on sequences from diverse fungal species (*Ustilago maydis*, *Schizosaccharomyces pombe*, and others) was used. Blast analysis determined 704 genes thought to be unique to *F. graminearum*. The presence of at least one InterPro domain was required to help eliminate falsely predicted proteins. The *F. graminearum* gene complement was also compared with *F. asiaticum*, *F. boothii*, *F. culmorum* and *F. pseudograminearum* by hybridising genomic DNA of these species to a *F. graminearum* microarray. This approach determined 382 genes that appear to be *F. graminearum*-specific. 7,132 genes were detected during barley infection. Detection of 408 of these was specific to infection, 126 of which are predicted to be secreted. Comparison of the sequenced PH-1 and GZ3639 (= NRRL 29169 sequenced by the Torrey Mesa Research Institute/Syngenta) strains was used to determine the position of SNPs.

Total predicted genes	11,640
Predicted <i>F. graminearum</i> -specific genes	704 (Blast) 382 (Microarray)
Genes expressed exclusively in barley infection	408
Percent of these genes predicted to encode secreted proteins	31%
Number of predicted secreted plant cell wall degrading enzymes	32
SNPs between strains PH-1 and GZ3639	10, 495

1.4.14 Fungicide Targets

Investigations into possible fungicide control measures for *F. graminearum* and *F. culmorum* have provided some promising yet mixed results. The success of fungicide application is complicated by its effects being apparently dependent upon other fungal species present and the effect of the fungicide upon these species (Aldred and Magan, 2004). Another problem arises from reports that certain fungicides may actually stimulate mycotoxin production when applied under certain conditions (Aldred and Magan, 2004). While several fungicide studies have reported a correlation between disease extent and mycotoxin level (Homdork *et al.*, 2000; Mennitti *et al.*, 2003; Mesterhazy *et al.*, 2003; Haidukowski *et al.*, 2005), one point of concern is that fungicide application

reduces visible disease symptoms while having little effect on mycotoxin level, leading to apparently healthy but contaminated grain (Aldred and Magan, 2004).

An example of such a situation was presented by Nicholson *et al.* (2003). When azoxystrobin (which inhibits respiration by binding to the Q_o site of cytochrome bc₁) was applied to fields infected with both *Fusarium* species and the non-toxin producing FEB species *Microdochium nivale*, disease levels were reduced but DON level in grain increased. This was thought to be due to selective inhibition of *M. nivale* by azoxystrobin, reducing competition on the toxigenic *Fusarium* species. In contrast, other fungicides including tebuconazole (a sterol biosynthesis inhibitor) were selective against the *Fusarium* species only and effects were generally dose- dependent but none of the applications used could reduce DON to a level below 0.75 ppm. Other fungicides that have been found to increase DON production include tubiconazole, difenoconazole, epoxiconazole and propiconazole (Simpson *et al.*, 2001; Magan *et al.*, 2002; Aldred and Magan, 2004).

Tebuconazole has been suggested to be a useful fungicide against FEB in several other studies (Homdork *et al.*, 2000; Cromei *et al.*, 2001; Mennitti *et al.*, 2003; Mesterhazy *et al.*, 2003), but applications may be insufficient when disease pressure is high (Mesterhazy *et al.*, 2003). The extent of disease and DON reduction by tebuconazole appears to vary with conditions, sometimes being highly effective – two applications at 189 g ai/ha around the flowering stage by Cromei *et al.* (2001) were reported to reduce FEB incidence by up to 90%. However, Milus and Parsons (1994) found that tebuconazole application at 140 g ai had little effect on disease or DON levels in heavily inoculated plants. In addition, Covarelli *et al.* (2004) found tebuconazole to be poorly effective against *F. culmorum* DON production *in vivo*.

Tebuconazole has very poor solubility (Mauler-Machnik and Sutý, 1997). Its effects are short-lived and a slow-release method is required to allow increased solubility and longer periods of availability (Balmas *et al.*, 2006). Balmas *et al.* (2006) synthesised a complexation of tebuconazole with β -cyclodextrin for controlling foot and crown rot of Durum wheat in soil inoculated with *F. culmorum*. Applied as a seed dressing, the complexation reduced disease and

increased grain yield comparable to a commercial tebuconazole formulation. It allowed release of the fungicide without hampering its effectiveness.

Other fungicides reported to show efficacy against FEB include trifloxystrobin, which, *in vitro*, may act to inhibit initiation of trichothecene biosynthesis (Covarelli *et al.*, 2004), the sterol biosynthesis inhibitors prochloraz and bromoconazole (Mennitti *et al.*, 2003), the mitosis β -tubulin assembly inhibitors carbendazim, (Jones, 2000; Cromey *et al.*, 2001), the osmotic signalling MAP kinase inhibitor fludioxonil (Jones, 2000) and a range of fungicide mixes. The development of resistance against such fungicides is, however, an ongoing concern. A recent study of carbendazim resistance in *F. graminearum* in China noted a correlation between resistance and increased trichothecene production (Zhang *et al.*, 2009).

Currently, no clear fungicidal preventative or cure has so far been found. Disease and mycotoxin reduction is often only partial or requiring several applications at high dosage, leading to high cost and potential safety concerns. Grain may still contain mycotoxins at levels above those set by regulations and so be hard to market (Jones, 2000). The effects of fungicide application are also very dependent on application rate and timing and prevailing conditions in the field (discussed in Jones, 2000), with the complexity of *Fusarium* biology meaning application at the correct time is difficult to achieve (Mauler-Machnik and Sutý, 1997). Systemic fungicides may prove more successful as they are absorbed into tissues and less easily removed by moisture (Jones, 2000). New fungicides, combinations and fungicide targets need to be investigated to obtain a much better degree of FEB control at a suitable cost and safety level.

In addition, the application of fungicides to a vertical cereal ear is difficult with substances easily running or washing off the ears. Flowering also tends to occur over a period of around two weeks and to control this period of maximum susceptibility with a single spray is problematic. Finally, with *Fusarium* exhibiting symptomless spread through ears (Brown *et al.*, 2010), by the time symptoms are noted, then fungicide application will be too late with a large percentage of the ear already infected.

In the future, new EU regulations revising Directive 91/414/EEC which controls the use of plant protection products will further limit the number of chemistries permissible for use on crops. Substances previously used to treat wheat crops that could be subject to exclusion include the triazoles, mancozeb and prochloraz among others (Clarke *et al.*, 2008). As a result, alternative methods of control need to be found and combating wheat diseases may prove much more difficult. According to a report by the environmental consultancy ADAS, the percent loss of UK wheat crop will greatly increase under such proposals (from around 6-16% to 20-30%), with at least a 20% decrease in production, an increase in the land required to maintain current UK production of over 500,000 hectares and a significant economic impact (Clarke *et al.*, 2008).

1.4.15 Other *Fusarium* Species and Important Related Species

Fusarium verticillioides

Fusarium verticillioides infects maize, causing ear mould and stalk rot, especially in warmer areas (White, 1999). *F. verticillioides* grows optimally at a higher temperature than *F. graminearum* and this affects the geographical distribution of the diseases these species cause (discussed in Miller, 2001). Fumonisin mycotoxins are produced by *F. verticillioides* during infection of maize and are known to disrupt sphingolipid biosynthesis, posing a serious health threat to certain livestock (Duvick, 2001). Fumonisin B1 has also been shown to inhibit the plasma membrane H⁺ ATPase of germinating maize embryos (Gutierrez-Najera *et al.*, 2005) and fumonisins have been linked to oesophageal cancer (reviewed in Miller, 2001). However, while the FUM1 polyketide synthase is required for fumonisin production, it is dispensable for ear rot infection, indicating that fumonisins are not required for pathogenesis (Desjardins *et al.*, 2002). The genome of *F. verticillioides* has been sequenced and compared to that of *F. graminearum*, The *F. verticillioides* genome is contained within 12 chromosomes (Ma *et al.*, 2010).

Fusarium oxysporum

The vascular wilt pathogen *F. oxysporum* is found in a large number of host-specific forms, known as *formae speciales* (discussed in Michielse and Rep, 2009). Recently, lineage-specific (LS) regions of the *F. oxysporum* f sp. *lycopersici* (*Fol*, a tomato pathogen) genome were found to confer host selectivity (Ma *et al.*, 2010). Such regions appear to be acquired horizontally, vary between different *F. oxysporum formae speciales*, and are absent in non-pathogenic strains. The genome of *F. oxysporum* f sp. *lycopersici* is far larger than those of other *Fusarium* species, primarily because of an abundance of transposon sequences. The genome is composed of a number of core chromosomes and lineage-specific chromosomes (Ma *et al.*, 2010).

Fusarium solani

The *F. solani* (teleomorph *Nectria haematococca*) species complex contains many saprophytic fungi found in a wide range of habitats and pathogens able to colonise a very large range of host plants species and also causes opportunistic infections in humans (Coleman *et al.*, 2009). *F. solani* species complex members can survive in extremely harsh environments and degrade a wide assortment of compounds for nutrition. The most extensively studied member is *N. haematococca* mating population VI, in which supernumerary chromosomes have been shown to confer the ability to colonize a series of different habitats (Coleman *et al.*, 2009).

Fusarium circinatum

F. circinatum is the fungal pathogen responsible for pitch canker disease of pine trees (discussed in Iosif *et al.*, 2009). Symptoms of infection include cankers on branches, roots and trunks with resinous exudates ('pitch'). Severe infections can lead to crown dieback and death of the tree. The disease is a serious problem and reduces growth, timber quality and production in the Southern hemisphere. Pitch canker threatens both native forest and plantations and established infections may prove difficult to eradicate. Recently a sensitive detection method has been developed for *F. circinatum* in pine seed (Iosif *et al.*,

2009). This technique comprises an enrichment stage in complete media to increase *F. circinatum* biomass followed by real-time PCR-based detection. By detecting the pathogen in seed it is hoped to prevent long-distance spread of the disease via contaminated seeds.

Trichoderma reesei

Trichoderma species are free-living fungi, common in most soil types (reviewed Harman *et al.*, 2004). As well as existing as avirulent plant symbionts, often colonising just a couple of root epidermal cell layers, they can also parasitise other fungi, a basis of their beneficial role to the host plant. Benefits to the host plant include protection against pathogens, better growth and development, and increased productivity, nutrient uptake and use.

Epichloë festucae

Epichloë are mutualistic temperate grass symbionts (endophytes) (reviewed in Schardl, 2001). The grass benefits from this interaction by gaining increased resistance to herbivores, parasites and drought and also enhanced growth and nutrient acquisition. For obtaining nutrients from the host plant, *Epichloë* do not produce specialised structures such as haustoria or “intracellular” hyphae and are instead confined in the apoplast. A secreted β -1,6-glucanase that may be involved in nutrient acquisition has also been identified (Moy *et al.*, 2002; Bryant *et al.*, 2007). Secreted proteinases may allow the acquisition of other nutrients. Reactive oxygen species (ROS) also appear to play a role in maintaining the symbiosis between *E. festucae* and the host plant. Disruption of the NADPH oxidase gene *noxA* led to unregulated hyphal growth and rendered the fungus pathogenic and causing death of the host plant (Tanaka *et al.*, 2006). NoxA is regulated by NoxR, together with the small GTP-binding protein RacA (Takemoto *et al.*, 2006).

Neurospora crassa

Neurospora crassa is a well-studied model fungus. Perhaps most famously used in early studies to illustrate the 1:1 relationship between genes and

enzymes, this species is now routinely used for the study of regulation of the circadian clock (Horowitz, 1991; Heintzen and Liu, 2007). An ongoing process to create single gene deletion strains for each gene in the *N. crassa* genome is being co-ordinated by the Neurospora Genome Project (www.dartmouth.edu/~neurosporagenome). This is providing an extremely useful genetic resource as such strains can, for example, be tested for complementation of phenotypes by the reinsertion of genes from heterologous species.

Chapter 2. Materials and Methods

2.1 Naming Conventions and Abbreviations

Gene names are given in the format *ABC1*, the protein encoded by that gene as *Abc1* and deletion strains of said gene as *abc1*. Species identifier prefixes are not used for gene names, the species in question being identified separately. A list of abbreviations used in the thesis is shown in **Table 2.1**. A list of accession numbers is provided in **Appendix 1**.

Table 2.1. List of abbreviations used in the thesis.

FEB	Fusarium Ear Blight
HGCA	Home-Grown Cereals Authority
LSD	Least Significant Distance
SEM	Standard Error of the Mean
ANOVA	Analysis of Variance
REML	Restricted Maximum likelihood
DON	Deoxynivalenol
NIV	Nivalenol
3-ADON	3-Acetyldeoxynivalenol
15-ADON	15-Acetyldeoxynivalenol
ZEA	Zearalenone
PCR	Polymerase Chain Reaction
SNA	Synthetic nutrient-poor agar
PDA	Potato dextrose agar
CM	Complete medium
MM	Minimal Medium
gDNA	Genomic DNA
GluCP	Glutamate carboxypeptidase
Pol	DNA polymerase epsilon subunit B
Hyd	HAD superfamily hydrolase
dpi	Days post inoculation

2.2 Fungal Strains and Growth Conditions

The sequenced *Fusarium graminearum* strain PH-1 (Cuomo *et al.*, 2007) was used as the wild-type control strain in this study and for targeted single gene deletion experiments. *F. graminearum* was routinely cultured on 2% agar plates of either Synthetic Nutrient-poor Agar (SNA, 0.1% KH₂PO₄, 0.1% KNO₃, 0.1%

MgSO₄ x 7H₂O, 0.05% KCl, 0.02% glucose, 0.02% sucrose and 2% agar Urban *et al.*, 2002) as minimal medium or Potato Dextrose Agar (PDA, Oxoid, made according to manufacturer's instructions) as complete medium. *Fusarium* synthetic complete medium (FSCM) was prepared as described (Leslie *et al.*, 2006). For the removal of old conidia and production of fresh conidia, plates were scraped with an overlay of TB3 (0.3% yeast extract, 0.3% Bacto Peptone and 20% sucrose) and conidia were harvested after 24 hr in sterile water (Brown *et al.*, 2010). Plates were incubated at room temperature under constant illumination from one near-UV tube (Phillips TLD 36W/08) and one white light tube (Phillips TLD 36W/830HF) (Baldwin *et al.*, 2010). *Neurospora crassa* strains were obtained from the Fungal Genetics Stock Centre (www.fgsc.net) and grown on race tubes containing Vogel's (minimal) medium (Vogel, 1956) with 2 g/l of NH₄Cl and KNO₃ in place of NH₄NO₃, 15 g/l sucrose and 15 g/l agar in the dark at 37°C.

2.3 Fungal stocks

Fungal stocks were maintained either as water solutions of conidia from freshly harvested TB3-treated plates, frozen at -80°C, or as soil stock tubes (Urban *et al.*, 2002; Brown *et al.*, 2010). These were made by placing a small amount of doubly-sterilised soil particles into 2 ml cryotubes (Nunc), adding an agar piece from an SNA plate culture of *F. graminearum*, covering with 200 µl PDB and incubating at 28 °C in the dark for 14 days prior to freezing at -80 °C. Small amounts of soil were removed from these stocks when needed, the tubes being kept on ice and out of the freezer for the minimum possible time during this process.

2.4 Agarose Gel Electrophoresis

Gel electrophoresis was performed using 1% agarose in 1x Tris/Borate/EDTA (TBE: 90 mM Tris-base, 90 mM boric acid, 2 mM EDTA). 5 µl /100 ml ethidium bromide solution was added to gels prior to setting (BioRad, 10 mg/ml). Gels were run with TBE containing ethidium bromide as running buffer in gel tanks

(BioRad) at 60-90V for 60-90 min depending on gel size and number of well combs used. Gels were imaged under UV irradiation using a Gene Genius imager with GeneSnap software (Syngene). Lambda *Bst*EII DNA ladder (New England Biolabs) was used for size estimation.

2.5 Targeted Deletion Fusion PCR

For the targeted deletion of the *FGSG_09891*, *FGSG_09893*, *FGSG_09896* (*ICL1*), *FGSG_09900*, *FGSG_09905*, *FGSG_09906*, *FGSG_09907* and *FGSG_09908* (*PKAR*) genes, a polymerase chain reaction (PCR) fusion-based split marker deletion method (Catlett *et al.*, 2003, **Figure 2.1**) was used. DNA regions flanking the target gene were amplified using Hotstar Taq DNA Polymerase (Qiagen) with primers incorporating a 24 bp region of the *hph* hygromycin resistance gene (primers described in **Table 2.2**). Two overlapping sections of the *hph* gene were also amplified (**Table 2.3**). All PCR products were cleaned using a PCR purification kit (Qiagen). Flanks and overlapping *hph* sections were then mixed in a 1:1 ratio and fused in a second PCR using two of the original PCR primers (**Table 2.4**). In some cases, nested primers were used to increase fusion product amounts (**Table 2.5**). The concentration of each DNA fusion product was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

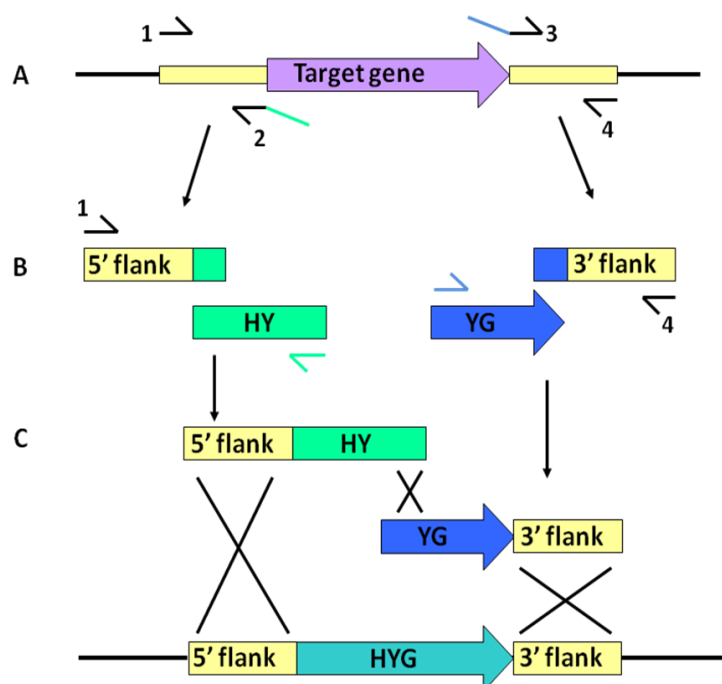


Figure 2.1. Split-marker targeted single gene deletion (Catlett et al., 2003). **A.** Amplification of regions either side of the target gene (flanks) using primers which incorporate a short overlap to an antibiotic resistance gene (in this case for hygromycin, *Hph*) allows fusion in a second PCR reaction. The resistance gene is amplified separately in two overlapping sections. **B.** One resistance gene section is joined to each of the two flanking regions by PCR. **C.** The two DNA fusion products made are then used for transformation of fungal protoplasts, resulting in deletion of the target gene and replacement with the antibiotic resistance gene via DNA recombination. The antibiotic can then be used to select for successful deletion events.

2.6 DNA Primers

DNA oligonucleotide primers were designed using the Vector NTI software (Invitrogen) with melting temperature set at 55 ± 2 °C and purchased from Sigma-Aldrich as dry samples. Upon receipt, primers were diluted in sterile H₂O to 100 µM for storage and an aliquot further diluted to 10 µM for use in polymerase chain reaction (PCR) experiments. Both primer concentrations were frozen at -20 °C until required.

2.7 Polymerase Chain Reaction (PCR)

PCR reactions were performed with either Hotstar Taq Polymerase (Qiagen), REDTaq PCR ReadyMix (Sigma), REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich) or Ex-Taq (Takara) as indicated and according to manufacturer's instructions. PCR reactions were performed in either a G-Storm GS4 (AlphaMetrix Biotech) or GeneAmp 9700 (Applied Biosystems). Reaction mixtures and cycle parameters were used according to the manufacturer's instructions with an annealing temperature of 55°C and extension time of 1 kb per minute.

2.8 PCR Product Purification

PCR product purification was performed using a QIAquick PCR Purification Kit according to the manufacturer's instructions with a final elution volume of 30 µl.

2.9 Gel Extraction

Gel extraction was performed using a QIAquick Gel Extraction Kit according to the manufacturer's instructions with a final elution volume of 30 µl.

2.10 Molecular Cloning for the Targeted Deletion of the *NTH1* Gene

For the targeted deletion of *NTH1*, a molecular cloning approach was used. *Escherichia coli* strain DH5α was used for cloning steps and grown in Luria-Bertani (LB) or 2 x yeast tryptone (2x YT, Formedium) medium at 37°C +/- 100 µg/ml ampicillin. Stocks of strains were stored at -80°C in 2x YT containing 15% glycerol.

All restriction enzyme digestion steps were performed at 37°C overnight unless otherwise stated with reaction mixtures and buffers according to manufacturer's instructions. In some cases, sequential digestion was performed with the reaction mixture being purified with a Qiagen QIAquick PCR purification kit prior

to the second digestion step. Restriction enzymes were obtained from New England Biolabs (NEB – *HindIII*, *XbaI*), Promega (*SacI*) and GIBCO (*BamHI*, *XhoI*)

Flanking regions of the *F. graminearum* *NTH1* gene were amplified by PCR as described above. A scheme depicting the cloning procedure used is shown in **Figure 2.2**. Flank PCR products were ligated into the pCR2.1-TOPO™ vector (Invitrogen) using T4 DNA ligase (MBI Fermentas) according to the manufacturer's instructions, stored at 15°C overnight and transformed into *E. coli*.

Transformed *E. coli* were selected on LB agar containing 100 µg/ml ampicillin, 40 µl 100 mM IPTG and 80 µl 20 mg/ml X-Gal for blue-white selection. Colonies of each transformation (5' or 3' flank) were grown overnight in LB media containing 100 µg/ml ampicillin, DNA was extracted (Qiagen QIAprep Miniprep Kit) and subjected to analytical restriction digestion with *HindIII*. Forward insert clones were then digested with *SacI* + *XbaI* (for 5' flank construct (pAB001-2)) or *BamHI* + *XhoI* (for 3' flank construct (pAB002-2)) to produce a fragment containing the flank region.

These fragments were then concentrated by ethanol precipitation, resuspended in 30 µl H₂O, gel purified with a Qiagen QIAquick gel extraction kit and ligated into the split marker hygromycin resistance vector pYG (for the 5' flank) or pHY (for the 3' flank) (Catlett *et al.*, 2003) prior to transformation of *E. coli*. The pYG vector had previously been cut with *SacI* and *XbaI* (supplied by Martin Urban) and pHY by *BamHI* and *XhoI*. Ligation reactions were as such: for the 3' flank (pAB002-2) fragment and pHY – 7 µl H₂O, 2 µl vector, 8 µl insert fragment, 2 µl 10x buffer, 1 µl T4 DNA ligase – stored at room temperature overnight; for the 5' flank (pAB001-2) fragment and pYG – 7 µl H₂O, 2 µl vector, 6 µl insert fragment, 2 µl 10x buffer, 1 µl T4 DNA ligase.

Colonies were analysed for ligation of the pAB001-2 fragment into pYG by growing overnight in 400 µl dYT containing 100 µg/ml ampicillin (dYT Amp) and screened using colony PCR with primers AB1 and AB2 and with pAB001-2 as positive control (Sigma REDTaq Readymix, according to manufacturer's instructions). One clone was selected and analysed by restriction digestion

using *SacI* and *XbaI* (four hour digest with *XbaI*). This clone was named pAB003 (see cloning scheme in **Figure 2.2**).

Colonies were analysed for the ligation of the pAB002-2 fragment into pHY by growing overnight in 400 µl dYT Amp and screened using colony PCR with primers AB3 and AB4 and with pAB002-2 as positive control (Sigma REDTaq Readymix, as above). Two clones, were retained and grown overnight in dYT Amp and DNA was extracted. This DNA was digested with *Bam*HI and *Xho*I (four hour digest for *Bam*HI). The two clones were named pAB004-1 and pAB004-2 (see cloning scheme in **Figure 2.2**).

The regions of pAB003 and pAB004-1 containing the flank region fused to the *hph* gene section were sequenced in both directions using the M13uni (-43) primer (for both pAB003 and pAB004-1) and A1 primer (for pAB003) or A2 primer (for pAB004-1). The flank-*hph* section regions were amplified from pAB003 and pAB004-1 by PCR using primers AB1 and YG+ for pAB003 and HY and AB4 for pAB004-1. 6x 50 µl reactions were performed, pooled and ethanol precipitated and the products quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). The concentration was adjusted to 1.7 µg/µl and used for transformation of *F. graminearum*.

A

800 bp 5' flank region of *NTH1*

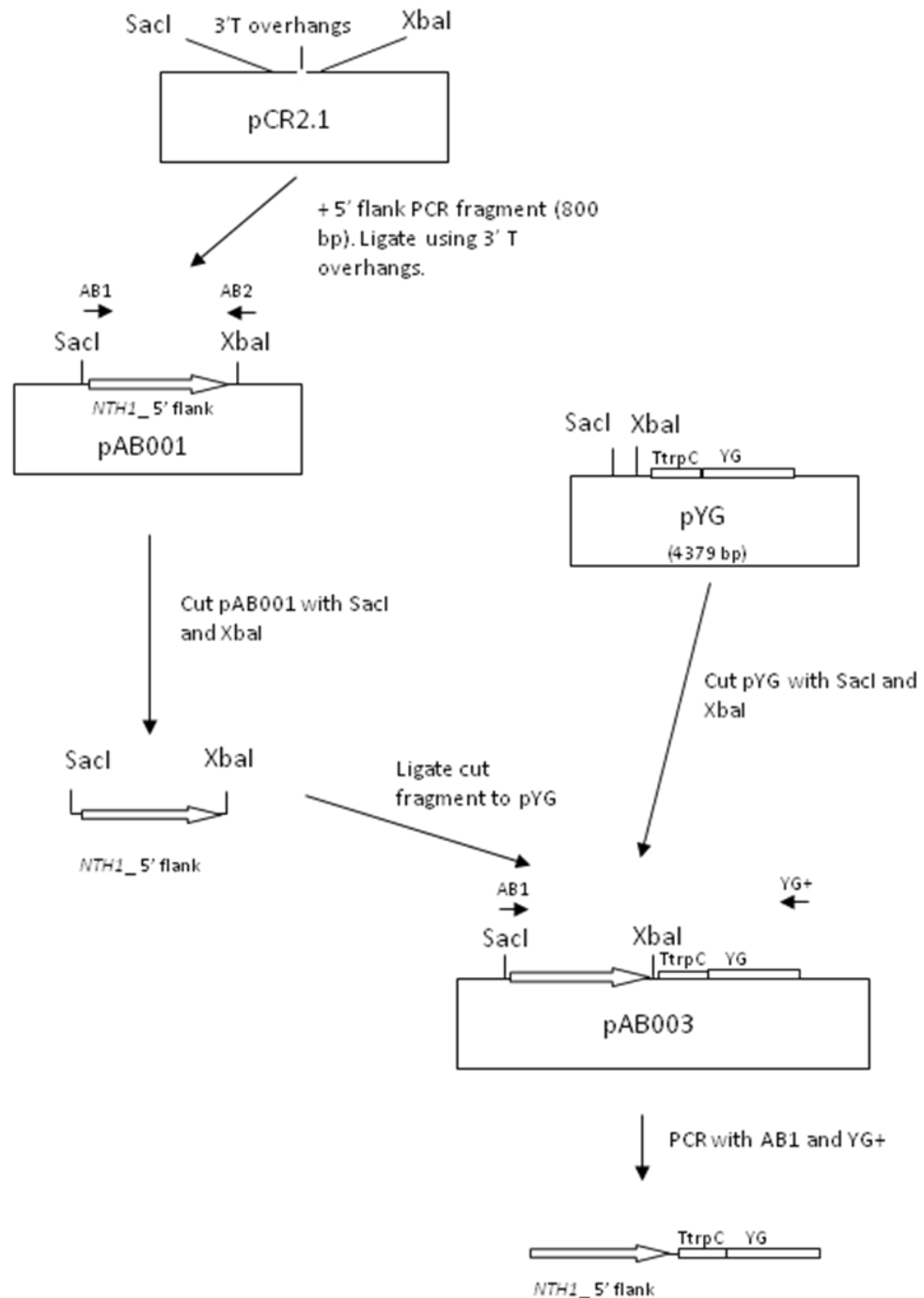


Figure 2.2A Cloning scheme for the *NTH1* 5' flank.

B

1000 bp 3' flank region of *NTH1*

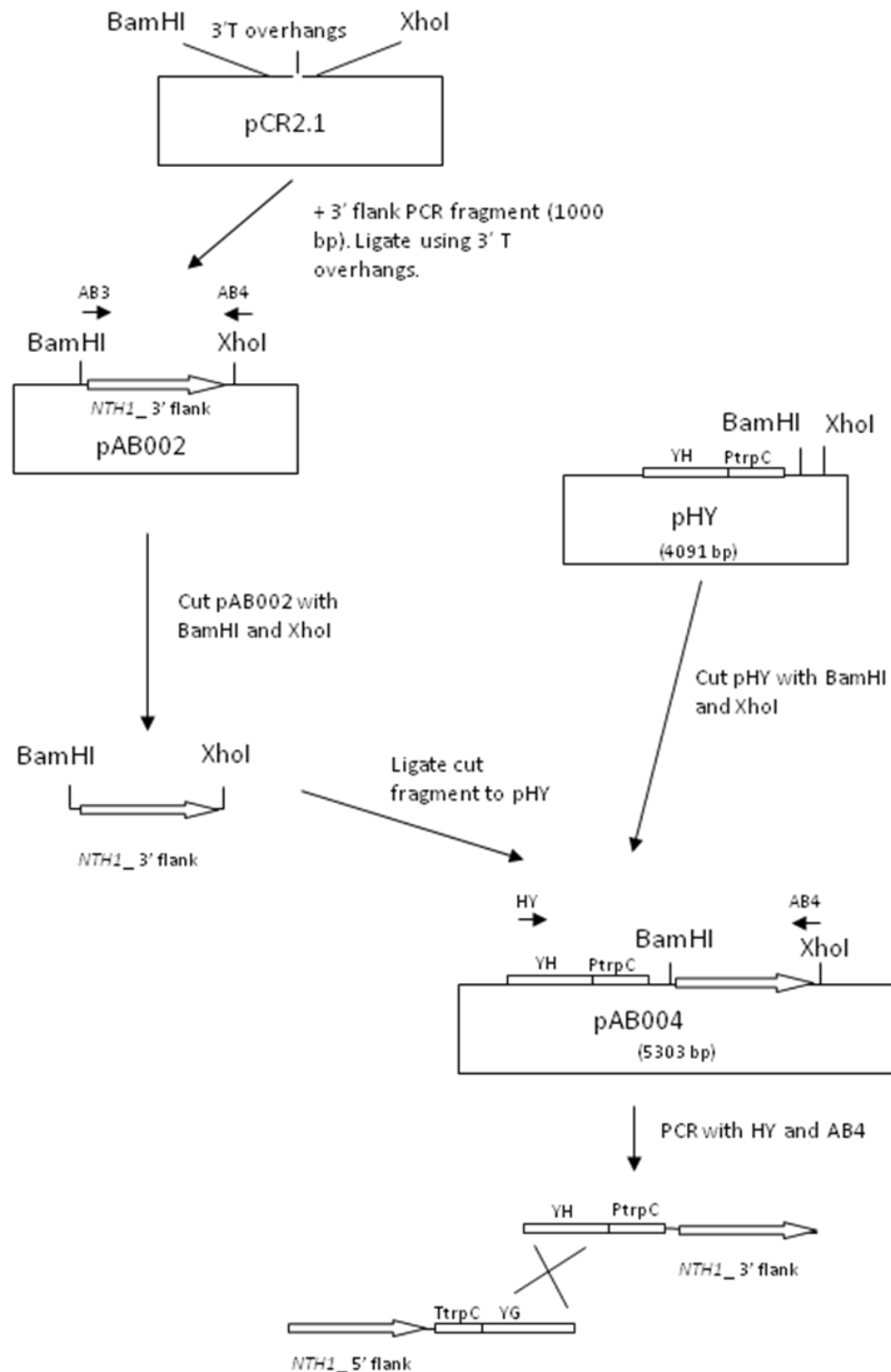


Figure 2.2B Cloning scheme for the *NTH1* 3' flank.

2.11 DNA Phenol:chloroform:isoamylalcohol (Ethanol) Precipitation

For the concentration of fusion DNA prior to transformation of *F. graminearum*, a phenol:chloroform:isoamylalcohol precipitation method was used. One volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to the DNA sample and mixed by vortexing briefly. The mixture was spun for 5 min at 13.2 k rpm and the top layer retained. 0.1 volume of 3 M NaOAc pH5.2 was added, followed by vortexing then either two volumes of absolute ethanol or one volume of isopropanol added followed by further vortexing. The mixture was incubated at -20 °C for 1 hr then spun for 15 min at 13.2 k rpm. The supernatant was poured off and the pellet retained. The pellet was washed by the addition of 1 vol 70% (v/v) ethanol and mixing by inversion followed by a second spin for 15 min at 13.2 k rpm. The liquid was then removed by pipetting and the DNA dried for 10 min at 37 °C. The DNA was then resuspended in a small volume of sterile H₂O at a concentration of 1-5 µg/ul.

2.12 Fungal Transformation

F. graminearum strain PH-1 was transformed as previously described (Hohn and Desjardins 1992, Proctor et al., 1997), but with a 1 hr incubation in 40% PEG8000 for *FGSG_09891*, *FGSG_09893*, *FGSG_09896* (*ICL1*), *FGSG_09900* and *FGSG_09906* deletion and glucose in place of sucrose in the regeneration medium for *PKAR*.

The transformation protocol is as follows: Two 90 mm SNA plates of *F. graminearum* were grown for seven days, 2 ml TB3 added to each plate, which were then surface scraped with a plastic plate spreader and the excess liquid containing the old conidia pipetted away. Each plate was then incubated for 1 day further and the fresh conidia harvested in 2 ml H₂O. The fresh conidia were spread on two large (20 mm) SNA plates and grow for three days. These plates were scraped with TB3 (10-20ml) as above and the excess liquid removed. After one further day incubation, the conidia were harvested in 10-20 ml H₂O by scraping the plates. The conidia were filtered through Miracloth (Calbiochem)

and germinated overnight in 300 ml PDB, stirred at 300 rpm in a baffled 1l flask at 15 °C. The resulting germlings were filtered through Miracloth and resuspended in 40 ml 1M sorbitol containing 1 g Driselase (Sigma) and 400 mg Sigma Lysing Enzymes (Sigma). The mixture was incubated at 30 °C, shaking at 80 rpm for 1 hr then after confirming protoplast formation and quality microscopically, was centrifuged for 10 min at 3200 rpm, the pellet resuspended in 25 ml STC (20% sucrose, 50 mM Tris/HCl, pH 8.0, 50 mM CaCl₂) and spun again for 10 min at 3200 rpm. The pellet was then carefully resuspended in a few ml STC, spun for 5 min at 5 k rpm, resuspended in 1 ml STC and spun again for 5 min at 5 k rpm. Finally, the protoplast pellet was resuspended in 400 µl STC.

For the transformation mixture, 10-20 µg transformation DNA construct (1:1 mixture of the two flank constructs if used) was added to 90 µl STC and mixed. 100 µl protoplast suspension was added, mixed and the mixture incubated at room temperature for 20 min. 1 ml of 40% PEG 8000 in STC was then added, mixed and incubated for a further 20 min. Finally, 5 ml of TB3 was added and the mixture placed on a tilting rack overnight.

1.4 g low melting point agar (Gibco) was dissolved in 100 ml 2x regeneration medium (0.4% yeast extract, 0.4% casein enzyme hydrolysate (N-Z-Amine A) (Sigma-Aldrich) and 100 ml 1.6M sucrose added. The mixture was cooled to 40 °C and hygromycin B added to a final concentration of 75 µg/ml. 37ml of the hygromycin media was added to 3 ml of the transformation mix, inverted to mix and poured into two 90 mm petri dishes. The plates were left to set then incubated at 28 °C for five days and colonies picked onto SNA containing 75 µg/ml hygromycin.

2.13 Selection of Transformants

Transformants were selected with 75 µg/ml hygromycin. Single hygromycin-resistant transformants were pre-screened by PCR analysis of crude DNA extracts using the REDExtract-N-Amp kit (Sigma-Aldrich) with 25 µl extraction solution and 25 µl neutralisation solution only, The presence of the *Hph* and

target genes was tested using primers given in **Table 2.6**. Transformants lacking the target gene but showing presence of *Hph* were retained for Southern blot analysis.

2.14 Genomic DNA Extraction from Fungi for Southern Analysis

200 ml of PDB was added to a 500 ml conical flask and then inoculated with a section of a SNA plate culture of *F. graminearum* and incubated in the dark at 28 °C shaking at 100 rpm for 3 days. The culture was filtered through Miracloth, frozen, freeze-dried, ground in a pestle and mortar and used for DNA extraction. DNA extraction was performed using the Nucleospin Plant XL Kit (Macherey-Nagel) with 7.5 ml buffer C1 and 100 µl RNase A or using the following protocol (adapted from one published by Xu and Leslie, 1996): 20 ml CTAB lysis buffer (20 g/l CTAB, 12.12 g/l TRISMA base, 2.92 g/l EDTA, 41 g/l NaCl) was added to 3 ml freeze-dried mycelium and vortexed quickly. 100 µl β-mercaptoethanol and 200 µl RNase (10 µg/µl) were added and the mixture incubated in a water bath at 65 °C for 30 min, inverting every 10 min then cooled on ice. 1 volume of CIA (chloroform:isoamylalcohol 24:1) was added and the mixture mixed gently on a tilting rack for 15 min then centrifuged for 5 min at 4.8 k rpm at 4 °C. The upper phase was removed and two volumes of absolute ethanol or one volume of isopropanol added and the mixture incubated at -20 °C for 1 hr prior to centrifugation for 15 min at 10 k rpm at 15 °C. The liquid was poured off and the pellet resuspended in the remaining liquid with heating at 60 °C. Two phenol:chloroform:isoamylalcohol extractions were then performed (as described) and one further with CIA in place of phenol:chloroform:isoamylalcohol. To precipitate the DNA, two volumes of ethanol were added to the DNA phase and incubated for 5 min followed by centrifugation for 5 min at 13.2 k rpm, the pellet washed in 70% (v/v) ethanol and resuspended in 400 µl TE pH8.0 (10 mM Tris.Cl, 1 mM EDTA).

2.15 Genomic DNA digestion

The reaction mixture for the digestion of genomic DNA (gDNA) for Southern analysis was as follows: 5-10 µg gDNA, 33 µl H₂O, 5 µl buffer, 5 µl 10x BSA, 2 µl restriction enzyme (Fermentas high concentration – 50 U/µl). The reaction mixture was incubated at 37°C overnight.

2.16 Southern Hybridisation Characterisation of Transformants

Transformants from the PCR-based pre-screen were grown from a single colony by inoculating a SNA plate with a very dilute solution of conidia in water and genomic DNA prepared from the samples. Southern hybridisation was performed using a standard alkaline protocol (Sambrook et al., 1989) with α-dCTP-labelled probes (Rediprime IITM random prime DNA Labelling System (Amersham)). The two original flanking regions of the target gene amplified using primers listed in **Table 2.2** were used to produce the labelled probes. Bands were imaged with a Typhoon phosphorimager. **Table 2.7** lists the restriction enzymes and hybridisation probes used in each experiment, together with the expected sizes of hybridising bands in the wild-type and gene deletion strains.

2.17 Fungal Growth Tests

Agar plugs 9mm in diameter were taken from the growing edge of fungal colonies growing on SNA using a cork borer and a single plug was placed in the centre of 90mm diameter growth test plates. The diameter of the new colony was recorded at 24 hour intervals. For initial growth tests of gene deletion strains on SNA and PDA, three independent transformants (two for *pkar*, *FGSG_09891* and *FGSG_09896*, one for *PKAR-e*) were analysed for each gene deletion. Percentage changes in growth rate are given as the average of the independent transformants compared to wild-type unless specified otherwise. For spore growth tests, 15 µl of a 10e⁶ spore ml⁻¹ solution was placed in the centre of the plate and allowed to dry. Plates were grown in

triplicate. For growth on different carbon sources, SNA low carbon (0.1% KH_2PO_4 , 0.1% KNO_3 , 0.1% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.05% KCl and 2% agar) was supplemented with either: 1% sucrose, glucose, trehalose or (v/v) ethanol, 0.4% olive oil or 6mM sodium acetate. For analysis of stress tolerance, H_2O_2 , menadione and NaCl were added to SNA to the concentration indicated. For the reduced water activity plates (A_w 0.98) containing glycerol, sterile glycerol was diluted to a final concentration of 7.31% (v/v) in sterile water containing 30 g/l sucrose, 1 g/l KH_2PO_4 , 0.5 g/l MgSO_4 , 0.5 g/l KCl, 0.01 g/l FeSO_4 , 2 g/l NaNO_3 , 2 ml/l trace element solution supplemented with uracil, leucine and lysine, and 2% agar (method provided by of N. Magan, Cranfield University). In the case of SNA low C or SNA low N, trace nutrients in the agar are thought to allow limited fungal growth when these elements are absent from the SNA mixture used to make the medium. Three plates were used per strain per media type.

2.18 Virulence Assays

Wheat ears of the susceptible hexaploid spring wheat cultivar Bobwhite were inoculated and scored as previously described (Urban et al., 2003) using either SNA agar plugs or a solution of conidia in water. In the case of conidial suspension inoculations, 5 μl of a 2×10^5 spore ml^{-1} suspension was placed in each inoculated floret. Plants were placed in a humid environment for 2 days post-inoculation with an initial 24 hour period of darkness. In preliminary infection tests, three independent transformants (two for *pkar*) were analysed for each targeted gene deletion on two wheat ears each. If a gene deletion strain (*nth1*, *pkar* and *fcv1*) exhibited reduced ear symptom spread in each of the transformants analysed, one representative transformant was selected for further comparison to the wild-type using a larger number of ears (typically between 10 and 20) and statistical analysis by ANOVA or REML.

2.19 Microscopy

Images of agar cultures of *Fusarium* were obtained using a Zeiss light microscope, while images of *Arabidopsis* were obtained using a Leica stereoscope. For images of single hyphae, the surface of an agar plate culture of *Fusarium* was agitated in a small volume (approx 200 µl) H₂O using a pipette tip and pipetted onto a glass slide for observation using a Zeiss Axiophot light microscope.

2.20 Sequence alignments

Protein sequence alignments were created using the ClustalX2 software (Larkin *et al.*, 2007) and coloured using GeneDoc software (Nicholas and Nicholas, 1997).

2.21 Statistical Analysis of Data

Means were compared using a Least Significant Difference (LSD) at the 5% level. Some datasets were transformed using square root or log to base 10 transformations to improve residuals plots prior to analysis. Analysis was performed using the statistical software package GenStat version 13 (VSN International). Graphs are shown with either the least significant difference (LSD) at the 5% level or error bars of +/- one standard error of the mean (SEM). In the case of graphs illustrating growth on a second medium type as a fraction of that on minimal medium alone, errors bars are given as +/- one standard error calculated for the ratio of the two values.

Growth tests:

Regression over time was used to obtain the growth rate on each plate (in mm day⁻¹). Rates of growth of different strains and media types were compared using a general Analysis of Variance (ANOVA) on the rates with a Completely Randomised Design. The treatment structure was Strain*Media and design structure Replicate number. In the case of growth tests comprising a series of

different concentrations of a particular chemical addition to the base medium, an additional ANOVA using a treatment structure of Strain*Concentration of chemical was used. In the case of growth tests with unequal replicate numbers for different strains or media types, Restricted Maximum Likelihood (REML- a type of maximum likelihood estimation where the likelihood function is calculated from the probability distribution of contrasts calculated from the data) analysis was used using the same design and block structure as above.

Wheat ear pathogenicity assays:

The percentage infection (the number of diseased spikelets below the inoculation point divided by the total spikelets below inoculation point) data over a series of time points at four day intervals was analysed using a split-plot in time ANOVA with design structure Experiment/Ear/Time and treatment structure Strain*Time. REML analysis was used to check for autocorrelation in the data with a random model of Experiment/Ear/Time and fixed model of Strain*Time. Comparison of deviance using Identity and Autoregressive Component of first order for Time revealed any autocorrelation. If detected, the dataset was analysed using the REML means output.

Other tests: General ANOVA using analogous design and treatment structures to above was used to compare DON mycotoxin production, sporulation data and other datasets unless otherwise stated.

Table 2.2. Primers used for the amplification of regions flanking the target genes. Sequences overlapping the hph hygromycin resistance gene are shown in grey. *5' is regarded as left and 3' as right of the gene indicated. **Location of the flank section relative to the target gene in bp (start of ORF for L flank and end of ORF for R flank).

Gene	Flank*	Flank length (bp)	Location**	Primer	Primer sequence (5'-3')
<i>FGSG_09891</i>	5'	800	-1	AB24	CTGTCTGGGCACTAGCAAGTGAA
				AB25	TCCTGTGTGAAATTGTTATCCGCTTTTGGCGGTGGAGGAGAAAG
	3'	383	+7	AB26	GTCGTGACTGGGAAAACCCTGGCGGGGAGTATTATGTGGATTACGCG
				AB27	GCTTACCGGCAGATTGAACG
<i>FGSG_09893</i>	5'	799	-1	AB7	GGTCGCGTGAGAGTAATGAGATC
				AB8	TCCTGTGTGAAATTGTTATCCGCTTTCTTTGCTTGGTGGACACG
	3'	676	+34	AB9	GTCGTGACTGGGAAAACCCTGGCGATGAAGAATGCGAACTGGGACT
				AB10	ATGGAAAGGCTAGGGTCCGA
<i>FGSG_09895</i> (<i>NTH1</i>)	5'	800	-93	AB1	CGTGTAAGTTGACGGCAACG
				AB2	CGATATGTGACGCTGTCCAA
	3'	1000	+1	AB3	GGTAATGTTATTGCAGACTTGG
				AB4	CAACTTACCCCAAACGTGTC
<i>FGSG_09896</i> (<i>ICL1</i>)	5'	750	-43	AB28	GGATTGGCTTGTGCTCGGT
				AB29	TCCTGTGTGAAATTGTTATCCGCTTCGAGGAATTGGACTGCGTG
	3'	637	+1	AB30	GTCGTGACTGGGAAAACCCTGGCGAGTGTTGCTGGGCGACTGAT
				AB31	AAGAGAGTGAGCGTGACGGC
<i>FGSG_09897</i> (<i>SNF1</i>)	5'	1000	+31	U21	GCAGATTGGAACCGTTCAGAT
				U22	CGAGGATAAGGATATGGTGAG

	3'	1212	-204	U23	GGCCATCACGCCAAGGAACCAT
				U24	TTGTCCAAGAGCCCGAGATGG
FGSG_09900	5'	267	-3	AB23	AAGCAACCACAGGAATAAGGGTG
				AB12	TCCTGTGTGAAATTGTTATCCGCTAGAGAATGGCTGCTCGATGG
	3'	708	+1	AB13	GTCGTGACTGGGAAAACCCTGGCGGGTTGCTTTGCCGGATAGTACT
				AB14	GATCTTGCGACTAGCCACGG
FGSG_09905	5'	768	-19	AB15	GATTGAGTCAGGGAGCGGTATC
				AB16	TCCTGTGTGAAATTGTTATCCGCTAAGTTGCAGTACAACCATGTGCTC
	3'	637	+1	AB17	GTCGTGACTGGGAAAACCCTGGCGCATGGTGGAACATCTACGATGTATG
				AB18	TTTGCGAGTGCGGTAATTGAG
FGSG_09906	5'	600	-1	AB36	GAAAGCTCCAGTCTGTTGGGG
				AB37	TCCTGTGTGAAATTGTTATCCGCTTTTGCAGGATGGAGAGGGTG
	3'	788	+8	AB38	GTCGTGACTGGGAAAACCCTGGCGACTGTATCTTTACTGAGGGATCGGC
				AB39	TGCTTCTGGTAAACCGGTCA
FGSG_09907 (FCV1)	5'	987	-13	U120	CGACGGATCAAACAATTTAGGG
				U121	TCCTGTGTGAAATTGTTATCCGCTTTCTGGGATTGGGACACGTG
	3'	428	+1	U122	GTCGTGACTGGGAAAACCCTGGCGAGCCAGGAGAAATAACGCACC
				U123	CAGGTTGTCTCGTGGCTTGG
FGSG_09908 (PKAR)	5'	1461	-7	AB19	CTCCATCGCCATGCAGAAAA
				AB20	TCCTGTGTGAAATTGTTATCCGCTGGACAGAACTTCAAGGAATGATCG
	3'	873	+5	AB21	GTCGTGACTGGGAAAACCCTGGCGCAAGGTAAGATGCAATTGGCG
				AB22	TTGAGCCCTTGATCCGAGGA

Table 2.3 Primers for the amplification of two overlapping sections of the *Hph* hygromycin resistance gene.

<i>Hph</i> gene section	Flank length (bp)	Target gene flank attached to	Primer	Reference
<i>HY</i>	1275	5'	M13R	Catlett <i>et al.</i> , 2003
			NLC37	Catlett <i>et al.</i> , 2003
<i>YG</i>	883	3'	M13F	Catlett <i>et al.</i> , 2003
			NLC38	Catlett <i>et al.</i> , 2003

Table 2.4. Templates and primers for fusion of target gene flanks to hph gene sections.

Gene	Templates	Primers
<i>FGSG_09891</i>	5' flank + HY	AB24 + NLC37
	3' flank + YG	AB27 + NLC38
<i>FGSG_09893</i>	5' flank + HY	AB7 + NLC37
	3' flank + YG	AB10 + NLC38
<i>FGSG_09896 (ICL1)</i>	5' flank + HY	AB28 + NLC37
	3' flank + YG	AB31 + NLC38
<i>FGSG_09900</i>	5' flank + HY	AB23 + NLC37
	3' flank + YG	AB14 + NLC38
<i>FGSG_09905</i>	5' flank + HY	AB15 + NLC37
	3' flank + YG	AB18 + NLC38
<i>FGSG_09906</i>	5' flank + HY	AB36 + NLC37
	3' flank + YG	AB39 + NLC38
<i>FGSG_09907 (FCV1)</i>	5' flank + HY	U120 + NLC37
	3' flank + YG	U123 + NLC38
<i>FGSG_09908 (PKAR)</i>	5' flank + HY	AB19 + NLC37
	3' flank + YG	AB22 + NLC38

Table 2.5. Nested primers used on flank-HY sections to increase product amounts. The generic reverse primer was used with the forward primer for *FGSG_09891.3*, *FGSG_09896.3 (ICL1)*, *FGSG_09900.3* and *FGSG_09906.3*.

Gene	Flank	Primer	Forward / Reverse	Primer sequence (5'-3')
<i>FGSG_09891</i>	5'	AB46	Forward	CAAGTGAAGGAAGAAGGGGAGA
<i>FGSG_09896 (ICL1)</i>	5'	AB48	Forward	GAAGGCCGAGCTAGGGAGTAGA
<i>FGSG_09900</i>	5'	AB58	Forward	AGGAATAAGGGTGCTCGTGGA
<i>FGSG_09906</i>	5'	AB59	Forward	GAGCTTCGTCAGCGTCCTTAGTAT
<i>Generic reverse primer</i>	5'	AB47	Reverse	TGCTGCTCCATACAAGCCAA
<i>FGSG_09908 (PKAR)</i>	5'	AB49	Forward	GAAAACCCGCTTCCAATCAA
		AB50	Reverse	TCCAGAAGAGGATGTTGGCG

Table 2.6. Primers to screen for the presence/absence of the target gene or *Hph* gene in transformants.

Gene	Primer	Primer sequence (5'-3')	Product length (bp)
<i>FGSG_09891</i>	AB68	TCATCTCCACCGAGGACGCT	1019
	AB69	TCTCAAGCTTCTCCTTTCTCG	
<i>FGSG_09893</i>	AB40	CTCCCCAGCTTGACGGTTTC	1476
	AB41	AAGTTTGAGGCTCCTCAGCAAC	
<i>FGSG_09895 (NTH1)</i>	AB5	CGCCACGTATCTACATCCC	800
	AB6	TTGGCACAGACTCCTTCGAG	
<i>FGSG_09896 (ICL1)</i>	AB70	GACAAACCCTTCTATTAACCCCG	1411
	AB71	AAATCGGGCAGCTTGGACTC	
<i>FGSG_09900</i>	AB51	GCCACACGATGTCAAAGATCAG	1490
	AB52	ATACAGCCGTGTCTCTGCCC	
<i>FGSG_09905</i>	AB42	AGAGTCCAGAGGCTGGGTCAA	423
	AB43	CTCCCAAGGCAAGTGCAACA	
<i>FGSG_09906</i>	AB53	ACATCTTCCCTGCCCCGTCTG	795
	AB54	CGGAAGAGTCGGTCTTGGTG	
<i>FGSG_09907 (FCV1)</i>	U130	CCGTCGCTCCTGCTTATCAC	650
	U131	ACTTCGGTTTCCAGCACTCG	
<i>FGSG_09908 (PKAR)</i>	AB44	TAGCCGGTGTGAAGTGGATCC	1243
	AB45	GGCCCTTTCTCCAGTCCCTT	
<i>Hph</i>	Hyg3	TCTCGGAGGGCGAAGAATCTC	834
	Hyg4	TTCTGCGGGCGATTTGTGTAC	

Table 2.7. Probes and restriction enzymes used for Southern hybridisation of wild-type and gene deletion strains.

Gene	Flank probe	Probe length (bp)	Restriction enzyme	Hybridising bands expected (bp)	
				Wild-type	Deletion
<i>FGSG_09891</i>	5'	800	<i>EcoRI</i>	5338	3351
	3'	383	<i>EcoRV</i>	2389	2998
<i>FGSG_09893</i>	5'	799	<i>SaI</i>	2127	3677
	3'	676	<i>SaI</i>	4314	3358
<i>FGSG_09895 (NTH1)</i>	5'	800	<i>EcoRV</i>	2.1 kb	0.75kb
	3'	1000	<i>HindIII</i>	0.9 kb and 3.8 kb	2.7 kb and 3.8 kb
<i>FGSG_09896 (ICL1)</i>	5'	750	<i>KpnI</i>	6646	3138
	3'	637	<i>PstI</i>	2653	3415
<i>FGSG_09900</i>	5'	267	<i>PstI</i>	5813	2547
	3'	708	<i>EcoRV</i>	1096 and 516	6263 and 516
<i>FGSG_09905</i>	5'	768	<i>EcoRI</i>	4143	1301
	3'	637	<i>BamHI</i>	3592	2373
<i>FGSG_09906</i>	5'	600	<i>EcoRI</i>	4143	2424
	3'	788	<i>HindIII</i>	2632 and 1869	707 and 1869
<i>FGSG_09907 (FCV1)</i>	5'	987	<i>SacI</i>	4223	3.9kb
	3'	428	<i>HindIII</i>	1869	747
<i>FGSG_09908 (PKAR)</i>	5'	1461	<i>HindIII</i>	1869	3317
	3'	873	<i>SaI</i>	3173	2462

Chapter 3. Location and Bioinformatic Analysis of the Chromosome I Micro-Region

3.1 Introduction

Numerous genes, proteins and metabolites have been shown, over the past 25 years, to play a role in the disease-causing ability of plant pathogens. In the earliest studies, either a biochemical or forward genetic approach to discovery was taken. However, there is now an increased availability of sequence information for plant pathogens and related saprobes, either as expressed sequence tags (ESTs) or as partial / fully sequenced genomes. As a result, in the vast majority of cases, a reverse genetics targeted gene deletion approach is now used in many species to reveal a requirement for the target gene in infection, symptom development and / or host range (Sexton and Howlett, 2006).

A pathogenicity factor is considered one whose requirement for the disease-forming ability of a pathogen is absolute. In contrast, a virulence factor contributes quantitatively rather than qualitatively to the disease development of the pathogen. Many common themes to pathogenicity and virulence have now been identified for plant- and animal-infecting pathogens, including particular protein families which co-ordinate intracellular processes, such as signal perception, signal transduction, transcription in the host and / or the production of host selective or host non-selective toxins (Examples may be found in Xu and Hamer, 1996; Kulkarni *et al.*, 2005; Egan and Talbot, 2008; Brefort *et al.*, 2009; Wilson and Talbot, 2009; Garcia-Pedrajas *et al.*, 2010). Increasingly, the selection of a gene for targeted deletion is made because that gene belongs to the same family as a previously determined pathogenicity or virulence factor in another species. The pathogen under investigation may have either a similar or dissimilar infection biology to that first species. Where functional redundancy is suspected, often the test pathogen chosen only has a single copy of the gene (Motteram *et al.*, 2009). Alternatively, searches for the presence / absence of specific protein domains (using Pfam or InterPro) or conserved motifs, such as the RXLR motif that is found in the secreted 'effector' molecules of oomycetes (Halder *et al.*, 2006), or the cysteine-rich Ecp proteins of *Cladosporium fulvum*

(DeWit and Joosten, 1999) are being used to identify related sequences that may play a role in virulence. These approaches, used either singly or in combination, may help to increase the rate of identification of pathogenicity or virulence factors of select protein families but do not help in the direct discovery of novel sequence unrelated factors that may also play a role in disease.

The bioinformatic comparison of fully sequenced genomes is now becoming possible due to the rapidly growing number of sequenced species (Comprehensive Phytopathogen Genomics Resource (CPGR, <http://cpgr.plantbiology.msu.edu/>); Genome OnLine Database (GOLD, www.genomesonline.org) and is providing a wealth of new data and investigation techniques. Comparisons of the genomes of different plant pathogenic species (Soanes *et al.*, 2007) have already revealed that large percentages of the predicted genes contained in pathogen genomes are unique to that particular species (Cuomo *et al.*, 2007; Wapinski *et al.*, 2007). In addition, many genes possess no functional annotation and may show only weak similarity to genes in other species. Retrospectively, a number of the previously identified avirulence genes have been identified as either species- or formae speciales-specific (Khang *et al.*, 2008; Ma *et al.*, 2010). Similarly, many biosynthetic clusters which result in the synthesis of specific host selective or host non-selective toxins have now been identified to be unique to just one or a few related plant pathogenic species (Brown *et al.*, 2002).

To identify new classes of virulence / pathogenicity determinants, other techniques in addition to forward genetics and mutant screening, which still has a success rate of only ~1% (Jeon *et al.*, 2007), should be considered. As the deluge of genomic sequence data continues to increase, this provides the opportunity for the development of novel bioinformatic/statistical-based methods to study pathogenicity. One way this can be achieved is to investigate the arrangement of pathogenicity and virulence genes across and within the chromosomes of individual pathogenic species and taxonomically related pathogenic species. Various gene types and gene families have been shown to be unevenly distributed in the genome. For example the *in planta* expressed genes of *Fusarium graminearum* are predominantly located in the sub-telomeric regions of the genome (Cuomo *et al.*, 2007). Very recently, some of the smallest chromosomes of the tomato-infecting vascular wilt pathogen *Fusarium*

oxysporum f sp *lycospersici* were shown to be transmittable between individual isolates, are rich in transposon sequences, and contain the genes required to alter the host species range of the recipient isolate (Ma *et al.*, 2010).

Several examples of clustering of genes important to virulence or pathogenicity are already known. In bacteria, genes required for pathogenicity are often grouped together in regions known as pathogenicity islands. These genomic regions, usually ranging in size from 10-200 kb, are characterised by a number of shared features, including a GC content and codon usage that differs from the rest of the genome, the presence of direct repeat sequences flanking the island and often contain genes or sequences enabling genetic mobility. Also these regions are frequently found to be absent from the genomes of closely related strains, exhibit instability and may be located close to tRNA loci (Hacker *et al.*, 1997; Hentschel and Hacker, 2001). Pathogenicity islands may contain genes encoding proteins for the production of adherence factors, metabolite acquisition, host cell entry and secretion systems and toxin production (Galán and Collmer, 1999; Hentschel and Hacker, 2001). For example, in the plant pathogenic bacterium *Pseudomonas syringae*, the *HRP* gene cluster encodes proteins that are used to form a type-III secretion system (T3SS) that is used for the secretion of effectors into the host (Brown, I. *et al.*, 2001; Jin and He; 2001; Li *et al.*, 2002).

Other types of gene clustering required for pathogenicity have been found in a number of fungal species. In several species, the clustered genes encode proteins required for biosynthesis or degradation of a particular metabolite. Examples include the *TRI* gene cluster of *F. graminearum* whose products are required for biosynthesis of B-type trichothecene mycotoxins, polyketide synthase clusters and non-ribosomal peptide synthetases (Brown, D.W. *et al.*, 2001, 2004; Varga *et al.*, 2005; Gaffoor and Trail, 2006). These biosynthetic clusters often contain transcriptional regulators which co-ordinate the expression of both the neighbouring genes as well as others elsewhere in the genome (Proctor *et al.*, 1995; Hohn *et al.*, 1999). The recent analysis of the *F. solani* (telomorph *Nectria haematococca*) genome (Coleman *et al.*, 2009) has confirmed the pea pathogenicity (*PEP*) genes are clustered together and are present on a 1.6 Mb supernumerary chromosome (Han *et al.*, 2001). The *PEP* genes include *PDA1*, which encodes a cytochrome P450 monooxygenase

enzyme known as pisatin demethylase, which serves to detoxify pisatin, an anti-microbial compound released from the pea host upon infection. In the basidiomycete fungus *Ustilago maydis*, the causal agent of corn smut, a large number of secreted protein genes (roughly one-fifth of the total secretome) are grouped together into twelve clusters containing from 3 to 26 genes (Kamper *et al.*, 2006; Dean, 2007; Howlett *et al.*, 2007). To date, five of these clusters have been shown to play a role in virulence of this pathogen. Unlike other fungal cluster types identified, no transcriptional regulators are found in the *U. maydis* secreted protein clusters and only a few of these clusters contain any genes which code for proteins which are not secreted. While most secondary metabolite clusters in the human pathogen *Aspergillus fumigatus* are found in heterochromatin near telomeric regions (Nierman *et al.*, 2005; Howlett *et al.*, 2007), the *U. maydis* secreted protein clusters are distributed randomly in the genome (Howlett *et al.*, 2007).

A genome-wide analysis approach was applied to *F. graminearum* to search for novel clusters of genes contributing to disease development. So far the only gene cluster types identified in *Fusaria* are those that encode proteins constituting biosynthesis systems for various secondary metabolites, such as deoxynivalenol, nivalenol and T-2 toxin (Hohn *et al.*, 1999, Brown *et al.*, 2001, 2004) and other clusters encoding proteins for the biosynthesis of polyketides and non-ribosomal peptide synthases (Varga *et al.*, 2005; Gaffoor and Trail, 2006). As described earlier, the ascomycete species *F. graminearum* is one of the main causal agents of the globally destructive Fusarium Ear Blight (FEB) disease (Snijders, 1994; Bai and Shaner., 2004), which lowers grain yield, reduces grain quality due to the loss of starch granules and cell walls, and contaminates grain with a range of mycotoxins (Gang *et al.*, 1998; Snijders, 2004). The sequenced genome of *F. graminearum* strain PH-1 with 10x coverage was made publicly available in 2003 at the Broad Institute and then successfully aligned to the four chromosomes (Cuomo *et al.*, 2007). Since then the sequenced genomes of two other plant pathogenic *Fusarium* species have become available, namely the tomato vascular wilt *F. oxysporum* f. sp. *lycopersici* and the cereal infecting *F. verticilloides* (Ma *et al.*, 2010). By aligning the sequence to the chromosome complement of each species and then inter-comparing the genome organisation of the three species, both species-specific

as well as conserved core regions of the fusaria genomes were identified (Ma *et al.*, 2010).

In this study, several bioinformatics resources were used in combination with statistics to locate putative disease development contributors in the genome of *F. graminearum*. As the starting point for this investigation, it was hypothesised that 'hotspots' for genes required for disease symptom spread are maintained in clusters in the genomes of closely related pathogenic species. By using a set of curated sequences, previously demonstrated to contribute to infection and disease formation in one or more plant or animal infecting species (Winnenburg *et al.*, 2006), several putative hotspots were identified.

The identification and bioinformatic analysis of one such hotspot is described in this chapter. Subsequent chapters describe the investigation of the function of several genes in this hotspot region including several that showed either no homology to any annotated genes or no homology to previously identified virulence determinants.

3.2 Methods

Fusarium graminearum homologues of Pathogen-Host Interactions Database (PHI-base, www.phi-base.org, Version 2.1) entries were located in the published PH-1 strain genome sequence (www.broadinstitute.org, www.helmholtz-muenchen.de/mips, version FG3) using a Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) with a expectation value cut-off of 1×10^{-100} . The location of hits was displayed on the *F. graminearum* chromosomes using the OmniMapFree software (J. Antoniw *et al.*, unpublished, www.omnimapfree.org). Putative gene function was assigned by BLAST search for homologues in the non-redundant protein sequences (nr) database held at the NCBI (www.ncbi.nih.gov, October 2007) with a cut-off score of $1e^{-40}$. Interspecies comparisons were made using a FASTA comparison tool at Rothamsted (<http://babnfs.fasta>, Lipman and Pearson, 1985) against selected published genome sequences (www.broadinstitute.org, www.jgi.doe.gov/genome-projects). Similarity scores were calculated as identities x overlap / query length. Relative genome location of reciprocal best

hits to the cluster genes in three sequenced *Fusarium* species, namely *F. verticillioides* (strain 7600, version FV2), *F. oxysporum f. sp. lycopersici* (strain 4287 version FOXY3) and *F. solani* (*Nectria haematococca*) (strain MPV1 77-13-4, version 2.0) was determined from the respective genome browser. Additional species used for comparative genomics in this manner were *Trichoderma reesei* (strain QM6a, version 2), *Neurospora crassa* (strain OR74A, version 4), *Magnaporthe oryzae* (strain 70-15, version 5), *Ustilago maydis* (strain 521, version 1) and *Mycosphaerella graminicola* (strain IPO323, version 3).

Repetitive elements in DNA were identified using a DotPlot software programme (J. Antoniwi). The nucleotide sequence of the region under study was plotted against itself and matching sequences highlighted to show repetitive sequence using a window of 20 nucleotides with a stringency of 15 matches. The percentage GC content was determined using the DNA motifs software programme (J. Antoniwi). The online TMHMM server www.cbs.dtu.dk/services/TMHMM/ was used to analyse the presence of predicted transmembrane protein segments. The online SignalP server www.cbs.dtu.dk/services/SignalP/ was used to search for predicted signal peptide sequences. The WoLF PSORT program (<http://wolfpsort.org>) was used to predict sub-cellular localisation of proteins. Global sequence alignments were performed using the ClustalW and EMBOSS tools at the European Bioinformatics Institute (www.ebi.ac.uk) with default parameters.

The distribution of virulence/pathogenicity factor homologues on chromosome I was analysed statistically by Sue Welham (Rothamsted Research) using a Chi-Square test. The Chi-Square test examined whether the proportion of the total number of genes identified as virulence/pathogenicity gene homologues is higher within the micro-region than outside it (i.e. the remainder of chromosome I of the *F. graminearum* genome). The null hypothesis is that such homologues exhibit a random distribution across the chromosome. The Chi-Square statistic assesses whether the observed frequency of virulence/pathogenicity homologues within the micro-region is greater than that expected if such genes show a random distribution. The test statistic shows an asymptotic (large-sample) distribution. However, this distribution does not hold when the expected frequencies are <5, as in the case of the micro-region. The permutation testing

method of Roff and Bentzen (1989) was therefore applied to obtain a valid p-value for the test statistic. The micro-region was defined as *FGSG_09891* to *FGSG_09908*.

3.3 Results

3.3.1 Identification of a Close Grouping of Virulence/Pathogenicity Gene Homologues in the *Fusarium graminearum* Genome

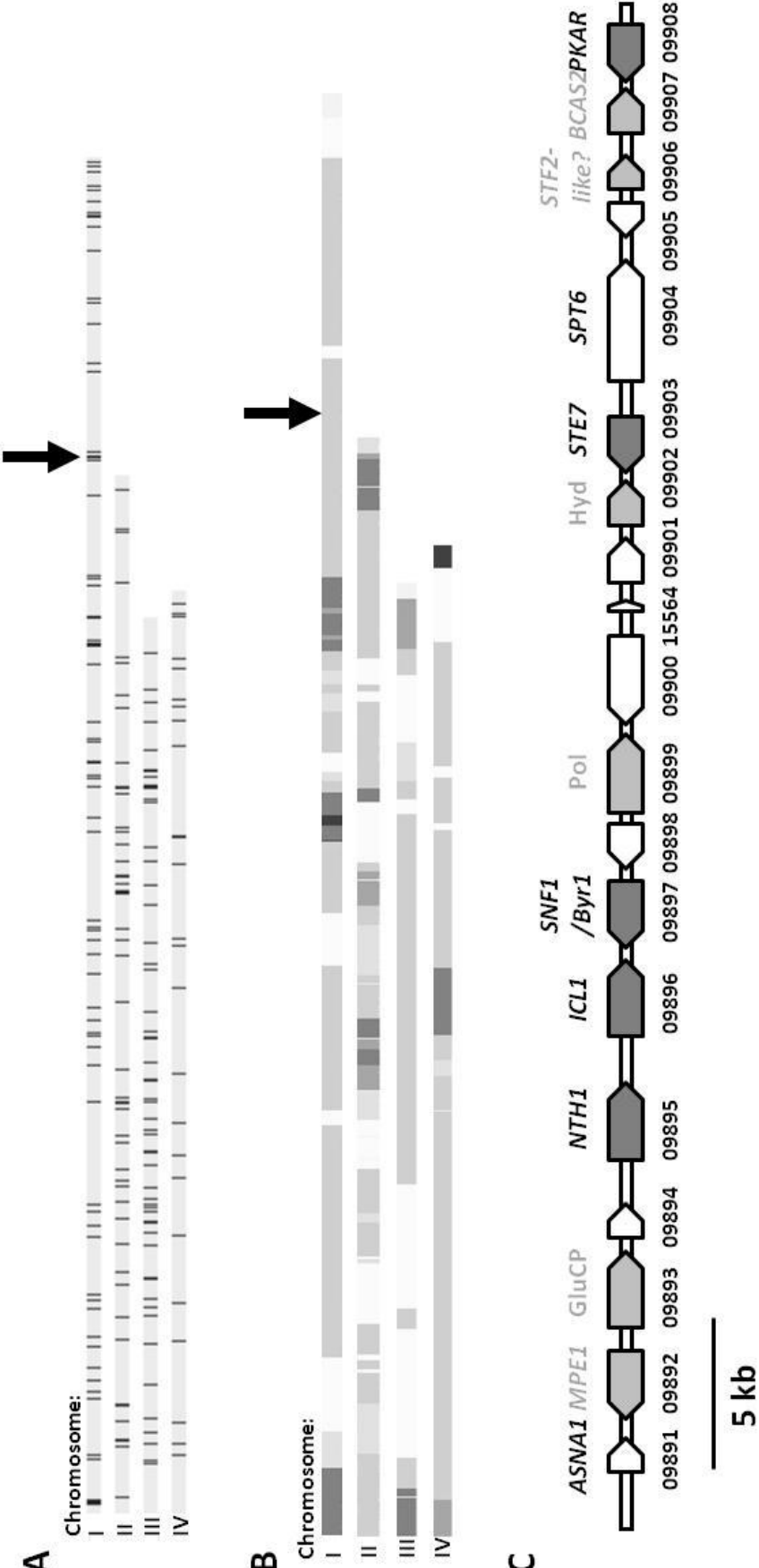
The location in the *F. graminearum* genome of homologues of verified pathogenicity / virulence genes in *Fusarium* and other species published prior to 2007 is shown in **Figure 3.1A**. A total of 211 hits were identified over the four chromosomes. A visual inspection of this gene distribution indicated the presence of a close grouping of five such gene homologues in a 37.6 kb, 15 gene region on chromosome 1 (shown by black arrow). A Chi-Square statistical analysis (see Methods) revealed that the presence of this number of homologues in the region deviated from the number expected for a random distribution of such homologues. The frequency of virulence/pathogenicity gene homologues within the micro-region (5 homologues in a 19 gene region, or an average of 0.26 homologues per gene) was significantly greater than that expected from a random distribution (0.4 homologues in a 19 gene region, or an average of 0.02 homologues per gene) ($p < 0.001$, **Table 3.1**). By comparing these results with the genetic map available for a cross between the sequenced strain PH-1 and a second strain from the same genetic lineage (Gale *et al.*, 2005) the identified micro-region was found to reside within a region of low recombination frequency (**Figure 3.1B**).

Table 3.1. Count (and percentage) of virulence/pathogenicity gene homologues and other genes on chromosome 1, categorized by presence in the micro-region. The micro-region on chromosome 1 (FGSG_09891-FGSG_09908) appears to contain a higher density of virulence/pathogenicity gene homologues than would be expected from a random distribution of such genes. This was confirmed by a chi-square test on the following table. The chi-square test statistic is 54.99 on 1 df ($p < 0.001$). Analysis by Sue Welham (Rothamsted Research).

	Outside micro-region	Inside micro-region
Vir/path homologues	87 (94.6)	5 (5.4)
Other genes	4314 (99.7)	14 (0.3)

Figure 3.1. (See over). Identification of a virulence gene homologue-enriched micro-region on chromosome I of *Fusarium graminearum*. **A.** The distribution of homologues of PHI-base entries in the *F. graminearum* genome is not random. Each horizontal grey bar represents one of the four *F. graminearum* chromosomes drawn to scale. The lengths of the chromosomes in Mbps are I - 11.685, II – 8.916, III – 7.766 and IV – 8.076. Each thin vertical line indicates the location of a gene homologous to a verified virulence gene contained in the PHI-base database. The thickness of vertical lines is representative of gene length. A significant ($p < 0.001$) grouping of such homologues occurs on chromosome I (indicated by black arrow). **B.** The virulence gene homologue micro-region is located in an area of low recombination frequency. The frequency of recombination is shown for a cross between the sequenced strain PH-1 and a second USA strain, MN00-676. Darker sections indicate increased recombination frequency. The absolute recombination frequency ranges from zero to >8 cM between consecutive genetic markers (Cuomo et al., 2007). The location of the virulence gene homologue micro-region is indicated by a black arrow. **C.** The gene content of the virulence gene homologue-enriched micro-region on chromosome I of *F. graminearum*. The genes homologous to known virulence genes are shaded dark grey. Five such genes are here contained in a 37.6 kb, 15-gene region. Genes with similarity to other annotated genes are shaded light grey. Gene IDs are shown in the format: FGSG_XXXXX.3. Genes are labelled with either names from publication or genome locus annotation (shown in black) or similarity to other annotated genes is indicated (shown in grey). Abbreviations: GluCP = glutamate carboxypeptidase, Pol = DNA polymerase epsilon subunit B, Hyd = HAD superfamily hydrolase.

Figure 3.1



The chromosome 1 pathogenicity/virulence gene homologue micro-region stretches from *FGSG_09895* to *FGSG_09908* (**Figure 3.1C**) and contains a total of 15 genes. The five genes homologous to the previously published pathogenicity/virulence genes are *FGSG_09895*, *FGSG_09896*, *FGSG_09897*, *FGSG_09903* and *FGSG_09908*. A small number of genes in the micro-region are annotated in the *F. graminearum* genome databases with a putative function for the proteins they encode. These are *FGSG_09895* (neutral trehalase), *FGSG_09896* (isocitrate lyase, published as *GzICL1* (Lee *et al.*, 2009a)), *FGSG_09897* (carbon catabolite derepressing protein kinase, also known as *GzSNF1* (Lee *et al.*, 2009b)), *FGSG_09902* (protein similar to HAD superfamily hydrolase), *FGSG_09903* (protein kinase *BYR1*, also published as *STE7* (Ramamoorthy *et al.*, 2007)), *FGSG_09904* (transcription elongation factor *SPT6*) and *FGSG_09908* (cAMP-dependent protein kinase regulatory subunit). To assess the possible function of the remainder of the genes in the micro-region, a BLASTp search was used to identify homologous genes in other species (see Methods). This revealed the similarity of *Fgsg_09899* to DNA polymerase epsilon subunit B, some limited similarity of *Fgsg_09906* to Stf2-like proteins (an ATP synthase regulatory factor) and of *Fgsg_09907* to Bcas2 family/domain proteins. *Fgsg_09900* was predicted to contain seven transmembrane spanning segments. Analysis of predicted sub-cellular localisation further suggested a plasma membrane location for *Fgsg_09900*. This analysis also predicted a peroxisome/glyoxysome location for *Icl1*. Isocitrate lyase functions in the glyoxylate cycle and this location is consistent with this function for *Icl1*. The remaining four genes in the micro-region did not show significant similarity to any previously annotated genes or any obvious motifs.

For the follow up bioinformatics analyses, the micro-region under study was expanded slightly to include *FGSG_09891* to *FGSG_09894*, situated immediately to the left of *FGSG_09895*, due to their proximity to the group of three virulence gene homologues (*FGSG_09895*-*FGSG_09897*). *FGSG_09891* is annotated as encoding an arsenical pump-driving ATPase (*ASNA1*), *FGSG_09892* as encoding a protein similar to retinoblastoma-binding protein and *FGSG_09893* as specifying a protein similar to glutamate carboxypeptidase. *Fgsg_09893* may possess a signal peptide sequence but the

match was of limited quality. A BLASTp search (see Methods) revealed similarity of Fgsg_09891 to the Get3/Arr4 protein of *S. cerevisiae* and of Fgsg_09892 to the protein Mpe1, which has been reported in *S. cerevisiae* as an essential component of the cleavage and polyadenylation factor in mRNA processing (Vo *et al.*, 2001). None of these additional protein sequences have functions previously linked to pathogenicity. These data are summarised in **Table 3.2**.

Table 3.2. Gene content of the *F. graminearum* chromosome I micro-region. Gene locus IDs are shown together with annotation from the *F. graminearum* genome browsers (see Methods), Similarity of the proteins encoded by these genes to other proteins determined by Blastp and any identified protein domains are also indicated (see Methods).

Gene	Genome annotation	Blastp	Domains (Pfam)
<i>FGSG_09891</i>	<i>ASNA1</i>	Similar to Get3/Arr4	Arsenite-activated ATPase (arsA) (PF02374)
<i>FGSG_09892</i>	<i>Retinoblastoma-binding protein-like</i>	Similar to Mpe1	DWNN (PF08783)
<i>FGSG_09893</i>	<i>Similar to glutamate carboxypeptidase</i>		Peptidase family M20/M25/M40 (PF01546) Peptidase dimerisation domain (PF07687) Yippee putative zinc-binding protein (PF03226)
<i>FGSG_09894</i>			Trehalase (PF01204)
<i>FGSG_09895</i>	<i>NTH1</i>		Neutral trehalase Ca ²⁺ binding domain (PF07492)
<i>FGSG_09896</i>	<i>ICL1</i>		Isocitrate lyase family (PF00463)
<i>FGSG_09897</i>	<i>SNF1</i>		Protein tyrosine kinase (PF07714) Protein kinase domain (PF00069) Ubiquitin associated domain (UBA) (PF08587)
<i>FGSG_09898</i>		Similar to DNA polymerase epsilon subunit B	
<i>FGSG_09899</i>			DNA polymerase alpha/epsilon subunit B (PF04042)
<i>FGSG_09900</i>			
<i>FGSG_15564</i>			
<i>FGSG_09901</i>			
<i>FGSG_09902</i>	<i>Similar to HAD superfamily hydrolase</i>		Haloacid dehalogenase-like hydrolase (PF00702)
<i>FGSG_09903</i>	<i>BYR1/STE7</i>		Protein kinase domain (PF00069)
<i>FGSG_09904</i>	<i>SPT6</i>		SH2 domain (PF00017) S1 RNA binding domain (PF00575)
<i>FGSG_09905</i>			
<i>FGSG_09906</i>		Similar to Stf2-like proteins	
<i>FGSG_09907</i>		Similar to Bcas2 family/domain proteins	Breast carcinoma amplified sequence 2 (BCAS2) (PF05700)
<i>FGSG_09908</i>	<i>PKAR</i>		Cyclic nucleotide binding domain (PF00027)

3.3.2 Comparison of Microsynteny with Other Fungal Genomes

A comparison of microsynteny of the micro-region across four sequenced *Fusarium* species and the closely related non-pathogenic species *Trichoderma reesei* (Goodwin, 2004; Martinez *et al.*, 2008; Coleman *et al.*, 2009; Ma *et al.*, 2010) revealed an exceptionally high degree of conservation of the micro-region across the five species (**Figure 3.2**). The order and orientation of the genes in the micro-region appear to be well conserved between the five species. In *F. oxysporum*, one, and in *F. solani*, five additional genes are present in the centre of the micro-region. The *F. graminearum* homologues of these additional genes are not located close to the micro-region in this species. A homologue of FGSG_15564 is present in *F. solani* but at a different genomic locus, while one gene, FGSG_09905, appeared to be specific to *F. graminearum*. However, a tBlastn analysis indicated that sequences of limited homology to FGSG_09905 are present in *F. verticillioides* and *F. oxysporum* at the corresponding locus, suggesting the possibility that a non-functional homologue of FGSG_09905 may be present in these two species or that an incorrect gene call has been made in one or more of the species. Similarly, tBlastn revealed sequences with limited similarity to FGSG_15564 at the corresponding locus in *F. verticillioides* and *F. oxysporum*. In *F. oxysporum* this sequence was present within the locus of the additional gene present in the micro-region. Overall, the micro-region site is highly conserved in the four *Fusaria* and also in the related fungus *T. reesei*. A wider analysis was also performed and included the rice blast fungus *Magnaporthe oryzae*, the wheat leaf pathogen *Mycosphaerella graminicola*, the model fungus *Neurospora crassa* and the maize pathogen *Ustilago maydis* (**Figure 3.3**). These species contain more fragmented versions of the micro-region, broken down into blocks of mostly 2 or 3 genes or single genes located at multiple different genomic loci. Therefore the intactness of the micro-region appears only to be retained between the *Fusaria* and very closely related taxa.

Figure 3.2. (See over). The micro-region exhibits a high degree of conservation across four sequenced *Fusarium* species and the related saprophyte *Trichoderma reesei*. Darker shading is used to indicate genes with greater similarity to their *F. graminearum* counterpart. Protein sequences were compared using the FASTA algorithm (Lipman and Pearson, 1985) and scores calculated as: Identities x Overlap / Length. *F. graminearum* homologues of known virulence genes are shaded grey. Gaps indicate genuine spaces in the genome where no gene is called. *FGSG_15564 is called only at the MIPS *F. graminearum* database (mips.helmholtz-muenchen.de/genre/proj/fusarium) and not at the Broad database (www.broad.mit.edu). The *F. solani* homologue of FGSG_09901 was located using a tBlastn search as a homologue of this gene but has so far not been called on the *F. solani* genome browser (genome.jgi-psf.org/Necha2/Necha2.home.html), yet this search yielded a high-scoring hit. *F. graminearum* genes are labelled with gene IDs in the format FGSG_098xx or FGSG_099xx where appropriate.

Figure 3.2

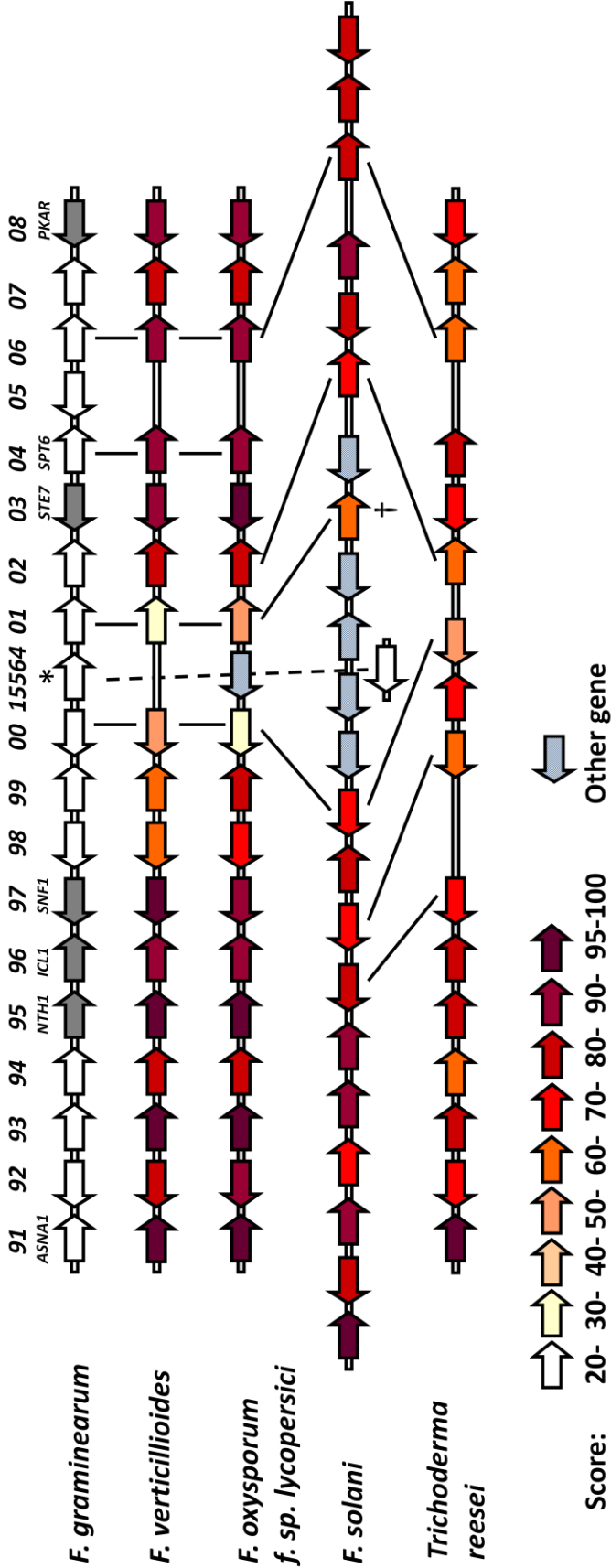
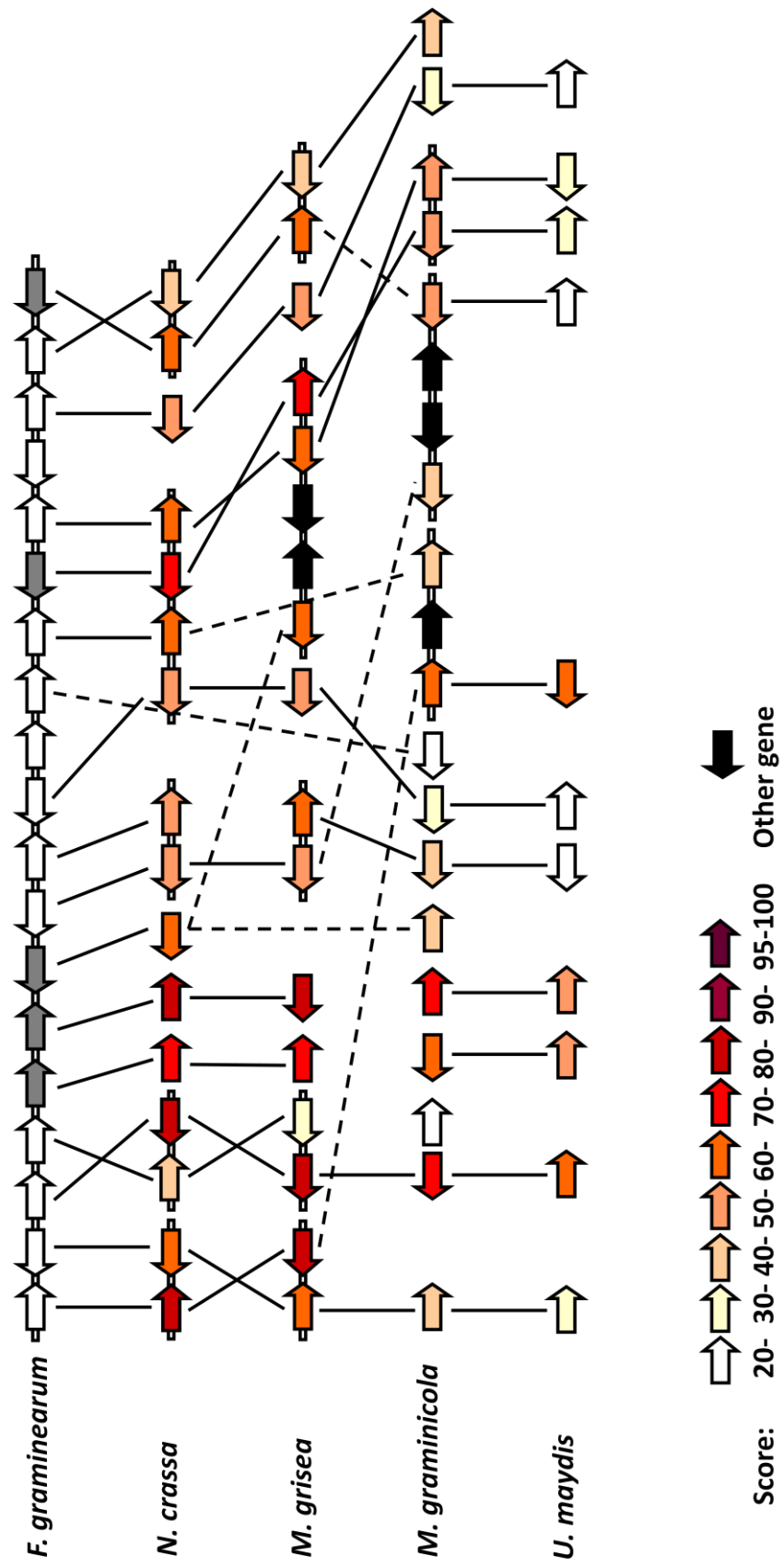


Figure 3.3. (See over). Conservation of the micro-region breaks down in more distantly related species. The cluster of *F. graminearum* was examined in the model filamentous fungus *Neurospora crassa*, together with three plant pathogenic species: the rice blast fungus *Magnaporthe oryzae*, *Mycosphaerella graminicola* the cause of Septoria tritici blotch disease on wheat leaves, and the Basidiomycete *Ustilago maydis*, the causal agent of corn smut. Protein sequences were compared using the FASTA algorithm (Lipman and Pearson, 1985) and scores calculated as: Identities x Overlap / Length.

Figure 3.3

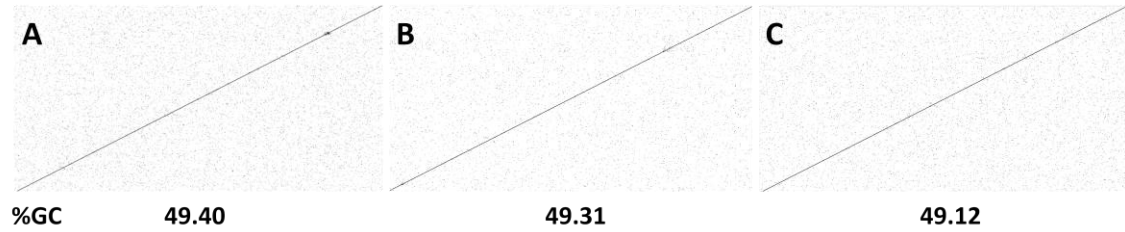


3.3.3 Other Features of the Identified Micro-Region

Bacterial pathogenicity islands are characterised by repetitive sequences that flank the island (Hentschel and Hacker, 2001). The genome of *F. graminearum* contains a low content of repetitive sequence, most probably due to the presence of the mechanism of Repeat-Induced Polymorphism (RIP), which acts to remove repetitive nucleotide sequence (Cuomo *et al.*, 2007). In common with the rest of the *F. graminearum* genome, the micro-region region possesses little repetitive sequence and unlike bacterial pathogenicity islands, no evidence was found for repetitive sequence-rich flanks adjacent to the micro-region (**Figure 3.4**). A 70 kb region immediately to the left of the micro-region and 60 kb immediately to the right of the micro-region were analysed for the presence of repetitive sequence. While small occasional (20-50 nt) repeats were noted, possibly representing promoter or protein elements, no extensive repetitive sequence was found. The densely dotted region in the plot for the region 'left' of the micro-region is found within the *FGSG_09887* gene and probably represent repeated protein elements. Pathogenicity islands in bacteria are also characterised by a GC content distinct from the rest of the genome (Hentschel and Hacker, 2001). GC content was also analysed for the micro-region and flanking regions. The average percent GC of the micro-region region was 49.31% (**Figure 3.4**), similar to the whole genome average of 48.33% (Broad Institute), and very similar to that of the two flanks. The micro-region also appears to be present in a region of low recombination frequency (**Figure 3.1B**).

The pattern of expression of the micro-region genes was analysed using published Affymetrix datasets (Güldener *et al.*, 2006). A heatmap depicting the expression of micro-region genes during infection of barley ears over a 144 h period (**Figure 3.5A**) indicates that the genes of the cluster are not subject to co-ordinate regulation during infection of this host species. By contrast, a number of genes of the *TRI* trichothecene biosynthesis cluster (**Figure 3.5B**), appear to exhibit co-ordinated expression and are highly induced after 48 h post infection.

Figure 3.4. The chromosome I micro-region is not flanked by highly repetitive sequences. Dotplots are shown for the following regions: **(A)** The 70.8 kb region immediately to the left of the micro-region. **(B)** The micro-region itself (from *FGSG_09891* to *FGSG_09908*, 47.4 kb). **(C)** The 58.2 kb region immediately to the right of the micro-region. A 200 nt undefined (NNNN) region was removed from the sequence for panel (A).



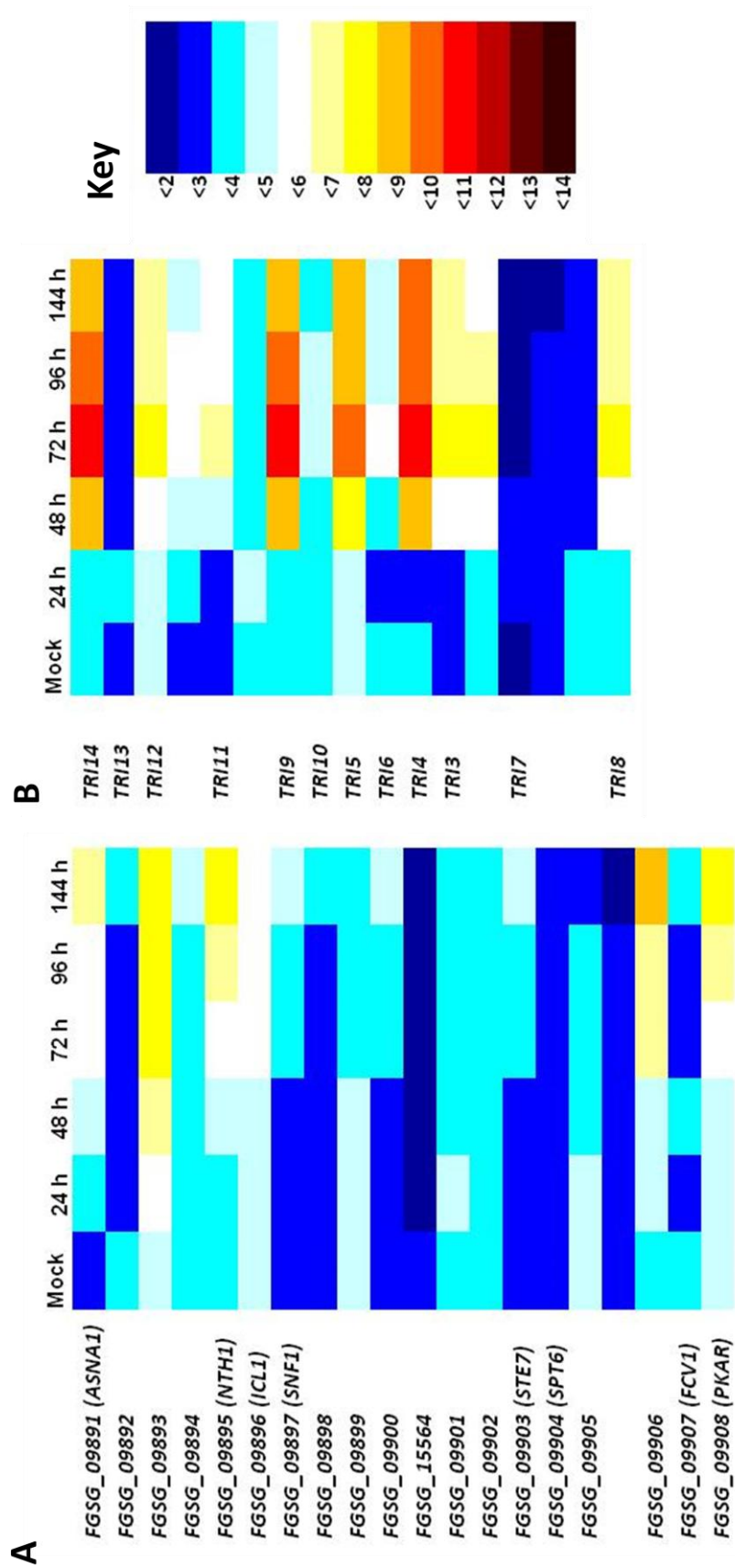


Figure 3.5. The expression of different genes in the micro-region appear not to be co-ordinate regulated. Expression of (A) the virulence gene homologue micro-region on chromosome 1 and (B) the trichothecene biosynthesis *TRI* cluster on chromosome 2 during *F. graminearum* PH-1 infection of barley ears over a 144 h period. Gene IDs/names are given at the left for each locus. Each line represents a single probe set. Values are coloured according to the normalised expression value data published in Guldener et al. (2006) (see key at right).

3.4 Discussion

A bioinformatics approach was used to identify a virulence gene-enriched region on chromosome I of the globally important plant pathogen *F. graminearum*. By plotting the homologues of known virulence/pathogenicity genes onto the chromosomes of *F. graminearum* using the PHI-base database, a micro-region exhibiting a grouping of virulence gene homologues significantly greater than expected from a random distribution was identified on chromosome I (**Figure 3.1**). The PHI-base database, since its launch in 2005, has also been used in a number of other publications to assist in the annotation of pathogenicity gene homologues. For example, Jeon *et al.* (2007) searched the contents of PHI-base to determine the homology of pathogenicity genes identified in a large forward genetics insertional mutagenesis experiment involving *Magnaporthe oryzae* to those in other species. In addition, DiGuistini *et al.* (2007) used PHI-base to annotate pathogenicity gene homologues in an EST collection from a pathogenic fungus. However, the method presented here, by combining the PHI-base database and OmniMap software, allows the prediction of possible virulence/pathogenicity gene location based on position relative to other such genes.

This new *F. graminearum* micro-region appears to be different to previously characterised fungal gene clusters. The *F. graminearum* micro-region, which is conserved across the four sequenced *Fusarium* species, does not contain a group of genes whose products are predicted to be secreted (only one possesses a very weak similarity to a signal peptide sequence), nor does it encode a series of enzymes for the biosynthesis of a particular metabolite. The genes of the micro-region are not subject to coordinate regulation and the micro-region appears not to possess a distinct GC content or repetitive sequence flanks. This micro-region could therefore represent the first example of a potentially novel type of virulence-associated gene cluster.

Previously characterised gene clusters in *Fusarium* species include the *TRI* trichothecene mycotoxin biosynthesis cluster for the production of secondary metabolites such as deoxynivalenol, nivalenol and T-2 toxin (Hohn *et al.*, 1999, Brown, D.W. *et al.*, 2001, 2004) and other clusters for the biosynthesis of other secreted metabolites, such as zearalenone, which require specific polyketides

synthases and / or non-ribosomal peptide synthases (Varga *et al.*, 2005; Gaffoor and Trail, 2006). In addition, a recently located cluster of *F. graminearum* genes (gene IDs *FGSG_08077* to *FGSG_08084*) appears to encode a biosynthesis cluster for the mycotoxin butenolide (Harris *et al.*, 2007). In *F. solani*, four genes contributing to pathogenicity on pea plants are located in a 25 kb cluster termed the *PEP* (pea pathogenicity) gene cluster (Han *et al.*, 2001). The *PEP* cluster possesses a GC content distinct from that of the rest of the *F. solani* genome, similar to bacterial pathogenicity islands, and also exhibits coordinated gene regulation (Han *et al.*, 2001; Liu *et al.*, 2003), indicating similarity of the *F. solani PEP* cluster to pathogenicity islands of bacteria and some of the fungal biosynthetic gene clusters. In both *F. solani* and *F. oxysporum* f sp *lycopersici* the genes providing factors to confer virulence towards different host plants are located on small supernumerary chromosomes (Han *et al.*, 2001; Ma *et al.*, 2010). The *F. graminearum* micro-region is distinct from these other two examples, being present on a core essential chromosome and this species appearing to lack supernumerary chromosomes. Bacterial pathogenicity islands are also usually flanked by highly repetitive DNA sequences, which, together with their atypical GC content, is considered evidence of their evolutionary movement by horizontal gene transfer. In addition, the genomic islands of the fungal pathogen *A. fumigatus* show an enrichment of repetitive elements (Fedorova *et al.*, 2008). No indication of highly repetitive DNA sequences flanking the chromosome I cluster in *F. graminearum* was found.

The *F. graminearum* genome comprises only four chromosomes, however these chromosomes are thought to be the result of the ancient fusion of smaller chromosomes of a progenitor species, as indicated by the chromosomal distribution of single nucleotide polymorphisms (SNPs) between two sequenced strains (Cuomo *et al.*, 2007). Regions of high SNP density in both the sub-telomeric regions and specific inner regions of each chromosome were correlated with a high level of recombination and the presence of genes specifically expressed *in planta* that would be expected to play a role in plant-fungal interactions (Cuomo *et al.*, 2007). In contrast, the new *F. graminearum* micro-region is present in a region of low SNP and low recombination frequency (**Figure 3.1B**, Beacham *et al.*, 2009; Cuomo *et al.*, 2007). The low

recombination frequency of the region could perhaps contribute to the maintenance of the micro-region by providing a 'quiet' region of low genomic activity to allow the collection and maintenance of important virulence-related genes. The low recombination frequency regions of the *F. graminearum* genome also appear to contain several gene clusters that, unlike the micro-region characterised here, contain genes that share transcriptional directionality, similar to bacterial operons, including one very nearby the characterised group at *FGSG_09910* to *FGSG_09914* (J. Antoniwi and K. Hammond-Kosack, unpubl.) Such gene clustering may represent either biosynthetic, virulence or another class of function.

The *F. graminearum* micro-region is highly conserved in several *Fusarium* species including *F. solani* and also in the closely related saprophyte *T. reesei*. This conservation in a non-pathogenic species could represent insufficient evolutionary distance between the *Fusaria* and *T. reesei* for breakdown of the micro-region to be noticeable. Alternatively, differential regulation of the micro-region between the pathogenic and non-pathogenic species may explain the presence of a highly sequence related micro-region in two fungal taxa with differing lifestyles (see General Discussion). The investigation of more distantly-related species indicates that these contained more fragmented copies of the micro-region (**Figure 3.3**). This finding suggests that this particular micro-region could possibly be specifically important in contributing to the virulence of *Fusarium* species. This however, leads to the questions of whether similar micro-regions containing a different complement of genes are key to the disease-causing ability of other pathogenic species.

With the micro-region identified, the next step was to characterise the function of the genes in the disease-causing ability of *F. graminearum*. Firstly, the role of the virulence/pathogenicity gene homologues in the micro-region in *F. graminearum* virulence was investigated to determine if this site contained genes verified to support FEB symptom development and this is described in the next chapter.

Chapter 4. Initial Characterisation of the Virulence/Pathogenicity Gene Homologues in the Micro-Region

4.1 Introduction

In locating the micro-region of pathogenicity/virulence gene homologues on chromosome 1 of *F. graminearum*, the first aim was to confirm the role of these homologues (*NTH1*, *ICL1*, *SNF1*, *STE7* and *PKAR*) in the disease-causing ability of this species. If a role of the micro-region in disease development was highlighted, this would then suggest the possibility that this site is important for harbouring other genes contributing to disease symptom spread. Progression could then be made to the targeted deletion of additional genes in the micro-region in the hope of revealing the presence of novel factors involved in this process.

Two of the virulence gene homologues residing within the micro-region, namely *SNF1* and *STE7* have been characterised elsewhere and have been shown to contribute to *F. graminearum* virulence (Lee *et al.*, 2009b; Ramamoorthy *et al.*, 2007). An independent deletion of the *SNF1* gene in the sequenced strain PH-1 has been performed by Martin Urban. The results are illustrated in **Figure 4.1** for completeness. This indicated that the *snf1* strain exhibited reduced virulence on wheat ears (**Figure 4.1A**), reduced growth *in vitro* on select carbon sources but not on complete medium (**Figure 4.1B** and data not shown) and retained the ability to produce the sexual fruiting bodies, perithecia (**Figure 4.1C**). The targeted deletion of the remaining three homologues, *NTH1*, *ICL1* and *PKAR* was undertaken in this project and is reported in this chapter together with initial characterisation of the deletion strains obtained.

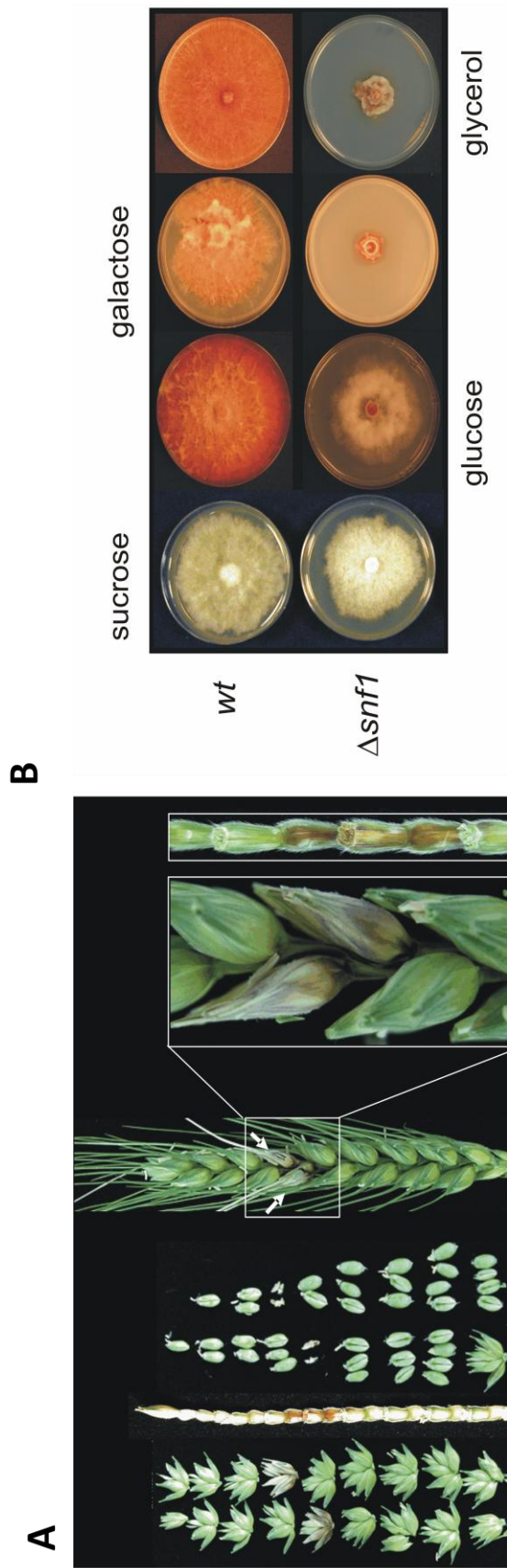


Figure 4.1. Effect of targeted deletion of the *SNF1* gene in *F. graminearum* PH-1. **A.** Reduced virulence of the *snf1* strain is shown by a restricted region of browning on infected wheat ears. Only the inoculated spikelets (white arrows) and adjacent rachis show a brown colouration that indicates FEB disease. **B.** *In vitro* growth of *snf1* on different carbon sources is reduced compared to wild type. **C.** *snf1* retains the ability to produce perithecia. Bar = 0.2 mm. Data generated by Martin Urban.

NTH1 is predicted to encode a neutral trehalase, an enzyme responsible for the breakdown of trehalose. This non-reducing disaccharide (α -d-glucopyranosyl- α -d-glucopyranoside) is a widespread sugar compound found in bacteria, plants, protozoa, insects and fungi. Trehalose has been implicated in the response of cells to a variety of stresses and, in addition, a role as a storage carbohydrate has been proposed (reviewed in Jorge *et al.*, 1997). In the yeast *Saccharomyces cerevisiae*, involvement of trehalose in thermotolerance (De Virgilio *et al.*, 1993, 1994; Hottiger *et al.*, 1987; Lewis *et al.*, 1995; Wera *et al.*, 1999), osmotic stress survival (Hounsa *et al.*, 1998), barotolerance (Iwahashi *et al.*, 1997), and protection from dehydration and dessication (Gadd *et al.*, 1987; Hottiger *et al.*, 1987) has been shown. Trehalose has also been shown to be involved in the heat protection of proteins (Hottiger *et al.*, 1994; Crowe *et al.*, 2006). Stress tolerance roles for trehalose have also been suggested for *Aspergillus nidulans* (Fillinger *et al.*, 2001), *Cryptococcus neoformans* (Petzold *et al.*, 2006), *Botrytis cinerea* (Doehlemann *et al.*, 2006), and arbuscular mycorrhizal fungi (Ocon *et al.*, 2007). A role for neutral trehalases in the virulence of fungal pathogens has been demonstrated for *Magnaporthe oryzae* (Foster *et al.*, 2003) but neutral trehalase is dispensable for virulence in *Candida albicans* (Eck *et al.*, 1997).

The *ICL1* gene of *F. graminearum* appears to encode an isocitrate lyase enzyme. Isocitrate lyase is a key enzyme of the glyoxylate cycle, which is used for the production of carbohydrates from fatty acids and other two-carbon compounds. Isocitrate lyase expression is regulated by carbon source. For example, in *C. neoformans*, *ICL1* expression is highly induced by ethanol and acetate but this induction is able to be suppressed by glucose (Rude *et al.*, 2002). In *L. maculans*, *ICL1* is induced under starvation conditions and by acetate but, interestingly, is not repressed by glucose (Idnurm and Howlett, 2002). Similarly, in *A. nidulans*, the addition of glucose to acetate-containing medium does not reduce *ICL1* expression (Bowyer *et al.*, 1994). Isocitrate lyase has been shown to be required for full virulence of the pathogenic fungi *M. oryzae*, *Colletotrichum lagenarium*, *Leptosphaeria maculans* and *C. albicans* (Lorenz and Fink, 2001; Idnurm and Howlett 2002; Wang *et al.*, 2003; Asakura *et al.*, 2006).

The *PKAR* gene is thought to encode the regulatory subunit of the cAMP-dependent protein kinase (protein kinase A, PKA). The PKA signalling cascade has been found to regulate a wide variety of processes in fungi, including virulence, sporulation, fertility and growth (for examples, see Gold *et al.*, 1997; Takano *et al.*, 2001; Staudohar *et al.*, 2002; Cassola *et al.*, 2004; Mehrabi and Kema, 2006; Zhao, W. *et al.*, 2006; Cervantes-Chavez and Ruiz-Herrera, 2007; Grosse *et al.*, 2008; Schumacher *et al.*, 2008; Ocampo *et al.*, 2009). PKA is comprised of a holoenzyme of two catalytic (PKAC) and two regulatory subunits (PKAR) (Corbin *et al.*, 1973; Corbin and Keely, 1977; Potter and Taylor, 1979). Cooperative binding of two cAMP molecules to two sites on each regulatory subunit leads to a conformational change and the resulting release of the two catalytic subunits, which are then able to phosphorylate downstream targets (Kopperud *et al.*, 2002).

Filamentous fungi are generally thought to possess one gene encoding the regulatory subunit of PKA and two genes that encode different catalytic subunits. One catalytic subunit is usually found to be more important than the other for processes such as growth and sporulation (Ni *et al.*, 2005). In *F. graminearum*, the PKA regulatory subunit is encoded by the gene *FGSG_09908* (*PKAR*) while the genes *FGSG_07251* and *FGSG_08729* appear to encode the two different catalytic subunits (Yu *et al.*, 2008). Recently, *Mucor circinelloides* has been found to be the first fungal species that possesses more than one *PKAR* gene. A *pkaR1* deletion strain showed affected growth, spore production and germination. Three other *PKAR* genes (*PKAR2-4*) were also found and shown to be expressed (Ocampo *et al.*, 2009). The regulatory subunit of PKA has been shown to be required for full virulence of a number of fungal species (Gold *et al.*, 1997; Takano *et al.*, 2001; Mehrabi and Kema 2006; Zhao *et al.*, 2006; Schumacher *et al.*, 2008), while in others, it appears to be essential for life (Cassola *et al.*, 2004; Cervantes-Chavez and Ruiz-Herrera, 2007).

This chapter describes the targeted deletion and initial characterisation of *NTH1*, *ICL1* and *PKAR* in *F. graminearum*. For *NTH1* a molecular cloning approach was used to produce nucleic acid constructs for transformation of *F. graminearum* protoplasts, while for *ICL1* and *PKAR*, a fusion PCR method (Catlett *et al.*, 2003) was used to provide such constructs. This study

demonstrated that *PKAR*, and to a much lesser extent, *NTH1*, but not *ICL1*, is required for a normal rate of FEB symptom spread by *F. graminearum*.

4.2 Methods

4.2.1 Targeted Deletion of *NTH1*

For the targeted deletion of *NTH1*, the entire coding region of *NTH1* (*FGSG_09895*) and a small (93 bp) upstream region were replaced with the resistance-conferring *Hph* hygromycin phosphotransferase gene using the split-marker technique (Catlett *et al.*, 2003). The cloning steps used to generate the *NTH1* deletion constructs are described in Chapter 2.

nth1 transformants that amplified the *Hph*, but not *NTH1* gene, were retained, grown from a single spore, and genomic DNA (gDNA) prepared (details in Chapter 2). gDNA was digested with *HindIII* or *EcoRV* (*Eco32I*) high concentration (50 U/μl) restriction enzymes (Fermentas) (reaction mixture as in Chapter 2). Digested DNA was then used for Southern gel blots using the original *NTH1* gene flank regions (amplified with primers AB1-4) as labelled probes (5' flank for *EcoRV* digest and 3' flank for *HindIII* digest). Details of Southern blotting and hybridisation are found in chapter 2.

4.2.2 Targeted Deletion of *ICL1* and *PKAR*

For the targeted deletion of the *ICL1* and *PKAR* genes, a fusion PCR approach was used (Catlett *et al.*, 2003). Using this technique, the 5' and 3' flanking regions of the target gene are amplified using primers that incorporate a 24 bp overlap with the corresponding *hph* section, which are produced in a separate amplification. Mixing the appropriate flank and *hph* sections (5' flank with *hph* section HY, and 3' flank with *hph* section YG) together allows hybridisation via the overlapping segment and the two sections become fused in a second PCR amplification reaction. Details of the PCR fusion protocol, primers used and PCR product sizes can be found in chapter 2. For *PKAR* and *ICL1*, nested

primers were used for the fusion of the 5' flank to the HY *hph* section to increase amplification efficiency (See Chapter 2).

Transformation of *F. graminearum* was performed as described in chapter 2, except for the transformation for targeted deletion of *PKAR*, which was performed by M. Urban using glucose in place of sucrose in the regeneration medium for protoplast embedding. Transformants were grown on SNA containing 75 µg/ml hygromycin B for four days then on PDA prior to screening by PCR. Screening of transformants by PCR, gDNA preparation, digestion and Southern gel blots were performed as described in chapter 2. Digestion of gDNA was performed using *HindIII* and *Sall* for *pkar* (for 5' and 3' flank probes respectively) and *KpnI* and *PstI* for *icl1* (for 5' and 3' flank probes respectively).

4.3 Results

To investigate the role of the micro-region in *F. graminearum* virulence, targeted gene deletion was used to produce mutants of the sequenced strain PH-1 lacking individual genes from within the micro-region. Initially only the homologues of virulence genes verified in other plant pathogenic species not previously described elsewhere (*SNF1* in Lee *et al.*, 2009b; Beacham *et al.*, 2010 (in preparation), *STE7* in Ramamoorthy *et al.*, 2007) were selected, namely *FGSG_09895* (designated *NTH1*), *FGSG_09896* (designated *ICL1*) and *FGSG_09908* (designated *PKAR*) to determine their role in the disease causing ability of *F. graminearum*.

4.3.1 Targeted Deletion of *NTH1*

For the targeted deletion of the *NTH1* gene, a molecular cloning approach was used. The 5' and 3' flanks of the gene were amplified (**Figure 4.2**) and then ligated separately into the Invitrogen pCR2.1 vector. Analytical restriction enzyme mediated digestion with *HindIII* confirmed integration of the flank PCR products into the pCR2.1 vector, with constructs showing the desired orientation of insertion selected for further procedures (**Figure 4.3**). Clones 5-4 (containing the 5' flank in a forward orientation) and 3-4 (containing the 3' flank in a forward orientation) were selected and the plasmids they contained named pAB001-2 and pAB002-2 respectively. pAB001-2 and pAB002-2 were then digested to release fragments containing the flank sequences (**Figure 4.4**) that were then ligated into the vectors pYG and pHY (**Figure 4.5**). The pAB001-2 (5' flank) fragment was ligated into pYG and the pAB002-2 (3' flank) fragment ligated into pHY.

Colony PCR was used to screen for ligation of the 5' flank fragment into pYG (**Figure 4.6**). All 10 colonies tested appear to amplify the 5' flank. 5'-1 was selected for analytical restriction digestion. This digestion showed the successful excision of the inserted pAB001-2 fragment (**Figure 4.7**). 5'-1 was renamed pAB003 (see cloning scheme in Chapter 2). Colony PCR was also used to screen for successful ligation of the 3' flank fragment into pHY (**Figure 4.8**). Two colonies, 3'-5 and 3'-10, appeared to amplify the 3' flank efficiently and were retained for analytical restriction enzyme digestion (to excise the

ligated pAB002-2 fragment). Digestion successfully excised the inserted fragment from 3'-5 and 3'-10 (**Figure 4.9**). 3'-5 and 3'-10 were renamed pAB004-1 and pAB004-2 (see cloning scheme in Chapter 2).

Successful ligation of the fragments containing the *NTH1* flanks into pHY and pYG was used to fuse the flank and *Hph* gene sections together. Sequencing results confirmed correct production of these final vector constructs. The flank-*Hph* section regions were amplified from pAB003 and pAB004-1 (see Chapter 2 and **Figure 4.10**) and used for transformation of *F. graminearum*.

Hygromycin-resistant transformants were screened for the presence of the intact *Hph* gene and absence of the *NTH1* gene by PCR (**Figure 4.11**, primers detailed in Chapter 2). This revealed five transformants (A4, A8-11) that amplified for the presence of the *Hph* gene and did not amplify the *NTH1* gene, suggesting a successful gene replacement event. These transformants were selected for Southern blot confirmation of targeted deletion of *NTH1*. The original *NTH1* 5' and 3' flank sequences used in the deletion construct cloning were used as labelled probes. An *EcoRV* digest was probed with the 5' flank and a *HindIII* digest with the 3' flank. Genomic DNA of transformants A4, A8, A10 and A11 exhibited the expected 0.75 kb and 2.7 kb hybridising bands in *EcoRV* and *HindIII* digests respectively, versus the 2.1 kb and 0.9 kb bands found in the wild-type progenitor strain (**Figure 4.12**), indicating successful single insertion of the two deletion constructs and targeted deletion of the *NTH1* gene.

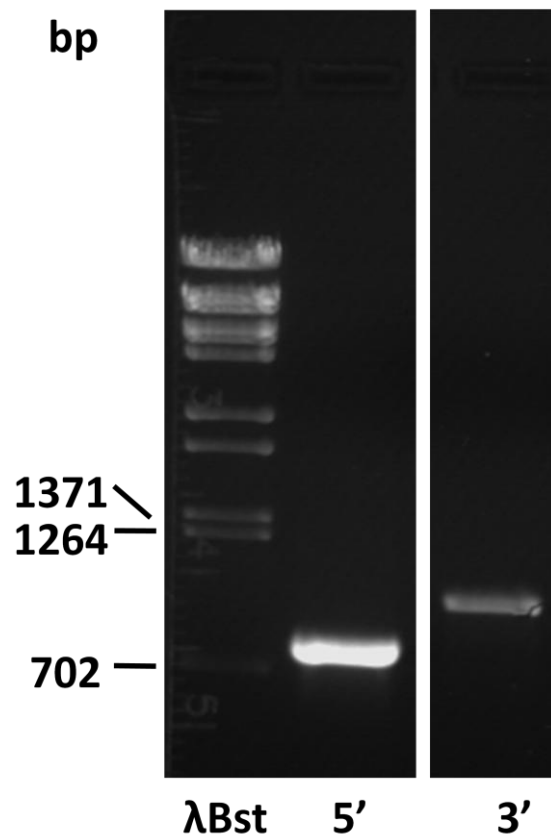


Figure 4.2. Amplification of the *NTH1* flanking regions. The 800 bp 5' flank (5') was amplified using primers AB1 and AB2, the 1000 bp 3' flank (3') was amplified using the primers AB3 and AB4.

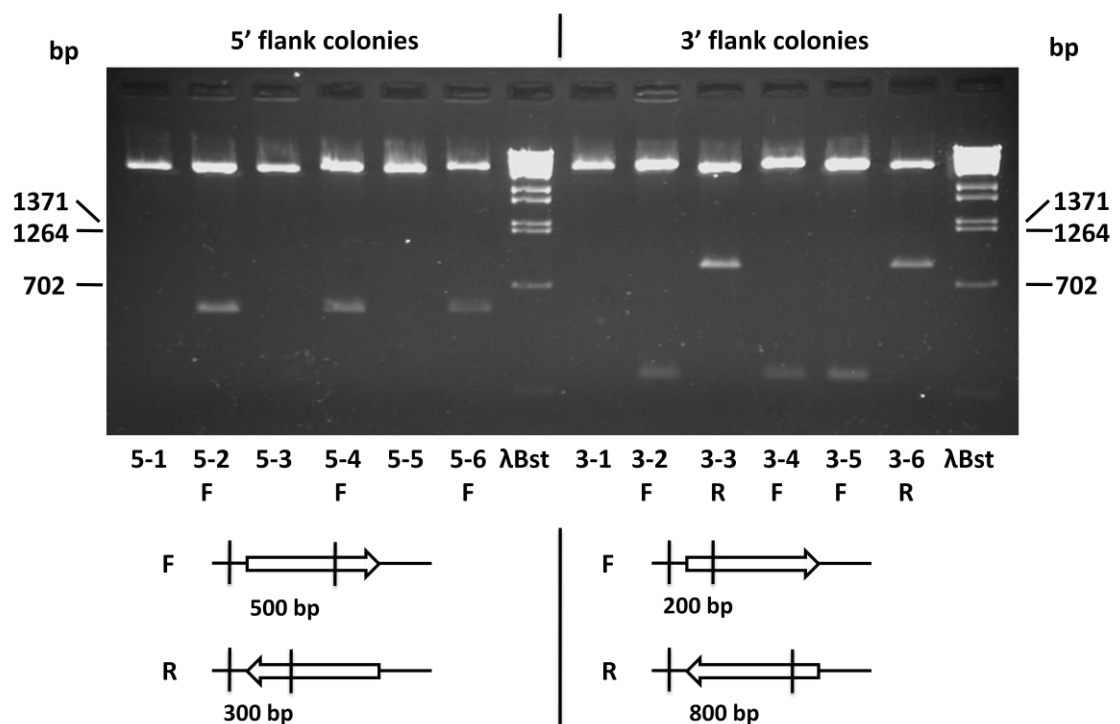


Figure 4.3. Confirmation of *NTH1* flank sequence insertion into the pCR 2.1 vector using *HindIII* digestion. Six colonies were analysed for each flank. As the PCR products could

be ligated to the pCR 2.1 vector in either a forward or reverse orientation, distinction was required between these two possibilities. *HindIII* digestion of pCR 2.1 containing the 5' flank in a forward orientation would yield a fragment near 500 bp in size, while a reverse orientation of the 5' flank would yield an approximately 300 bp fragment. For the 3' flank, a forward orientation in pCR 2.1 would show a roughly 200 bp *HindIII* fragment, with reverse orientation showing around 800 bp . Screening six colonies for each flank provided clones containing the required fragment size and therefore orientation for both flanks . Clones with forward (F) or reverse (R) insertions of the flank sequence are indicated.

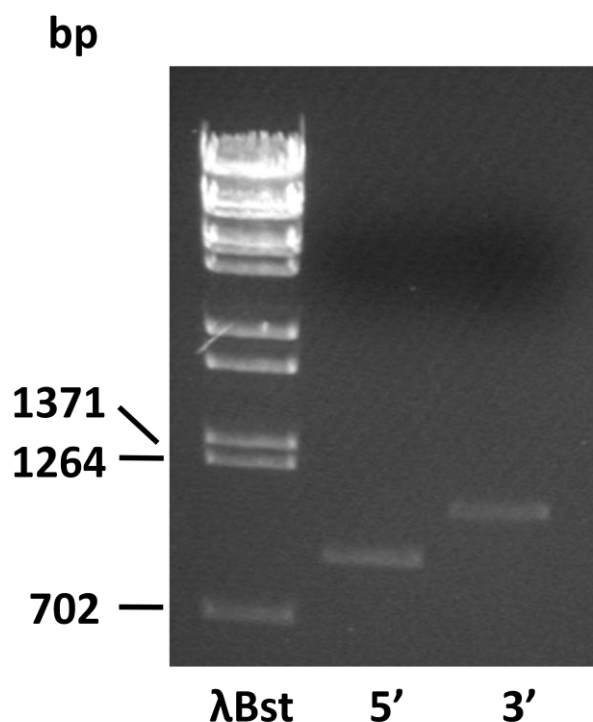


Figure 4.4. Excision of fragments containing the 5' and 3' flanks from pAB001-2 and pAB002-2 respectively by restriction digestion using *SacI* and *XbaI* (for pAB001-2) or *BamHI* and *XhoI* (for pAB002-2), for ligation into the pYG and pHY vectors. The digests were concentrated by ethanol precipitation and the excised fragments gel extracted.

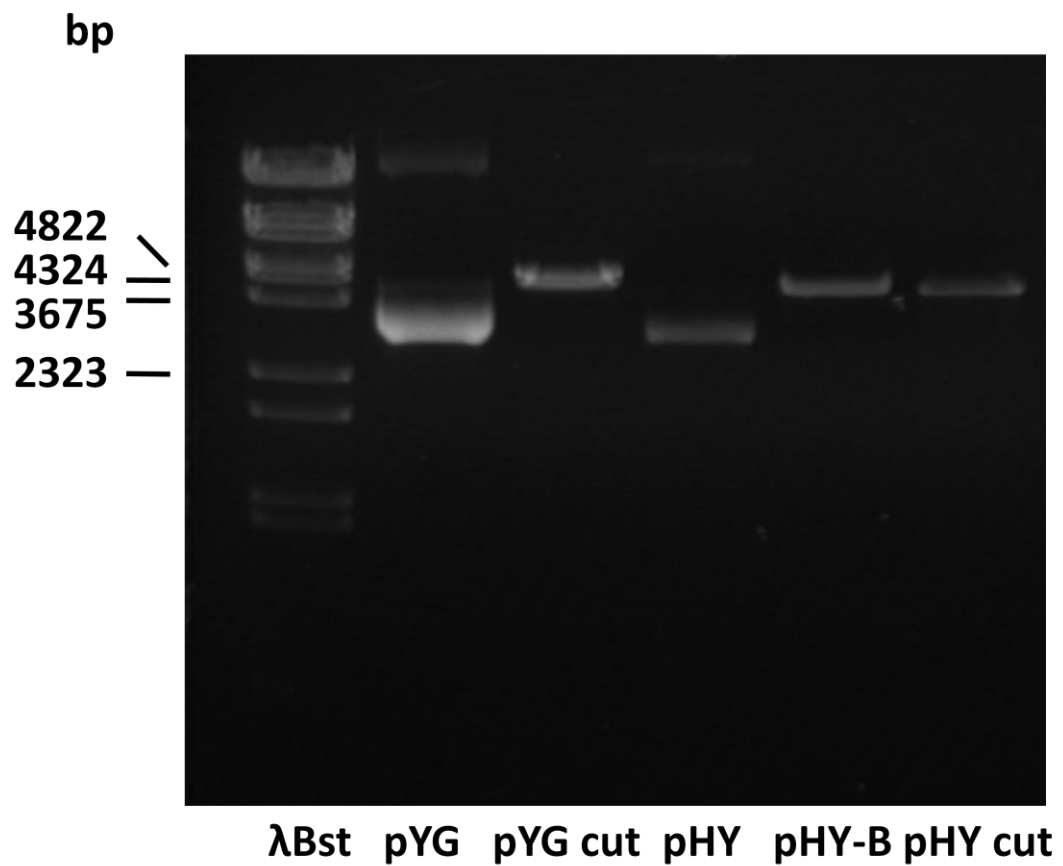


Figure 4.5. Digestion of pYG with *SacI* and *XbaI* and of pHY with *Bam*HI and *Xho*I in preparation for ligation to the flank-containing fragments cut from pAB001-2 and pAB002-2 respectively. Cut = double cut, pHY-B = *Bam*HI cut only.

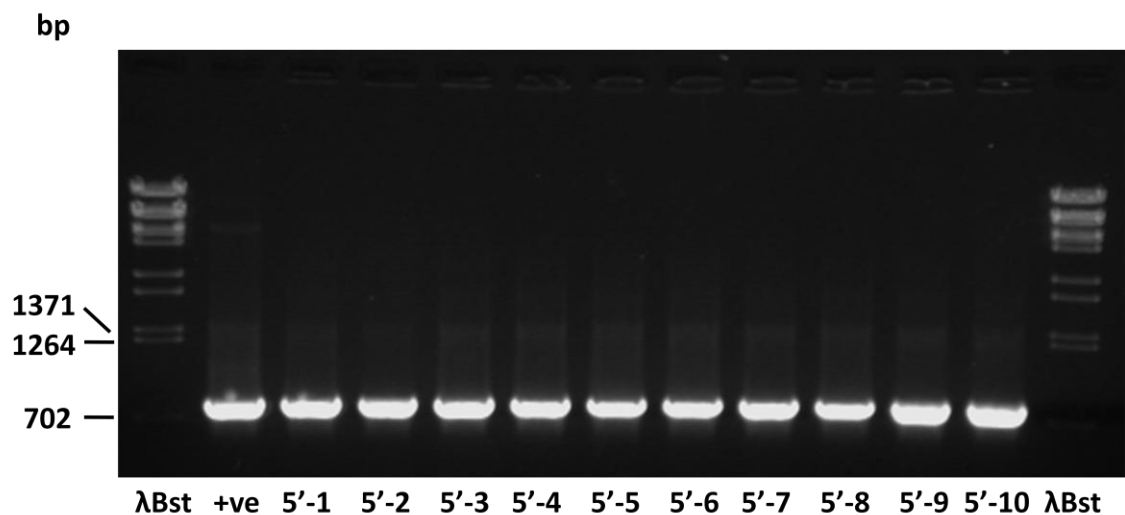


Figure 4.6. Colony PCR screening for ligation of the pAB001-2 (5' flank) fragment into pYG. pAB001-2 is used as template for the positive control (+ve).

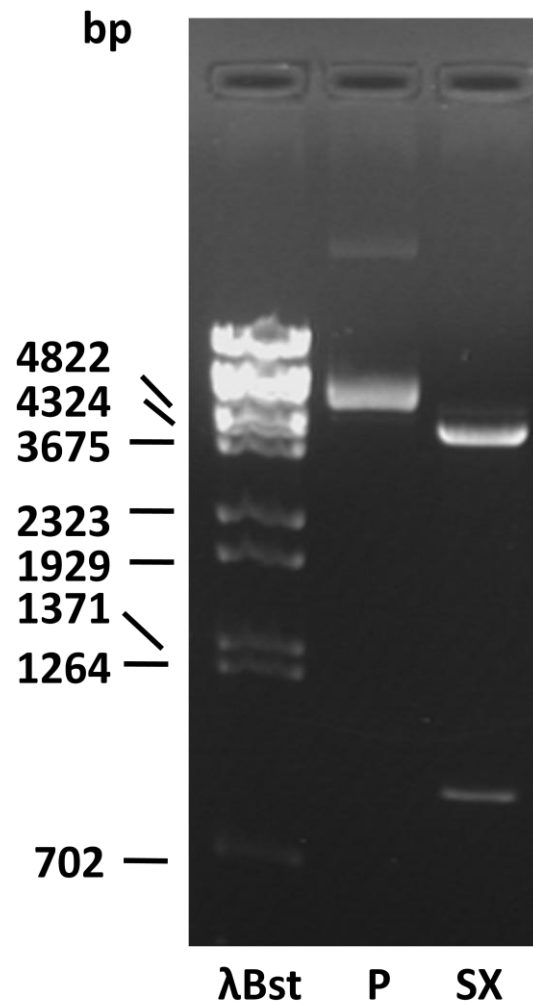


Figure 4.7. Analytical restriction digestion of clone 5'-1. P = plasmid prep, SX = *SacI*, *XbaI* sequential digest.

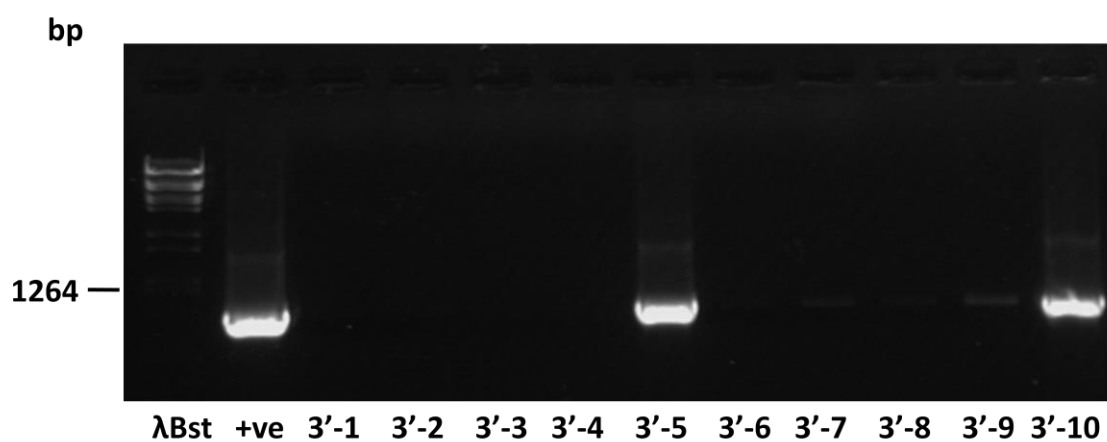


Figure 4.8. Colony PCR screening for ligation of the pAB002-2 (3' flank) fragment into pHY. pAB002-2 is used as template for the positive control (+ve).

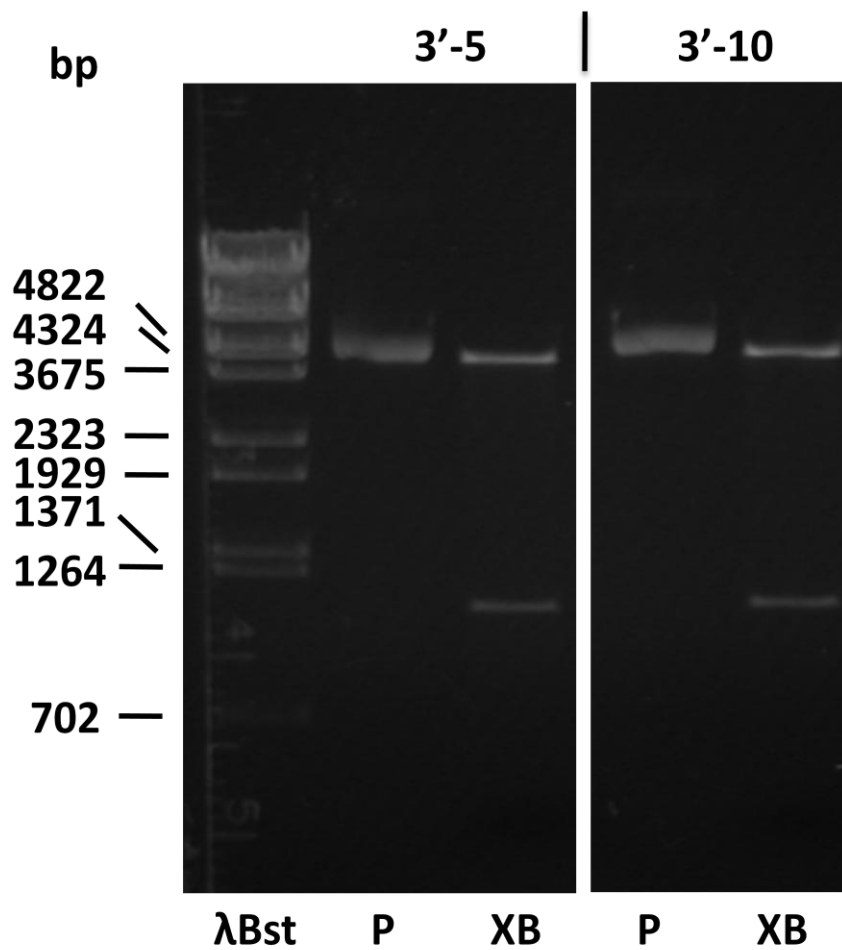


Figure 4.9. Analytical restriction digest of clones 3'-5 and 3'-10. P = plasmid prep, XB = *Bam*HI, *Xho*I sequential digest.

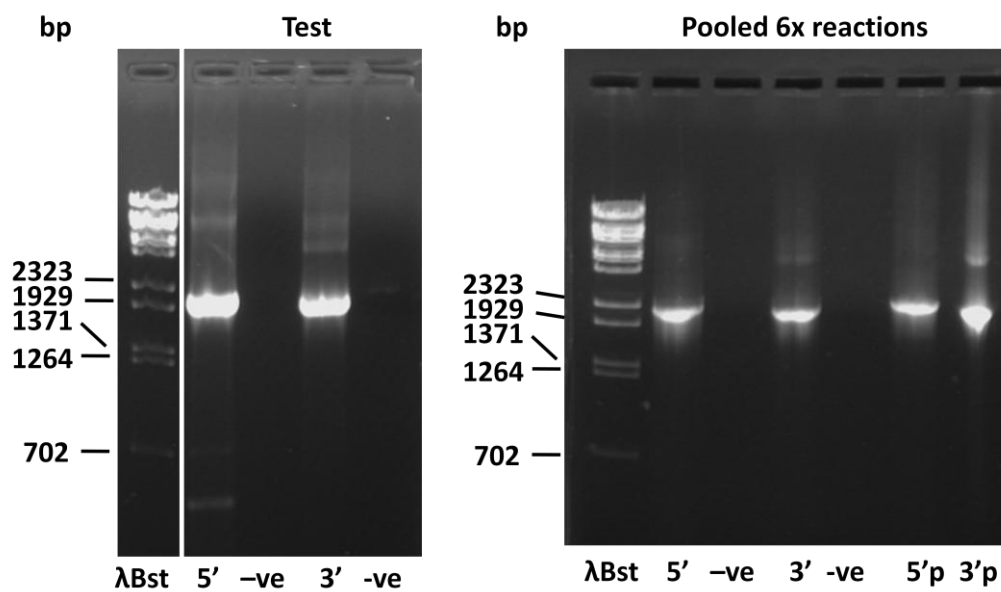


Figure 4.10. Amplification of PCR products from pAB003 and pAB004-1 for transformation into *F. graminearum*. 5' = pAB003 product, 3' = pAB004-1 product, 5'p, 3'p = after ethanol precipitation, -ve = no template.



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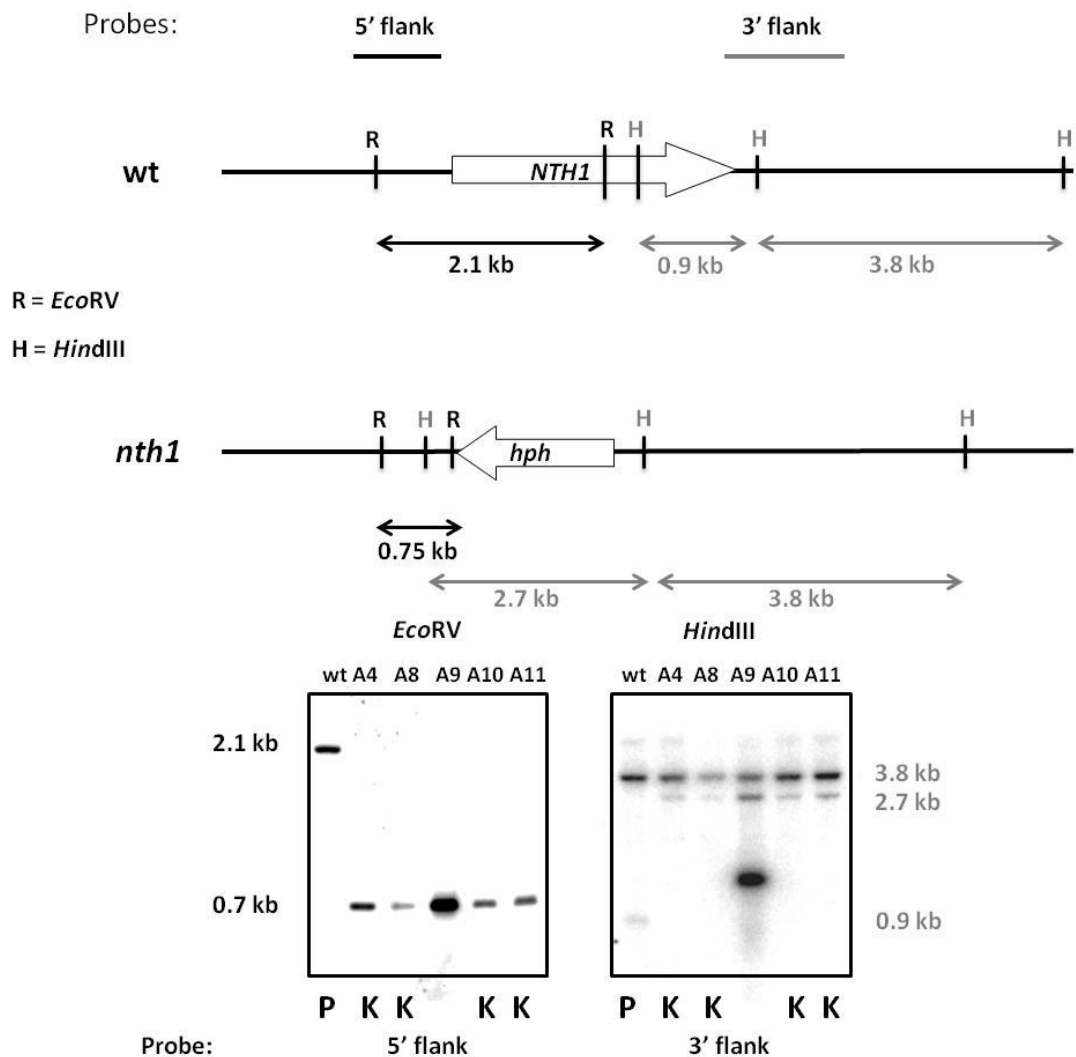


Figure 4.12. Targeted deletion of the *NTH1* gene in *F. graminearum*. Targeted deletion was confirmed by Southern hybridisation. The gene locus for the wild type and the deletion strain is shown. *EcoRV* and *HindIII* were independently used to digest genomic DNA (gDNA) of both the wild type and the deletion strain. Each restriction enzyme digest set of wild type and deletion strain gDNA was hybridised with a different DNA probe (5' flank or 3' flank). Colours (black/grey) indicate the pairing of restriction enzyme and probe and expected hybridising fragment size. Five independent transformants were analysed for *NTH1* and compared to the wild type strain. In each case the blots exhibited hybridising bands that indicated a single insertion of each of the two overlapping split marker DNA constructs and deletion of the target gene. P = Wild type, K = knockout strain. Transformant number is shown above the radiograph.

4.3.2 Targeted Deletion of *ICL1* and *PKAR*

PKAR and *ICL1* deletion constructs were produced by fusion PCR (see Chapter 2 for method and **Figures 4.13 and 4.14**). *pkar* and *icl1* transformants were screened for the presence of the target gene and *hph* gene (**Figures 4.15 and 4.16**, primer details in Chapter 2). This indicated 3 *pkar* transformants and 11 *icl1* transformants which did not amplify the target gene but did amplify the *Hph* gene, suggesting replacement of the target gene by *Hph*. Four *icl1* transformants, five *pkar* transformants plus one additional transformant (transformant 2) that appeared to possess both *PKAR* and *Hph* which may represent ectopic insertion of the deletion constructs were selected for Southern gel blots. The original 5' and 3' flank sections were again amplified by PCR and used for the construction of labelled probes for DNA gel blots.

Genomic DNA of three *icl1* transformants (3, 4 and 7) exhibited the expected 3.1 kb and 3.4 kb hybridising bands in *KpnI* and *PstI* digests respectively, versus the 6.7 kb and 2.7 kb bands found in the wild-type progenitor strain (**Figure 4.17A**) indicating successful single copy targeted deletion of *ICL1*. Genomic DNA of two *pkar* transformants (4 and 5) exhibited the expected 3.3 kb and 2.5 kb hybridising bands in *HindIII* and *SalI* digests respectively, versus the 1.9 kb and 3.2 kb bands found in the wild-type progenitor strain (**Figure 4.17B**), indicating single copy targeted deletion of *PKAR*. In addition, transformant 2 was confirmed to contain the *pkar* deletion constructs inserted into the genome in ectopic locations but with a wild type *PKAR* locus. This transformant was designated *PKAR-e*. Due to the poor ability of the *pkar* deletion strain to produce conidia (see later) and hence low possibility of genetic complementation, the *PKAR-e* strain was used as an extra control in addition to the PH-1 wild type in analyses of *pkar*.

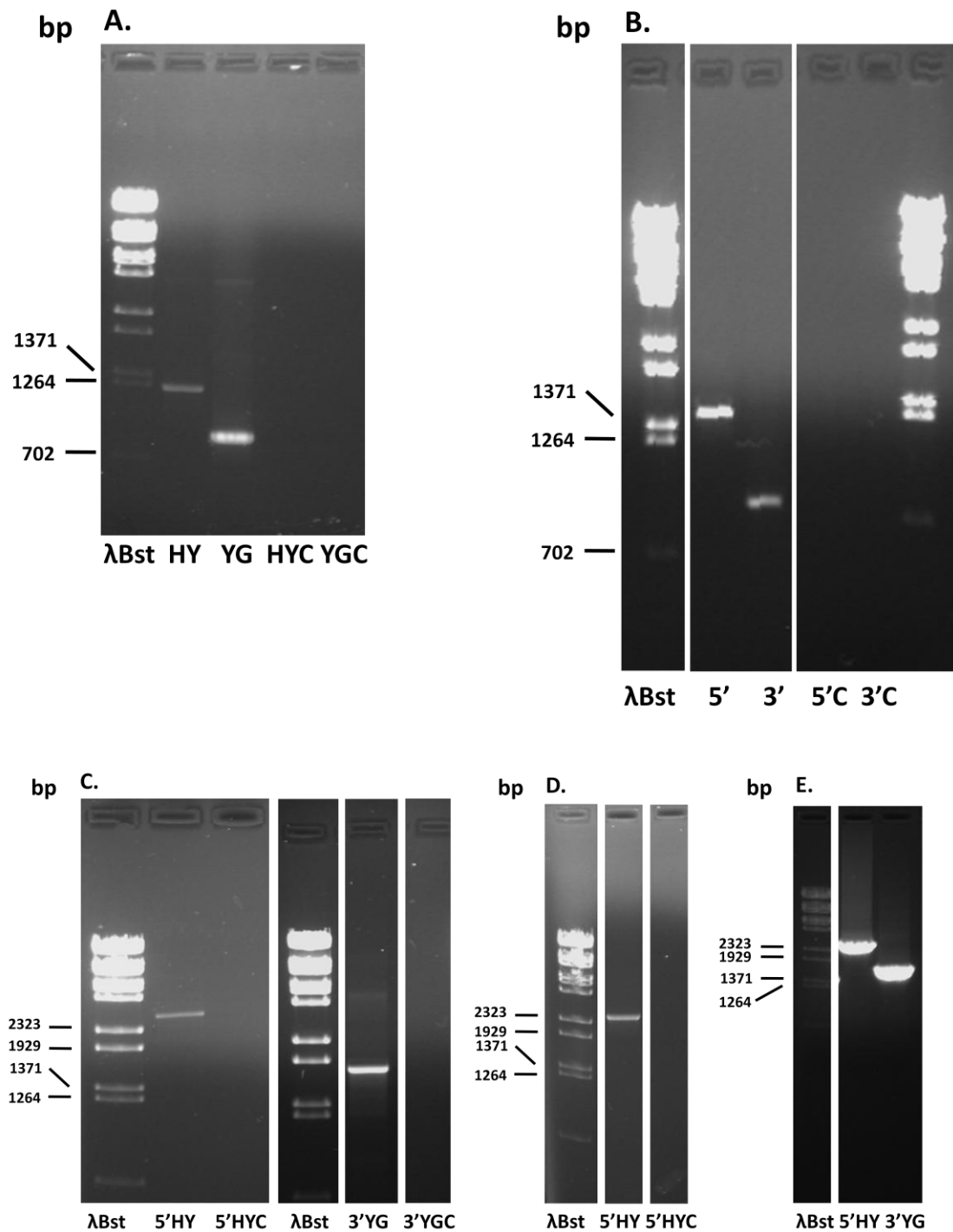


Figure 4.13. Fusion PCR manufacture of deletion constructs for targeted deletion of the *PKAR* gene. **A.** Amplification of the HY and YG sections of the *Hph* gene from pHYG1.4. **B.** Amplification of the 5' and 3' flanks of the *PKAR* gene. **C.** A test of the fusion of the 5' flank and HY and fusion of the 3' flank and YG by PCR. **D.** Nested PCR for 5' flank fusion to HY using the construct from (C) as template. **E.** Precipitation of DNA for transformation. Lanes labelled with 'C' are primer controls lacking template DNA.

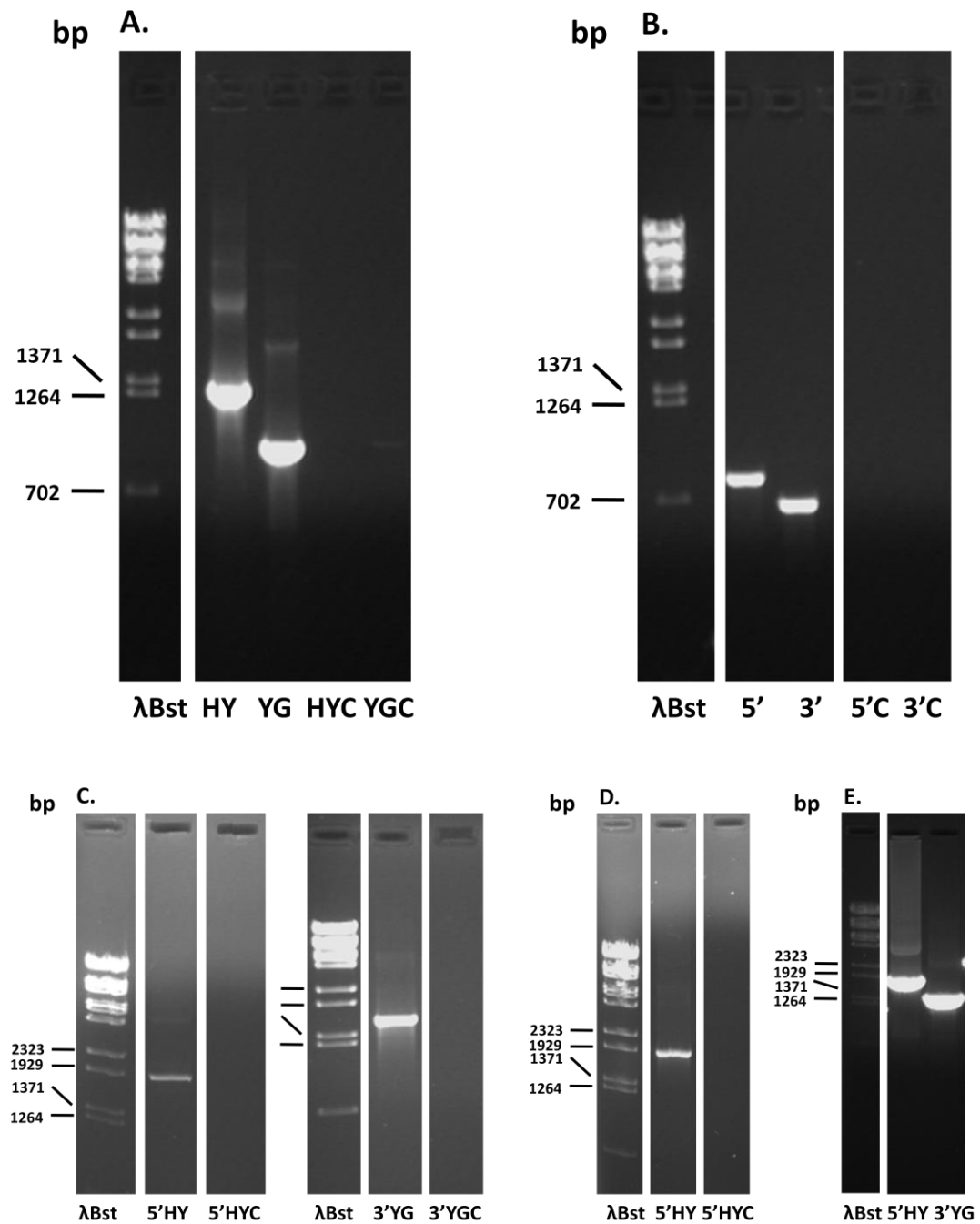


Figure 4.14. Fusion PCR manufacture of deletion constructs for targeted deletion of the *ICL1* gene. **A.** Amplification of the HY and YG sections of the *hph* gene from pHYG1.4. **B.** Amplification of the 5' and 3' flanks of the *ICL1* gene. **C.** A test of the fusion of the 5' flank and HY and fusion of the 3' flank and YG by PCR. **D.** Nested PCR for 5' flank fusion to HY using the construct from (C) as template. **E.** Precipitation of DNA for transformation. Lanes labelled with 'C' are primers controls lacking template DNA.

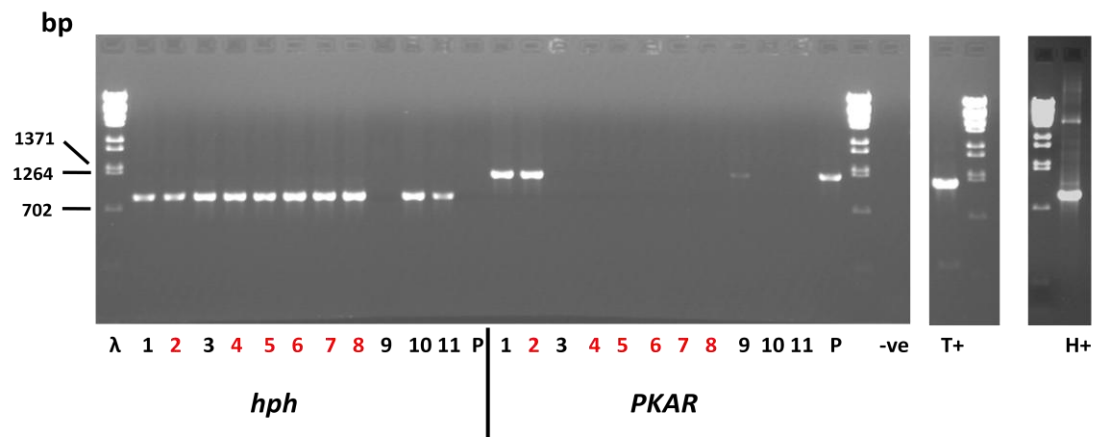


Figure 4.15. PCR screening of *pkar* transformants for the presence of the *PKAR* and *Hph* genes. Transformants are numbered 1-11. P = PH-1 wild type. λ = λ Bst ladder. -ve = water control (no template), T+ = *PKAR* positive control (PH-1 gDNA), H+ = *Hph* positive control (pHYG1.4). Transformants with red numbers proceeded to Southern blot characterisation as they appear to show successful targeted deletion of *PKAR* (with the exception of transformant 2, which appears to show insertion of the deletion constructs at an ectopic site in the genome).

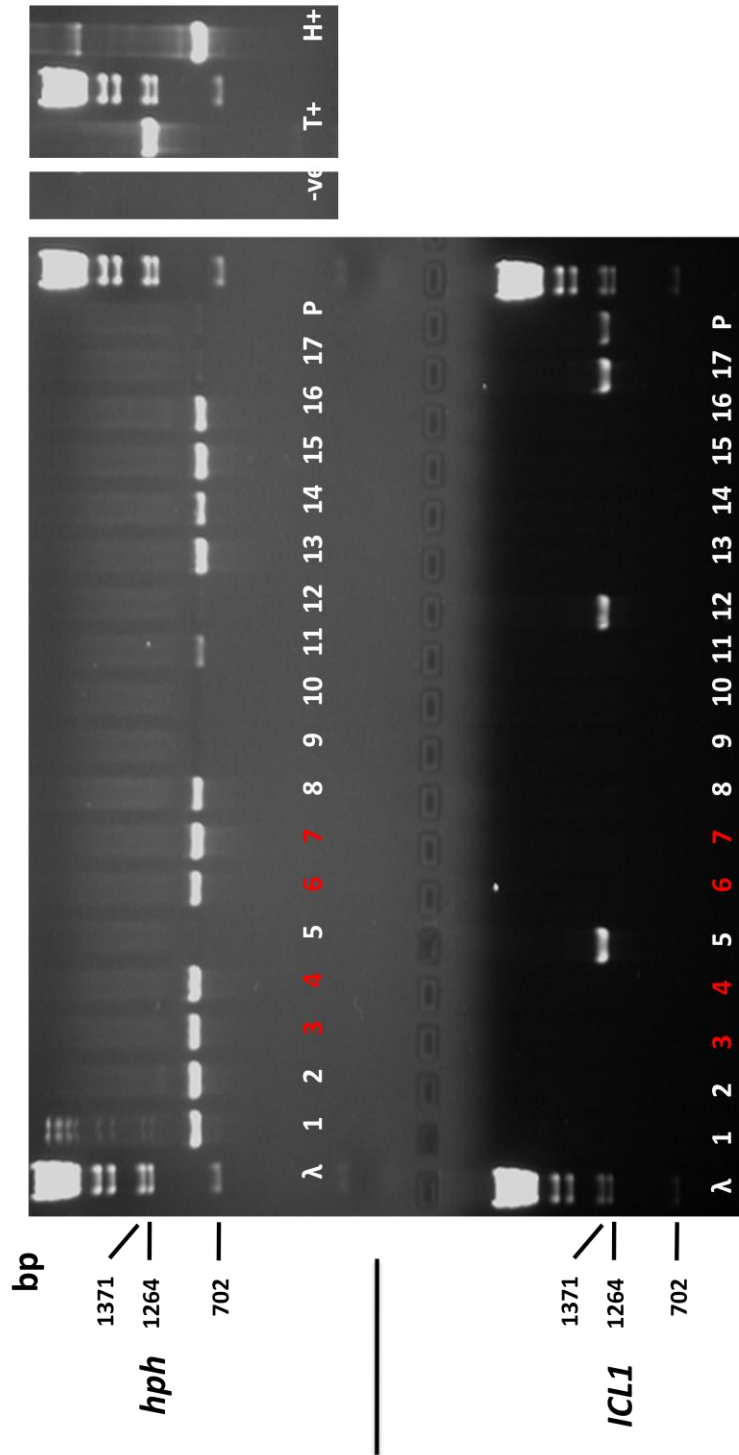
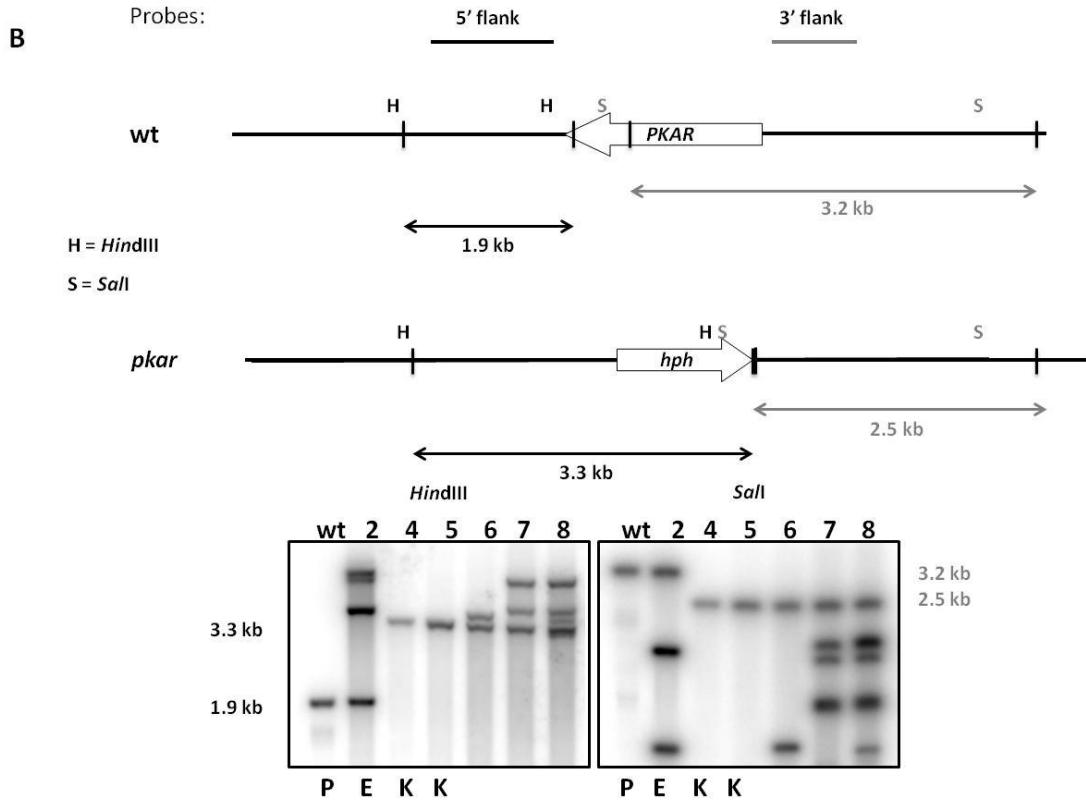
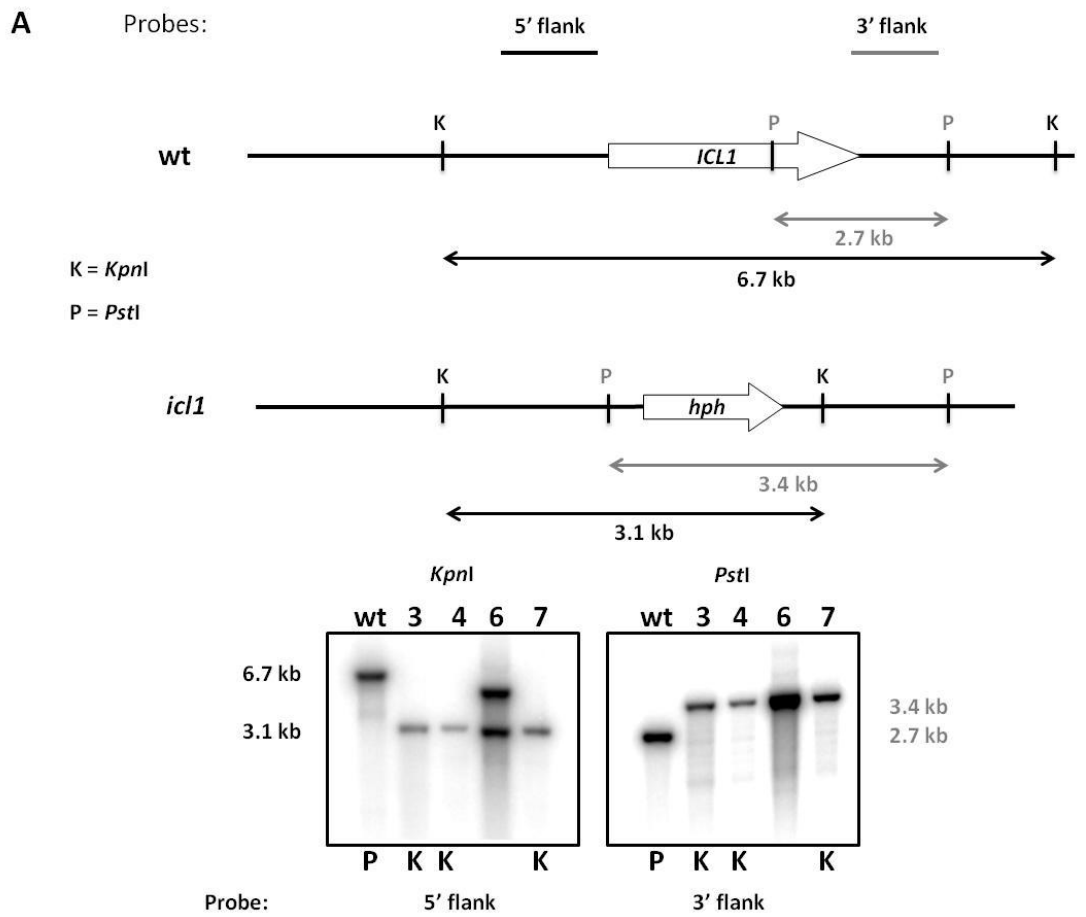


Figure 4.16. PCR screening of *icl1* transformants for the presence of the *ICL1* and *hph* genes. Transformants are numbered 1-17. P = PH-1 wild type. λ = λ Bst ladder. -ve = water control (no template), T+ = *ICL1* positive control (PH-1 gDNA), H+ = *hph* positive control (pHYG1.4). Transformants with red numbers proceeded to Southern blot characterisation as they appear to show successful targeted deletion of *ICL1*.

Figure 4.17. (See over). Targeted deletion of the (A) *ICL1* and (B) *PKAR* genes in *F. graminearum*. Targeted deletion was confirmed by Southern hybridisation. The gene locus for the wild type and deletion strain is shown in each case. Two different restriction enzymes were independently used to digest genomic DNA (gDNA) of both the wild type and deletion strain. Each restriction enzyme digest set of wild type and deletion strain gDNA was hybridised with a different DNA probe (5' flank or 3' flank). Colours (black/grey) indicate the pairing of restriction enzyme and probe and expected hybridising fragment size. Four and six independent transformants were analysed for *ICL1* and *PKAR* respectively and compared to the wild type strain. In each case the blots exhibited hybridising bands that indicated a single insertion of each of the two overlapping split marker DNA constructs and deletion of the target gene. P = Wild type, K = knockout strain, E = ectopic insertion of the two constructs. Transformant number is shown above the radiograph.



Initial Characterisation of the *nth1*, *icl1* and *pkar* Deletion Strains

4.3.3 Virulence on Wheat Plants

On wheat ears, the wild-type strain PH-1 causes browning and bleaching symptoms from 4 days post inoculation (dpi) which subsequently spread up and down the entire ear by 16 dpi (**Figure 4.18**). To assess the effect of gene deletion on pathogenicity, the rate of disease symptom spread between wild-type and the single-gene deletion strains was compared (**Figure 4.18 and 4.19**). Three independent transformants were initially tested on wheat ears in preliminary experiments for *nth1* and *icl1* strains, and two independent transformants for *pkar* (**Figure 4.19**). This preliminary analysis suggested a drastic reduction in the rate of ear colonisation for the *pkar* strain compared to wild-type. For *nth1*, only a modest reduction in the rate of symptom spread was noted. For *icl1*, little difference to the wild-type rate of disease symptom spread was noted, although transformant *icl1*-7 did show a very slight reduction in symptom spread. However, as only one of three transformants showed this very small reduction it was considered to be due to slight variation between the transformants and not due to an inherent role of *ICL1* in virulence.

Representative transformants of *nth1* (*nth1*-8) and *pkar* (*pkar*-5) (**Figure 4.18**) were selected after consideration of this preliminary test and *in vitro* growth analysis (see later) for further *in planta* study with a larger sample of wheat ears (referred to simply as *nth1* and *pkar*). The *pkar* strain showed the most drastically reduced symptom spread of the targeted deletion strains. While symptoms began to appear by 4 dpi, these were restricted to the inoculated spikelets and adjacent rachis and did not spread further down the ear even after a prolonged period of time. After 16 dpi, the wild-type has infected the entire ear, while the *pkar* strain still shows visible symptoms only at the inoculated spikelets, leading to an reduction in symptom spread by over 80%. The *PKAR-e* strain, by comparison, showed symptoms nearly identical to wild-type. For *nth1*, symptoms were present at 4 dpi and spread down the ear, causing browning of the rachis, but at a slower rate than the wild-type strain, such that by 12 dpi the percent infection of the ear below the inoculation point was significantly lower for *nth1* than wild-type (reduced by 17% (**Figures 4.19-4.21**)).

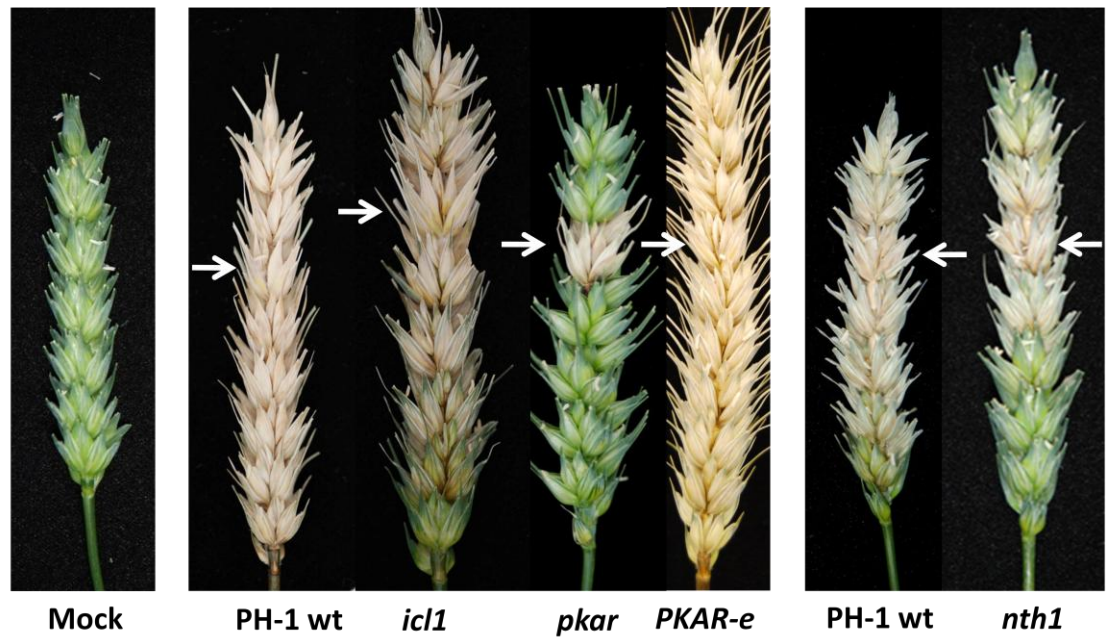


Figure 4.18. Disease symptoms of the *nth1*, *icl1*, *pkar* and *PKAR-e* strains on ears of the susceptible wheat cultivar Bobwhite. In the wild-type infection (PH-1 wt), bleaching of the ear begins at the inoculated spikelets and spreads up and down the ear resulting in a colour change from green to beige. Representative ears infected with each strain are shown. The point of inoculation is marked with white arrows. Photos taken at 16 days post infection (12 days post infection for *nth1* and adjacent PH-1 wt).

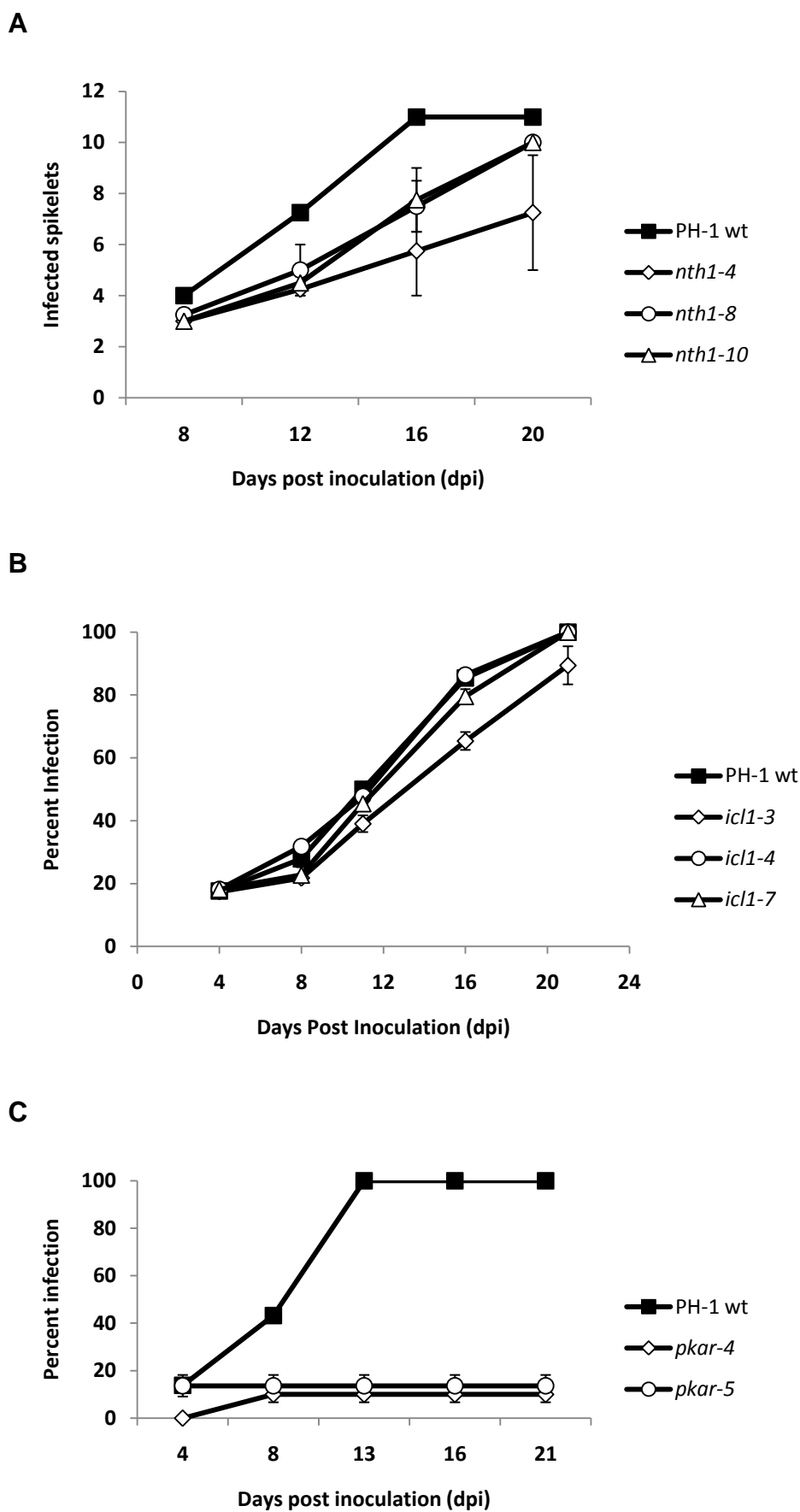


Figure 4.19. Preliminary experiments. Progression of wheat ears cv. Bobwhite inoculated with (A) *nth1*, (B) *icl1* or (C) *pkar* transformants. Error bars are shown as the standard error of the mean for two ears per strain (three for PH-1 wt in the *icl1* experiment).

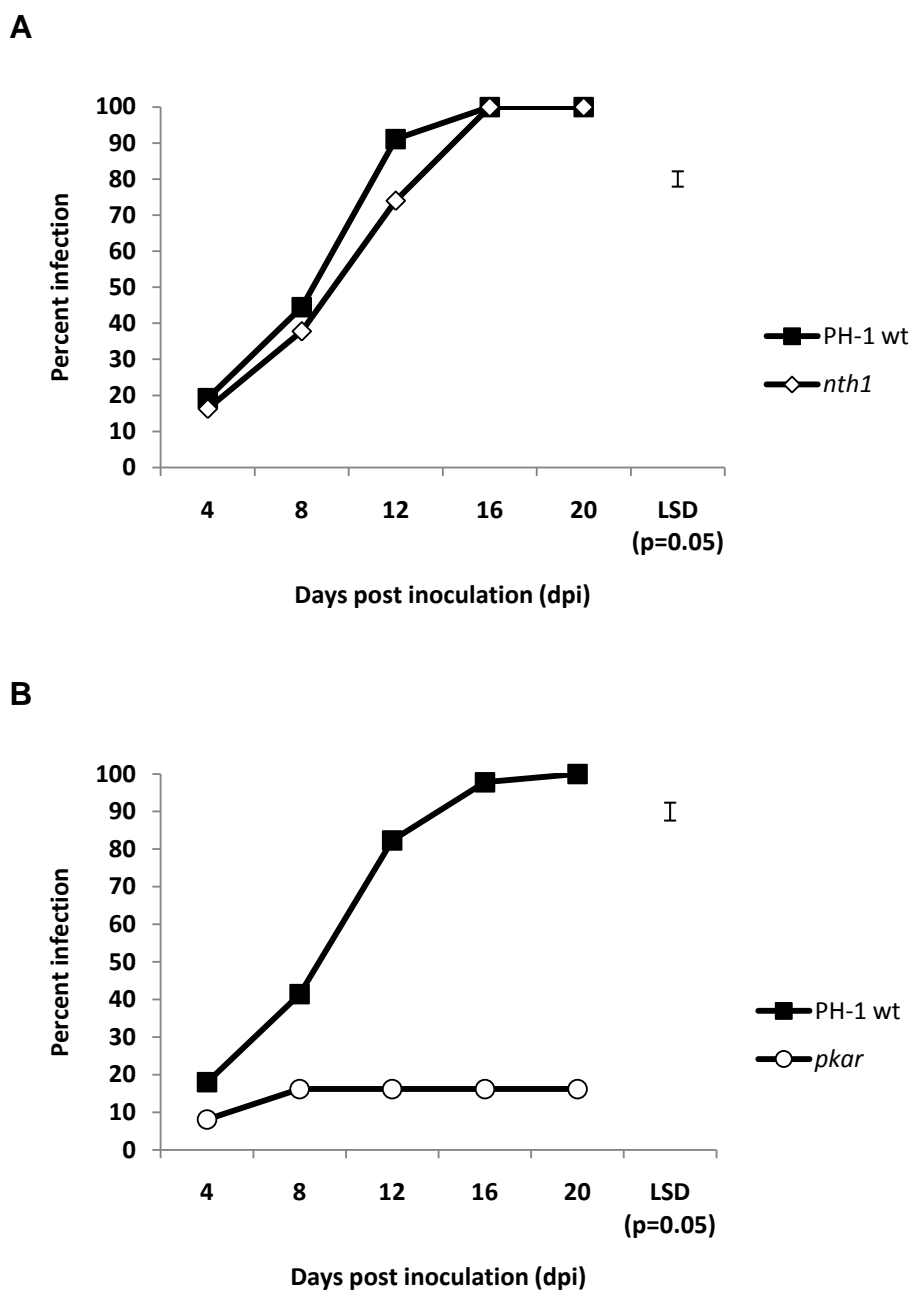


Figure 4.20. Large scale *in planta* analysis of virulence reduction of the **(A)** *nth1* and **(B)** *pkar* strains.

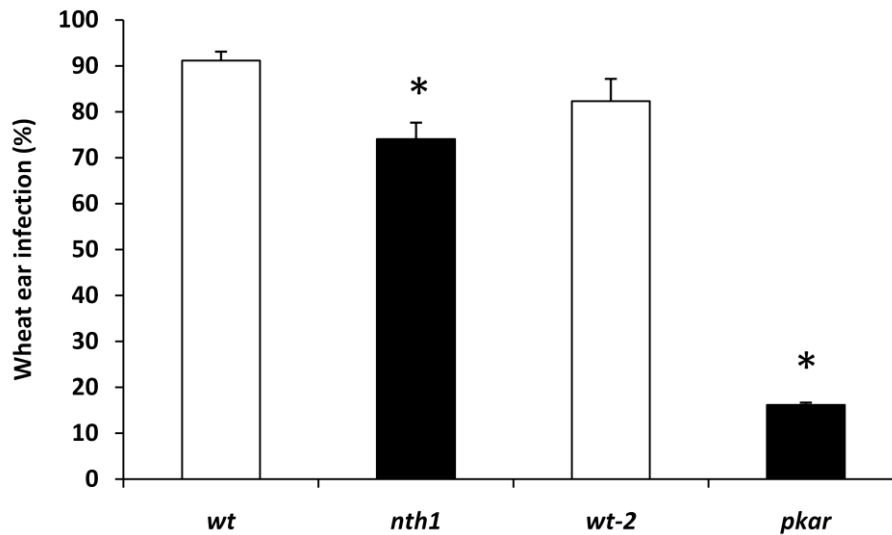


Figure 4.21. Further analysis of virulence. Percentage of ear downwards from the inoculation point exhibiting symptoms at 12 dpi for PH-1 wt, *nth1* and *pkar*. wt and *nth1* represent one experiment and wt-2 and *pkar*, a second experiment. Average of 10 PH-1 wt ears and 9 *nth1* ears for experiment 1, and 7 PH-1 ears and 7 *pkar* ears for experiment 2. * indicates significant difference from wild-type ($p < 0.05$).

The wild-type pathogenicity for the *icl1* mutant is comparable to the findings of a study of this gene in *F. graminearum* strain Z03643 (Lee *et al.*, 2009a). An earlier study had reported that the disruption of the mitogen-activated protein kinase kinase (MAPKK) *STE7* drastically reduces virulence on wheat ears for the *ste7* deletion strain compared to the wild type PH-1. Similarly, targeted deletion of *SNF1* drastically reduces virulence of *F. graminearum* (Lee *et al.*, 2009b; **Figure 4.1A**) The previously published *snf1* deletion strain made in *F. graminearum* GZ3639 showed limited symptom development on spikelets of spray-inoculated wheat ears (Lee *et al.*, 2009b). Collectively, these single-gene deletion experiments, results confirm a contribution to *F. graminearum* disease symptom spread for four of the five PHI-base pathogenicity gene homologues in the micro-region namely, *nth1*, *snf1*, *ste7* and *pkar*.

4.3.4 *In vitro* Growth

To determine the effect of *NTH1*, *ICL1* or *PKAR* gene deletions on the *in vitro* growth rate of *F. graminearum*, each strain was grown on both complete (PDA, 'CM') and minimal (SNA, 'MM') medium (**Figures 4.22 and 4.23**). The same set of transformants were analysed as for the preliminary wheat inoculation experiments. Percent reduction in growth is stated as the mean of the transformants analysed. For *icl1*, two of the three transformants (*icl1-4* and *icl1-7*) did not grow at a rate significantly different from the wild-type on minimal medium (**Figure 4.22B**), while *icl1-3* and *icl1-7* did not grow at a significantly different rate from the wild-type on complete medium (**Figure 4.22D**). In each case, the other transformant (*icl1-3* on MM and *icl1-4* on CM) grew at a rate slightly slower (8% and 9% reduction in growth rate respectively) than wild-type which was only just significant according to the statistical analysis at $p < 0.05$. This difference is slight and appears to reflect a small amount of variation between the *icl1* transformants. Indeed, if the *icl1* data is analysed independently of the other virulence-unaffected mutants then the *icl1-4* transformant result on complete medium is no longer significant.

When cultured on complete medium the growth rates were comparable to wild-type for *PKAR-e*, slightly reduced for *nth1* (15% reduction) and highly reduced for *pkar* (67%), which also produced much less aerial mycelium than the wild-type (**Figures 4.22 and 4.23**). When cultured on minimal medium the growth rates were comparable to wild-type for *PKAR-e*, slightly reduced for *nth1* (12% reduction) and highly reduced for *pkar* (73% reduction). The reduction in growth rate for *nth1* on minimal medium was not statistically significantly different from the wild type strain ($p < 0.05$).

The production of aerial mycelium on complete medium was also drastically reduced by the deletion of *pkar* (**Figure 4.23**). Interestingly, the morphology of *pkar* hyphae appeared different from the wild type and *PKAR-e* strains as well. Hyphae of the *pkar* strain exhibited much shorter distances between septa than the wild type and *PKAR-e* strains, leading to a 'string of cubes' appearance in *pkar* hyphae (**Figure 4.24**).

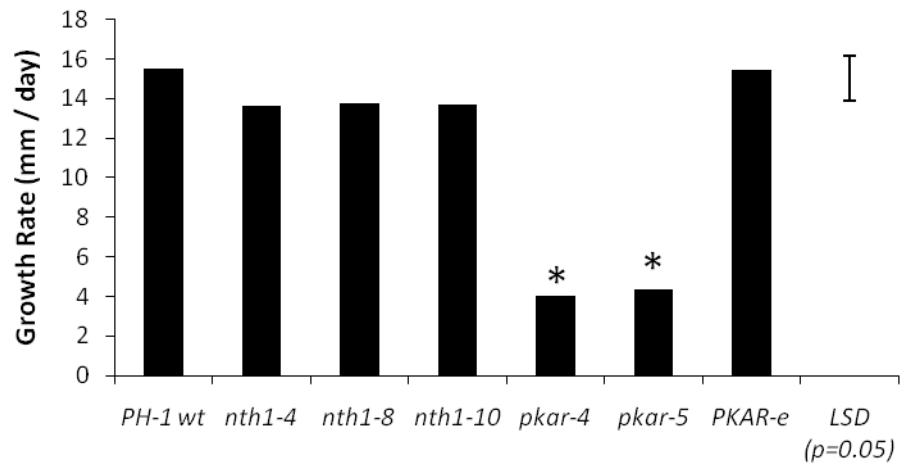
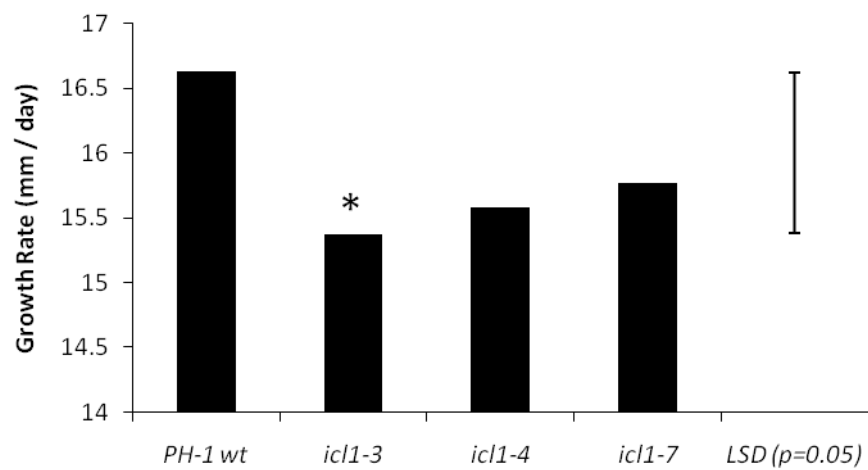
A**B**

Figure 4.22. In vitro growth rate of the (A) *nth1*, *pkar* and *E-pkar* strains and (B) *icl1* strains on minimal medium. The wild type (wt) strain PH-1 is included for comparison. LSD = least significant difference. Columns marked with * are significantly different from the wild type strain at the 0.05% level.

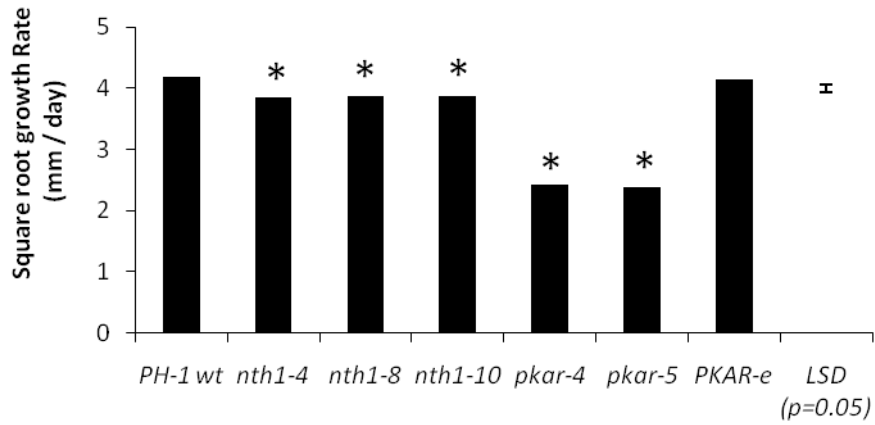
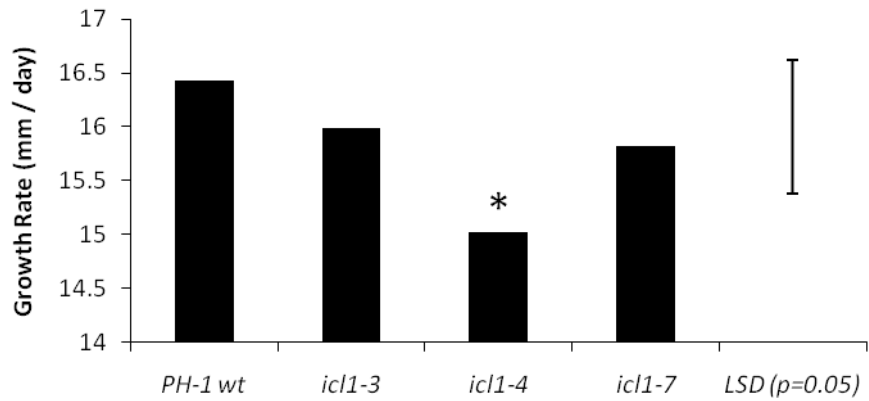
C**D**

Figure 4.22. (cont). In vitro growth rate of the (C) *nth1*, *pkar* and *E-pkar* strains and (D) *icl1* strains on complete medium. The wild type (wt) strain PH-1 is included for comparison. LSD = least significant difference. Columns marked with * are significantly different from the wild type strain at the 0.05% level.

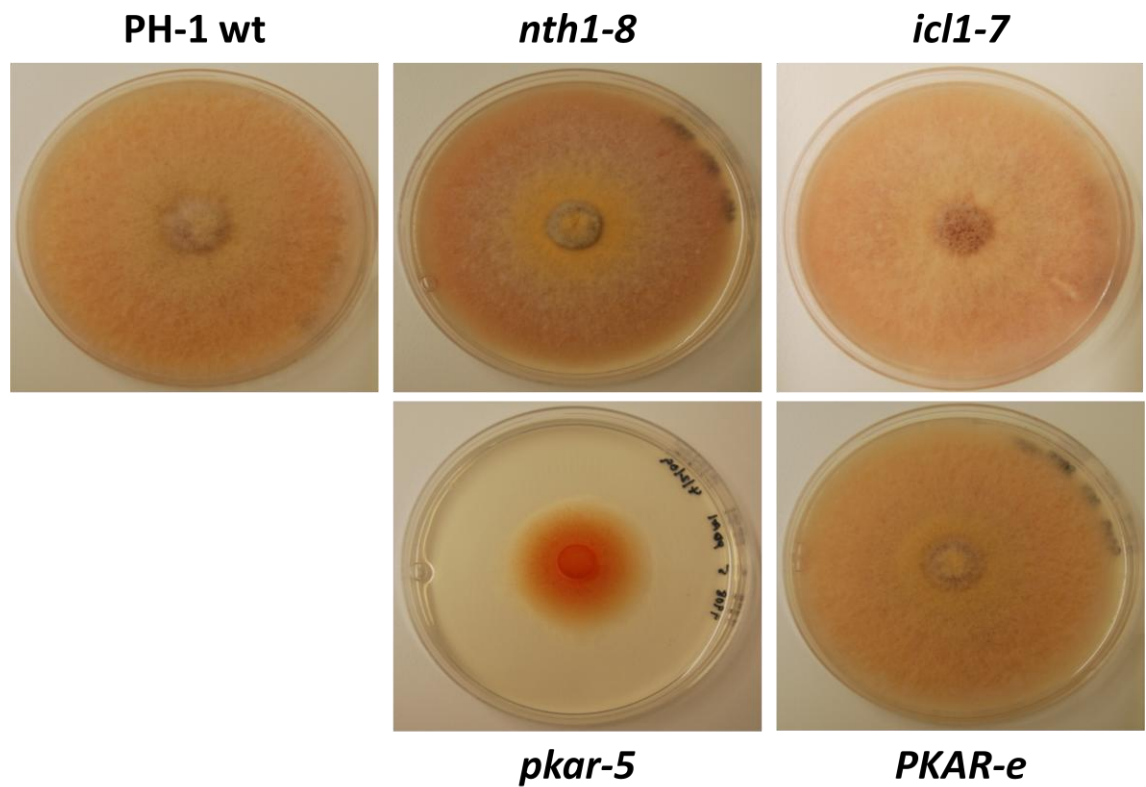


Figure 4.23. Growth of the *nth1*, *icl1*, *pkar* and *PKAR-e* strains on complete medium after six days. The PH-1 wild type is shown for comparison.

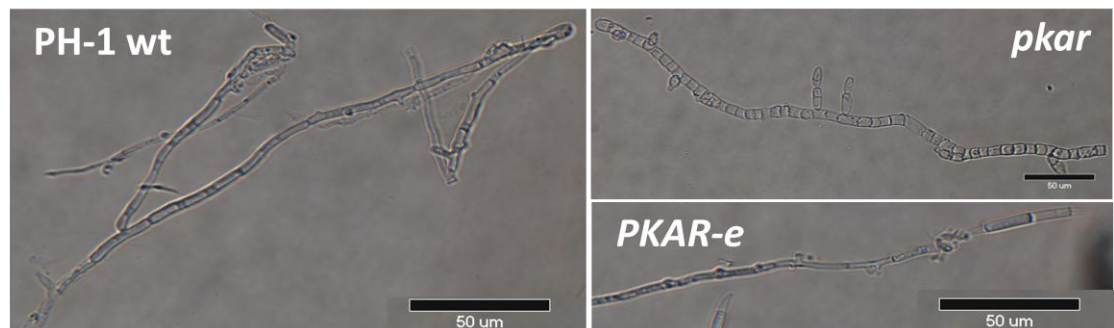


Figure 4.24. Hyphal morphology of the PH-1, *pkar* and *PKAR-e* strains. Hyphae of the *pkar* strain appear to be divided into smaller more cuboidal sections than the other strains, which show longer, more rectangular hyphal sections. Bar = 50 µm.

ICL1 is predicted to encode the enzyme isocitrate lyase, which catalyses the first committed step of the glyoxylate cycle which is used for the production of carbohydrates from fatty acids and other two-carbon precursors. It is hypothesised that a fungal strain lacking such an enzyme would be compromised in its ability to grow on such substances. For this reason we compared the ability of the *icl1* deletion strain and the wild-type strain to grow on sodium acetate, ethanol or olive oil as the sole carbon source. 1% glucose or sucrose as the sole carbon source and standard SNA medium, containing a 1:1 mixture of these two sugars at low concentration (1 mM), were used for comparison. As shown in **Table 4.1**, on standard SNA medium and glucose the two strains did not show a significant difference in growth rate. However, the *icl1* strain showed a significantly reduced growth rate compared to wild type on sodium acetate, ethanol and olive oil, reduced by 69%, 62% and 53%, respectively. This suggests that *ICL1* is important for the successful utilisation of these compounds by *F. graminearum*. Interestingly, on sucrose however, the *icl1* strain grew significantly faster than the wild type strain (increased by 25%).

The *nth1* gene is predicted to encode a neutral trehalase enzyme. Such enzymes are responsible for the breakdown of trehalose into glucose. We therefore compared the ability of the *nth1* and wild type strains to grow with glucose or trehalose as the sole carbon source (**Table 4.1**). On standard minimal medium containing a 1:1 ratio of 0.02% glucose and 0.02% sucrose, the *nth1* strain did not grow at rate significantly different from the wild type strain. However, with 1% glucose or trehalose as the sole carbon source, the growth rate of the *nth1* strain was significantly slower compared to the wild type strain on the same medium (reduced by 19% and 14% respectively). Compared to the growth rate of the *nth1* strain on minimal medium, growth of *nth1* was reduced by 17% and 12% with 1% glucose or trehalose as the sole carbon source respectively). The wild type strain grew at a similar rate on all three media types.

Table 4.1. Growth rate of *nth1* and *icl1* strains on additional media types (mm / day). * = growth rate significantly reduced compared to wild type (wt) ($p < 0.05$), † = growth rate significantly increased compared to wt ($p < 0.05$). MM contains glucose and sucrose as the carbon source, which are absent prior to the addition of any further carbon supplements.

Medium	wt	<i>nth1</i>
MM	14.71	14.08
MM + glucose	14.51	11.74*
MM + trehalose	14.32	12.38*
	wt	<i>icl1</i>
MM	14.51	13.63
MM + glucose	12.41	14.87
MM + sucrose	13.07	16.35†
MM + sodium acetate	14.81	4.55*
MM + ethanol	11.39	4.29*
MM + olive oil	12.91	6.07*

4.4 Discussion

The initial analysis of the *NTH1*, *ICL1* and *PKAR* genes has indicated that two of these genes, *NTH1* and *PKAR*, are required for a full rate of *F. graminearum* ear blight symptom spread on wheat, although to differing extents.

Infection of flowering wheat ears was slightly slowed in the *nth1* strain versus the wild type strain. The role of trehalose metabolism has been investigated in several important pathogenic fungal species. In the rice blast pathogen *Magnaporthe oryzae*, both the trehalose-6-phosphate synthase *TPS1* and *NTH1* are required for different stages of plant infection (Foster *et al.*, 2003). *TPS1* disruption caused a reduction in appressorial turgor pressure and poor production of penetration hyphae, while *NTH1* deletion led to poor post-invasive growth. In the glume blotch pathogen *Stagonospora nodorum*, the *TPS1* gene is required for full virulence (Lowe *et al.*, 2009). In addition, in the plant pathogen *Botrytis cinerea*, neither the T6PS gene, *TPS1*, nor the neutral trehalase gene, *TRE1*, are required for pathogenicity (Doehlemann *et al.*, 2006). It is interesting to note that in the publication of the *B. cinerea TRE1*, the authors could locate genes of only low homology to the *B. cinerea TRE1* gene in *F. graminearum* (20-21% similarity). In this study, alignment of *B. cinerea Tre1* and *F. graminearum Nth1* indicated 66.8% protein sequence identity. This discrepancy could perhaps be due to the software used in the analysis or the use of earlier versions of the annotated genomes used to obtain the sequences. The neutral trehalase gene *NTH1* is also dispensable for pathogenicity in another plant pathogenic species, *Leptosphaeria maculans* (Idnurm *et al.*, 2003). In the human pathogen *Cryptococcus neoformans*, the *TPS1* gene is required for pathogenicity in mice, rabbits and the nematode *Caenorhabditis elegans*, however, a null mutant of the neutral trehalase gene *NTH1* retains full pathogenicity in *C. elegans* (Petzold *et al.*, 2006). In the related pathogen *C. gattii*, the *TPS1* and *TPS2* genes but not the *NTH1* gene contribute to virulence (Ngamskulrungron *et al.*, 2009); while in another human pathogen, *Candida albicans*, the *TPS2* gene is required for full virulence but the neutral trehalase gene *NTC1* is not (Eck *et al.*, 1997; Maidan *et al.*, 2008). In the insect fungal pathogen *Metarhizium anisopliae*, the acid trehalase gene *ATM1* has been linked to pathogenicity (Zhao, H. *et al.*, 2006; Xia *et al.*, 2002).

Interestingly, *M. oryzae* possesses a novel type of ‘mixed’ trehalase, *TRE1*, with characteristics of both neutral and acidic trehalases (Foster *et al.*, 2003). *TRE1* encodes the trehalase activity important during spore germination and hyphal growth and is required for growth on trehalose as the sole carbon source). In contrast, *M. oryzae NTH1*, while expressed during germination, is not required for growth on trehalose as the sole carbon source. This study has shown that a deletion strain of *F. graminearum Nth1*, which shows 75% protein sequence identity to the *M. oryzae Nth1*, is also able to grow with trehalose as the sole carbon source, although the rate of growth was slightly reduced compared to the PH-1 wild type strain. While *M. oryzae nth1* growth on minimal medium plus 10 g/l glucose or trehalose is comparable to the wild type (Foster *et al.*, 2003), *F. graminearum nth1* growth on the same concentration of glucose or trehalose as the sole carbon source is slightly but significantly slower than the wild type strain. On standard minimal medium containing 0.02% glucose and 0.02% sucrose, the difference in growth rate between *F. graminearum nth1* is not significant, however a difference is noted for the richer complete medium. The greater concentration of carbon source in the complete medium and 1% glucose test may help to exacerbate the defects of the *nth1* strain and reveal a difference in carbon source utilisation ability that is not apparent with the lower concentrations of sugars found in the standard minimal medium. By contrast, on complete medium the *M. oryzae nth1* strain grew normally. In addition, *M. oryzae tps1* is unable to grow with glucose or trehalose as the sole carbon source, while *C. neoformans tps1* shows a slight reduction in growth rate on complete medium containing 2% glucose (Petzold *et al.*, 2006). In *B. cinerea*, *tps1* and *tre1* do not show defects in vegetative growth (Doehlemann *et al.*, 2006). This suggests that, similar to *M. oryzae*, *NTH1* in *F. graminearum* plays only a minor role in trehalose utilisation.

An alignment of the trehalase sequences from *F. graminearum*, *F. verticillioides* and *F. oxysporum* plus select trehalase sequences from other fungal species is shown in **Figure 4.25**. This indicates the presence of three trehalase types – neutral, acid and mixed that group together in the alignment. The neutral trehalases of *F. graminearum* (Fgsg_09895), *F. verticillioides* (Fveg_02610) and *F. oxysporum* (Foxg_05530) align well with those of *M. oryzae Nth1*, *B. cinerea Tre1* and *A. nidulans TreB*. An acid trehalase is present in the genomes of *F*

verticillioides (Fveg_13500) and *F. oxysporum* (Foxg_14683) but not *F. graminearum*. *F. graminearum* appears to possess a second trehalase (Fgsg_05622), which may be important for trehalose mobilisation during germination and for utilisation of trehalose. This trehalase appears distinct from the acid trehalase of *F. verticillioides* and *F. oxysporum* and is also only somewhat similar to *F. graminearum* Nth1 (Fgsg_09895) (19.4% protein sequence identity) so may encode a mixed type trehalase akin to Tre1 of *M. oryzae*. In fact, the Fgsg_05622 protein aligns with *M. oryzae* Tre1 with an identity of 59.1%, indicating it may well encode a mixed type trehalase. Similar proteins are found in *F. verticillioides* (Fveg_06865) and *F. oxysporum* (Foxg_09264). Interestingly, in *B. cinerea*, Tre1, which shares 66.8% protein sequence identity and *A. nidulans* TreB, which shares 63.8% identity with *F. graminearum* Nth1, are required for trehalose mobilisation during spore germination (d'Enfert *et al.*, 1999; Doehlemann *et al.*, 2006). Trehalases with mixed features of the neutral and acid enzymes have also been identified in *C. albicans* (Sanchez-Fresneda *et al.*, 2009) and thermophilic fungi (Lucio-Eterovic *et al.*, 2005).

Figure 4.25. (See over). Multiple sequence alignment of trehalase proteins from a series of fungal species. Possible 'mixed' type trehalases Foxg_09264, Fveg_06865, Fveg_05622 and Mo Tre1 (light grey), neutral trehalases Fveg_02610, Foxg_05530, Fgsg_09895, Mo Nth1, Bc Tre1 and An TreB (blue), and acid trehalases Fveg_13500 and Foxg_14683 (dark grey) are shown. Shading indicates well-conserved residues (red – conserved in all sequences, orange – conserved in at least 80% of the sequences and yellow – conserved in at least 60% of the sequences). Foxg_14683 is truncated at 669 residues (full length 1010 residues). Foxg = *F. oxysporum*, Fveg = *F. verticillioides*, Fgsg = *F. graminearum*, Mo = *M. oryzae*, Bc = *B. cinerea*, An = *A. nidulans*.


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          *          260          *          280          *          300
Foxg_09264 : -----SFVVAGGRFRPEYY : 175
Fveg_06865 : -----SFVVAGGRFRPEYY : 175
Fgsg_05622 : -----SFVVAGGRFRPEYY : 175
Mo_Trel : -----TFVVAGGRFRPEYY : 176
Fveg_02610 : LDVQMLPEKITPDLVRDMNDAPGLLAVDVQEVPEPEHPSGWTGKMPFVVPGGRFNELYG : 284
Foxg_05530 : LDVQMLPEKITPDLVRDMNDAPGLLAVDVQEVPEPEHPSGWTGKMPFVVPGGRFNELYG : 284
Fgsg_09895 : LDVQMLPEEITTDLVRDMNDAPGLLAVDVQEVSEPEHPSGWTGKMPFVVPGGRFNELYG : 284
Mo_Nth1 : LDVVLPETITPEYVVGINKAPGLLAVDMEETVDP-KTGERVMSGRPFVVPGGRFNELYG : 285
Bc_Trel : LDVCWLPEKITPEVVRDMNSKPGLLAVAMEEVIDP-STGKTLKGLPFVVPGGRFNELYG : 286
An_TreB : LDVQELAAEITPEYVRDLNEKPGLLALAMEEKYDE-KTGKTDFAGVPFVVPGGRFNELYG : 295
Fveg_13500 : -----KGSVEIKYRLEAHKLH : 219
Foxg_14683 : -----KGSVEIKYRLEAHKLH : 187
                    5v6 ggrF e y

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          *          320          *          340          *          360
Foxg_09264 : WDSYWIIIEGLLRTGGSFVNTIAKNTIENELDFIEEYGFVPNGARIYYLNRSQPPLLSQMIK : 235
Fveg_06865 : WDSYWIIIEGLLRTGGSFVNTIAKNTIENELDFIEEYGFVPNGARIYYLNRSQPPLLSQMIK : 235
Fgsg_05622 : WDSYWIIIEGLLRTGGSFVNTIAKNTIENELDFIEEYGFVPNGARIYYLNRSQPPLLSQMIK : 235
Mo_Trel : WDSYWILEGLLRTGGAFNTISKNTIENELDLVETIGFVPNGARIYYKNRSQPPLLSQVVR : 236
Fveg_02610 : WDSYMASLGLLIN--DRVDLAKSMVINCFCEIEHYGKILNATRSYYLCRSQPPLFLTDMAL : 342
Foxg_05530 : WDSYMASLGLLIN--DRVDLAKSMVINCFCEIEHYGKILNATRSYYLCRSQPPLFLTDMAL : 342
Fgsg_09895 : WDSYMASLGLLIN--DRVDLAKSMVINCFCEIEHYGKILNATRSYYLCRSQPPLFLTDMAL : 342
Mo_Nth1 : WDSYMESLGLLVN--DKVYLAKSMVINCFCEIKHYGKILNATRSYYLCRSQPPLFLTDMAL : 343
Bc_Trel : WDSYMESLGLLVN--DKVYLAKSMVINCFCEIEHYGKILNATRSYYLCRSQPPLFLTDMAL : 344
An_TreB : WDSYMESLGLLAS--NRVDLAKAMVINCFCEIKHYGKILNANRSYYLTRSQPPFLTDMAL : 353
Fveg_13500 : VNQAIVDLTVVPSVDSEATIVN-VIDGYSAVRSDFVKSGEDDDGAVFSAVRPVGIPNNTA : 278
Foxg_14683 : VNQAIVDLTIVPSTDSEATVVN-VIDGYSAVRSDFVKSGQDEDDGIFSAVREVGIANNTA : 246
wlsy      g66      6 k 6 n5      g      n r yy      rsqPp 6 6

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          *          380          *          400          *          420
Foxg_09264 : -AYIEHTNDTDILERALPLLVQEHEFFMTNRSVPVYINN-ETYYLNTYNNVSNTRPRPESY : 293
Fveg_06865 : -AYIEHTNDTDILERALPLLVQEHEFFMTNRSVPVYINN-ETYYLNTYNNVSNTRPRPESY : 293
Fgsg_05622 : -AYVE-----YNVSNTRPRPESY : 252
Mo_Trel : -IYVEHTNDTSILGRAVPLLIKEHEFFINNRSIDVEASNGKTYRLQRYAVTNTQPRPESY : 295
Fveg_02610 : RVYEKIKHEPDAKEFLRRSILAAIKEYHVSVMSEPRLDPE-----STGLSRYRPEGRGV : 395
Foxg_05530 : RVYEKIKHEPDAKEFLRRSILAAIKEYHVSVMSEPRLDPE-----STGLSRYRPEGRGV : 395
Fgsg_09895 : RVYEKIKHEPDAKEFLRRSILAAIKEYHVSVMSEPRLDPE-----TGGLSRYRPEGRGV : 395
Mo_Nth1 : RVYDKIRHEPDATEFLRTAILAAIKEYHVSVMVAEPRLDPE-----VTGLSRYRPEGTGV : 396
Bc_Trel : RVFDKIKHEPGSLDFLKTAILAAIKEYHVSVMVAEPRYDPE-----VTGLSRYRPEGLGV : 397
An_TreB : RVYDRIQNEPGAMDFLRHAILAAIKEYHVSVMVAEPRLDPE-----VSGLSRYRSPGIGV : 406
Fveg_13500 : YIIAQVNG-SKSLDLSKRQLVHGKPYVHTNESSIAQAIP-----VKFSTGVPVRI : 327
Foxg_14683 : YIIAQVNG-SKSLDLSKRQLVHGKPYVHTNESSIAQAIP-----VKFSAGVPVHI : 295
                    5                                6

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          *          440          *          460          *          480
Foxg_09264 : REDYVTANNESYYSP-SGEVYSGGEEINFKTKREALGNLASGAESGLDYSVKQWVARPDDA : 352
Fveg_06865 : REDYVTANNESYYSP-SGEVYSGGEEINFKTKREALGNLASGAESGLDYSVKQWVARPDDA : 352
Fgsg_05622 : REDYITAENTSYYSPESGKVYKGGEEISFKQKEALGNLASGAESGLDYTVKWIARPEDA : 312
Mo_Trel : REDYITASNRSYYSPSGIIPESHQINSEKAVLYSHLASGAESGWYDTSRWLSTPSDA : 354
Fveg_02610 : PPETEATHFVHILDE----YIKKHMTFEQFVRAY-NHGEVEEPELDEYFMHDAVRES : 449
Foxg_05530 : PPETEATHFVHILDE----YIKKHMTFEQFVRAY-NHGEVEEPELDEYFMHDAVRES : 449
Fgsg_09895 : PPETEATHFVHILDE----YIKKHNTTFEDFVRKY-NHGEIEEPELDEYFMHDAVRES : 449
Mo_Nth1 : PPETEADHFLHILEF----YKKNHMTFKEFVEAY-NFGRIREPELDEYFLHDAVRES : 450
Bc_Trel : PPETEASHFEHLLAF----YAEKYNMTFKEFVDAY-NNGRVVEKELDDYFLHDAVRES : 451
An_TreB : PPETEASHFLHLLTF----YAEKHGMEFKEFVQAY-NYGVKVEPELDEYFMHDAVRES : 460
Fveg_13500 : TKYVGAASSDAFDDP----GKIAKEASRRAMEEGYEKSLLSHVREWESVMPSDSVDSYA : 382
Foxg_14683 : TKYVGAASSDAFEDP----EKTAKEASNRALLEEGYEKSLLSHLKEWESVMPSDSVDSYA : 350
                    P          y          Y          e          d

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      *           500           *           520           *           540
Foxg_09264 : --IRNYFPLRYLNTRNIIPVDLNSILYGNEIAIAEFYEQTGNSSASRQWREVAANRSFA : 410
Fveg_06865 : --IRNYFPLRYLNTRNIIPVDLNSILYGNEMAIAEFYEQTGNSSASRQWREVAANRSFA : 410
Fgsg_05622 : --IRNYFPLRYLNTRNIIPVDLNSILYGNEIAIADFYEQTGNSSASEQWREVAANRSYA : 370
Mo_Trel : --VRNYFPLRSINTNINPVDLNSILYANEVAIAEFILNRTGNSTGASDWMDLAKQRSEA : 412
Fveg_02610 : --GHOTSYRLEGVCAN--LATIDLNSLLFKYETDIARTIRSVFN--DRLTMPEEFCAGTPY : 504
Foxg_05530 : --GHOTSYRLEGVCAN--LATIDLNSLLFKYETDIARTIRSVFN--DRLTMPEEFCAGTPY : 504
Fgsg_09895 : --GHOTSYRLEGVCAN--LATIDLNSLLFKYETDIARTIRSVFN--DKLTMHEEFCAGTPY : 504
Mo_Nth1 : --GHOTSYRLEGVCAD--LATVDLNTLLFKYETDIARTIRNVFG--DKLVIPAEYCVGS-L : 504
Bc_Trel : --GHOTSYRLERVCAD--LATIDLNSLLYKYEKDIAYTIRTFFQ--DKLEVPAEFCVGD-M : 505
An_TreB : --GHOTSYRLERVCGN--LATVDLNSLLYKYEVDIARVIRVYFK--DKLEIPVEFRTPA-T : 514
Fveg_13500 : SPENTLPDEYIIDSIIAVTNTYYLLQNTVGKNAQKKVSGAPVNVDSISVGGLTSDSY : 442
Foxg_14683 : FPENTLPDDEYIIDSIIAVTNTYYLLQNTVGKSAQKKAVSGAPVNIDSIISVGGLTSDSY : 410
      1       1 6       6 6dlN L e ia

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      *           560           *           580           *           600
Foxg_09264 : MHAFMWNETHSYFDYNLTSKAQSIFYPTDNSTAEIDKEN-----APKGKQVFFSPTQ : 463
Fveg_06865 : MHAFMWNETHSYFDYNLTSKAQSIFYPTDNSTAEIDKEN-----APKGKQVFFSPTQ : 463
Fgsg_05622 : MHGFLWNETLWSYFDYNLTSKAQQIIYFVDENTTVVDED-----APKGQQVFFSPTQ : 423
Mo_Trel : MYALMWNETLWSYFDYNMTSKTQNRFIPVDEDAVSIETNN-----APAGQQVFFFHVAQ : 465
Fveg_02610 : QPGEVLSSAANDRRAKRRKLTVDKLMWDEKE-GMFFDYDT-----AKRERCTYESCTT : 556
Foxg_05530 : QPGEVLSSAANDRRAKRRKLTVDKLMWDEKE-GMFFDYDT-----PKRERCTYESCTT : 556
Fgsg_09895 : QPGEVLSSAANDRRAKRRKLTVDKLMWDEKE-GMFFDYDT-----AKRERCTYESCTT : 556
Mo_Nth1 : QPGQVETSAIWDRRSKRRKLAIDKYLWNEEA-GMYFDYDT-----AKRQCCNYESCTT : 556
Bc_Trel : TPGQLQTSSMWDRRARGRKLAIDKYLWNKEK-GMYFDYNT-----LKKEQCTYESATT : 557
An_TreB : KDIQSESSSVWDRRARRRKMRMDTYLWDEEK-GMYFDYDT-----VKQERTNYESATT : 566
Fveg_13500 : AGLIFWDADLFMQPGLTTSHPEAAQRITNYRVAKYDQAKKNIATSYAGSQNKTKESDSAA : 502
Foxg_14683 : AGLIFWDADLFMQPGLTTSHPEAAQRITNYRVAKYDQAKKNIATSFAGSQNKTKESASAA : 470
      5                                     5

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      *           620           *           640           *           660
Foxg_09264 : FYPFWLGAAPDYLKNNPYAVLNAYKRVSYLLDTRQGGIPASN---VESGQQWDQPNVWP : 519
Fveg_06865 : FYPFWLGAAPDYLKNNPYAVLNAYKRVSYLLDTRQGGIPASN---VESGQQWDQPNVWP : 519
Fgsg_05622 : FYPFWLGAAPDYLKNNPFAVYTAYKRVEYLDNRDGIPASN---VETGQQWDQPNVWP : 479
Mo_Trel : YYPFWTGAAPRSLKNNPLAVLRAYERIDAYLDIKRGAIPATN---LKTGQQWDEPNVWP : 521
Fveg_02610 : LWALWAGIATPKQA--AEMVRKALPKFEAYGGLVSGTEESRGAVGLRPNRQWDYPYGWP : 614
Foxg_05530 : LWALWAGIATPKQA--AEMVRKALPKFEAYGGLVSGTEESRGAVGLRPNRQWDYPYGWP : 614
Fgsg_09895 : LWALWAGIATPKQA--AEMVRKALPKFEAYGGLVSGTEESRGAVGLRPNRQWDYPYGWP : 614
Mo_Nth1 : FWALWAGVASPKQA--AIMVTRALPKFEAYGGLLSGTEESRGQIGLDRPNRQWDYPYGWA : 614
Bc_Trel : FWAMWAGVASPQQA--ASLVTNALPKFEAAAGLLSGTEESRGAVGLRPNRQWDYPYGWA : 615
An_TreB : LWAMWAGLVTPRQA--SAMITKALPKFEEFGGIVSGTEESRGAVGLRPNRQWDYPYGWA : 624
Fveg_13500 : VYPWTSCRFGNCTATGPCWDYEYHLNGDIGISLVNQWVTSGDTDFFKETLLPIYDSVANL : 562
Foxg_14683 : VYPWTSCRFGNCTATGPCWDYEYHLNGDIGISLVNQWVTSGDTDFFKETLLPIYDSVANL : 530
      5 w G

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      *           680           *           700           *           720
Foxg_09264 : PLMHLMSGLEKVPATFGIMDPSFIEVRRLALRLAQRYLDSTFCTWRATGGTTSDLPRIQ : 579
Fveg_06865 : PLMHLMSGLEKVPATFGIMDPSFIEVRRLALRLAQRYLDSTFCTWRATGGTTSDLPRIQ : 579
Fgsg_05622 : PLMHLLMAGLERVPPTFGIRDPSFVEVRRLALRLGQRYLDSTFCTWYATGGSTSETPKLIQ : 539
Mo_Trel : PLMHLLMEGLTRVPATFGEDDVAWTEIQDLALRLGQRYLDSTFCTWYATGGSTSETPQLQ : 581
Fveg_02610 : PQQMLAWTGLIRY-----SFTEEAERIAYKWLFMVTKAFVDFHGVVVEKYDVT : 662
Foxg_05530 : RNGGLAWSGRPRN-----GITDNGT-----IPTAGLH : 617
Fgsg_09895 : PQQMLAWTGLIRY-----SFTEEAERIAYKWLFMVTKAFVDFHGVVVEKYDVT : 662
Mo_Nth1 : PQQMLAWTGLYRY-----SFTEEAERLAYKWLFMITKAFVDFNGVVVEKYDVT : 662
Bc_Trel : PQQMLAWTGLLRY-----NYQEDAERLAYKWLFMITTAFVDFNGVVVEKYDVT : 663
An_TreB : PQQMLAWTGFARY-----GYQEEAERLAYKWLYMITKAFVDFNGVVVEKYDVT : 672
Fveg_13500 : FADLLKPNGSSWT-----ITNMTDPDEYANHIDAGGFTMALASETLIQANKIRRQ : 612
Foxg_14683 : FADLLKPNGSSWT-----ITNMTDPDEYANHIDAGGFTMALASETLIQANQIRRQ : 580
      6 G

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		*	740	*	760	*	780	
Foxg_09264	:	SAAEGADGIMFEKYADNATNVAGGGGEYEVVEGFGWTNGVLIWAVDEFGNRLKQPQC-N-F	:	638				
Fveg_06865	:	SAAEGADGIMFEKYADNATNVAGGGGEYEVVEGFGWTNGVLIWAVDEFGNRLKQPECN-F	:	638				
Fgsg_05622	:	STSDEEEGIMFEKYADNATNVAGGGGEYEVVEGFGWTNGVLIWAVDEFKNRLTRPKCDNL	:	599				
Mo_Trel	:	GLNAEDKGIMFEKYGDNSTNVAGSGGEYEVVEGFGWTNGVLMWVADTFNNKLTRPDCGNI	:	641				
Fveg_02610	:	RPVDPHR--VDAEYGNQGLGFRG-----VNKEGFAWVNASYIYGLQIINAHMRR-----	:	709				
Foxg_05530	:	SKCWPGR--V-----	:	625				
Fgsg_09895	:	RPADPHR--VDAEYGNQGLGFRG-----VNKEGFAWVNASYIYGLQIINAHMRR-----	:	709				
Mo_Nth1	:	RPVDPHR--VDAEYGNQGLGFKG-----VAKEGFGWVNASYIYGLQIINAHMRR-----	:	709				
Bc_Trel	:	RVVDPHK--VDAEYGNQGSDFKG-----VAKEGCGWVNASYVYGLQIINAHMRR-----	:	710				
An_TreB	:	RPIDPHR--VDAEYGNQGVDFKG-----APREGFGWVNASYVYGLEMLNAHQRR-----	:	719				
Fveg_13500	:	FGMTENKTQDEIASDVLFIENGITLEFTTMNGSAIVKQADVVLMSFPLGYNDN-----	:	666				
Foxg_14683	:	FGMTENKTQDEIASDVLFIENGITLEFTTMNGSAIVKQADVVLMSFPLGYNDN-----	:	634				

g g

		*	800	*	820	*	840	
Foxg_09264	:	TGDSSN-----ERRDTNSAVMLHARDAARVKKFGNRKRAAEKAAHKRSSRLFHF----	:	687				
Fveg_06865	:	TGDSSN-----ERRDTDSAVMLHARDAARVKKFGNRKRAAEKAAHKRSSRLFHF----	:	687				
Fgsg_05622	:	ESAHSN-----DKRDP-SAVMLNARDAKRVKKFGRRKRAAEKAAHKRSSRVFYF----	:	647				
Mo_Trel	:	TAANVHSDGSQARKRGEMWSALEMHPYDAAWTKEFGARKVRRDKAEARALGNVMGGV---	:	698				
Fveg_02610	:	-----ALGALTPTYQTLLRAIEKNEEKTLAGLLAA----	:	738				
Foxg_05530	:	-----	:	-				
Fgsg_09895	:	-----ALGALTPTYDTLMKAIIEQNEEKTLAGLLSS----	:	738				
Mo_Nth1	:	-----ALGTLTPYDTFIKALEDNRRNALSEMV-----	:	736				
Bc_Trel	:	-----TLGTLTPWDQYNKAMNL-----	:	727				
An_TreB	:	-----ALGAVTPWETYSKAVSAQGSDTVLENRSE----	:	748				
Fveg_13500	:	-----YTDQNGLDDLDYYANKQSPDGPAMT-----	:	691				
Foxg_14683	:	-----YTDQNGLDDLDYYANKQSPDGPAMTWAISIVADE	:	669				

Isocitrate lyase has been shown to be required for full virulence of a number of plant pathogenic fungi including the rice blast fungus *M. oryzae* (Wang *et al.*, 2003). This species produces a melanised infection structure known as an appressorium. The appressorium develops an extremely high turgor (Howard *et al.*, 1991) which is used to drive penetration of the host leaf surface and initiate infection. Targeted deletion of the *ICL1* gene encoding isocitrate lyase affected infection-related development of this species at the prepenetration stage. Spore germination was reduced together with delayed appressorium formation and maturation, resulting in reduced disease symptoms on rice leaves. In *Colletotrichum lagenarium*, the causal agent of cucumber anthracnose, appressoria of *icl1* disruption mutants retained melanin biosynthesis but penetration hyphae did not develop, leading to a reduction in virulence (Asakura *et al.*, 2006). Inoculation of wounded host plants indicated, however, that post-invasive growth was unaffected in the *icl1* mutant. Appressorium formation and host penetration in both *M. oryzae* and *C. lagenarium* involves lipid degradation (Thines *et al.*, 2000; Yamauchi *et al.*, 2004), however, lipolysis is unaffected in the *C. lagenarium icl1* mutant. In *Leptosphaeria maculans*, the causal agent of blackleg disease of canola, a plasmid insertion event resulting in the deletion of *ICL1* and adjacent sequence produced a strain with reduced virulence and *in planta* hyphal growth on *Brassica napus* and *B. juncea* cotyledons. Introduction of the wild type *ICL1* gene into the *icl1* strain partially recovered virulence on *B. napus* (Idnurm and Howlett, 2002). In both *C. lagenarium* and *L. maculans*, virulence of the *icl1* strain could be at least partially restored by the addition of glucose or sucrose, implying a role for gluconeogenesis via the glyoxylate cycle in host invasion. Malate synthase, another key glyoxylate cycle enzyme, is required for pathogenicity of the wheat leaf pathogen *Stagonospora nodorum* (Solomon *et al.*, 2004b). In *F. graminearum*, appressorium formation is not used for entry into the wheat ear. Also in the point assay used, the spores were placed directly inside the wheat floret. Therefore, it would appear that unlike *L. maculans* and *S. nodorum*, where the glyoxylate cycle contributes to leaf colonisation, this pathway is not required by *F. graminearum* to successfully colonise the wheat ear. The observation that *in vitro* the *icl1* *F. graminearum* mutants could grow more rapidly than the wild-type strain in the presence of sucrose as the sole carbon source may have influenced the disease progression of the *icl1* deletion strain. In the wheat ear the soluble sugar

arriving in the phloem is sucrose, so this strain would be able to utilise this carbon source more effectively than the wild-type strain. This might have contributed to the unaffected virulence phenotype of the *icl1* strain.

In fungal pathogens of humans and animals, however, isocitrate lyase appears to be frequently dispensable for complete virulence. While *ICL1* deletion in *C. albicans* prolongs the survival of mice (Lorenz and Fink, 2001), in *A. fumigatus*, which causes invasive aspergillosis in immunocompromised individuals, *ICL1* is not required for virulence in a murine model and *icl1* deletion strains exhibit invasive growth (Schobel *et al.*, 2007). In addition, *ICL1* is not required for virulence in an isolate of *S. cerevisiae* that is pathogenic to mammals (Goldstein and McCusker 2001). While expression of the *ICL1* gene in the human and animal pathogen *C. neoformans* was found to be upregulated during infection, null mutants of this gene did not show reduced virulence (Rude *et al.*, 2002).

The *ICL1* gene has also been deleted in another strain of *F. graminearum*, namely Z03643 (Lee *et al.*, 2009a). The Z03643 *icl1* mutant grew similar to the wild type strain on complete medium, as seen for the PH-1 *icl1* mutant, which showed similar growth rates to the wild type strain on both complete and minimal medium. However, the Z03643 *icl1* mutant produced much whiter aerial mycelium on complete medium than the wild type, a phenotype not present for the PH-1 *icl1* mutant, which produces peach-coloured aerial mycelium similar in appearance to the wild type PH-1 strain (data not shown). Growth of the Z03643 *icl1* mutant was reduced with acetate as the sole carbon source, consistent with findings of the PH-1 strain *icl1* mutant, but completely abolished on other C₂ compounds and fatty acids tested. That the PH-1 *icl1* deletion strain retained the ability to grow at around 50% of the wild type rate on olive oil may be due to the presence of extra compounds in this substance not found in the purified fatty acids used in the Z03643 study. A reduced growth rate for *icl1* mutants on fatty acids or two-carbon compounds is consistent with results obtained for *C. lagenarium*, *M. oryzae*, *C. neoformans*, and *L. maculans* (Idnurm and Howlett, 2002; Rude *et al.*, 2002; Wang *et al.*, 2003; Asakura *et al.*, 2006).

For both *F. graminearum* strains, the isocitrate lyase mutant showed disease symptoms on susceptible wheat comparable to the wild type. However, it should be noted that in the Z03643 study, a spray inoculation method was used to

assess disease symptom severity unlike the point inoculated method employed with PH-1 and PH-1 *icl1*. The wild type Z03643 strain does not produce symptoms that spread through the adjacent spikelets when a conidial suspension is point inoculated onto the ear. This prevents any reduced virulence phenotype of mutants that may show a slower rate of spread through the ear from being noted. These results are comparable to those previously described for animal pathogenic fungi yet unusual for plant pathogenic fungi, as described above.

The effect of *ICL1* deletion on *F. graminearum* virulence may be complicated by the presence of a second isocitrate lyase gene, as found when the methylisocitrate lyase gene, *MCL1*, was deleted in a Z03643 *icl1 mcl1* double mutant in *F. graminearum*. *MCL1* functions in the methyl isocitrate lyase cycle that metabolises propionyl-CoA to pyruvate. The Z03643 *mcl1* mutant showed slightly reduced disease severity compared to the wild type Z03643 strain on barley but not wheat, however the Z03643 *icl1 mcl1* double deletion strain showed greatly reduced virulence on both wheat and barley ears, with only the formation of a few small necrotic spots. In addition, the double mutant could no longer grow on acetate. As may be expected, the Z03643 *mcl1* mutant was able to grow on acetate and fatty acids but not on propionate. Species such as *M. oryzae* and *C. lagenarium* appear to possess only one *ICL1* gene (Asakura *et al.*, 2006), which may be the reason a reduced virulence phenotype is observed upon disruption of the isocitrate lyase gene.

The deletion of the *pkar* gene revealed that *PKAR*, the regulatory subunit of the *F. graminearum* protein kinase A is required for a full rate of FEB symptom spread on wheat ears. The rate of symptom spread was drastically reduced in the *pkar* strain and symptoms remained confined to the inoculated spikelets and adjacent rachis. The effect of deletion of the PKA regulatory subunit on the virulence of fungal pathogens appears to be highly variable. Disruption of *PKR1*, the regulatory subunit of PKA in *C. neoformans* var. *grubii* resulted in hypervirulence (D'Souza *et al.*, 2001), while deletion of the *PKAR* gene of *A. fumigatus* (Zhao *et al.*, 2006) or the *PKAR* gene of *B. cinerea* reduces virulence, however, no PKA activity is detected in the *pkar* strain (Schumacher *et al.*, 2008). In *C. albicans*, meanwhile, homozygous deletion of the PKAR-encoding gene is lethal (Cassola *et al.*, 2004).

Partial losses of virulence resulting from PKAR deletion are found for species such as *C. lagenarium*, where, while the *rpk1* deletion mutant is non-pathogenic on cucumber, it can form lesions when inoculated onto wounds (Takano et al., 2001) suggesting that the defect in virulence in this case is at the pre-penetration or penetration stage of development. In *Mycosphaerella graminicola*, *BCY1* disruption produces a mutant that is able to germinate, penetrate the host leaf and colonise the mesophyll, but is unable to produce pycnidia (Mehrabi and Kema, 2006). In each case, different stages of the infection process appear to be most affected by loss of the *PKAR* gene. In *F. graminearum*, the *pkar* mutant is able to cause bleaching of the inoculated spikelets but then does not spread to other spikelets and by 20 days post-inoculation shows only some limited browning on the rachis close to the inoculated spikelets. In this species too, the fungus may be able to complete the initial infection stages in the inoculated spikelets but later infection-related processes that would allow colonisation of the whole ear may be unable to be completed in the absence of the *PKAR* gene. In *U. maydis*, mutation of *UBC1* (*PKAR*), a gene which is required for filamentous growth, produces strains which can colonise plants and cause localised symptoms similar to wild type but cannot form galls (Gold et al., 1997), although a dikaryon that is homozygous for *UBC1* deletion would be expected to be non-pathogenic due to its lack of filamentous growth.

The catalytic subunit of PKA (here termed PKAC) has also been investigated in a number of fungal species. Disruption of a single PKAC gene in fungi is often sufficient to produce a series of phenotypic changes including a reduction in virulence of animal-, plant- and insect-pathogenic species. Disruption of *PKA1*, considered the major PKA catalytic subunit gene in *C. neoformans* var. *grubii*, resulted in a complete loss of virulence (D'Souza et al., 2001). In *A. fumigatus*, *PKAC1* deletion reduces expression of the *PKSP* virulence factor and *pkac1* mutants are rendered almost avirulent (Liebmann et al., 2004) while in *Candida albicans*, *TPK2* (*PKAC*) deletion results in reduced virulence (Sonneborn et al., 2000).

In *U. maydis* disruption of the *ADR1* (*PKAC*) gene causes a loss of pathogenicity. However, deletion of the second PKAC-encoding gene, *UKA1*, has little effect on virulence (Dürrenberger et al., 1998). In *F. verticillioides* *fpk1*

(PKAC) disruptant also shows reduced virulence (Pei-Bao *et al.*, 2010). In *M. oryzae* *CPKA* deletion causes delayed appressoria formation and reduced virulence (Mitchell and Dean, 1995, Xu *et al.*, 1997). The *cpkA* mutants form smaller appressoria that are defective in penetrating plant cells, but inoculation of wounds still allows lesion formation indicating that post- penetration disease development may be unaffected (Xu *et al.*, 1997). The insect pathogen *M. anisopliae* PKAC disruption mutant, *pka1*, also shows greatly reduced virulence due to a delay in appressoria formation and in addition has a reduction in appressorial turgor pressure (Fang *et al.*, 2009). *Verticillium dahlia* *PKAC1* disruptants were still able to infect tomato and eggplant but disease severity was reduced. Ethylene is thought to be important in symptom induction in this species and the *pkac1* disruption strains were shown to produce reduced amounts of ethylene (Tzima *et al.*, 2010). In *Trypanosoma cruzi*, meanwhile, use of a PKAC inhibitor is lethal (Bao *et al.*, 2008).

Deletion of the PKAR gene would be expected to result in constitutive, unregulated PKA activity, while PKAC deletion resulted in reduced or abolished PKA activity (dependent on the deletion of one or both PKAC genes), so the PKAR and PKAC mutants may be expected to have opposing phenotypes. While this result is found for example with *C. neoformans* var. *grubii* virulence (D'Souza *et al.*, 2001), this does not seem to be the case in some species. In *M. graminicola*, both *BCY1* (PKAR) and *TPK2* (PKAC) disruptants are defective in the later stages of disease progression (Mehrabi and Kema, 2006). In *A. fumigatus*, deletion of *PKAR* reduces virulence (Zhao *et al.*, 2006) and deletion of *PKAC1* almost completely abolishes virulence (Liebmann *et al.*, 2004). In *B. cinerea*, both *pka1* and *pkaR* strains show reduced virulence, although this may be due to the lack of PKA activity detectable in the *pkaR* strain (Schumacher *et al.*, 2008). *B. cinerea* *pka2* mutants show no obvious phenotypes (Schumacher *et al.*, 2008).

The *in vitro* growth rates of the *F. graminearum* *pkar* mutant were severely reduced on both minimal and complete medium. Growth defects have been noted for a number of PKAR mutants in fungi. For example, the *A. fumigatus* and *A. niger* *pkaR* and *C. lagenarium* *rpk1* mutants show reduced growth (Takano *et al.*, 2001; Staudohar *et al.*, 2002; Zhao *et al.*, 2006). In *U. maydis*, *UBC1* (PKAR), is required for filamentous growth (Gold *et al.*, 1997). The *B.*

cinerea *pkaR* and *M. circinelloides* *pkaR1* mutants, however, show reduced growth on solid medium but not in liquid culture (Schumacher *et al.*, 2008; Ocampo *et al.*, 2009). This is in contrast to the *F. graminearum* *pkar* mutant which also shows reduced growth in liquid complete medium (data not shown). In addition, overexpression of *PKAC1* in *A. fumigatus* also causes reduced growth (Grosse *et al.*, 2008). In *A. nidulans*, however, overexpression of *PKAB* enhances growth and rescues the growth defects of *PKAA* deletion (Ni *et al.*, 2005). In *C. albicans*, *bcy1* (PKAR) homozygotes could not be obtained, suggesting this gene is probably essential for viability (Cassola *et al.*, 2004).

Growth reduction has also been noted in PKAC mutants, for example *M. anisopliae* *pka1*, *A. fumigatus* *pkaC1*, *A. nidulans* *pkaA*, *M. graminicola* *tpk2* and *N. crassa* *pkac-1* (Fillinger *et al.*, 2002; Liebmann *et al.*, 2004; Banno *et al.*, 2005; Mehrabi and Kema, 2006; Fang *et al.*, 2009). The *B. cinerea* *pka1* mutant shows reduced growth on solid medium but not in liquid culture (Schumacher *et al.*, 2008). In *Fusarium verticillioides*, *FPK1* disruption causes reduced growth with reduced production of aerial mycelium on complete medium. (Pei-Bao *et al.*, 2010). In *A. niger* meanwhile, a loss of PKA activity leads to reduced growth (Staudohar *et al.*, 2002).

Other effects on growth include disruption of the *U. maydis* PKAC-encoding *ADR1* gene, which causes constitutively filamentous growth (Dürrenberger *et al.*, 1998) and the *C. albicans* *tpk1* mutant, which has defective hyphal morphogenesis on solid medium but only a slight effect on hyphal formation in liquid medium. Deletion of *TPK2* causes only a partial defect in growth on solid medium but defective growth in liquid medium. The yeast form of *tpk2* but not *tpk1* was unable invade agar. Homozygous *tpk1* and *tpk2* mutants appeared unaffected except for a strain lacking *TPK2* but with a single regulatable *TPK1* which was expressed at low levels. This strain was severely reduced in growth (Bockmuhl *et al.*, 2001). In *V. dahlia*, *PKAC1* disruption does not appear to affect growth (Tzima *et al.*, 2010), while in *Y. lipolytica* the *tpk1* mutant has normal growth but always grows filamentously (Cervantes-Chavez and Ruiz-Herrera, 2009).

Deletion of *pkar* in *F. graminearum* also led to altered hyphal morphology with very short, almost cuboidal segments in the *pkar* hyphae. Hyphal segments in

the wild type strain appeared much longer and more rectangular in shape, with those of the *PKAR-e* strain also appearing slightly longer than those of the *pkar* mutant. Altered hyphal morphology was also noted in a temperature-sensitive mutant of the PKAR-encoding gene, known as *mcb*, in *N. crassa* (Bruno *et al.*, 2006). At 25 °C, below the restrictive temperature, where defined hyphae can be formed (at the restrictive temperature growth polarity is lost), conidia of the *mcb* mutant growing on sucrose minimal agar germinate and produce hyphae with swollen tips. These gave rise to a series of bulbous compartments that are separated by septa somewhat resembling those found in the *F. graminearum* *pkar* mutant. These compartments could then generate further hyphal tips that develop into typical hyphae. A shift to the restrictive temperature caused a loss of growth polarity and all regions grew, causing an increase in hyphal length and diameter until the hyphae burst. This was thought to be due to cell wall growth rather than swelling due to weakened cell walls and turgor pressure as thick walls were observed after bursting, walls of the *mcb* mutant at the restrictive temperature were more resistant to hydrolytic enzymes and the fact that wild type growth was not restored by the addition of 1M sorbitol, nor was lysis prevented.

In addition, morphological abnormalities were noted for the *pkaR* deletant of *A. fumigatus* which possesses early hyphae that are wider, thicker and darker than the wild type strain (Zhao *et al.*, 2006). The *A. niger* *pkaR* mutant shows a loss of growth polarity in submerged culture with the formation of swollen hyphal tips (Staudohar *et al.*, 2002), while fresh hyphae of the *F. verticillioides* *fpk1* mutant are stubby and lack branches (Pei-Bao *et al.*, 2010). Overexpression of PKAC-encoding genes in *C. albicans* leads to the production of short, curved, bulged filaments (Bockmuhl *et al.*, 2001). In *M. graminicola*, colony budding morphology is also dependent on PKA signalling (Mehrabi and Kema, 2006), as is the yeast-hyphal transition in *C. albicans* (Sonneborn *et al.*, 2000) and in *U. maydis*, disruption of *UBC1* causes a multiple budding phenotype (Gold *et al.*, 1994).

The cellular compartments seen in the *F. graminearum* *pkar* mutant could result from increased septa formation, or alternatively from an increased rate of nuclear division. This phenotype does not appear to involve the bursting of fungal cells suggesting that they maintain a functional cell wall. In yeast, PKA is

known to be involved in control of the cell cycle (Sonneborn *et al.*, 2000). The *pkaR* deletant of *A. fumigatus* shows an increased expression of cell cycle regulatory genes and in the number of nuclei present in hyphae (Fuller *et al.*, 2009). In *M. anisopliae* PKA is responsible for up-regulating approximately one-third of the genes induced by insect cuticle, including some of those responsible for cell cycle control and the cytoskeleton (Fang *et al.*, 2009). In *A. nidulans* however, addition of a PKA activator appeared to slow the duplication cycle and allow polarisation (Vanzela and Said, 2002).

Summary

The targeted deletion of the mitogen-activated protein kinase kinase (MAPKK) *STE7* has been published elsewhere (Ramamoorthy *et al.*, 2007). These studies reported drastically reduced pathogenicity on wheat ears for the *ste7* deletion strain compared to wild type. In addition, deletion of *SNF1* leads to drastically reduced virulence of *F. graminearum* on wheat ears (Beacham *et al.*, 2010; Lee *et al.*, 2009b). Together with the data presented here, these results confirm a role in symptom spread rate for four of the five PHI-base pathogenicity gene homologues in the micro-region (*nth1*, *snf1*, *ste7* and *pkar*).

The reduction of *in vitro* growth rate of the *nth1* and *pkar* strains was seen for each transformant tested and appears to explain their defects in *in planta* symptom spread rates to differing extents. The wheat ear is considered to be a nutrient-rich environment for the fungus, and so the slightly reduced growth rate of *nth1* on complete medium (reduced by 15%) would appear to explain its reduced rate of FEB symptom spread (reduced by 17%). The symptom coverage of the wheat ear by *pkar* is over 80% less than for the wild-type at later time points, while the difference in growth rate between the two strains on complete medium is 67%. This suggests that the inherent growth rate defect accounts for a large portion of the reduction in disease development rate by the *pkar* strain. It is possible that an additional factor is inhibiting disease formation by *pkar in planta* in addition to its growth rate defect but further investigation may be required to determine the cause and nature of any such defect. The *snf1* and *ste7* strains appear to only be marginally affected in growth on complete medium.

With the micro-region having been demonstrated to contribute to *F. graminearum* growth and spread on wheat ears, the next objective was to determine the role of additional micro-region genes in disease symptom spread. By this approach, the possibility that this micro-region was a hotspot for genes contributing to the successful establishment and / or spread of Fusarium ear blight could be explored.

Chapter 5. Initial Characterisation of Additional Genes in the Micro-Region

5.1 Introduction

Loss of two of the three homologues of verified virulence genes present in the micro-region was shown in Chapter 4 to slow the spread of *F. graminearum* through wheat ears, in addition to the two previously characterised genes. This suggested an important role for this micro-region of chromosome I in contributing to the disease-causing ability of this species. The micro-region may therefore harbour additional genes whose deletion results in a slower rate of FEB symptom spread on wheat and would not previously have been identified due to a lack of homology to known virulence genes in other species. Therefore, six further genes in the cluster were selected for targeted deletion that are either not homologous to any known virulence genes or showed no homology to any annotated genes. Single gene deletion strains of *FGSG_09891*, *FGSG_09893*, *FGSG_09900*, *FGSG_09905*, *FGSG_09906* and *FGSG_09907* (later designated *FCV1*) were generated.

FGSG_09893 shows similarity to peptidases, while *FGSG_09900* appears to encode a membrane-integral protein of unknown function (see Chapter 3). *FGSG_09905* and *FGSG_09906* do not show similarity to any annotated protein sequences. *FGSG_09905* may represent a *F. graminearum*-specific sequence (see Chapter 3).

Fgsg_09891 shares 48.5% protein sequence identity with *S. cerevisiae* Get3, a highly conserved ATPase protein with homology to the ArsA subunit of bacterial arsenical transporters (Boskovic *et al.*, 1996, Shen *et al.*, 2003). Get3 (also known as Arr4) has been suggested to be involved in a number of different processes including stress resistance (Shen *et al.*, 2003), metal ion homeostasis (Metz *et al.*, 2006) and secretory protein trafficking (Schuldiner *et al.*, 2005; Auld *et al.*, 2006; Schuldiner *et al.*, 2008).

Fgsg_09907 (*Fcv1*) shows similarity to Spf27/Bcas2 (Breast Carcinoma Amplified Sequence 2) proteins, some of which have been shown to function in splicing of pre mRNAs (Ohi *et al.*, 2002). *Fgsg_09907* also shows similarity to the *Arabidopsis thaliana* Mos4 protein (this study; Palma *et al.*, 2007), which

may act to regulate plant disease resistance (*R*) gene expression in defence against pathogens mediated by the late acting signal-transducing protein Npr1 (Zhang *et al.*, 2003; Palma *et al.*, 2007).

5.2 Methods

The PCR fusion, transformation of *F. graminearum*, screening of transformants, Southern blots, wheat ear pathogenicity assays and *in vitro* growth tests are described in chapter 2.

5.2.1 Complementation of *fgsg_09907.3 (fcv1)*

The wild type *FGSG_09907 (FCV1)* coding region plus upstream and downstream sequence was amplified from PH-1 genomic DNA and the *Neo* neomycin phosphotransferase geneticin resistance gene was amplified from pSK666 (S. Kang, Pen State University, primers listed in **Table 5.1**). PCR products were cleaned using a PCR purification Kit (Qiagen). The *Neo* gene was fused to the wild type *FGSG_09907* gene in a second PCR reaction. 6 x 50 µl reactions were performed, pooled, precipitated and resuspended in a small volume of water. DNA concentration was determined using a Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies). Transformation of *fcv1* protoplasts was performed as described in Chapter 2 but with a longer growth time for *fcv1* plates to allow sufficient spore production and 75 µg/ml geneticin and 75 µg/ml hygromycin as selection. DNA extraction was performed on mycelia samples using a XNAT REExtract-N-Amp Tissue PCR Kit (Sigma). Transformants were screened by PCR using primers listed in **Table 5.2**.

Table 5.1. Primers for amplification of the wild type *FGSG_09907* (*FCV1*) gene and *Neo* geneticin resistance gene. The two resulting products were mixed and fused using primers AB80 and AB77.

Gene	Primers	Primer sequence (5'-3')
<i>FGSG_09907</i> (<i>FCV1</i>)	AB80	TCATTTACAGCTACGAAACGAGATG
	AB81	ACATGGTGGAGTGAGGGGTACCTATTGAACGCAGTTGGGGCA
<i>Neo</i>	AB76	GGTACCCCTCACTCCACCATGT
	AB77	CGCCAGCAGTAGACACTTGG

Table 5.2. Primers for PCR analysis of *FGSG_09907* (*FCV1*) complementation transformants.

Gene	Product length (bp)	Primer	Primer sequence (5'-3')
<i>FGSG_09907</i> (<i>FCV1</i>)	650	U130	CCGTCGCTCCTGCTTATCAC
		U131	ACTTCGGTTTCCAGCACTCG
<i>Neo</i>	1430	AB76	GGTACCCCTCACTCCACCATGT
		AB77	CGCCAGCAGTAGACACTTGG
<i>Hph</i>	834	Hyg3	TCTCGGAGGGCGAAGAATCTC
		Hyg4	TTCTGCGGGCGATTTGTGTAC
<i>FGSG_09900</i>	1490	AB51	GCCACACGATGTCAAAGATCAG
		AB52	ATACAGCCGTGTCTCTGCCC

5.2.2 Nucleotide Sequencing

The region from -597 bp to +143 bp relative to the start of the *PKAR* ORF was amplified from *fgsg_09907-1*, *fgsg_09907-3* and *fgsg_09907-4* using primers AB82 and AB83 (see **Table 5.3**). This region is located between the *PKAR* gene and *hph* gene inserted into the *fgsg_09907* (*fcv1*) knockout strains and overlaps the *fgsg_09907* 3' flank sequence by 514 bp. The PCR products were cleaned using a Qiagen PCR purification Kit and dried. PCR products were sequenced by Eurofins MWG Operon (Germany) in the forward and reverse direction using primers AB82 and AB83.

Table 5.3. Primers for sequencing the *PKAR* region in *fgsg_09907* (*fcv1*) strains.

Primers	Primer sequence (5'-3')
AB82	CGGTACCCAATTGCGCCCTAT
AB83	GCTGCGTCACCAGTCAAGGT

5.2.3 *Neurospora crassa* Analysis

Neurospora crassa wild-type strains of mating type A and a and *ncu01167* knockout mutants of mating type A and a (FGSC17004 and FGSC17003, respectively) were obtained from the Fungal Genetics Stock Centre (FGSC, University of Missouri, USA). Strains were grown on agar slopes of Vogel's medium (see Chapter 2) at 37°C prior to inoculation of 90 mm Vogel's medium agar plates and growth overnight at 37°C. Spores were harvested in sterile water and a 10 µl aliquot of a 2.5×10^5 spores per ml solution was used to inoculate race tubes of Vogel's medium made using 25 ml pipettes containing 15 ml agar laid horizontally and capped at the pointed end, with the pipette filter remaining in place at the other end. The tubes were incubated at 37°C and growth recorded twice daily.

5.2.4 Conidial Germination Viability

Conidia were diluted to a concentration of 1×10^4 per ml in either synthetic nutrient-poor medium (SNA), SNA minus carbon (SNA-C), SNA minus nitrogen (SNA-N) or Fusarium synthetic complete medium (FSCM, Leslie *et al.*, 2006) containing 1% low melting point agar (Gibco) cooled to 40°C. One millilitre of the resulting mixture was spread on a glass slide and placed in a high humidity environment in the dark. After 15 hours, 50 spores on each slide were counted to determine that percentage that had germinated. Three slides per strain per medium type were analysed.

5.2.5 Bioinformatic Analysis of *FCV1*.

Predict NLS Online was used to search for nuclear localisation sequences in *FCV1* (cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl) and NetNES1.1 Server (www.cbs.dtu.dk/services/NetNES, la Cour *et al.*, 2004) to search for nuclear export sequences. Other bioinformatics methods are described in Chapter 3.

5.3 Results

5.3.1 Targeted Deletion of Additional Genes in the Micro-Region

PCR fusion was used for the production of constructs for the targeted deletion of *FGSG_09891*, *FGSG_09893*, *FGSG_09900*, *FGSG_09905*, *FGSG_09906* and *FGSG_09907* (*FCV1*). Details of primers used and product sizes are to be found in Chapter 2. **Figures 5.1 and 5.2** show the amplification of regions flanking the target genes. The HY and YG overlapping sections of the *hph* gene were also amplified from pHYG1.4 (**Figures 5.1 and 5.2**). The initial 5' flank region chosen for *FGSG_09900* (**Figure 5.1**) proved to be too long to fuse to the HY section of the *hph* hygromycin resistance gene and so primers for the amplification of a new shorter 5' flank region were designed and used to amplify the required fragment for this gene (**Figure 5.2**). Fusion of flank regions to *hph* sections was tested in a second PCR (**Figures 5.3 and 5.4**). For the 5' flank-HY fusion constructs of *FGSG_09891*, *FGSG_09900* and *FGSG_09906*, nested primers were used to increase amplification efficiency (**Figures 5.5 and 5.6**). Fusion PCR was repeated to increase DNA yield, the products precipitated (**Figure 5.7**) and used for transformation of *F. graminearum*. Construct production, targeted deletion and PCR screening of *FGSG_09907* (*FCV1*) was performed by M. Urban (Rothamsted Research) and is shown in **Figure 5.8**.

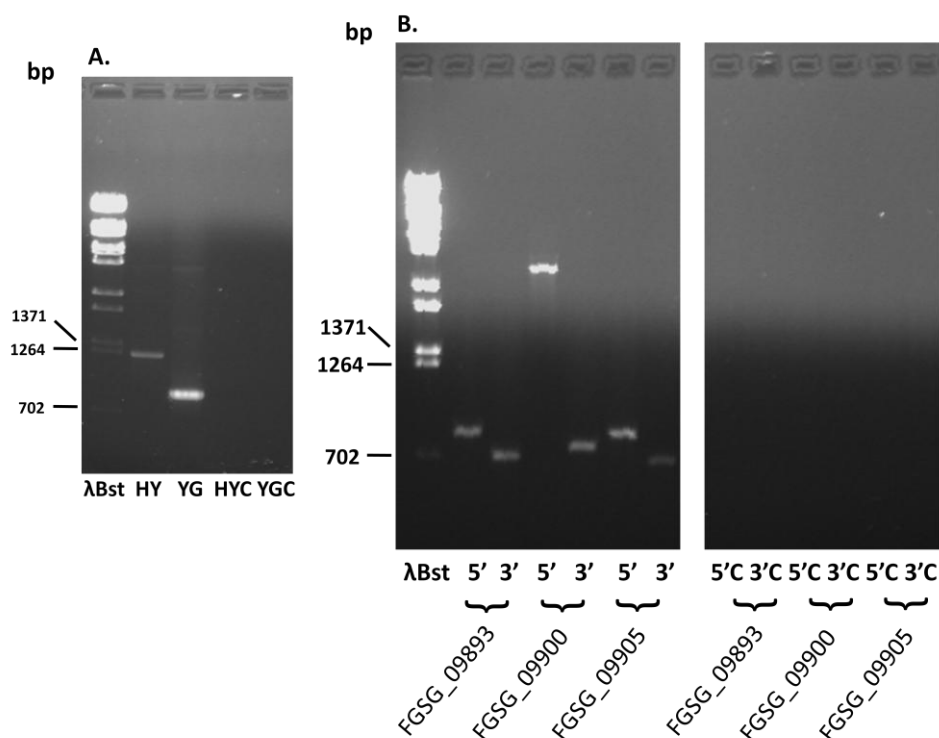


Figure 5.1. PCR amplification of (A) the *hph* HY and YG sections and (B) the flank regions for *FGSG_09893*, *FGSG_09900* and *FGSG_09905* for the targeted deletion of these genes. Lanes labelled with 'C' are primer controls lacking template DNA. The *FGSG_09900* 5' flank here was replaced by a new flank shown in figure 5.2.

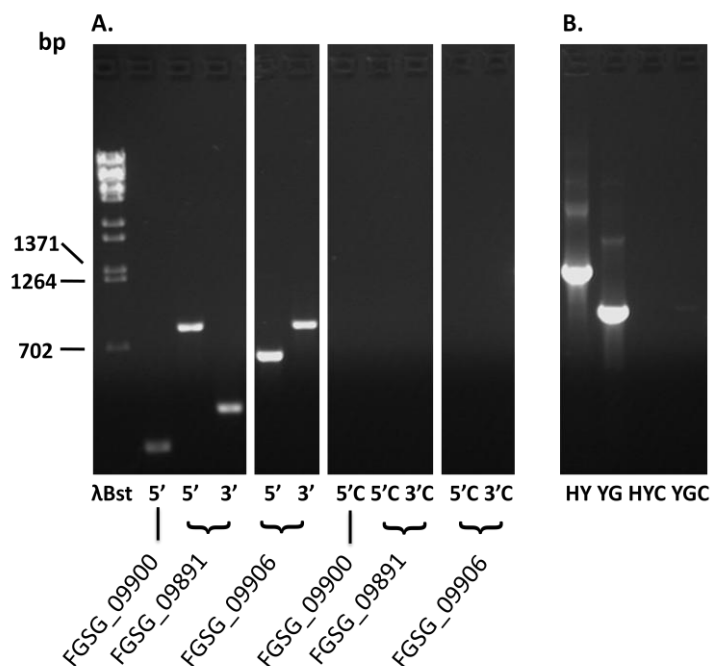


Figure 5.2. PCR amplification of (A) the *hph* HY and YG sections and (B) the flank regions for *FGSG_09891* and *FGSG_09906* plus the new 5' flank region for *FGSG_09900*. Lanes labelled with 'C' are primer controls lacking template DNA.

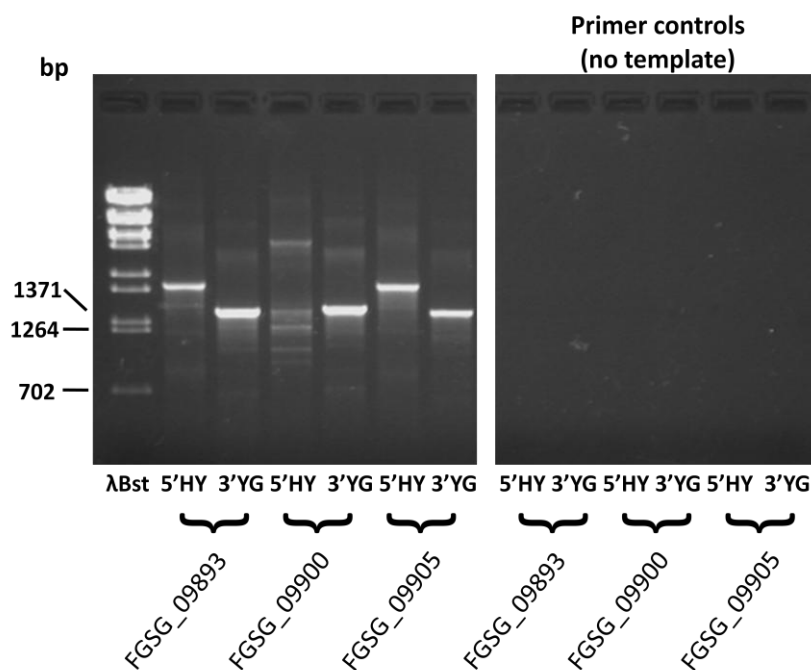


Figure 5.3. A test of the fusion of the 5' flank and HY section and fusion of the 3' flank and YG section by PCR for *FGSG_09893*, *FGSG_09900* (3' and YG only), and *FGSG_09905*.

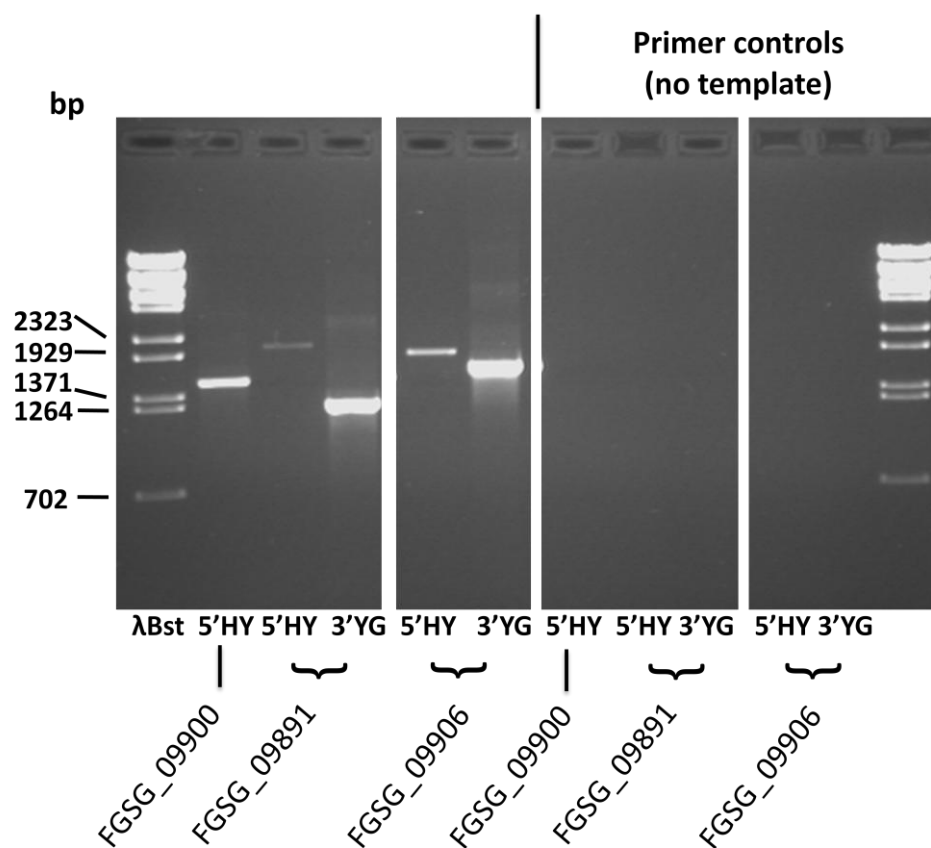


Figure 5.4. A test of the fusion of the 5' flank and HY section and fusion of the 3' flank and YG section by PCR for *FGSG_09900* (5' and HY only), *FGSG_09891* and *FGSG_09906*.

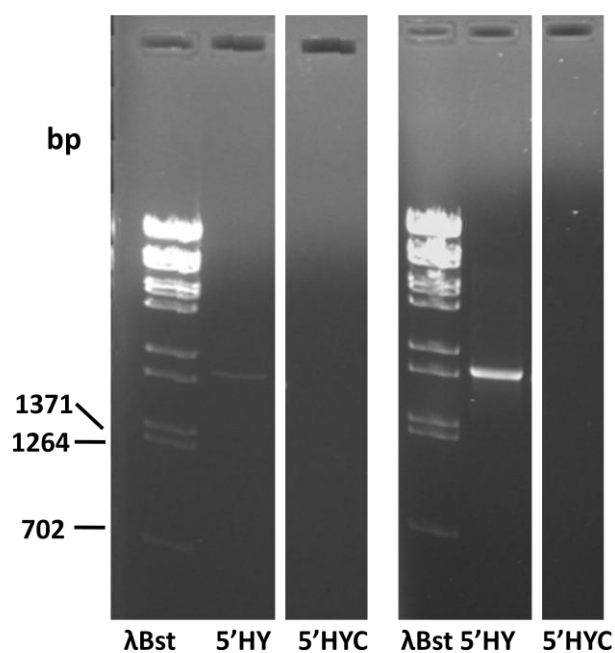


Figure 5.5. **A.** Test of *FGSG_09891* 5' flank fusion to HY. **B.** Nested PCR for *FGSG_09891* 5' flank fusion to HY using the construct from (**A**) as template.

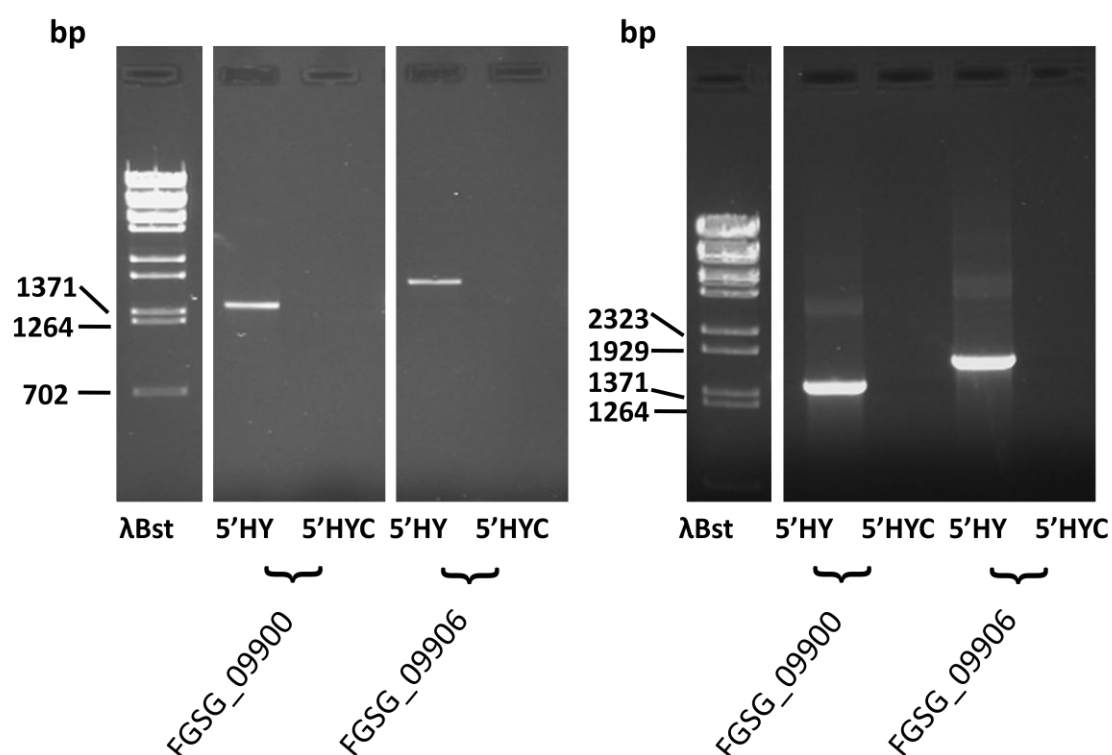


Figure 5.6. **A.** Test of *FGSG_09900* and *FGSG_09906* 5' flank fusion to HY. **B.** Nested PCR for *FGSG_09900* and *FGSG_09906* 5' flank fusion to HY using the constructs from (**A**) as template.

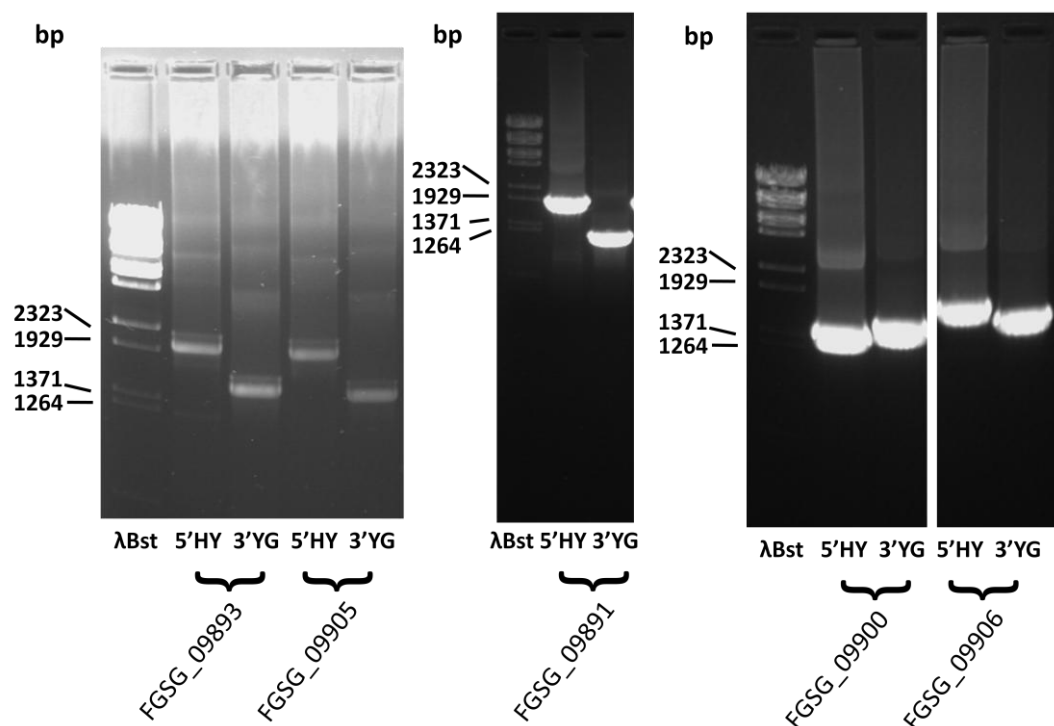


Figure 5.7. Fusion DNA used for transformation of *F. graminearum* protoplasts

Transformants were screened by PCR for the presence of the target gene and *hph* gene. Six out of ten transformants screened for *fgsg_09891* amplified the *hph* gene only (**Figure 5.9**). Six out of seven *fgsg_09893* transformants (data not shown) and 13 out of 18 *fgsg_09900* transformants amplified *hph* only (**Figure 5.10**). Nine transformants were screened for *fgsg_09905* with three amplifying the *hph* gene only (**Figure 5.11**), while for *fgsg_09906*, 16 out of 20 transformants appeared successful (**Figure 5.12**). For *fgsg_09907* 3 out of 4 transformants appeared successful (**Figure 5.8**).

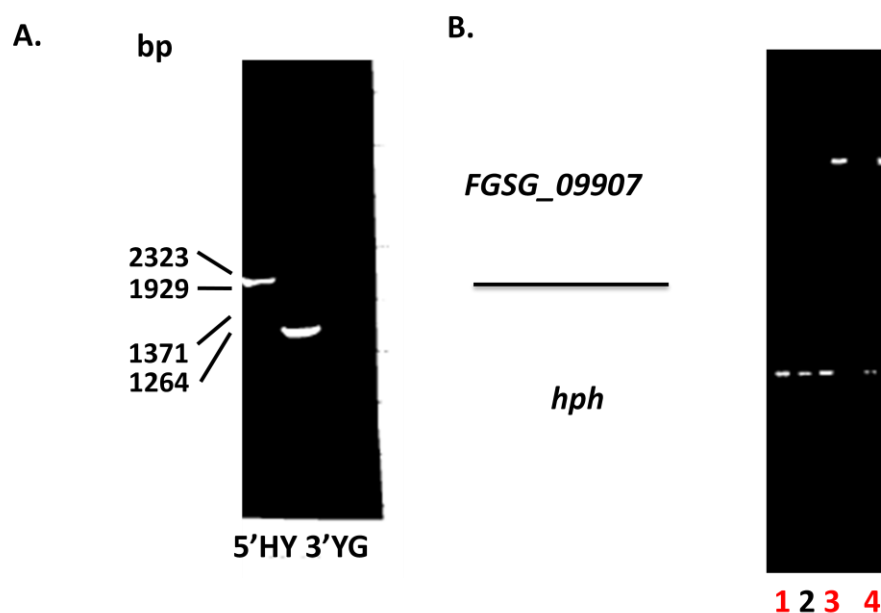


Figure 5.8. A. Flank-HY/YG fusion construct production for targeted deletion of *FGSG_09907*. B. PCR screen of *fgsg_09907* (*fcv1*) transformants. Transformants numbered in red were selected for characterisation by Southern hybridisation (data kindly provided by M. Urban).

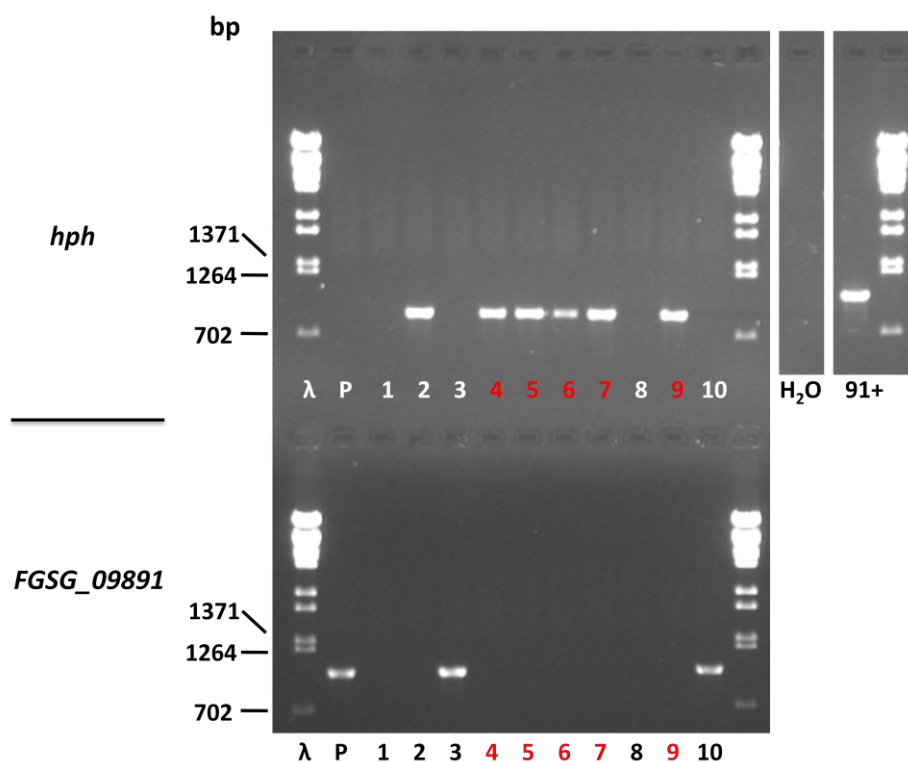


Figure 5.9. PCR screen of *fgsg_09891* transformants. Ten transformants (1-10) are depicted. Primers are used to amplify the *hph* or *FGSG_09891* genes, 91+ = positive control. H₂O = water control (no template). P= PH-1 wild type. Transformants numbered in red were selected for characterisation by Southern hybridisation.

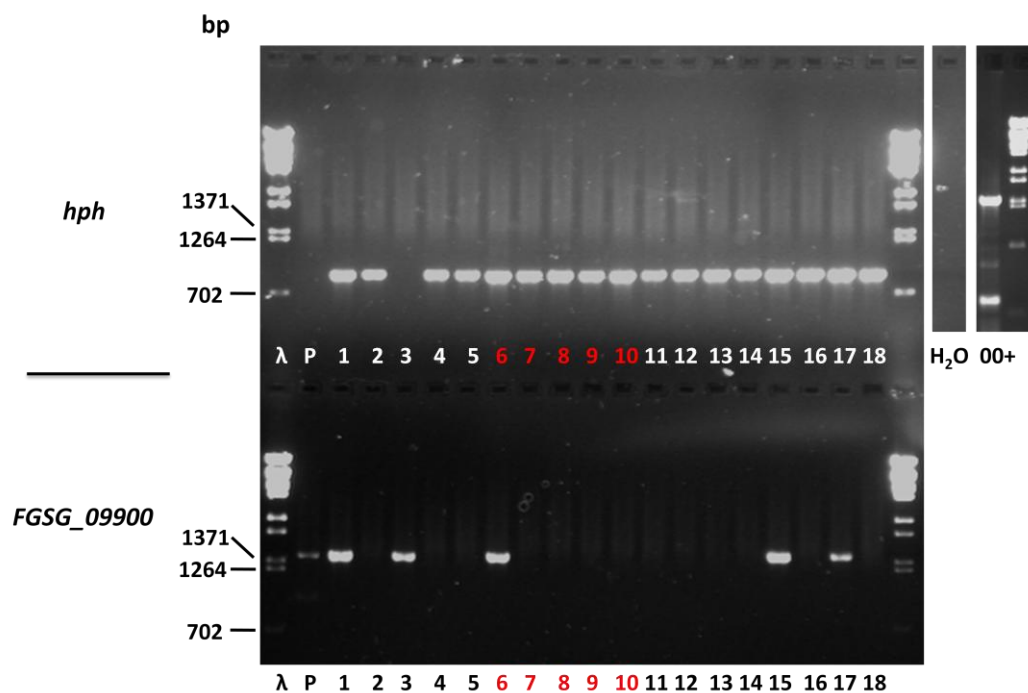


Figure 5.10. PCR screen of *fgsg_09900* transformants. 18 transformants (1-18) are depicted. Primers are used to amplify the *hph* or *FGSG_09900* genes, 00+ = positive control. H₂O = water control (no template). P= PH-1 wild type.

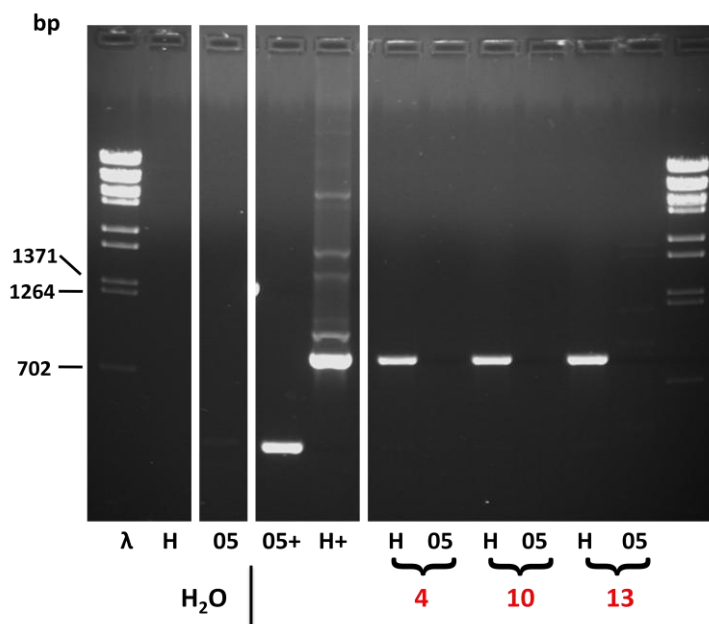


Figure 5.11. PCR screen of *fgsg_09905* transformants. Three transformants (4, 10, 13) are depicted. Primers are used to amplify the *hph* (H) or *FGSG_09905* (05) genes. + = positive control. H₂O = water control (no template). Transformants numbered in red plus an additional putative ectopic insertion transformant were selected for characterisation by Southern hybridisation.

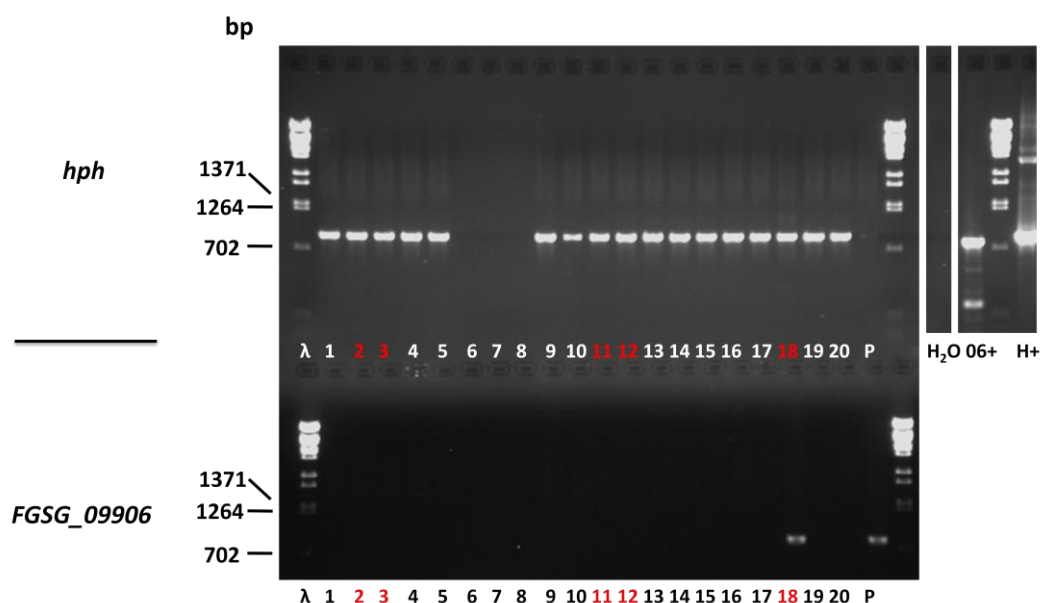


Figure 5.12. PCR screen of *fgsg_09906* transformants. 20 transformants (1-20) are depicted. Primers are used to amplify the *hph* (H) or *FGSG_09906* genes, 06+, H+ = positive control. H₂O = water control (no template). P= PH-1 wild type.

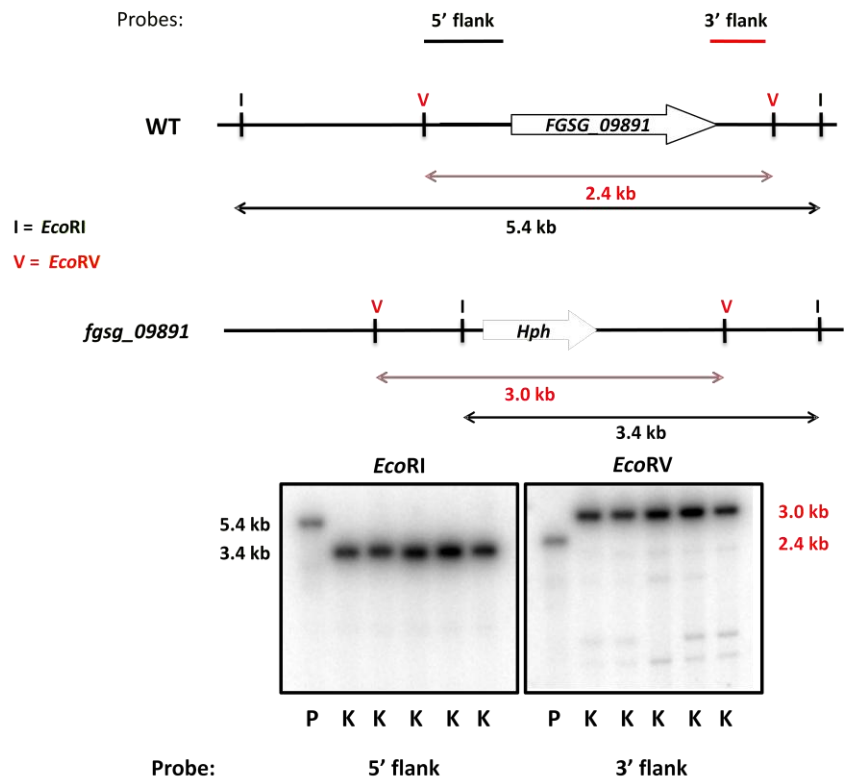
Southern hybridisation was again used to confirm targeted deletion of genes and single-copy insertion of the deletion constructs. In total, five, five, five, four, five, and three transformants were selected for characterisation by this technique for *fgsg_09891*, *fgsg_09893*, *fgsg_09900*, *fgsg_09905*, *fgsg_09906* and *fgsg_09907* (*fcv1*), respectively. Genomic DNA was digested by restriction enzymes (listed in Chapter 2) and used for Southern gel blot analysis (**Figure 5.13**).

For *fgsg_09891*, all five transformants showed the expected 3.0 and 3.4 kb bands in the *EcoRV* and *EcoRI* digests, respectively, compared to 2.4 and 5.4 kb bands for PH-1 in the *EcoRV* and *EcoRI* digests, respectively (**Figure 5.13A**). For *fgsg_09893*, all five transformants showed the expected 3.7 and 3.4 kb bands in *SaII* digests compared to 2.1 and 4.3 kb bands for PH-1 (**Figure 5.13B**). For *fgsg_09900*, four transformants (7-10) showed the expected 2.5 and 6.3 kb bands in the *PstI* and *EcoRV* digests, respectively, compared to 5.8 and 1.1 kb bands for PH-1 in the *PstI* and *EcoRV* digests, respectively (**Figure 5.13C**). The fifth transformant (6) appeared to show ectopic insertion of the deletion constructs. For *fgsg_09905*, three transformants (4, 10, 13) showed the expected 1.3 and 2.4 kb bands in the *EcoRI* and *BamHI* digests, respectively,

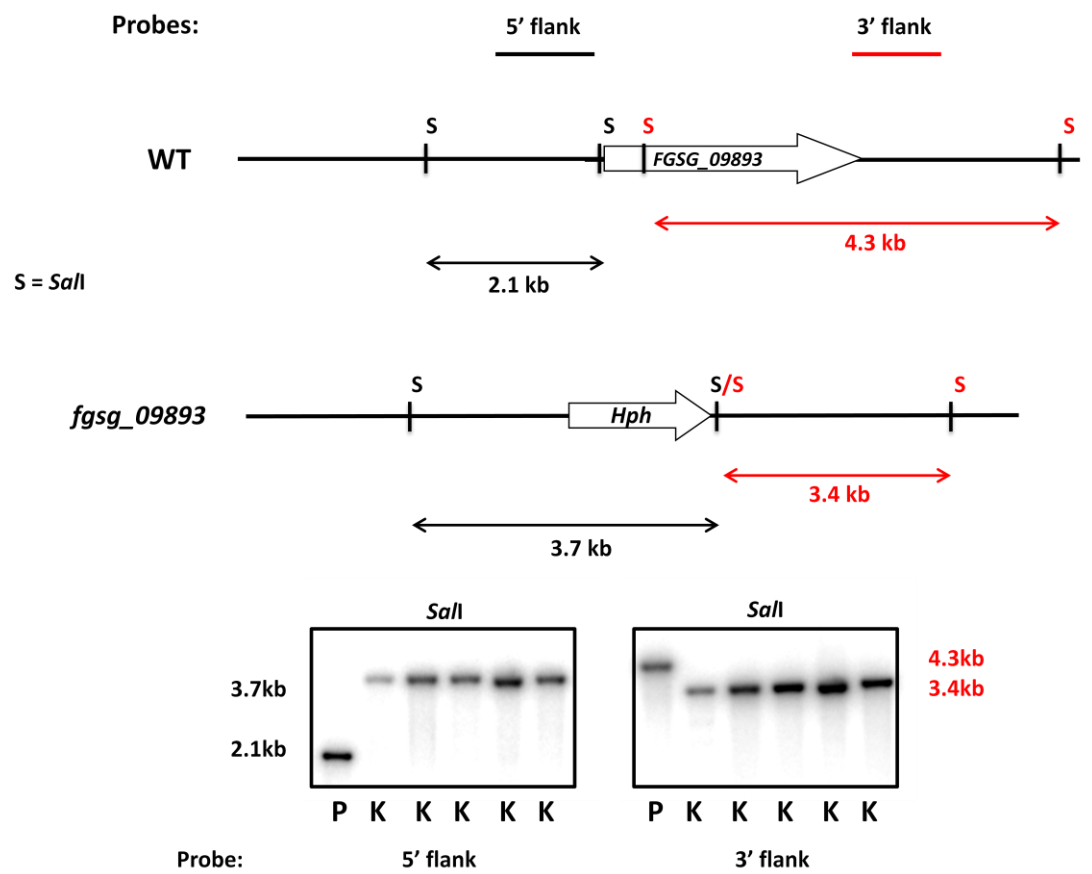
compared to 4.1 and 3.6 kb bands for PH-1 in the *EcoRI* and *BamHI* digests, respectively (**Figure 5.13D**). Transformant 2 appears to be identical to the wild type strain at the *FGSG_09905* locus. For *fgsg_09906*, four transformants (2, 3, 11, 12) showed the expected 2.4 and 0.8 kb bands in the *EcoRI* and *HindIII* digests, respectively, compared to 4.1 and 2.6 kb bands for PH-1 in the *EcoRI* and *HindIII* digests, respectively (**Figure 5.13E**). Transformant 18 appears to show ectopic insertion of the deletion constructs. For *fgsg_09907* (*fcv1*), three transformants showed the expected 3.9 kb and 0.7 kb bands in *SacI* and *HindIII* digest respectively, compared to 4.2 kb and 1.9 kb in the wild-type (**Figure 5.13F**).

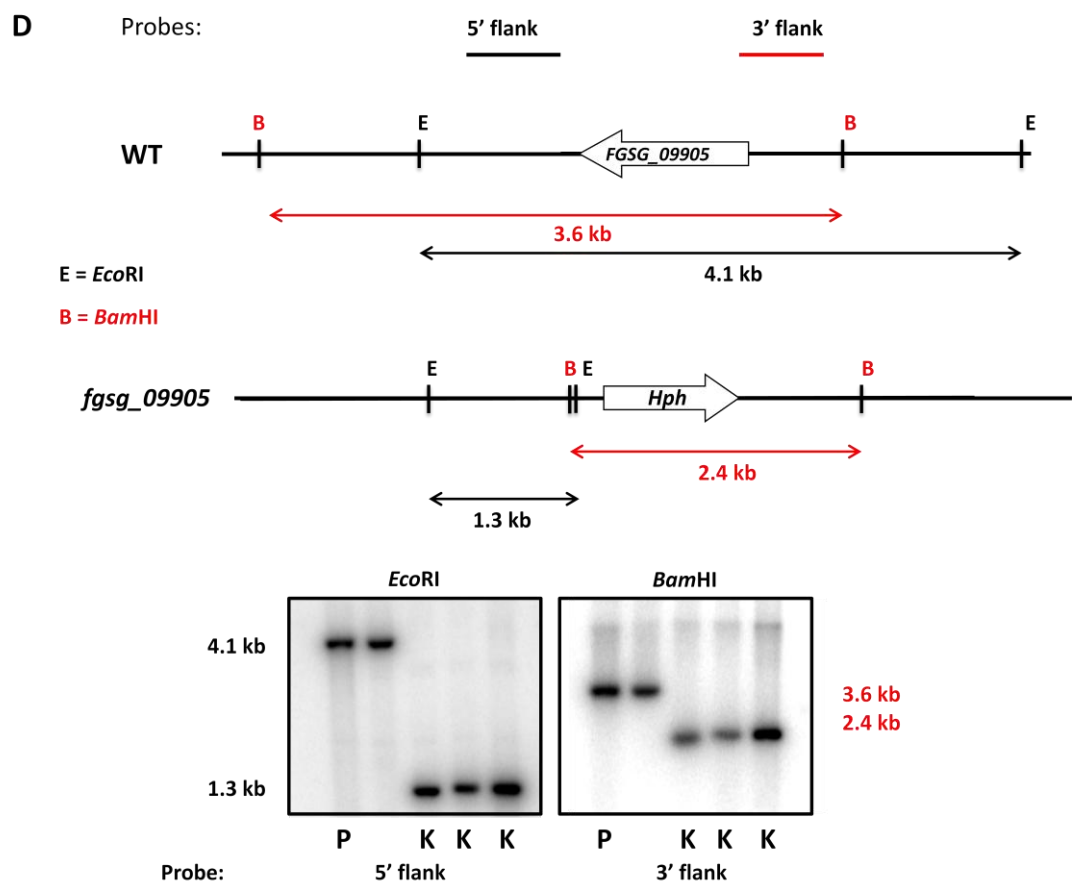
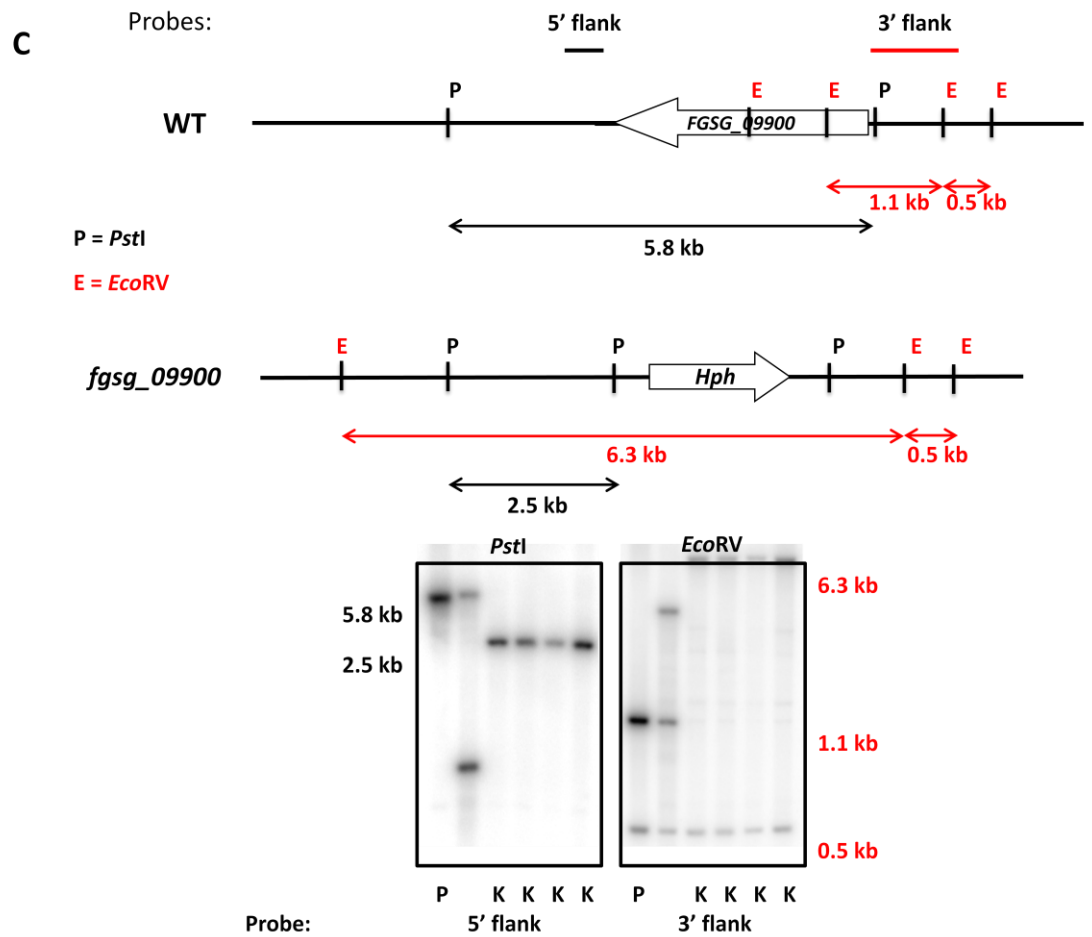
Figure 5.13. (See over). Targeted deletion of the (A) *FGSG_09891.3*, (B) *FGSG_09893.3*, (C) *FGSG_09900.3*, (D) *FGSG_09905.3*, (E) *FGSG_09906.3* and (F) *FGSG_09907.3* (*FCV1*) genes in *F. graminearum*. Targeted deletion was confirmed by Southern hybridisation. The gene locus for the wild type and deletion strain is shown in each case. Two different restriction enzymes were independently used to digest genomic DNA (gDNA) of both the wild type and deletion strain. Each restriction enzyme digest set of wild type and deletion strain gDNA was hybridised with a different DNA probe (5' flank or 3' flank). Colours (black/red) indicate the pairing of restriction enzyme and probe and expected hybridising fragment size. Five, five, five, four, five and three independent transformants were analysed for each of *FGSG_09891.3*, *FGSG_09893.3*, *FGSG_09900.3*, *FGSG_09905.3*, *FGSG_09906.3* and *FGSG_09907.3* (*FCV1*) respectively and compared to the wild type strain. In each case the blots exhibited hybridising bands that indicated a single insertion of each of the two overlapping split marker DNA constructs and deletion of the target gene. P = Wild type, E = ectopic insertion, K = knockout strain. Transformant numbers are shown above the radiograph.

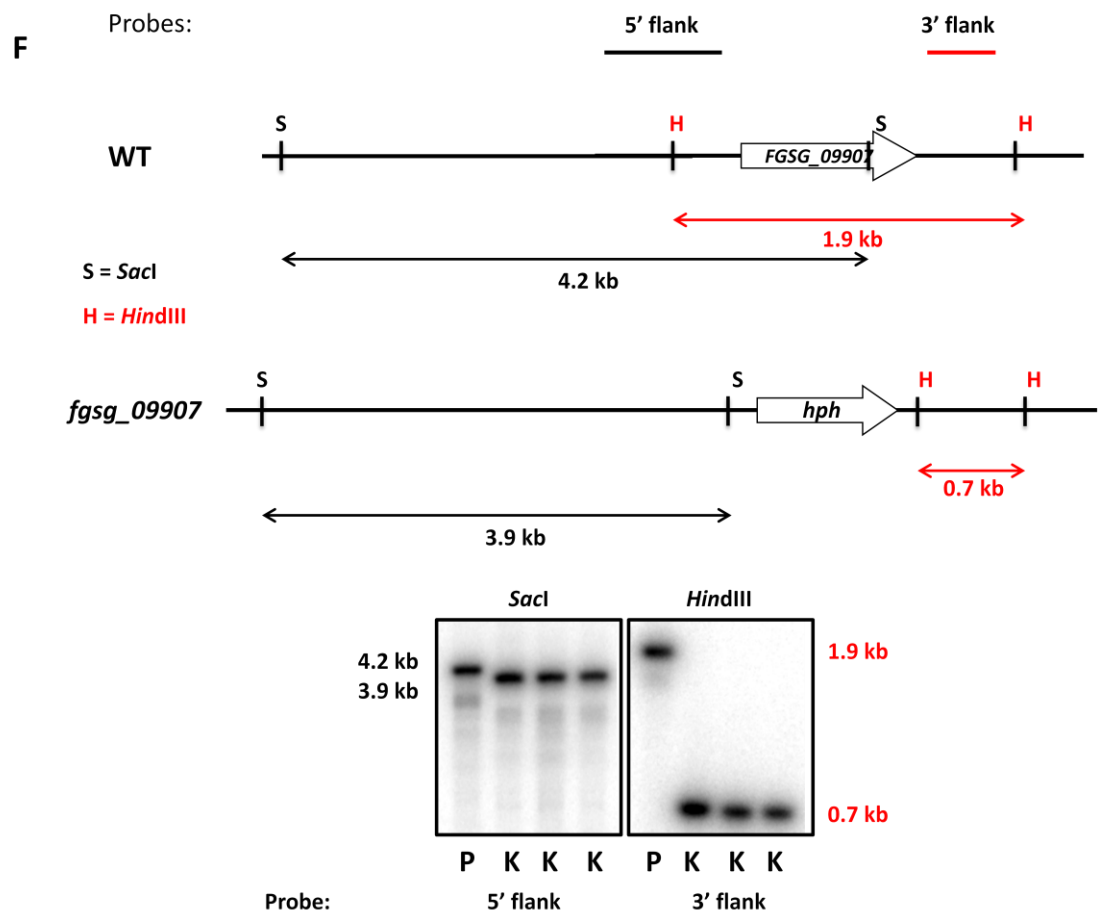
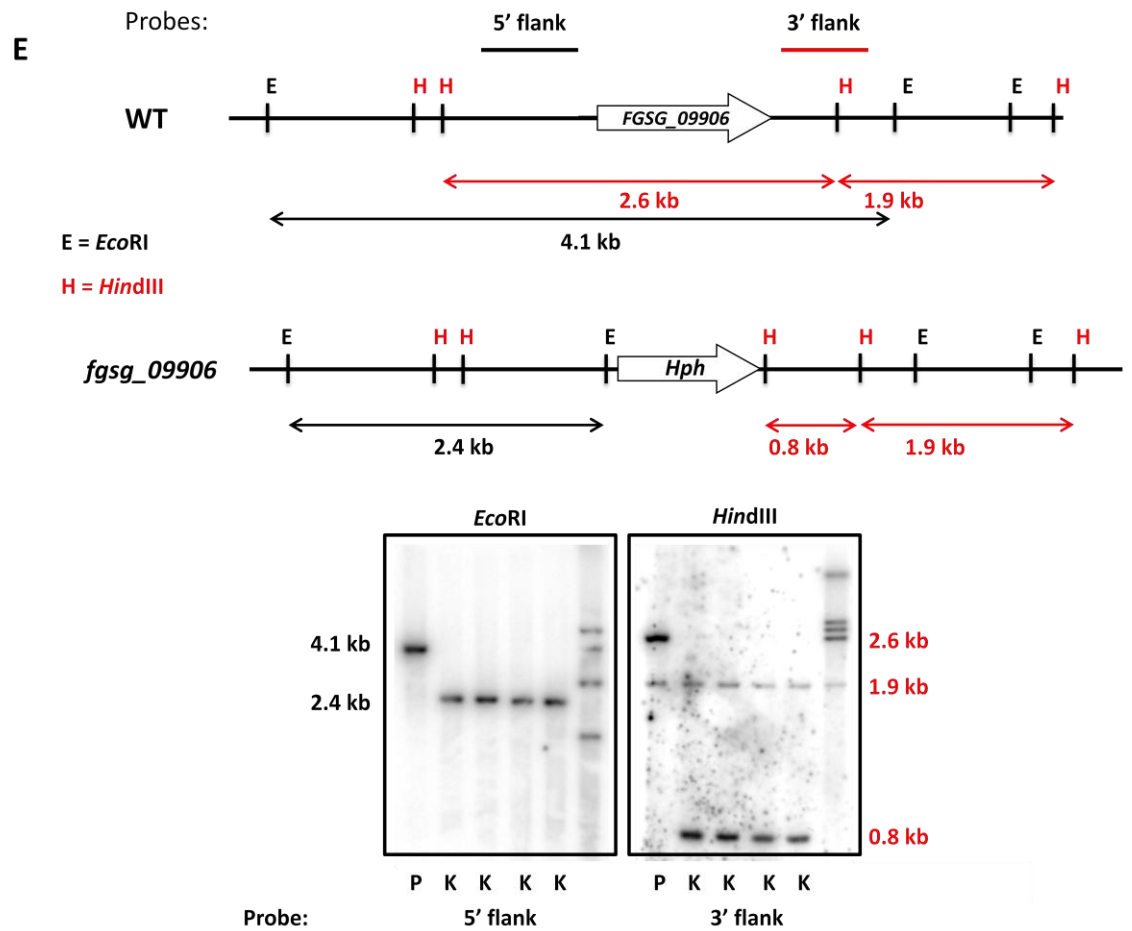
A



B







As *PKAR* is located adjacent to *FGSG_09907 (FCV1)* at the 3' end of the *FGSG_09907 (FCV1)* locus, this raised the possibility that insertion of the *hph* gene and 3' *FGSG_09907 (FCV1)* flank at this site may have affected the functioning of the *PKAR* gene by causing sequence alterations at the point of insertion of the 3' flank. To eliminate this possibility, a 740 bp region between the *hph* and *PKAR* genes, encompassing part of the *fgsg_09907 (fcv1)* 3' flank and *PKAR* ORF in transformants 1, 3 and 4 was sequenced. By comparison to the published genome sequence (Cuomo et al., 2007, www.broad.mit.edu, www.mips.helmholtz-muenchen.de/genre/proj/fusarium). This analysis indicated no changes in the sequence between the *FGSG_09907 (FCV1)* 3' flank and *PKAR* due to the targeted deletion of *FGSG_09907 (FCV1)* in transformants 3 and 4. In transformant 1, a single nucleotide change was noted. This could represent a sequencing error, however to remove any ambiguity, transformant 3 was selected for further analyses of the *FGSG_09907 (FCV1)* deletion.

The *fgsg_09907* transformant 3 (later known as *fcv1-3*) strain was complemented by reinsertion of the *FGSG_09907 (FCV1)* gene. The wild-type *FCV1* gene plus 1 kb upstream and 0.4 kb downstream sequence was amplified from genomic DNA of the wild-type PH-1 strain using primers that incorporate a 25 bp overlap with the *Neo* neomycin phosphotransferase geneticin resistance gene (**Figure 5.14A**). The *Neo* gene was also amplified separately. These two sections were fused in a second PCR (**Figure 5.14B to E**) and the construct used to transform *fcv1-3*. PCR screening of transformants that showed resistance to both geneticin and hygromycin and had been grown from a single spore was used to select strains with successful reinsertion of the *FGSG_09907 (FCV1)* gene. Transformants were assayed for the presence of *FGSG_09907 (FCV1)*, the geneticin and hygromycin resistance markers (Gen and *Hph* respectively), and a positive control gene (*FGSG_09900*) (**Figure 5.15**). Of the six transformants analysed, four (*fcv1 + FCV1-1* to 4) successfully amplified all four genes indicating reinsertion of the *FGSG_09907 (FCV1)* gene together with the geneticin marker into the *fgsg_09907 (fcv1)* strain. Transformant 2 was selected for further analysis.

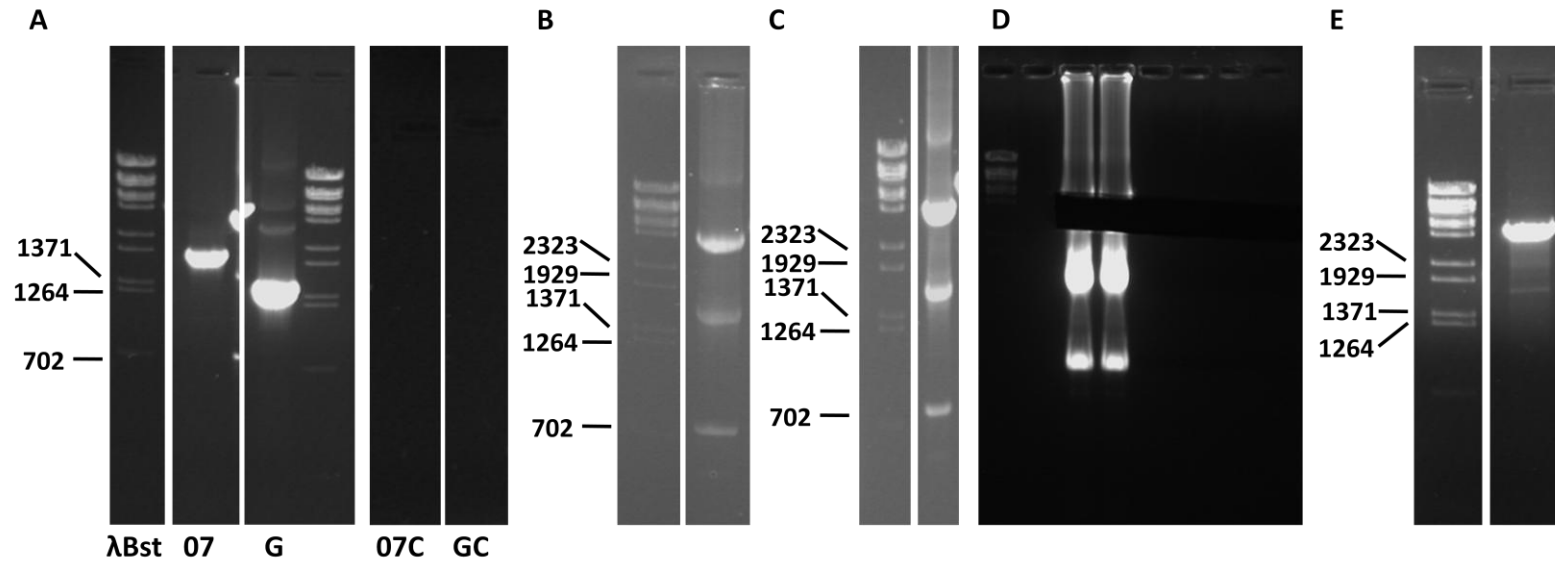


Figure 5.14. Production of DNA constructs for the genetic complementation of *fgsg_09907* (*fcv1*). **A.** Amplification of the wild-type *FGSG_09907* (*FCV1*) gene (07) and geneticin resistance gene (G) is shown with primer controls (07C and GC respectively). **B.** The *FGSG_09907* (*FCV1*) and geneticin resistance genes are fused in a second PCR. The band of size 2.5 kb is required. **C.** Ethanol precipitation of the fusion products is followed by gel extraction (**D.**) to yield the final fusion complementation construct (**E.**). The ladder in each case is λBst .

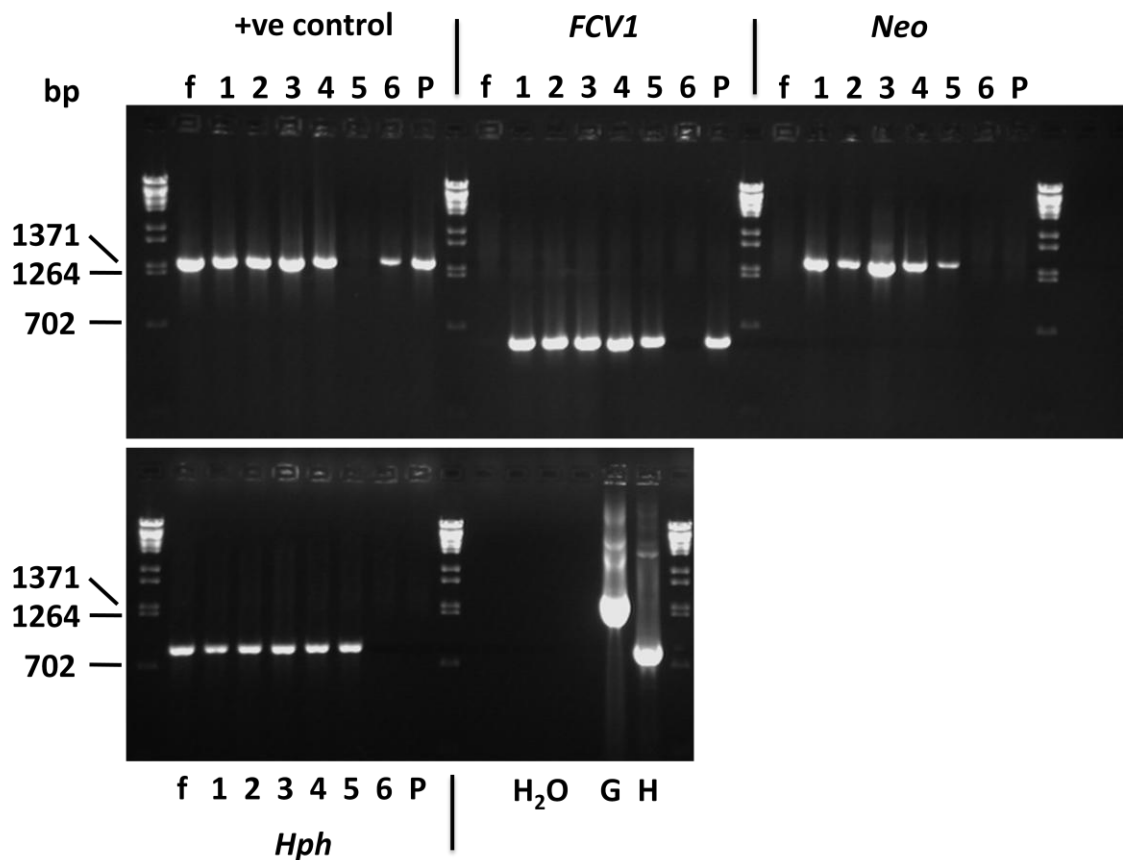


Figure 5.15. Confirmation of *fgsg_09907* (*fcv1*) complementation. *fgsg_09907* (*fcv1*), six independent putative complementation transformants and the PH-1 wild type strain were assayed by PCR for their ability to amplify *FGSG_09900* (+ve control), *FGSG_09907* (*FCV1*), the geneticin resistance marker (*Neo*) and hygromycin resistance marker (*Hph*). f = *fcv1*, P = PH-1 wt, 1-6 = six independent putative complementation transformants. H₂O = primer controls (no template) for *FGSG_09900*, *FGSG_09907* (*FCV1*), *Neo* and *Hph*, G = *Neo* positive control, H = *Hph* positive control. Transformant 2 was selected for further analyses.

Initial Characterisation of the Gene Deletion Strains

5.3.2 Virulence on Wheat Plants

Initially three independent transformants per gene deletion were analysed in preliminary wheat inoculation experiments (**Figure 5.16**). The virulence of the *fgsg_09893*, *fgsg_09900*, *fgsg_09905* and *fgsg_09906* transformants on wheat ears following point inoculation did not appear different from the wild-type. For *fgsg_09891*, transformants 5 and 6 did not appear to show a significant difference in virulence compared to the wild-type, while transformant 7 appeared to show slightly reduced virulence (**Figure 5.16**). This transformant also displayed an *in vitro* growth behaviour distinct from transformants 4 and 5 *in vitro* (see later) and so was not considered representative of the *fgsg_09891* targeted deletion strain and not used in further experimentation. Representative wheat ears are shown in **Figure 5.17**.

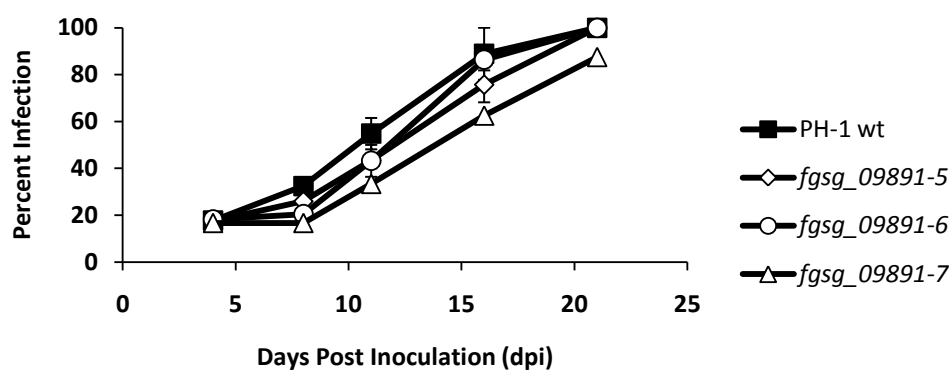
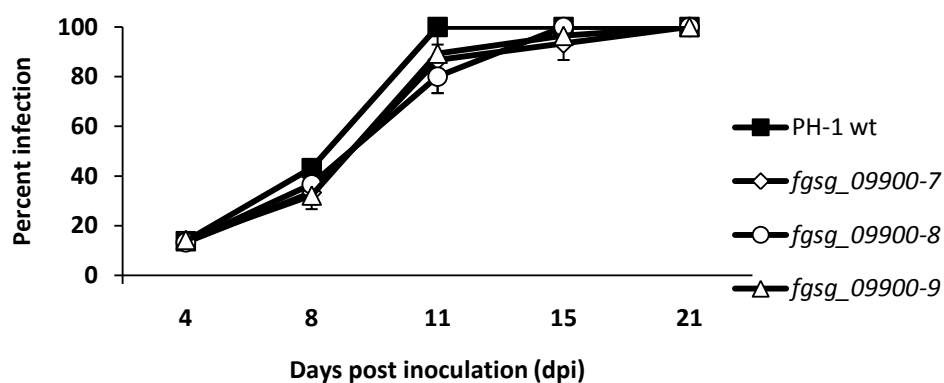
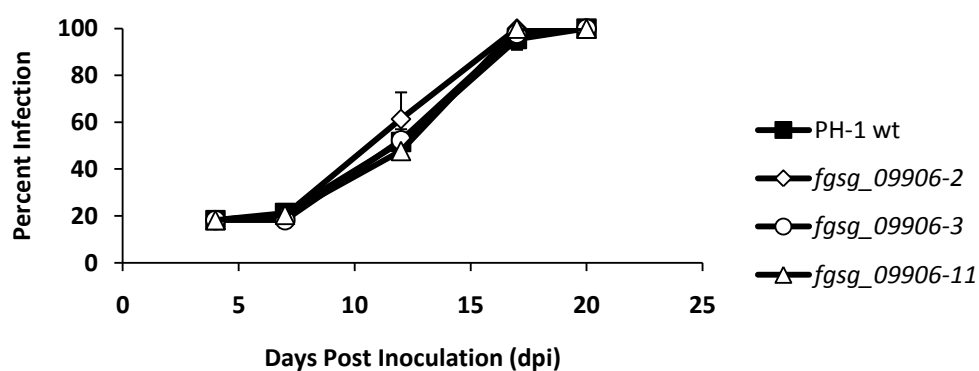
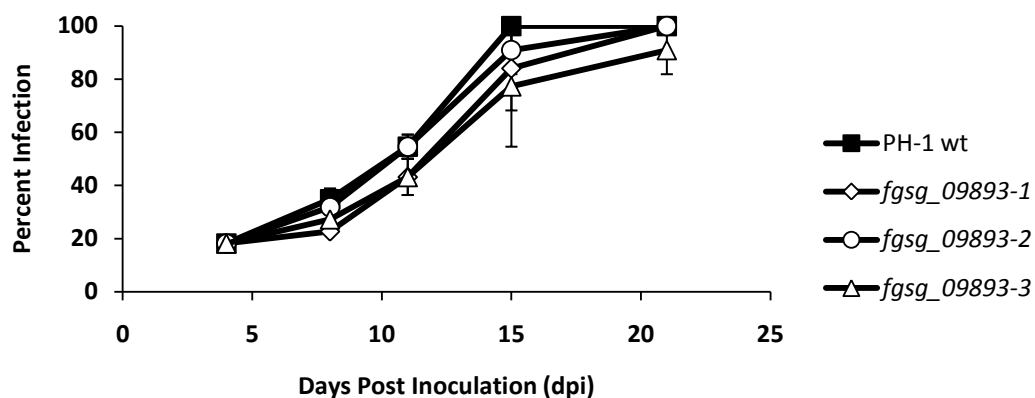
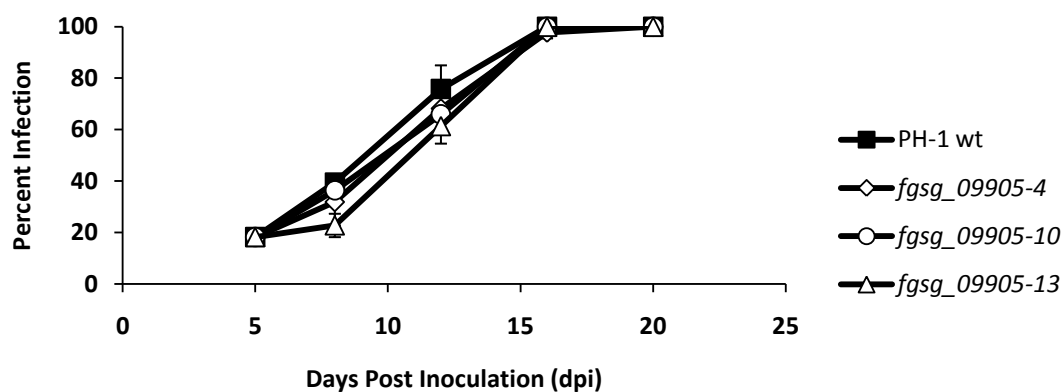
A**B****C**

Figure 5.16. Preliminary experiments. Progression of wheat ears cv. Bobwhite inoculated with (A) *fgsg_09891*, (B) *fgsg_09893* or (C) *fgsg_09900*, transformants. Error bars are shown as the standard error of the mean for two ears per strain (three for PH-1 wt, one for *fgsg_09891-3*).

D



E



F

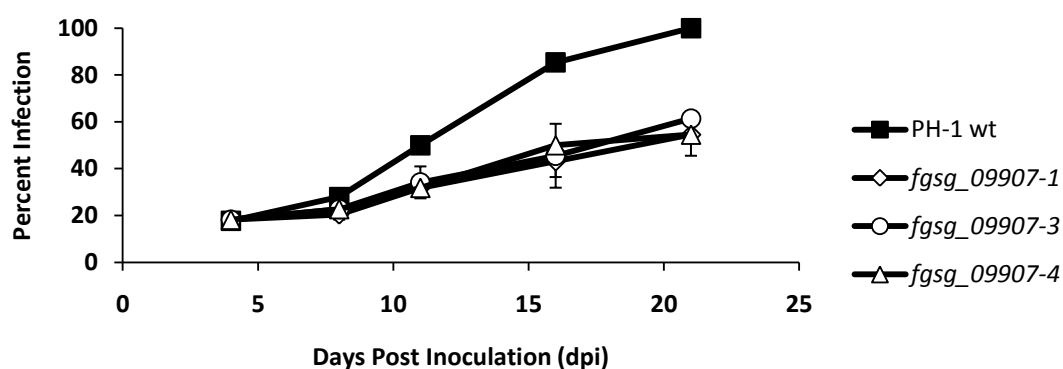


Figure 5.16. (cont). Preliminary experiments. Progression of wheat ears cv. Bobwhite inoculated with (D) *fgsq_09905*, (E) *fgsq_09906* or (F) *fgsq_09907* (*fcv1*) transformants. Error bars are shown as the standard error of the mean for two ears per strain (three for PH-1 wt, one for *fcv1-3*).

nodes, and the adjoining spikelets was reduced significantly by day 8 (**Figure 5.18A**, $p < 0.05$). At the end of the infection time course, dissection of the entire ear revealed that, unlike in the wild-type colonised ear, from the *fgsg_09907-3* colonised ear, although a lot of small brown aborted grain were present in the middle of the ear, plump green grain could be recovered from the base of the ear and small immature white grain were present above the point of inoculation. From each of the PH-1 infected ears only a few very shrivelled brown grain or no grain at all were recovered (**Figure 5.19**). These additional analyses further suggest that the loss of the gene *FGSG_09907* (*FCV1*) leads to a reduced rate of symptom development. Therefore, the gene *FGSG_09907* has been designated *FCV1* for *Eusarium graminearum* Contributor to Virulence 1. Strain *fgsg_09907-3* is hereafter referred to as *fcv1*. Targeted deletion of six additional genes in the cluster has therefore revealed a further one gene, *FCV1*, which appears to be required for a wild-type rate of FEB symptom spread on wheat ears.

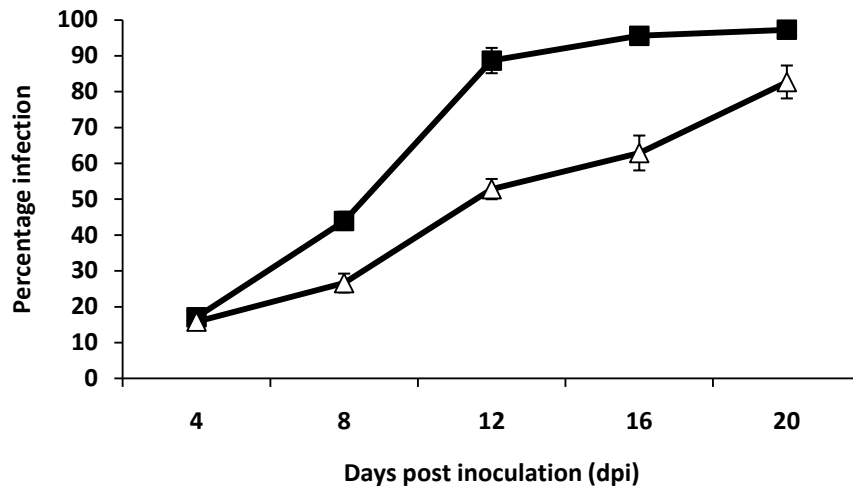
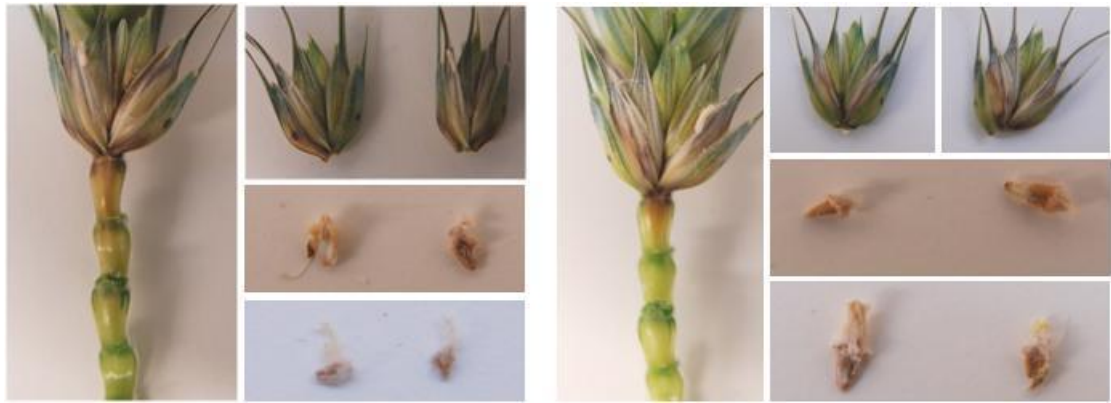
A**B**

Figure 5.18. The *fgsg_09907-3* (*fcv1*) strain shows slower spread of disease symptoms in wheat ears. **A.** A large scale test using the representative transformant *fgsg_09907-3* (hereafter called *fcv1*). Black squares – wild-type, white triangles – *fgsg_09907-3*. Average of 13 wild-type and 10 *fgsg_09907-3* inoculated ears. Error bars are given as +/- one standard error of the mean. **B.** The early infection process of *fgsg_09907-3* is similar to wild type but with a slower spread of visible symptoms. (Left) Images of wild type infected wheat ear at 5 dpi showing browning of the inoculated spikelets and rachis and shrivelled grain contained within the inoculated spikelets. (Right) Images of *fgsg_09907-3* infected wheat ear at 5 dpi showing browning of inoculated spikelets but reduced rachis browning compared to wild type. Grain from inoculated spikelets are shrivelled but some are larger than those from ears infected with wild type *F. graminearum*. In this panel, some spikelets have been removed to shown the disease symptoms on the rachis.



Figure 5.19. The (A) mock (B) wild type (C) *fgsg_09907-3 (fcv1)* and (D) *fcv1+FCV1* infected wheat ears at 19 dpi. The three *Fusarium* strains destroy the developing grain in infected spikelets to different extents. Inoculated spikelets (black arrows) have become fully bleached for all three strains. The images show the central portion of the ear only. Grain collected from (E) mock (F) wild type (G) *fgsg_09907-3 (fcv1)* and (H) *fcv1+FCV1* infected wheat ears at 19 dpi. The mock ear (E) contains healthy plump green grains and several small white grains not yet developed. The wild type, *fgsg_09907-3 (fcv1)* and *fcv1+FCV1* infected ears (F-H) contain small shrivelled brown grain. The *fgsg_09907-3 (fcv1)* infected ear (G) contains a few plump green grains from uninfected spikelets (red arrow). Grain from the top of the ear, above the inoculation point are small but still green in *fgsg_09907-3 (fcv1)* infected ears (G) but are brown or shrivelled in wild type infected ears (F) (white arrows).

To confirm that the phenotypes observed in the *fcv1* strain were due to deletion of the *FCV1* gene, the *fcv1* strain was genetically complemented by reinserting a copy of the wild type *FCV1* gene. Reinsertion ectopically of the *FCV1* gene into the *fcv1* strain restored a wild-type rate of symptom spread on wheat ears (**Figure 5.20**). These data further indicate that the *in planta* phenotypes noted in the *fcv1* strain were due to the deletion of *FCV1*. Four independent complemented strains exhibited successful reinsertion of the *FCV1* gene and recovery of the *fcv1* reduced symptom spread rate phenotype on wheat ears (**Figure 5.20**). Complementation transformant 2 was selected for further analysis in a larger scale test (**Figure 5.21**) which confirmed the restoration of symptom spread rate by complementation of the *fcv1* strain.



Figure 5.20. *fcv1+FCV1* complementation transformants (1-4) show comparable symptom spread to the wild-type strain. Images taken at 10 dpi.

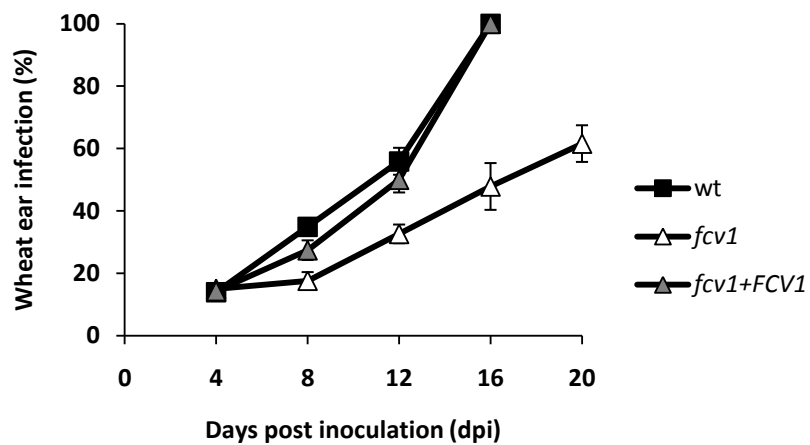


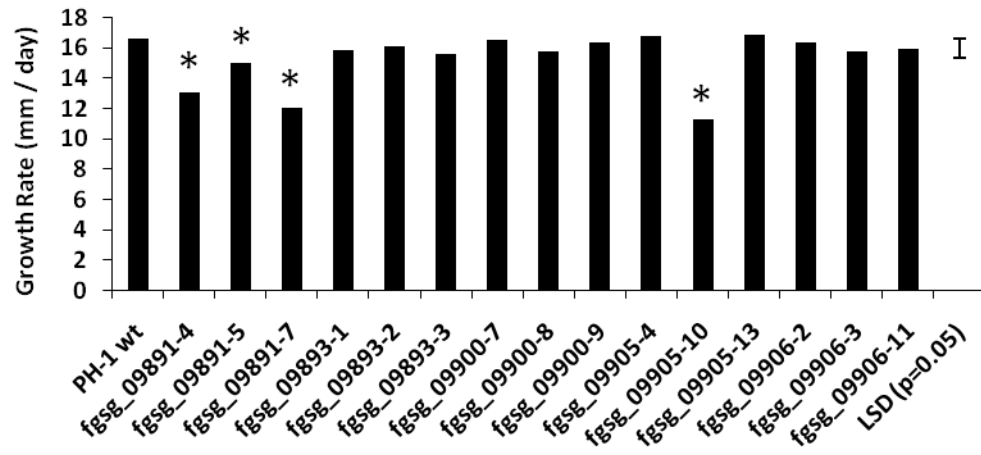
Figure 5.21. Disease progression of wild type, *fcv1* and *fcv1+FCV1* strains on wheat ears. The disease progress of the wild-type and *fcv1+FCV1* strains was not significantly different. Three ears were used per strain.

5.3.3 *In vitro* Growth

Three transformants per gene deletion were assessed for their rate of *in vitro* growth on minimal and complete medium compared to wild-type (**Figure 5.22**). For *fgsg_09893*, *fgsg_09900* and *fgsg_09906*, all three transformants did not exhibit a growth rate significantly different from the wild type on either minimal or complete medium. For *fgsg_09891*, only transformant 7 grew at a significantly reduced rate on complete medium. Given the different behaviour of this isolate to other *fgsg_09891* isolates *in planta* as well as *in vitro*, it was decided to discontinue use of *fgsg_09891-7* as it did not appear representative of targeted deletion of the *FGSG_09891* gene. All three *fgsg_09891* transformants grew at a significantly reduced rate on minimal medium, *fgsg_09891-7* again exhibited the lowest rate of growth. The average growth rate reduction for *fgsg_09891-4* and *fgsg_09891-5* was 16% compared to wild-type. For *fgsg_09905*, transformants 4 and 13 did not grow at a significantly different rate to wild-type on either complete or minimal medium, however, transformant 10 was significantly slower on both media types. This transformant was not considered representative of the *fgsg_09905* deletion and was not used further.

When grown *in vitro*, the three independently generated transformants of the *fcv1* strain (*fcv1-1,3* and *4*, formerly *fgsg_09907-1, 3* and *4*) showed an altered colony morphology with reduced hyphal density and a significantly reduced growth rate compared to the wild type strain on both minimal and complete medium (**Figure 5.22C and D**). When cultured on complete medium an average 23% reduction in growth rate relative to wild-type was observed for the *fcv1* transformants. On minimal medium, the growth of the *fcv1* transformants was far more restricted (average 66% reduction compared to wild-type). In a second experiment incorporating the *fcv1+FCV1* complemented strain, the *fcv1* strain showed a 29% reduction in growth rate on complete medium and a 62% reduction on minimal medium compared to the wild-type. The complemented strain grew at the same rate and with a similar appearance to the wild-type strain on both minimal and complete medium (**Figure 5.23**).

A



B

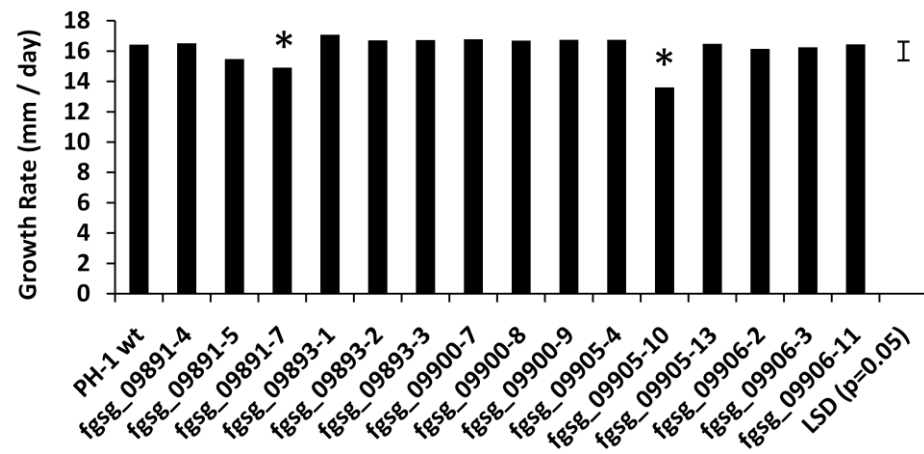


Figure 5.22. In vitro growth rate of the *fgsg_09891*, *fgsg_09893*, *fgsg_09900*, *fgsg_09905* and *fgsg_09906* strains on minimal medium (A) and complete medium (B). The wild type (wt) strain PH-1 is included for comparison. Columns marked with * are significantly different from the wild type strain at the 5% level.

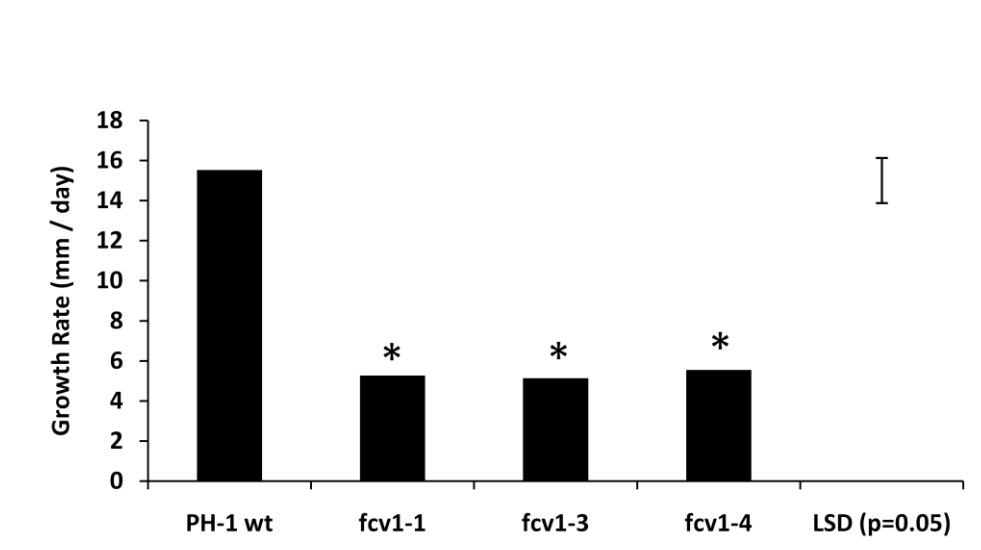
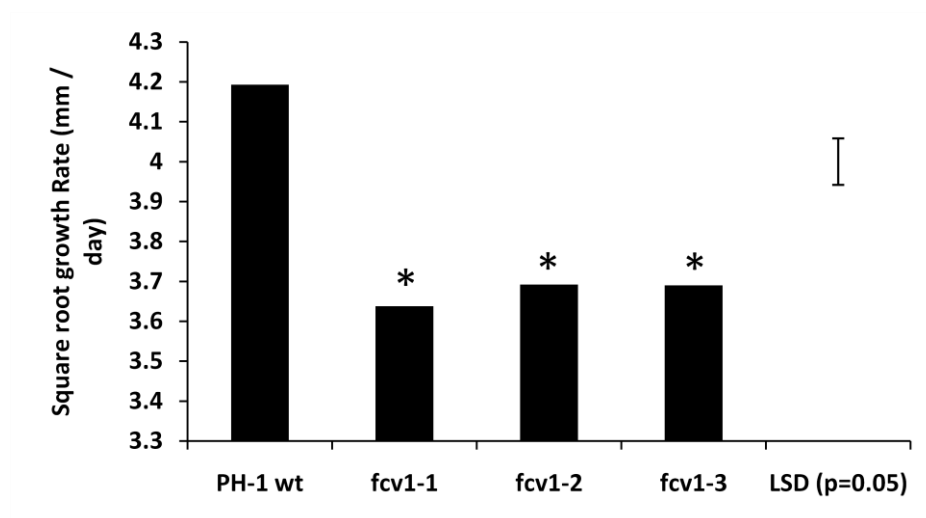
C**D**

Figure 5.22. (Cont). In vitro growth rate of the *fcv1* strains on minimal medium (**C**) and complete medium (**D**). The wild type (wt) strain PH-1 is included for comparison. Columns marked with * are significantly different from the wild type strain at the 5% level. In (D) the square root of the growth rate is shown to indicate the data transformation used to permit analysis by ANOVA.

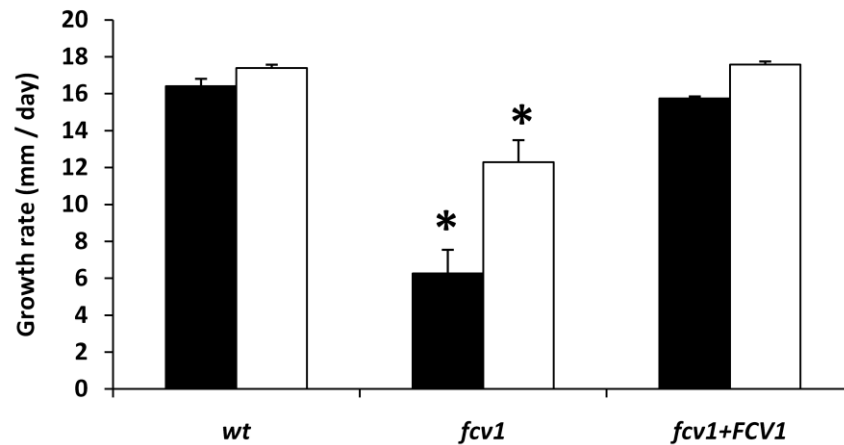


Figure 5.23. Growth rate of wild type, *fcv1* and *fcv1+FCV1* on minimal medium (black bars) and complete medium (white bars). * indicates significant difference from wild type at $P < 0.05$. All error bars are presented as \pm one standard error of the mean (SE).

When growing on minimal medium the *fcv1* strain showed an unusual phenotype, whereby hyphal advance through the solid medium rather than on the surface was favoured, accompanied by production of many clusters of conidia within the agar. This gave the growing colonies a novel ‘feathery’ appearance (**Figure 5.24**). By contrast, the wild type strain, *fcv1+FCV1* strain and all the other single-gene deletion mutants showed a much denser growth on the agar surface of the minimal medium and only limited growth and production of conidial clusters within the agar. As a consequence the number of *fcv1* conidia on the plate surface was visibly less than for the wild type strain. On complete medium, the production of aerial mycelium was greatly reduced in the *fcv1* strain, leading to a darker colony appearance than wild type (**Figure 5.24 A and B**).

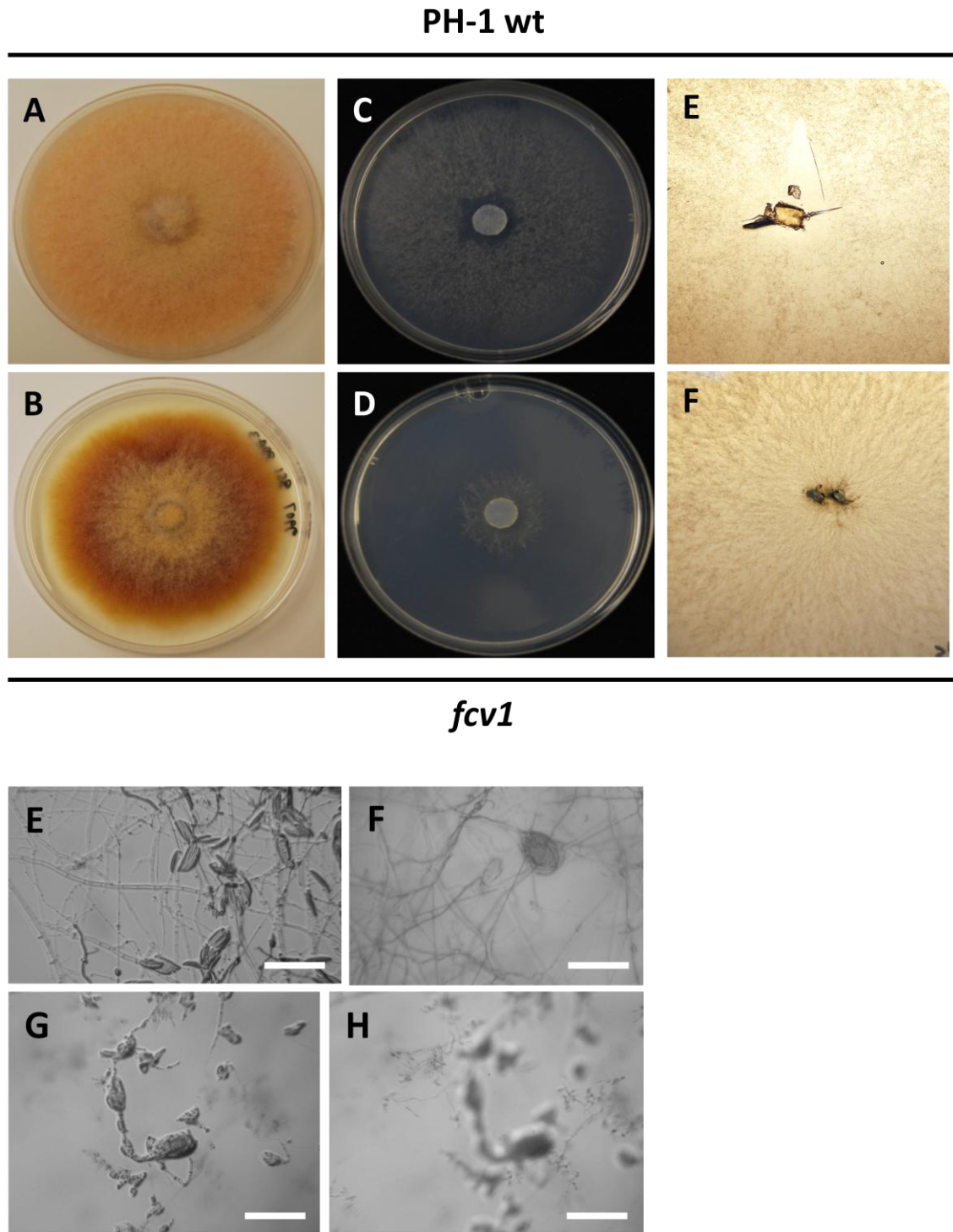


Figure 5.24. In vitro growth of wild-type (top) and *fcv1* (bottom) on complete medium (A-B) and minimal medium (C-D). A close-up of the minimal medium plates is shown in E and F to illustrate the differing colony morphologies. E. Wild-type, F. *fcv1*+*FCV1* and G. and H. *fcv1* colony appearance on the surface of a minimal media agar plate. Note the higher hyphal density of wild type and *fcv1*+*FCV1* strains compared to *fcv1*. The *fcv1* images show two different focal planes of the same colony region. (3) illustrates sparse surface growth and (4) shows conidial abundance within the agar. Bar = 40 μ m. Images E and F by M. Urban.

To check that the reduced hyphal density of the *fcv1* in the initiating agar plug was not the direct cause of the *in vitro* reduced growth, germinating conidia instead of plugs of mycelium were used as the starting inocula. This resulted in a more pronounced change in growth rate compared to the wild-type: complete medium (45% reduction), minimal medium (94% reduction), minimal medium with low carbon (84% reduction) and minimal medium with low nitrogen (84% reduction) (**Figure 5.25**). These results again indicated a more severe growth defect of *fcv1* on minimal compared to complete medium. The germination efficiency of *fcv1* and wild-type conidia was also tested on four different medium. This indicated an slight but significant overall reduction in germination rate of *fcv1* compared to the wild-type strain on minimal medium types (minimal medium, minimal medium with low carbon and minimal medium with low nitrogen, 5.6% average reduction) but no significant difference in germination efficiency between the two strains on complete medium (**Table 5.4**). Collectively, these experiments reveal that there was an intrinsic lower growth rate of the *fcv1* hyphae which was particularly pronounced when the colony was growing under nutrient limiting conditions.

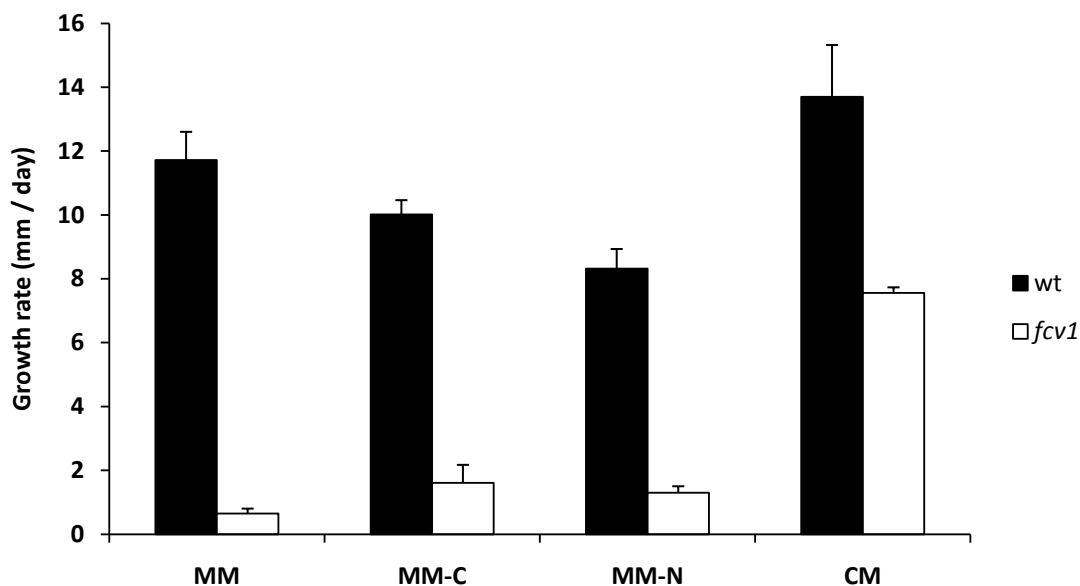


Figure 5.25. *fcv1* has an inherently reduced growth rate compared to wild type. Growth rate of spore-inoculated plate cultures of wt (black bars) and *fcv1* (white bars) on minimal medium (MM), minimal medium with low carbon (MM-C) or low nitrogen (MM-N) or complete medium (CM). * = significant difference from wild type at $p < 0.05$.

Table 5.4. Spore germination efficiency of *fcv1* and wild-type. * = significantly different to wild-type ($p < 0.05$).

Medium	wt	<i>fcv1</i>
MM	99.33	92*
MM-C	99.33	94.67*
MM-N	99.33	94.67*
Overall (MM)+(MM-C)+(MM-N)	99.33	93.78*
CM	96	97.3

The result of targeted deletion of the *fcv1* homologue in the model filamentous fungus *Neurospora crassa* was also investigated. FASTA analysis indicated the best hit for *fcv1* in *N. crassa* to be *NCU01167*, with a score of 49.7. The growth rate of the wild-type and *ncu01167* deletion strains for both *N. crassa* mating type A and a on minimal medium was compared. In the case of both mating types, the *ncu01167* strain grew significantly slower than the wild-type (43% and 40% reduction in growth rate for mating type A and a, respectively, **Figure 5.26**). This indicates that the *fcv1* gene and its homologues are required for the full growth potential of both these species of fungi yet are not essential for life.

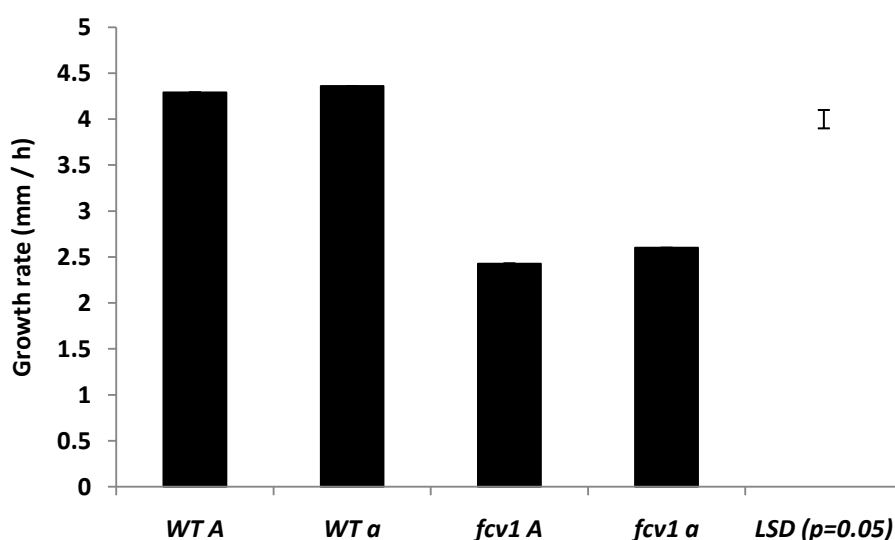


Figure 5.26. *In vitro* growth rate of the wild-type and *fcv1* homologue deletion strains of the model filamentous fungus *Neurospora crassa*. In each case the mating type (A or a) is indicated.

In the nutrient-rich environment of the developing wheat ears, the disease development of *fcv1* following point inoculation by agar plugs was only 50% of that shown by the wild-type by day 8, 58% by day 12 and 48% by day 16 in the experiment shown in **Figure 5.18A** and 58% of that of the wild-type by day 8, 57% by day 12 and 62% by day 16 in the experiment shown in **Figure 5.21**. By the 16 dpi time point the wild-type isolate had produced disease symptoms throughout the ear (**Figure 5.21**). The *in vitro* growth rate of *fcv1* on agar plug inoculated complete medium plates was 77% of that of the wild-type in the experiment shown in **Figure 5.22D** and 70% in the experiment shown in **Figure 5.23**. This growth defect appears to contribute to the observed reduction in disease development.

Interestingly, microscopic observations revealed that during the early phase of *fcv1* induced disease development up to day 4, both the macroscopic external and internal symptom development were similar to the wild-type strain in the initially inoculated spikelet. It is presumed that at the time of inoculations the spikelets are potentially the most nutrient rich tissue in the ear because of the phloem connections are continuously unloading sucrose to the sink tissue, the five developing grain. Also up to 15 anthers containing mature pollen are also present within each spikelet and may act as a further nutrient source.

5.3.4 Bioinformatic Analysis of the *FCV1* Gene

Bioinformatic analysis of the *FCV1* sequence revealed a number of features. The subcellular location of Fcv1 was predicted to be intracellular, with limited homology to a number of nuclear, cytoplasmic and mitochondrial proteins being reported (**Table 5.5**). A search for signal peptide sequence in Fcv1 did not locate a predicted signal peptide, consistent with an intracellular protein location for Fcv1 (**Figure 5.27A**). Fcv1 also appears to lack transmembrane elements, further suggesting a location inside the cell (**Figure 5.27B**).

In planta, the expression of *FCV1* remained low over a 144 h time course of *F. graminearum* strain PH-1 infecting the ears of the fully susceptible barley cultivar Morex and exhibited little fluctuation (**Figure 3.5**, Gldener *et al.*, 2006). This pattern of expression is in contrast to other genes in the cluster such as

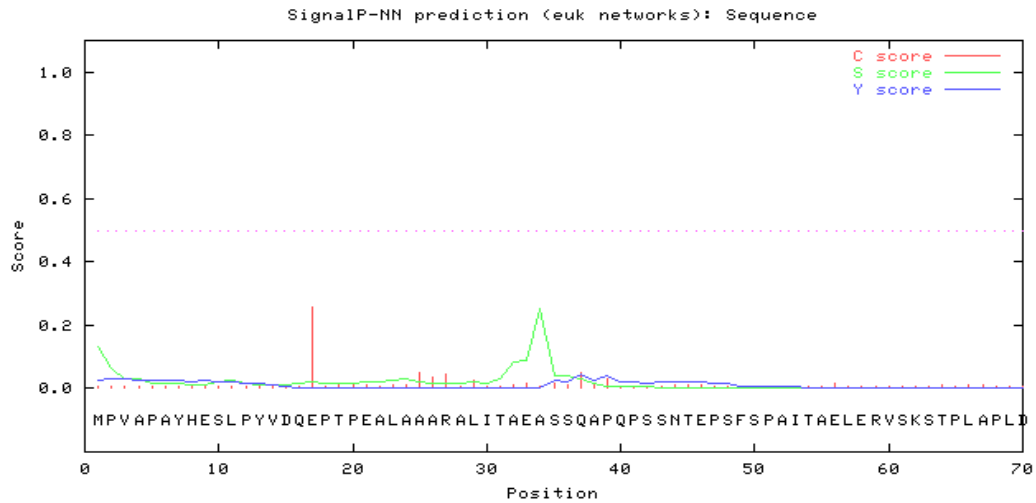
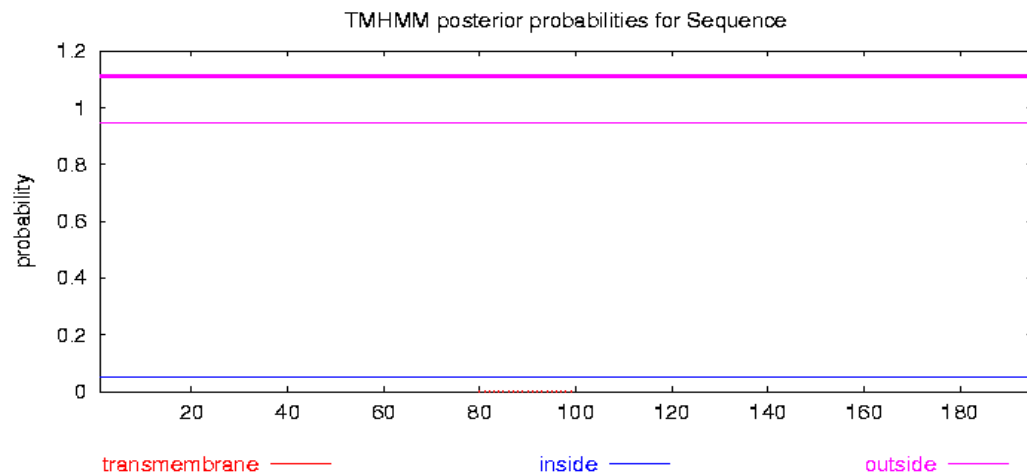
FGSG_09891, *FGSG_09893*, *NTH1*, *FGSG_09906* and *PKAR* which show a large increase in transcript abundance over the time course, and is more akin to that of genes such as *FGSG_09892*.

A multiple sequence alignment comparing Fcv1 and its closest homologues in the fungal species analysed in Chapter 3 indicates a region of higher conservation towards the C-terminal end of the protein (**Figure 5.28**). The greatest sequence conservation is found between the *Fusarium* species, and, like the cluster synteny analysis in Chapter 3, a gradual breakdown of conservation is noted when less closely related species are compared to *F. graminearum*.

A BLAST search for homologues of Fcv1 revealed the presence of a Bcas2 domain that encompasses most of the Fcv1 sequence. Breast Carcinoma Amplified Sequence 2 (Bcas2) was identified as a protein upregulated in breast cancer (Nagasaki *et al.*, 1999, Worsham *et al.*, 2006) but has since been characterised as a pre mRNA splicing factor and is also known as Spf27 or Cwf7 (Neubauer *et al.*, 1998, Ajuh *et al.*, 2000; Maass *et al.*, 2002; Ohi *et al.*, 2002). Fcv1 exhibits a degree of sequence identity to Bcas2/Spf27 proteins. A protein sequence comparison Fcv1 and a number of Bcas2/Spf27 proteins is shown in **Table 5.6**. Further analysis revealed that Fcv1 is not predicted to contain either a nuclear localisation sequence or nuclear export sequence.

Table 5.5. Fcv1 WoLFPSORT prediction. Number of hits: Nuclear: 13.0, Cytoplasmic: 8.5, Cytoplasmic/Mitochondrial: 7.5, Mitochondrial: 5.5

ID	Site	Identity (%)	Comments
MAZ3_SCHCO	Nuclear	8	[Uniprot] SWISS-PROT45:Nuclear.
CCHL_NEUCR	Mitochondrial	16	[Uniprot] SWISS-PROT45:Mitochondrial inner membrane.
LEU3_PHACH	Cytoplasmic	12	[Uniprot] SWISS-PROT45:Cytoplasmic.
CPC1_NEUCR	Nuclear	14	[Uniprot] SWISS-PROT45:Nuclear.
ARIS_PENRO	Cytoplasmic	14	[Uniprot] SWISS-PROT45:Cytoplasmic.
DYNA_NEUCR	Cytoplasmic	6	[Uniprot] SWISS-PROT45:Cytoplasmic.
MAZ4_SCHCO	Nuclear	8	[Uniprot] SWISS-PROT45:Nuclear.
PTH_YEAST	Mitochondrial	12	[Uniprot] SWISS-PROT45:Mitochondrial. GO:0005739; C:mitochondrion
LEU3_PICST	Cytoplasmic	12	[Uniprot] SWISS-PROT45:Cytoplasmic.
RPB3_SCHPO	Nuclear	14	[Uniprot] SWISS-PROT45:Nuclear.
BIMB_EMENI	Nuclear	4	[Uniprot] SWISS-PROT45:Nuclear.
B7_USTMA	Nuclear	12	[Uniprot] SWISS-PROT45:Nuclear.
LEU3_CANAL	Cytoplasmic	15	[Uniprot] SWISS-PROT45:Cytoplasmic.
COX6_NEUCR	Mitochondrial	17	[Uniprot] SWISS-PROT45:Mitochondrial inner membrane.
DODA_AMAMU	Cytoplasmic	15	[Uniprot] SWISS-PROT45:Cytoplasmic.
MB11_COPCI	Nuclear	9	[Uniprot] SWISS-PROT45:Nuclear.
LEU3_CANMA	Cytoplasmic	14	[Uniprot] SWISS-PROT45:Cytoplasmic.
ZUO1_YEAST	Nuclear	14	[Uniprot] SWISS-PROT45:Nuclear. GO:0005840; C:ribosome
MK16_YEAST	Nuclear	15	[Uniprot] SWISS-PROT45:Nuclear. GO:0005730; C:nucleolus
CPC1_CRYPA	Nuclear	15	[Uniprot] SWISS-PROT45:Nuclear.
SPT4_YEAST	Nuclear	13	[Uniprot] SWISS-PROT45:Nuclear. GO:0008023; C:transcription elongation factor complex
SSN6_YEAST	Nuclear	7	[Uniprot] SWISS-PROT45:Nuclear. GO:0005634; C:nucleus
ATPD_AGABI	Mitochondrial	15	[Uniprot] SWISS-PROT45:Mitochondrial.
LEU3_ASHGO	Cytoplasmic	14	[Uniprot] SWISS-PROT45:Cytoplasmic.
LEU1_YEAST	Cyto/Mito	11	[Uniprot] SWISS-PROT45:Mitochondrial and cytoplasmic. GO:0005739; C:mitochondrion
SW10_SCHPO	Nuclear	13	[Uniprot] SWISS-PROT45:Nuclear.

A**B**

TMHMM prediction:

Sequence Length: 195
 # Sequence Number of predicted TMHs: 0
 # Sequence Exp number of AAs in TMHs: 0.00556

Figure 5.27. Bioinformatic analysis of the Fcv1 protein sequence. Prediction results for signal peptide (top) and transmembrane elements (bottom) indicate the absence of both of these features in Fcv1. The signal peptide C score is a “cleavage site” score which should only be high at a predicted signal peptide cleavage site, the S score is a “signal score” provided for each amino acid with high scores indicating that residue is likely to form part of the signal peptide. The Y score is a derivative of the C and S scores to provide better cleavage site prediction. The TMHMM plot calculates the probability for each residue that it resides in a helix, inside or outside the cell. The prediction, which considers the most probable overall structure suggests a lack of transmembrane helices for Fcv1 .

Table 5.6. Protein sequence identity of Fcv1 toBcas2/Spf27 proteins and Arabidopsis Mos4.

Protein	Species	Identity (%)	Similarity(%)
Bcas2	<i>Aspergillus fumigatus</i>	43.5	57.9
Bcas2	<i>Talaromyces stipitatus</i>	43.4	57.0
Bcas2	<i>Penicillium marneffe</i>	42.7	57.8
Cwf7	<i>Schizosaccharomyces pombe</i>	26.5	38.9
Spf27	<i>Lepeophtheira salmonis</i>	26.1	40.9
Bcas2	<i>Xenopus laevis</i>	24.2	43.6
Bcas2	<i>Homo sapiens</i>	23.6	43.2
Spf27	<i>Homo sapiens</i>	23.6	43.2
Mos4	<i>Arabidopsis thaliana</i>	23.4	34.0

Figure 5.28. (See over). Multiple sequence alignment of Fcv1 (Fgsg_09907.3) and its closest homologues in the fungal species analysed in Chapter 3. The highest level of sequence conservation is with the other *Fusarium* species, *F. verticillioides*, *F. oxysporum* and *F. solani*. Species identifiers are as follows: Foxg (*F. oxysporum*), Fveg (*F. verticillioides*), Fgsg (*F. graminearum*), Nh (*F. solani*), Tr (*T. reesei*), Mgg (*M. oryzae*), Ncu (*N. crassa*), Mg (*M. graminicola*), Um (*U. maydis*). Shading indicates well-conserved residues (red – conserved in all sequences, orange – conserved in at least 80% of the sequences and yellow - conserved in at least 60% of the sequences). The full titles of the hits in *F. solani*, *T. reesei* and *M. graminicola* are: jgi|Necha2|80338|fgenes1_pg.sca_5_chr5_3_0000137, jgi|Trire2|57923|e_gw1.4.1012.1 and jgi|Mycgr3|83408|fgenes1_pm.C_chr_1000118 respectively.

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*          20          *          40          *          60
Foxg_05518 : -----MPT : 3
Fgsg_09907 : -----MPV : 3
Fveg_02621 : -----MPI : 3
Nh80338 : -----MAV : 3
Tr57923 : -----MAV : 3
Ncu01167 : -----MPL : 3
Mg83408 : -----MPL : 3
Mgg_07336 : -----MSS : 3
Um01682 : MARARGGCASEGSAASNEPDTAQSASAAANTLAATSTSSENGDTETSAAGGPSKDSFSYHA : 60
                                         m

*          80          *          100          *          120
Foxg_05518 : PP-AYHESLPYVD--QEPNLEALAAARDLIAAEASSQPPLP-TSNP-----EPSFTP : 51
Fgsg_09907 : AP-AYHESLPYVD--QEPTPEALAAARALITAEASSQAPQP-SSNT-----EPSFSP : 51
Fveg_02621 : PP-AYHESLPYVD--QEPTPEALTAARDLIAAEASSQPPLP-TSNP-----EPSFTP : 51
Nh80338 : PP-AYHESLPYVD--PEPSPEALAAARALITAEAAATQLEKP-SSHP-----EPSFSP : 51
Tr57923 : AP-AYHESLPYID--PEPSPEALSAAARALIAEQSTSSSSSS-SSSSSAALPPLKEPSFSP : 59
Ncu01167 : IT-TIHESLPYID--PEPTPERSAAEALIAAERSLQPD--PFHALLPPPLTPSSHITP : 58
Mg83408 : IL-SSSDALPYID--AAPSVDALAAANALIEAELDPEHMN--TSHPSIPAMRESK--YSD : 56
Mgg_07336 : IRRTVHESLPYVD--ADFTPTQRAAAEALIAAELEASSATTVPAPHDLPPAYTPN--FTP : 59
Um01682 : VELAPTDAIPYFDRELQLQGLRSRVDALIAEEQASMSBIS--STSSRLPPVYELFSFRP : 118
          hesLPY D   ep p   aa aLI aE           s           p

*          140          *          160          *          180
Foxg_05518 : AMIAELERVSK-----STP-LAPLDLSRY---EAPSPSAPPAT----- : 85
Fgsg_09907 : AITAELEERVSK-----STP-LAPLDLSRY---EAPSPSAPPAT----- : 85
Fveg_02621 : AMIAELERVSK-----STP-LAPLDLSRY---EAPSPSAPPAT----- : 85
Nh80338 : AIAAELARVSN-----STP-LAPLDLSRY---EAPSPSAPPVE----- : 85
Tr57923 : AISAQLSRIQS-----SQEPQPPDLDSRY---EAQELPPPPSSDSSSPS : 101
Ncu01167 : LLESEFDRIASHHVQAANPDGTQAPPKLSALDLARYSSLPEI PSASELAGMD-----S : 113
Mg83408 : LIEATHARIAAG-----QKSGGLDSRY---ELLDP-----A : 87
Mgg_07336 : AIAAELDRISS---SADP-----SRPAKLSAIDTKRY---ELEDDDEEGQ-----P : 100
Um01682 : DIRTLEERVAS-----GQESTHTLDTHRY---TLPSPTSGEAAS----- : 154
          6 a2l R6           p           6Dl RY           e

*          200          *          220          *          240
Foxg_05518 : -----ALPAAATAHSYLSSRLTNLELLEKW-GKNAWLLGNHGLEAELQVLERELAA : 135
Fgsg_09907 : -----ALPAAAVAHSYLSSRLTNLELLEKW-GKNAWLLGNHGLEAELQALERELAV : 135
Fveg_02621 : -----ALPAAATAHSYLSSRLTNLELLEKW-GKNAWLLGNHGLEAELQALERELAA : 135
Nh80338 : -----ALPLAAVAHSYLSSRLTNLELLDKW-GKNAWLLGNHGLEAELQALERELAA : 135
Tr57923 : EATAQATRRALQNAFVSSSYLSSRAQNLALLDAH-GRNAWLLSNYHLEAELRSLERDLAA : 160
Ncu01167 : SEATLLSSLGKAYTDHAYVAQRRRAHALLDAY-GKNAWLIGNWQLEGELKATEKELAE : 172
Mg83408 : KGDLEAWKVALQKAYASAEYLRGREINLSLLETY-GKNAWLIGNSALEDELRALEKEVEA : 146
Mgg_07336 : APTADYLASALSKEYTSATHIRLRHHALLDAPEGRNAWLVANWQLEGELRALEAELAA : 160
Um01682 : ---LSDWQAQAVDSHAQLGHMDVRMKNIELLKKY-GSNAWRLSNFQQEQNIRLLSEQIDL : 210
          a6 A           y6 R   n6 LL           G NAWl6 N   lE e6   6e   6a

*          260          *          280          *          300
Foxg_05518 : VKREIDIVNLERQKRONAVGAEIKTLDLTWRTGVGRVLETEVAVEELRKRKIREELTRRAT : 195
Fgsg_09907 : TKREVDIVNLERQKQRTAVGAEIKTLDLTWRAGVGRVLETEVAVEELRKRKIREELARRAV : 195
Fveg_02621 : VKREIDIVNLERQNRONAVGAEIKTLDLTWRTGVGRVLETEVAVEELRKRKIREELTRRAT : 195
Nh80338 : TKREVDIVNLERQKRQAVGAEIKALDDTWRAVGGRVLETEVAVEELRGRIREELARRAT : 195
Tr57923 : AKRDIDLVAARAARQTDVKAEMQTEHNNWREGVGRVLETEIAVQELREQIRHELRTAA : 220
Ncu01167 : AKREIDLVTLQKQAQDEVGPEILGLLEDTWKKGVGRVLETEAAVEGLRRQVLEVRGME- : 231
Mg83408 : AKIEGEQTQQSRRTVQANAAGEMQGLEEGWKTGVGRMVETQAAGERLRMEILERKRQGAS : 206
Mgg_07336 : AKRQVDILAQRRAQDDVSGELRGLEDSSWKRGVGVLEIEVATHRLRAEVESRRNEALV : 220
Um01682 : VKAETSEINRLBQKNHLEAGGKIATLNKRRTELISRGLEEVANITTSQEVDMLKSKK : 270
          Kr d 6   R   q   v   e6   L   W   g6gr 62t2 A   lr   6

*
Foxg_05518 : DDQGG----- : 200
Fgsg_09907 : ----- : -
Fveg_02621 : DGQEQ----- : 200
Nh80338 : QGDEQAQGQEQ : 206
Tr57923 : AAEPQS----- : 227
Ncu01167 : ----- : -
Mg83408 : ----- : -
Mgg_07336 : AQAAAGA----- : 227
Um01682 : LETELSLLE-- : 279

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5.4 Discussion

Targeted deletion of the homologues of verified virulence genes that are present in the micro-region confirmed a role for four of these five genes in the determining the rate of FEB symptom spread by *F. graminearum* on wheat ears. Having confirmed the importance of this region of chromosome 1 in disease progression by *F. graminearum*, additional genes in the region were selected for targeted deletion in the search for novel virulence determinants. Six further genes were successfully deleted – *FGSG_09891*, *FGSG_09893*, *FGSG_09900*, *FGSG_09905*, *FGSG_09906* and *FGSG_09907* (*FCV1*).

The *fgsg_09891*, *fgsg_09893*, *fgsg_09900*, *fgsg_09905* and *fgsg_09906* targeted deletion mutants did not show disease symptom progression significantly different to the wild type strain. However, deletion of *FGSG_09907* caused a significant reduction in the rate of disease symptom spread and this gene has been named *Eusarium graminearum* *Contributor to Virulence 1* (*FCV1*). Disease progression of *F. graminearum* on wheat ears was restored by reinsertion of a copy of the wild-type gene into the *fcv1* strain.

FCV1 deletion also alters the filamentous growth and morphology of *F. graminearum*. The growth rate of the *fcv1* strain was reduced compared to the wild-type and complemented strains on both complete and minimal medium. The reduction in growth rate of *fcv1* on complete medium appears to explain a large portion of its defect in FEB symptom spread rate. Like *pkar*, an additional *in planta*-specific effect may also be present that leads to the larger *in planta* vs *in vitro* defect in these strains. However, further investigation may be required to confirm any additional effects during host invasion.

The growth morphology of the *fcv1* strain on minimal medium exhibited an unusual degree of preference for growth within the solid medium as oppose to surface growth, leading to an altered colony appearance. On minimal medium the *F. graminearum* MAP kinase deletion strain *gpmk1* (*map1*) has been reported to exhibit growth under the surface of the solid medium with few long hyphae (Jenczmionka *et al.*, 2003). Hyphae of strain 8/1 harbouring a deletion of the *GPMK1* gene are short, highly branched and produce conidia. The *gpmk1* disruption mutants are reduced in conidiation on SNA medium, however as almost all conidia are contained within the medium and unable to be

harvested from the medium surface, this result appears to be misleading (Jenczmionka *et al.*, 2003). By covering the SNA plates with cellophane foil to include the conidia produced under the surface, *gpmk1* still exhibited a reduction level of conidiation compared to the wild type strain (Jenczmionka *et al.*, 2003). However, Urban *et al.* found no reduction in spore production by *gpmk1* mutants (Urban *et al.*, 2003).

Jenczmionka *et al.* noted just three to four conidia were produced per conidiophore in *gpmk1* mutants and were found under the surface throughout the whole plate, unlike the typical large bundles of conidia normally observed (Jenczmionka *et al.*, 2003). The Urban *et al.* study of *GPMK1* also found that *gpmk1* mutants produce conidia that are evenly spread across the colony rather than contained in discrete sporodochia, although in this case many conidia were present on the surface of the solid medium (Urban *et al.*, 2003). The viability of conidia was unaffected by the disruption of *GPMK1* (Jenczmionka *et al.*, 2003; Urban *et al.*, 2003). The similarities between the growth patterns of the *gpmk1* and *fcv1* mutants suggests that the proteins they encode may function in an overlapping set of processes in *F. graminearum* involved in growth morphology.

The bioinformatics analysis revealed a predicted intracellular location for Fcv1 and a low level of expression *in planta*. Fcv1 also was found to contain a Bcas2 domain and showed a degree of similarity to Spf27/Bcas2 proteins. Bcas2 was originally identified as a protein upregulated in breast cancer and breast cancer cell lines (Nagasaki *et al.*, 1999, Worsham *et al.*, 2006) but ubiquitously expressed in many organs (Nagasaki *et al.*, 1999). Upregulation of Bcas2 in cancer cells appears to be specific for breast cancer (Maass *et al.*, 2002) and Bcas2 appears to be localised to the nucleus (Maass *et al.*, 2002, Qi *et al.*, 2005). Bcas2 has also been suggested to interact with estrogen receptor α and modulate its transcriptional regulation activity (Qi *et al.*, 2005). The human Bcas2 sequence was found to be identical to that of a putative spliceosome-associated protein identified by mass spectrometry, known as Spf27 (Maass *et al.*, 2002; Neubauer *et al.*, 1998, Ajuh *et al.*, 2000). Fcv1 also shows similarity to the fission yeast (*Schizosaccharomyces pombe*) Spf27 homologue Cwf7 (Ohi *et al.*, 2002, NP_595665) and other Spf27 proteins.

Cwf7 was identified in *S. pombe* as a protein that co-immunoprecipitated with the protein Cdc5 as part of a 40S snRNP-containing complex (McDonald *et al.*, 1999). Cdc5, together with its *S. cerevisiae* and human homologues Cef1 and hCdc5L respectively, are required for pre-mRNA splicing (Burns *et al.*, 1999; McDonald *et al.*, 1999; Tsai *et al.*, 1999; Ajuh *et al.*, 2000, 2001) and are present in spliceosome-associated complexes that may function in spliceosome assembly (Neubauer *et al.*, 1998; McDonald *et al.*, 1999; Tsai *et al.*, 1999, Ajuh *et al.*, 2000; Ohi *et al.*, 2002; Chan *et al.*, 2003). In *S. cerevisiae*, the complex containing Cef1 is known as the PRP NTC (Nineteen Complex) due to the presence of the Prp19 protein (Tsai *et al.*, 1999). In humans, the NTC is also known as the NMP200 Complex or PSO4 complex (Ohi and Gould 2002). Interaction of Cwf7 with Cdc5 was confirmed by a further study using mass spectrometry and co-immunoprecipitation to analyse the content and interactions of the Cdc5-associated complex components (Ohi *et al.*, 2002). The *CWF7* gene was found to be essential for viability and by analysing cells lacking the Cwf7 protein, it was shown that levels of pre-mRNAs of *TFIID* and *HIS3* increased, while levels of the corresponding mature mRNAs decreased (Ohi *et al.*, 2002). This indicated that Cwf7 is required for pre-mRNA splicing in *S. pombe*. The human homologue of Cwf7, Spf27, co-purifies with hCdc5L (Ajuh *et al.*, 2000), but no homologue of Cwf7 is found in the *Saccharomyces cerevisiae* Cef1-associated complex (Ohi *et al.*, 2002). However, Cwf7 interacts with Cwf8, the *S. pombe* homologue of the *S. cerevisiae* pre-mRNA splicing factor Prp19 (Ohi and Gould, 2002). Prp19 interacts with both Cef1 and Snt309 in *S. cerevisiae* using the same conserved sequence element as the Cwf7-Cwf8 interaction (Chen *et al.*, 1998; Tsai *et al.*, 1999; Ohi and Gould, 2002). Cwf7 overexpression is able to rescue growth of *snt309* cells, suggesting that Cwf7 is a functional homologue of Snt309 (Ohi and Gould, 2002).

Human Prp19 may also act as an E3 ubiquitin ligase (Hatakeyama *et al.*, 2001) and complexes containing Prp19 and Spf27, and also Cdc5 have been suggested to be involved in the response to DNA damage (Lu and Legerski, 2007; Zhang, N. *et al.*, 2009). Prp19 overexpression in human cells reduces DNA damage-induced apoptosis (Lu and Legerski, 2007).

Bcas2 has been reported to interact with the p53 tumour suppressor protein and reduce its transcriptional activity. Bcas2 deprivation induced apoptosis in cells containing p53 by retaining p53 in the nucleus and increasing p53 transcriptional activity (Kuo *et al.*, 2009). Cdc5 is also required for G₂/M progression in *S. pombe* (Nurse *et al.*, 1976; Ohi *et al.*, 1994; McDonald, *et al.*, 1999). Interestingly, in human cells lacking p53, or containing a mutant p53, depletion of Bcas2 caused cell cycle arrest at the G₂/M checkpoint (Kuo *et al.*, 2009).

Fcv1 also shows 23.4% protein sequence identity to the *Arabidopsis thaliana* Mos4 protein (At3g18165, NP_566599.1) (this study; Palma *et al.*, 2007). The *MOS4* (*modifier of snc1*, 4) gene was identified in a screen for suppressors of the *snc1* (suppressor of NPR1-1, constitutive 1) gain-of function mutation which leads to constitutive defence activation and enhanced resistance (Zhang *et al.*, 2003; Palma *et al.*, 2007). The *mos4-1* mutation abolishes the *snc1* defence and resistance phenotypes. The *mos4-1* mutant *Arabidopsis* plants show delayed flowering time and a reduced number of seeds per silique compared to the wild type Col-0 (Palma *et al.*, 2007). GFP fusion showed that Mos4 is nuclear localised.

Arabidopsis plants harbouring the *snc1* mutation show constitutive expression of the *PR-1* and *PR-2* pathogenesis-related genes. Double mutant *mos4-1 snc1* plants however, lose constitutive *PR-2* expression and also show a partial reduction in *PR-1* expression. . In addition, *mos4-1* also recovers the small curly leaves seen in *snc1* to a wild type appearance (Palma *et al.*, 2007). *PR-1* expression is regulated by salicylic acid (SA) via the Npr1 protein which controls basal resistance (Dong, 2004). That *mos4-1 npr1* plants are more susceptible than either *mos4-1* or *npr1-1* plants suggests that two separate basal defence pathways could be affected in *mos4-1 npr1* plants. This implies a role for *MOS4* in regulating *NPR1*-independent basal resistance (Palma *et al.*, 2007). However, *mos4-1 snc1* double mutation also partially reduces the high *PR-1* expression of *snc1* plants so *MOS4* may also play a role in *NPR1*-dependent responses. The *mos4-1* mutation also restores approximately wild type levels of salicylic acid (SA) to *snc1* plants, which show elevated SA. However, *mos4-1* plants still show an increase in SA in response to avirulent pathogens. The

reduced SA levels of *mos4-1 snc1* plants may be due to regulation of SA synthesis by the SA-independent pathway (Palma *et al.*, 2007).

In common with its *S. pombe* and human homologues, Mos4 interacts with AtCdc5, which shows significant homology to human Cdc5L. AtCdc5 also interacts with Prl1, a nuclear-localised homologue of the Plrg1 protein that interacts with hCdc5L in the NTC (Ajuh *et al.*, 2001). *AtCDC5* mutation partially suppresses the morphological, enhanced resistance and enhanced *PR-1* and *PR-2* expression of the *snc1* mutation.

Each of the *mos4*, *Atcdc5* and *prl1* single gene mutants is more susceptible to a subclinical concentration of the bacterial pathogen *Pseudomonas syringae* pv *maculicola* (*P.s.m.*) that does not cause symptoms in the wild type Col-0 and also to *Pseudomonas syringae* pv *tomato* DC3000 (*P.s.t.*) and the oomycete *Hyaloperonospora parasitica* Noco2, indicating that all three genes are involved in basal resistance in *A. thaliana* (Palma *et al.*, 2007). Each mutant also shows reduced expression of *PR-1* compared to wild type when inoculated with a clinical dose of *P.s.m.*, but all could accumulate SA in response to infection. Inoculation with *P.s.t.* DC3000 *hrpA*⁻ which is unable to secrete effectors did not lead to significant growth in either the wild-type or mutant plants, suggesting that *MOS4*, *AtCDC5* and *PRL1* do not play a role in non-host resistance (Palma *et al.*, 2007).

Due to their similar phenotypes and demonstrable interaction, Mos4, AtCdc5 and Prl1 were proposed to act together in a multiprotein complex similar to the NTC to regulate immunity in Arabidopsis, termed the Mos4-associated complex (MAC) (Palma *et al.*, 2007). The authors suggest that the individual components of the MAC are not essential for splicing as a total loss of splicing would most likely be lethal and the *mos4*, *Atcdc5* and *prl1* plants are only mildly affected by mutation of these genes, although they acknowledge that the complex as a whole could prove essential for splicing as *mos4-1Atcdc5-1* and *mos4-1 prl1-1* double-homozygous mutants appeared to be lethal. It has been suggested that the MAC could function in alternative splicing but assays of several Arabidopsis genes known to undergo alternative splicing showed no difference in splicing pattern in the *mos4*, *Atcdc5* or *prl1* mutants compared to wild type (Palma *et al.*, 2007). As AtCdc5 exhibits *in vitro* sequence-specific DNA-binding activity

(Hirayama and Shinozaki 1996), it has been suggested that the MAC acts as a transcriptional regulator (Palma *et al.*, 2007). Alternatively, the MAC could contribute to regulation mediated by microRNA (miRNA) and small interfering RNA (siRNA) (Palma *et al.*, 2007).

More recently, the entire MAC was identified by immune-affinity purification and 24 MAC proteins including Mos4, AtCdc5 and Pel1 were identified (Monaghan *et al.*, 2009). Of these, 19 MAC proteins show homology to NTC proteins including two functionally redundant proteins named Mac3A and Mac3B that show homology to Prp19. Many MAC proteins are predicted to play a role in splicing from their homology to known splicing proteins including snRNPs. Like the previously-characterised MAC components, Mac3A and Mac3B are required for basal and R protein-mediated resistance in Arabidopsis and suppress the various *snc1* phenotypes. However, unlike Mos4, AtCdc5 and Prl1, race-specific resistance conferred by the R protein Rpm1 was not affected by *MAC3* mutation. Similarly, resistance mediated by another race-specific resistance R protein Rpp2 was also unaffected by *MAC3* mutation. But both Rps4 and Rps5 mediated race –specific resistance was found to be reduced when assessed in the *mac3a mac3b* double mutant background. In plants, R proteins are involved in the direct or indirect detection of pathogens (often via their secreted ‘effector’ molecules) and activation of defence responses (reviewed, for example, in Hammond-Kosack and Jones, 1997; Bent and Mackey, 2007). Both Mac3A and Mac3B are localised to the nucleus and Mac3A was shown to interact with AtCdc5 *in planta*.

Mutation of *MOS4* or *PRL1* in a *mac3a mac3b* background is lethal, further suggesting that the entire MAC is required for an essential process such as splicing. However, Mac3B was recently shown to have *in vitro* E3 ubiquitin ligase activity (Wiborg *et al.*, 2008). E3 ubiquitin ligases are known to play roles in plant defence (reviewed, for example in Craig *et al.*, 2009) and so the MAC may contribute to immunity via ubiquitin targeted protein degradation (Monaghan *et al.*, 2009).

It is currently unknown if Fcv1 contributes to growth, morphology, and FEB symptom spread of *F. graminearum* by participating in the correct splicing of pre-mRNAs of genes required for specific cellular processes. Further investigation will be required to determine the connection between *FCV1* and these processes in fungi. So far the bioinformatics analysis indicates that the Fcv1 protein is most likely cytoplasmically localised in *F. graminearum*. Therefore, interacting proteins would be required to target the protein to the nucleus if this is the site of its functioning.

Deletion of *FGSG_09893*, *FGSG_09900*, *FGSG_09905* and *FGSG_09906* appeared to have little effect on the *in vitro* growth of *F. graminearum*. *FGSG_09891* encodes a protein with sequence similarity to *S. cerevisiae* Get3. Get3 is a highly conserved ATPase protein with homology to the ArsA subunit of bacterial arsenical transporters (Boskovic *et al.*, 1996, Shen *et al.*, 2003), but appears not to possess an arsenic binding site (Metz *et al.*, 2006). A number of roles have been suggested for Get3 (also known as Arr4) including stress resistance to As^{3+} , As^{5+} , Co^{2+} , Cr^{3+} , Cu^{2+} or VO_4^{3-} salts and temperature (Shen *et al.*, 2003), metal ion homeostasis via copper-dependent interaction with and possible antagonisation of the CLC chloride transport protein Gef1 in yeast (Metz *et al.*, 2006), and in secretory pathway protein sorting in a complex with the proteins Get1 and Get2 (Schuldiner *et al.*, 2005; Auld *et al.*, 2006). *GET3* mutation rescues some of the phenotypes of the *npl4* mutant, which is afflicted in a gene encoding a component of the Cdc48-Npl4-Ufd1 complex that regulates several membrane-associated processes, such as ER-associated degradation (ERAD) via the proteasome (Bays *et al.*, 2001; Auld *et al.*, 2006). *GET3* is co-regulated with both the Cdc48-Npl1-Ufd1 complex and the proteasome. Get3 is localised to the ER and nuclear membranes and cytosol, and localisation at the ER membrane requires the Get1 and Get2 proteins. Interestingly, deletion of *GET1* or *GET2* alters Get3 distribution to a small number of punctuate sites, as found during stress conditions or a switch from rich to minimal medium (Shen *et al.*, 2003, Schuldiner *et al.*, 2005), although this change of localisation has also been shown to be dependent on the presence of Get1 (Schuldiner *et al.*, 2005). Growth of the *fgsg_09891* strain was significantly slower than the wild-type on minimal but not complete medium, suggesting that loss of *FGSG_09891* may restrict growth of *F. graminearum* in

nutrient-limiting conditions but in higher nutrient environments such as growth on complete medium or *in planta*, the *fgsg_09891* strain is able to grow in a manner comparable to wild-type, by comparison, *get3* exhibited a growth defect in iron-limiting conditions (Metz *et al.*, 2006).

Chapter 6. Further Characterisation of the Single Gene Deletion Strains

6.1 Introduction

The functional investigations of the Chromosome I gene micro-region described in Chapters 3 and 4 revealed the presence of four homologues of verified virulence genes that are required for the full rate of FEB symptom spread caused by *F. graminearum* on wheat, and an additional gene, named here as *FCV1*, which is also required for a normal rate of symptom development. This chapter describes further characterisation of the *nth1*, *pkar* and *fcv1* single gene deletion strains of *F. graminearum*.

6.2 Methods

6.2.1 *In vitro* Growth Tests and Wheat Virulence Assays

These are described in Chapter 2. In the case of the H₂O₂ oxidative stress combined analysis, four datasets were analysed together. The number of replicates used is shown in **Table 6.1**. The combined dataset was analysed by REML (see Chapter 2).

Table 6.1. Number of replicates used in the combined hydrogen peroxide stress analysis.

	mM hydrogen peroxide		
	0	2	5
PH-1 wt	12	9	9
<i>fcv1</i>	12	9	9
<i>fcv1+FCV1</i>	3	3	3

6.2.2 *In vitro* Conidia Production

A 2 ml aliquot of mung bean liquid medium (M. Urban) was placed in each well of a 6 well plate (Nunc). Each well was inoculated with either 400 conidia (in the case of PH-1 wt, *nth1* and *fcv1*) or a 9 mm plug of an SNA agar dish culture (in the case of PH-1, *pkar* and *PKAR-e*). The plate was incubated at 28°C, shaking

at 170 rpm in the dark for 7 days. Spore concentration was determined using a haemocytometer. Four wells were used per fungal strain.

6.2.3 Perithecia Induction and Ascospore Discharge

Perithecia production was assayed using carrot agar (Proctor *et al.*, 1997). After five days of growth, the total aerial mycelium was removed and the plate spread with 0.7 ml of 2.5% Tween 60. Perithecia development was recorded for up to 2 weeks. Ascospores were collected by placing 4 ml sterile water in the lid of the upturned plates and incubating for a further 24 hours. The sterile water was then pipetted onto microscope slides and observed for ascospores. Three plates were used per strain.

6.2.4 DON Mycotoxin Production

To analyse the *in planta* production of DON, wheat ears (cv. Bobwhite) were inoculated as described above for PH-1 wt, *nth1* and *fcv1*) by placing the conidial suspension into two florets of ten adjacent spikelets of each ear. Alternatively, small plugs of an SNA agar plate culture of PH-1, *pkar* or *PKAR-e* were added into 10 paired florets per ear using a Pasteur pipette tip. After 10 days the ears were collected, frozen at -80°C and then freeze-dried. The infected portion of each ear was ground with liquid nitrogen, diluted 1:5 with sterile water and blended for 20 s. The resulting mixture was centrifuged at 4800 rpm for 5 minutes and the supernatant diluted in an appropriate amount of 50 mM Tris pH8.0 for DON quantification using the EZ-Quant DON ELISA kit (Diagnostix). Three ears were analysed separately per fungal strain.

6.2.5 *Arabidopsis thaliana* Virulence Assays

For inoculation of *Arabidopsis thaliana*, plants of the ecotype Landsberg *erecta* (Ler) were inoculated according to Urban *et al.*, (2002) using a conidial suspension spray. Disease progression was scored using a modification of the protocol described in Cuzick *et al.* 2008. Plants were sprayed with either 0.5 ml

of 1×10^5 *Fusarium* conidia ml⁻¹ or water (control). Twenty plants were used per fungal strain over two separate experiments and the data pooled. Inoculated plants were placed in trays and then inside a perspex inoculation box containing 1 cm of water. These boxes were sealed and placed in the dark for 16 h before uncovering. Plant growth and inoculation experiments were performed inside controlled growth facilities (Weiss Gallenkamp) at Rothamsted Research. Plants were scored for disease progression at 9 days post inoculation (dpi) using a numerical scoring scale system for flowers (F) and siliques (S). Scores were assigned as described in **Table 6.2**. The number of counts per score for each fungal strain used were determined and the data analysed using a Generalised Linear Model. The model fitted (see chapter 2) was (Experiment/Block/Rep) + Strain*Scale. Apical dominance and height data were analysed by General ANOVA.

Table 6.2. Disease scoring system used for Arabidopsis (adapted from Cuzick et al., 2008).

Organ	Score	Description of disease phenotypes
Flowers (F)	0	Normal
	1	Aerial mycelium visible on flower
	3	Drying of flowers
	5	Stem constriction within flower head
Siliques (S)	0	Normal
	1	Aerial mycelium on silique surface
	3	Drying of silique surface
	5	Drying of pedicel surface or pedicel constriction
	7	Main stem constriction (including loss of siliques caused by stem colonization)

6.2.6 *Neurospora crassa* Analysis

This is described in Chapter 5. Hydrogen peroxide was added to the medium prior to pouring to the concentration indicated.

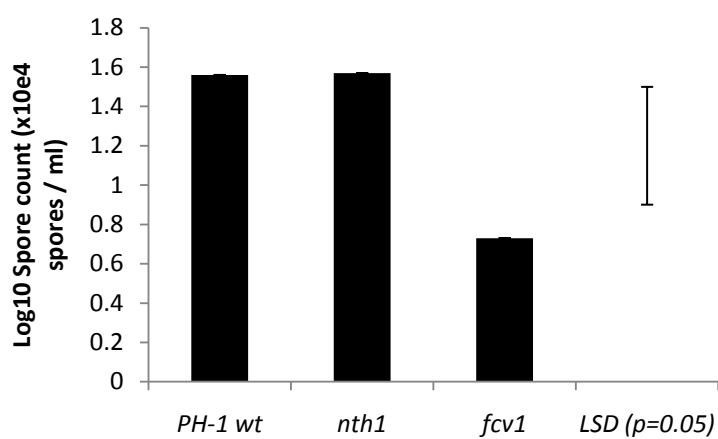
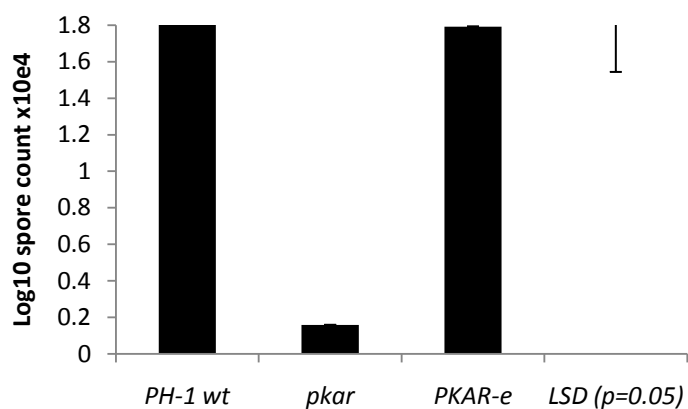
6.3 Results

6.3.1 Further Analysis of the *nth1*, *fcv1* and *pkar* Strains

The three micro-region genes identified in this study that contribute to *F. graminearum* symptom spread rate on wheat, *nth1*, *fcv1* and *pkar*, were analysed further to determine whether these genes and their gene products had additional roles in *F. graminearum* in asexual and sexual spore production, DON mycotoxin production in planta and when grown in the presence of various stresses.

6.3.2 Conidia Production

The production of asexual spores, namely macroconidia, was assayed for the reduced virulence mutants (**Figure 6.1A and B**). The *nth1* and *PKAR-e* strains produced similar numbers of conidia to the wild-type strain. The *fcv1* and *pkar* strains meanwhile, showed a significant reduction in the number of conidia produced compared to the wild type strain. Conidia production in *fcv1* was reduced by about 77% compared to wild type, while *pkar* showed a 98% reduction in conidia production. However, in a second experiment, the conidia production defect of *fcv1* was unable to be rescued in the complemented strain. Given that, in this particular experiment some of the replicates of each strain failed to show induction of conidiation, even for the wild-type (**Figure 6.1C**), it is considered that a problem with the experimental set-up was responsible. This outcome prevents observation of possible complementation of the *fcv1* conidiation defect.

A**B****C**

Rep	Strain	Spores (x10 ⁴ ml ⁻¹)
1	PH-1 wt	3
2	PH-1 wt	27.5
3	PH-1 wt	36.5
4	PH-1 wt	48
1	<i>fcv1</i>	4.5
2	<i>fcv1</i>	2.5
3	<i>fcv1</i>	8
4	<i>fcv1</i>	16.5
1	<i>fcv1</i> + FCV1	13.5
2	<i>fcv1</i> + FCV1	9.5
3	<i>fcv1</i> + FCV1	0.5
4	<i>fcv1</i> + FCV1	0.5

Figure 6.1. Production of conidia by the (A) *nth1* and *fcv1* and (B) *pkar* and *PKAR-e* strains compared to wild-type. (C) A second experiment fails in conidia induction for some replicates of each strain preventing complementation from being observed.

The appearance of *fcv1*, *fcv1* + *FCV1* and wild-type conidia was compared (**Figure 6.2**). The conidia of all three strains appeared similar with no obvious differences in shape or the number of cells contained within the conidia. Occasionally, some cells, usually the end cells of the conidium, appeared to lack contents. This was noted for all three strains.

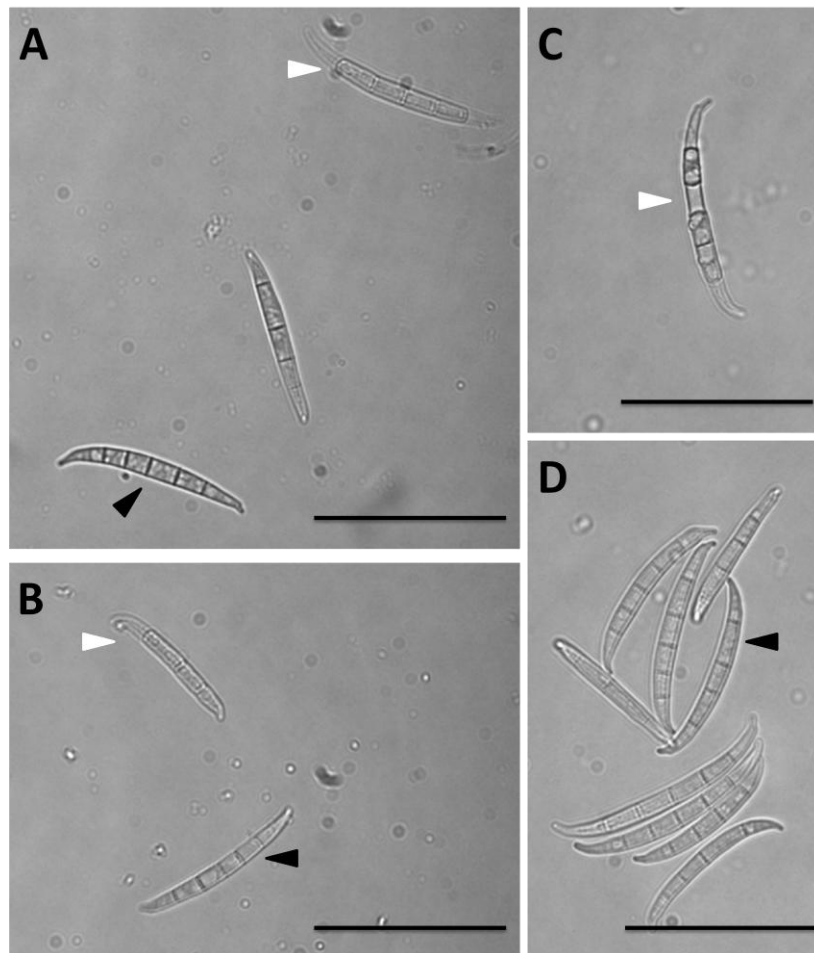


Figure 6.2. Conidia of wt (**A**), *fcv1*+*FCV1*, (**B**) and *fcv1* (**C-D**). All three strains produce conidia of normal appearance (black arrows) and those that appear to lack contents in some cells (white arrows). The end cells of the conidia most often appeared to lack contents. Bar = 20 μ m.

6.3.3 Sexual Reproduction

F. graminearum produces ascospores within perithecia. To determine the effect of gene deletion on sexual spore production, each single-gene deletion strain generated was assayed for perithecia and ascospore production (**Figure 6.3**). All strains were competent to produce perithecia and discharge ascospores into the air. It was not possible to determine the effect of the *PKAR* deletion on ascospore discharge due to a loss of perithecial production in subsequent experiments thought to derive from mutation in the *pkar* strain. The *fgsg_09891* (*get3/arr4*) strain, however, tended to produce more aerial mycelium than the other strains.

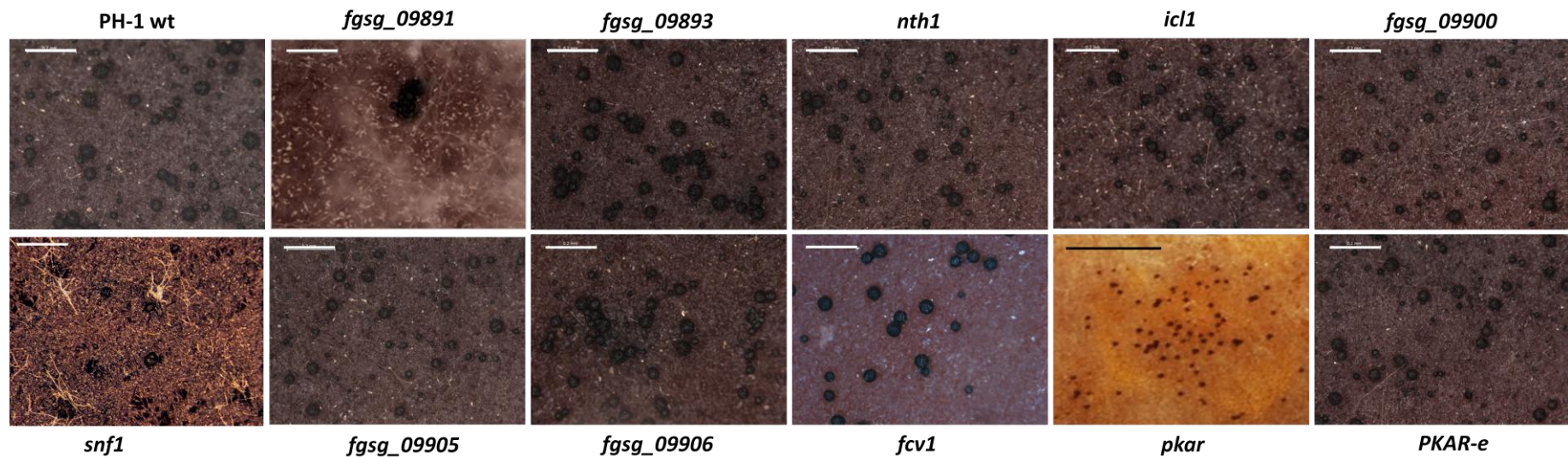


Figure 6.3. Production of perithecia by single-gene deletion mutants of *F. graminearum* micro-region genes. All the targeted gene deletion strains are competent to produce perithecia. Black spherical structures in the images are the perithecial fruiting bodies that contained ascospores. Bar = 0.2mm, except *pkar* image shown at lower magnification, where bar = 5 mm.

6.3.4 Production of the DON Mycotoxin

The ability of the *fcv1*, *nth1*, *pkar* and *PKAR-e* strains to produce the DON mycotoxin during infection of wheat ears was assayed. (**Figure 6.4**). For PH-1, *nth1*, and *fcv1* inoculations were performed by using a solution of conidia. Due to the poor conidia production of the *pkar* strain, for the inter-comparison of PH-1, *pkar* and *PKAR-e* a plug-inoculated method was used. The *pkar* strain produced much lower levels of detectable DON in infected tissue compared to the *PKAR-e* and wild type strains. The *PKAR-e* strain appeared to show an over-production of DON in this experiment, suggesting ectopic insertion of the *PKAR* gene deletion constructs resulted in altered regulation of DON biosynthesis, possibly via the disruption of other regulatory factors. The *fcv1* and *nth1* strains, meanwhile, produced DON levels comparable to that of the wild type (**Figure 6.4**).

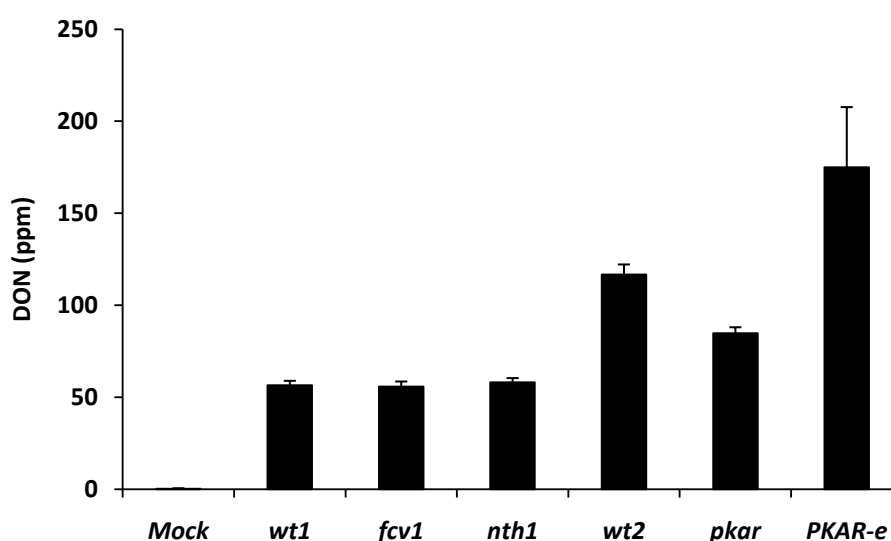


Figure 6.4. Production of the deoxynivalenol (DON) mycotoxin in planta by the reduced virulence strains. Two separate methods using conidia (*wt1*, *fcv1*, *nth1*) or agar plugs (*wt2*, *pkar*, *PKAR-e*) were used (see text).

6.3.5 Arabidopsis Floral Tissue Infection

Fusarium graminearum can infect the floral tissue of the model plant species *Arabidopsis thaliana* (Urban *et al.*, 2002). The virulence of the *nth1* and *fcv1* strains towards *Arabidopsis* was therefore explored. At 9 days post-infection (dpi), the *nth1* strain showed a slight but not significant reduction in disease severity on *Arabidopsis* flowers and siliques compared to the wild type strain while for the *fcv1* strain there was a significant reduction in symptom severity on both flowers and siliques (**Figures 6.5 and 6.6**). For the wild-type and *nth1* infected plants, the floral tissue became brown and shrivelled. The stems supporting the individual flowers were also shrivelled and had turned a dark brown colour and the entire flower cluster was enveloped in mycelium. Siliques also exhibited some browning and loss of structure. In contrast, *fcv1*-infected plants showed browning and shrivelled tissue that was predominantly restricted to the flowers. The upper stems supporting the flowers remained green and full size, comparable to water-inoculated control plants, while the discolouration of siliques was very limited.



Figure 6.5. *FCV1* but not *NTH1* is required for full symptom development of *F. graminearum* on *Arabidopsis* floral tissue. Images taken at 10 days post inoculation. wt = wild type. Bar = 5 mm.

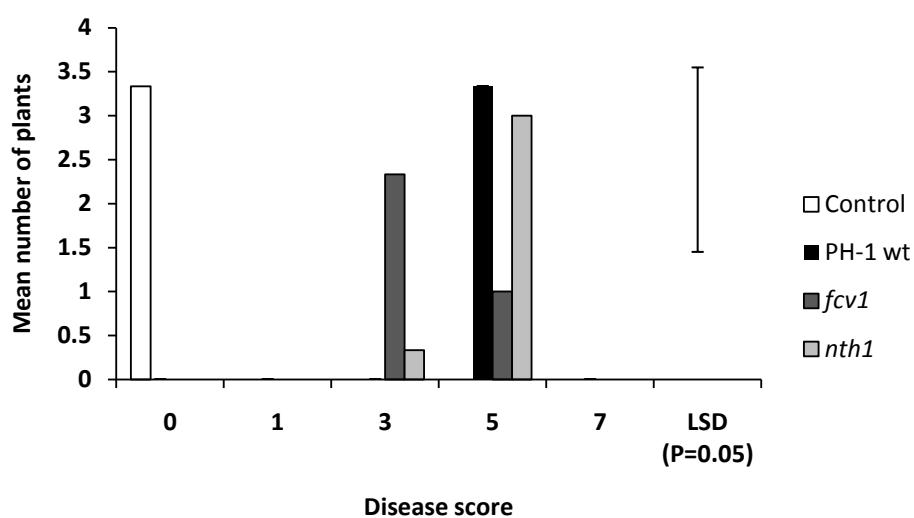
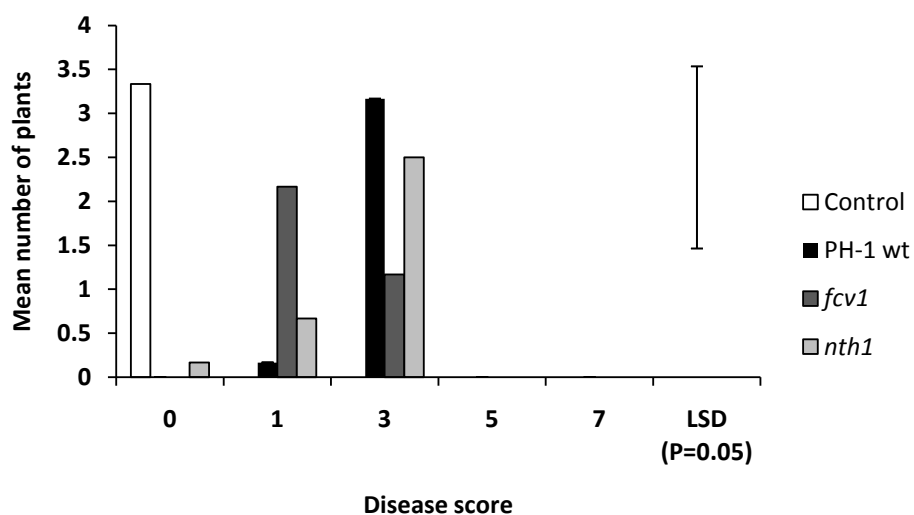
A**B**

Figure 6.6. Infection of Arabidopsis (A) Flowers and (B) Siliques by *nth1* and *fcv1*. The mean count for each disease score is shown for each strain.

6.3.6 Wounded Wheat Ear Virulence Assay for *fcv1*

If the reduction in the rate of FEB symptom spread in the *fcv1* strain is at least partly due to impaired penetration of the host, inoculation of wounded wheat ears should allow some recovery of symptom spread rate. When wounded wheat ears were inoculated with a conidial suspension of the *fcv1* or wild-type strain, (**Figure 6.7**) the spread of the *fcv1* mutant and wild-type from the cut rachis and spikelet surface was comparable to that for the intact ears, with *fcv1* showing a reduced rate of disease spread compared to the wild-type. This suggested the impairment in *fcv1* lies at the post-penetrative stage.



Figure 6.7. Infection of wounded wheat ears by wild-type strain (**A, C**) and the *fcv1* strain (**B, D**). In each ear the disease symptoms spread downwards in a manner comparable to those seen when intact ears were inoculated. At the wound surface/inoculation site (**C-D**), the host tissue is bleached similar to the rest of the diseased portion of the ear. Images taken at 12 dpi.

6.3.7 Stress Tolerance

In the drying tissue of the infected wheat ear, the invading fungus will likely be subjected to increasing osmotic stress and, prior to this, may suffer an oxidative attack as part of the host defence systems. To investigate the tolerance of the *nth1* and *fcv1* strains to cellular stresses, the strains were subjected to growth tests with different media providing oxidative or osmotic stress conditions. Hydrogen peroxide (H_2O_2) or menadione (a glutathione antagonist) was used to provide two different types of oxidative stress when added to minimal medium (SNA). Sodium chloride (NaCl) or glycerol was added to minimal medium to provide osmotic stress conditions. Due to the already very poor *in vitro* growth rate of the *pkar* strain it was not included in this analysis as further growth defects would likely prove hard to quantify.

The preliminary experiment (**Figure 6.8**) confirmed that the growth rate of the *fcv1* strain on minimal medium was significantly less than the wild-type and *nth1* strains. The addition of 1 M NaCl or 7.31% glycerol (A_w 0.98) lead to a significant reduction in the growth rate of the wild type strain (44% and 23% reduction, respectively) and *nth1* (48% and 26% reduction, respectively) ($p < 0.05$). However, for *fcv1*, neither 1 M NaCl nor glycerol (A_w 0.98) caused a significant reduction in growth rate. Instead, the addition of 1 M NaCl or glycerol caused a slight increase in growth rate of *fcv1* (28% and 37%, respectively), which was just statistically significant for glycerol ($p < 0.05$). The addition of 2 mM H_2O_2 led to a significant decrease in the growth rate of all three strains, but this change was most dramatic for the *fcv1* strain (75% reduction in growth rate compared to 11% for the wild-type and 16% for *nth1*). 50 μM menadione addition led to a similar percent reduction in growth rate for all three strains (18% for wild-type, 20% for *nth1* and 24% for *fcv1*), yet this change was significant for the wild-type and *nth1* strains but not of *fcv1* ($p < 0.05$). The preliminary experiment suggested a similar stress response of *nth1* to the wild-type for all stresses tested, reduced sensitivity to osmotic stress for the *fcv1* strain compared to wild-type and *nth1* yet increased sensitivity of *fcv1* to H_2O_2 . The response to menadione appeared similar for all three strains but may suggest reduced sensitivity to this chemical for *fcv1*.

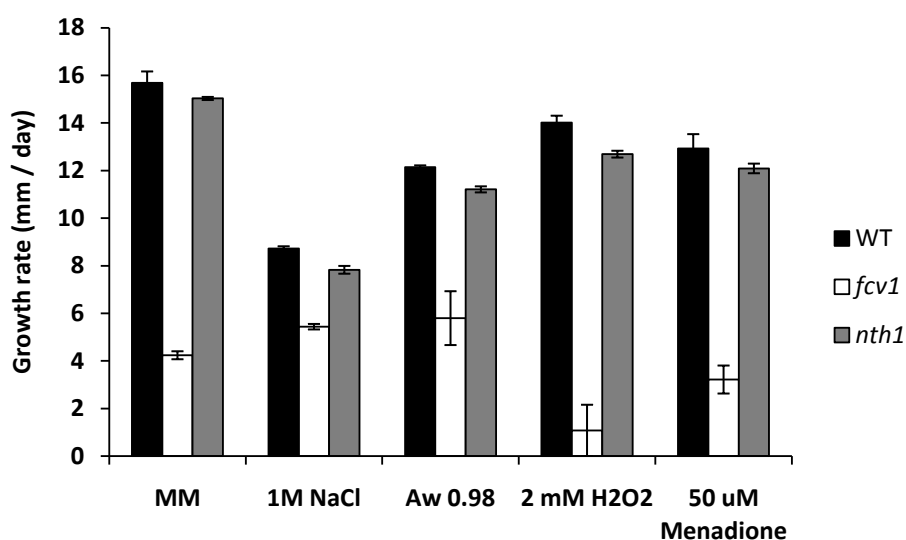


Figure 6.8. Preliminary stress tolerance analysis of the wild-type, *fcv1* and *nth1* strains.

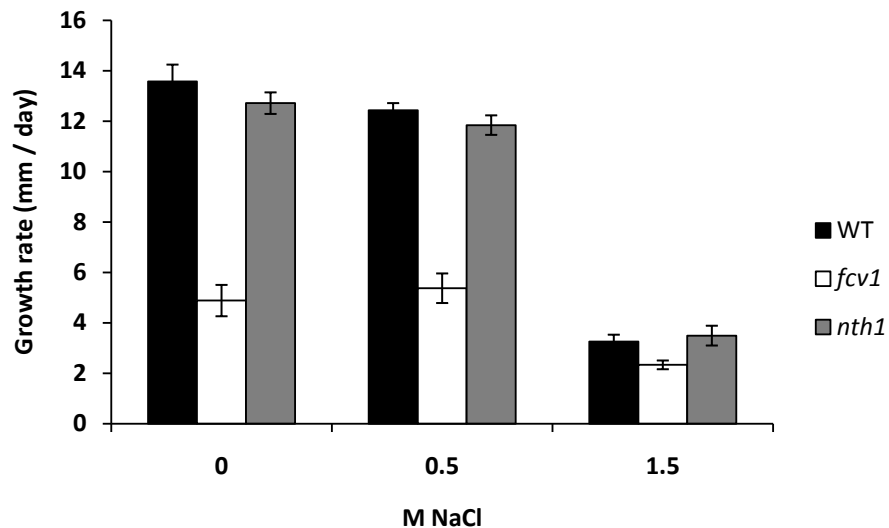
The following series of experiments used a series of different concentrations of H₂O₂, menadione and NaCl were performed to investigate the effect of oxidative and osmotic stresses of differing magnitudes on the *F. graminearum* strains. Some results varied slightly between the experiments (described below). By combining experiment datasets in the case hydrogen peroxide sensitivity, an overall analysis was made of sensitivity to this particular stress over a specific range of chemical concentrations. For clarity a summary table from all the following analyses is described in full at the end of the section.

6.3.7.1 Osmotic Stress Provided by NaCl

In the NaCl osmotic stress experiment 1 (**Figure 6.9A**), the addition of 0.5M NaCl had little effect on growth rate of the wild type strain, *fcv1* or *nth1*. However, the addition of 1.5 M NaCl caused a more dramatic reduction in the growth rate of the three strains, although this reduction is less severe for *fcv1* compared to the other two strains (by 76% for the wild type, 73% for *nth1* and 52% for *fcv1*). This can be more clearly observed in the plot depicting the growth rate on 0.5 M and 1.5 M NaCl as a percentage of the growth rate on minimal medium alone (**Figure 6.9B**). The growth of *fcv1* on 1.5 M NaCl as a

fraction of the growth on minimal medium alone was higher than for the wild type strain or *nth1*. As in the preliminary experiment, this suggested that the osmotic stress sensitivity of *nth1* to NaCl is not significantly different from the wild-type strain, while *fcv1* may show reduced sensitivity to osmotic stress.

A



B

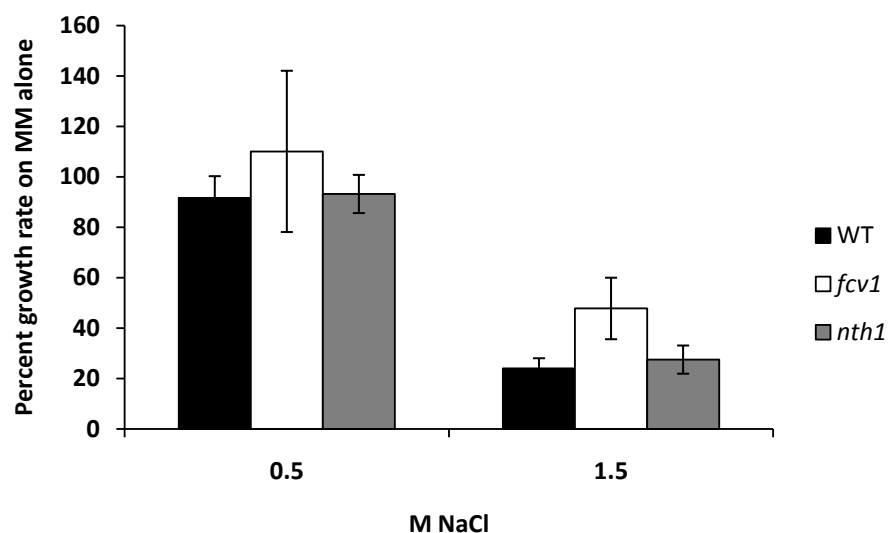
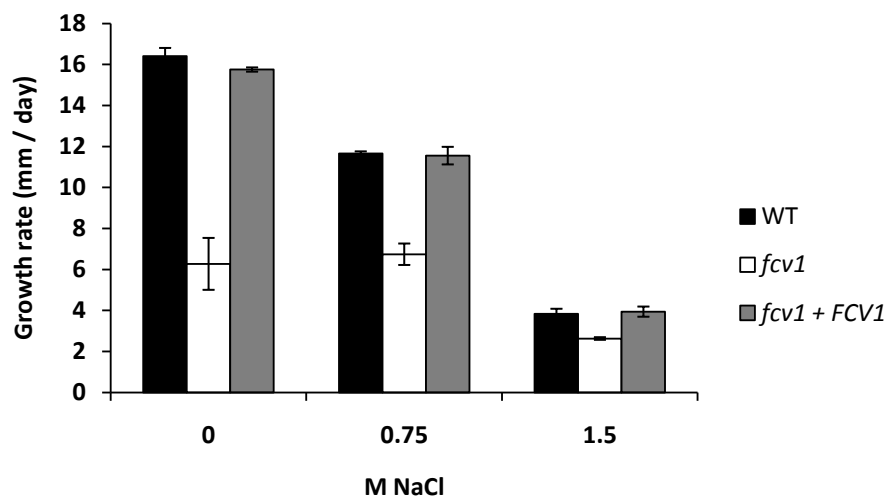


Figure 6.9. A. Means plot and **B.** Fraction of growth on minimal medium alone for osmotic stress experiment 1.

A second NaCl osmotic stress experiment was performed with the inclusion of the *fcv1* + *FCV1* strain in place of the *nth1* strain and with 0.75 M NaCl in place of 0.5 M (**Figure 6.10A and B**). The percent reduction in growth rate of all three strains on 0.75 M NaCl was similar. However, addition of 1.5 M NaCl caused a reduction in growth rate of 77% and 75% for the wild-type and *fcv1*+*FCV1* strains respectively but only 58% for *fcv1*. This experiment confirmed that the *fcv1* strain is less sensitive to osmotic stress provided by 1.5 M NaCl than the wild-type strain and that this phenotype is eliminated by the reinsertion of the wild-type *FCV1* gene in the *fcv1*+*FCV1* strain.

A



B

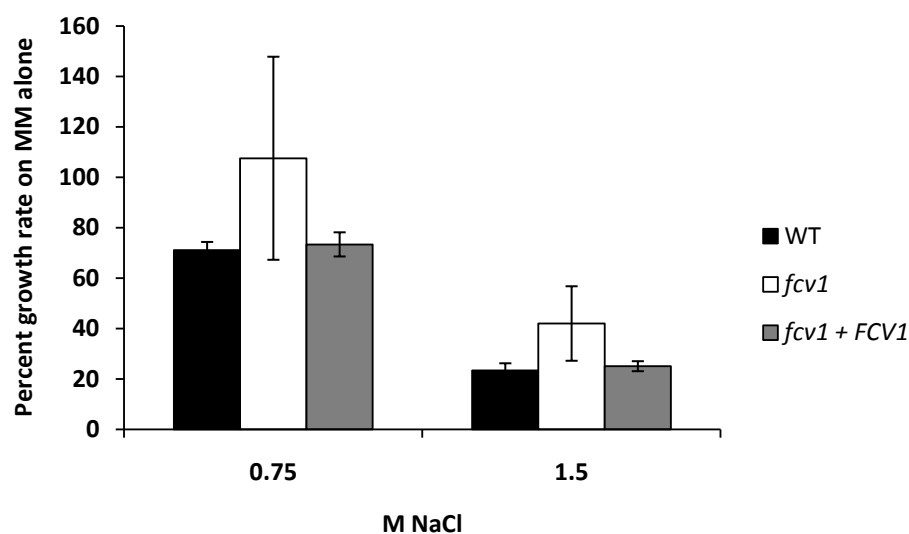
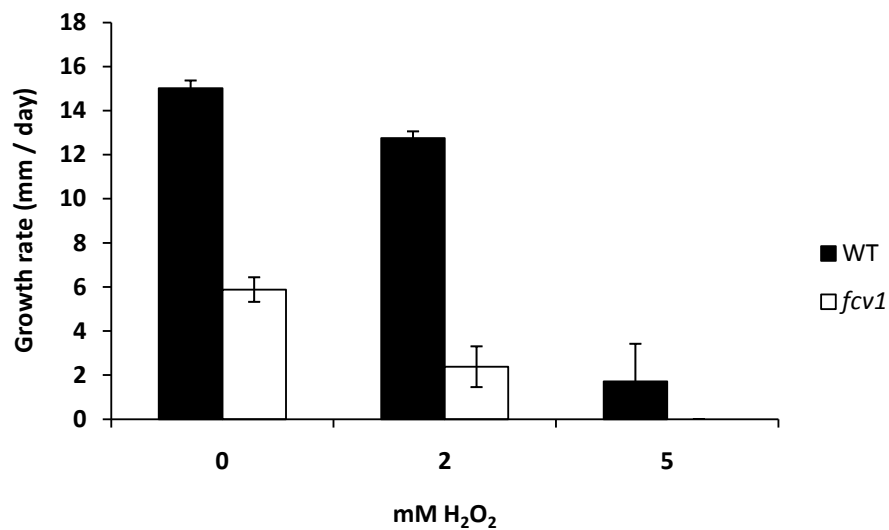


Figure 6.10. A. Means plot and **B.** Fraction of growth on minimal medium alone for osmotic stress experiment 2.

6.3.7.2 Oxidative Stress Provided by H₂O₂

In the first H₂O₂ oxidative stress experiment (**Figure 6.11A and B**), the addition of 2 mM H₂O₂ caused a significant reduction in the growth rate of *fcv1* (60%) but not of the wild-type strain, while 5 mM H₂O₂ is sufficient to prevent growth of *fcv1* but not wild-type. This suggested an increased sensitivity of *fcv1* to H₂O₂ compared to wild-type.

A



B

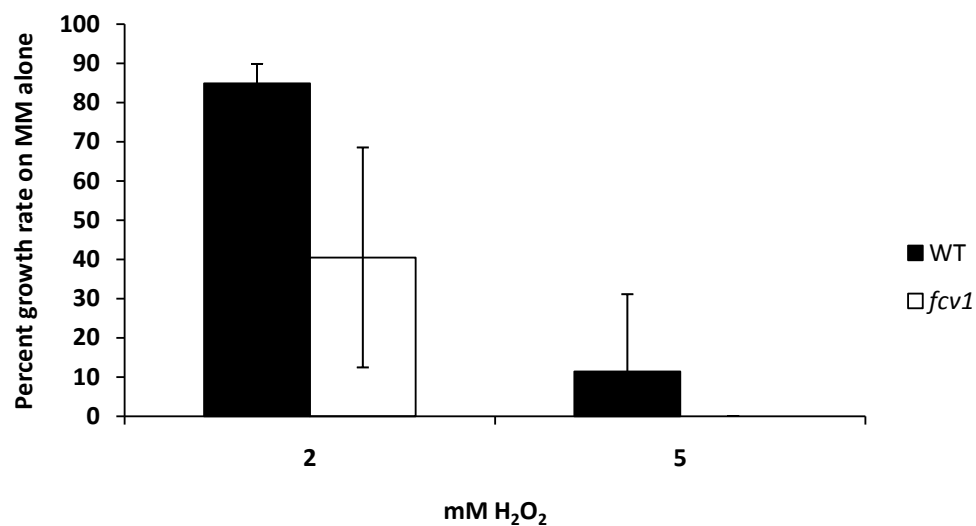


Figure 6.11. A. Means plot and **B.** Fraction of growth on minimal medium alone for H₂O₂ oxidative stress experiment 1.

However, the statistical analysis of a second experiment (**Figure 6.12**), which uses the same concentrations of H_2O_2 as experiment 1 but included the complemented strain) revealed a different outcome. There was no significant interaction of H_2O_2 addition and fungal strain, suggesting no significant differences between the response of the different strains to H_2O_2 in this particular experiment.

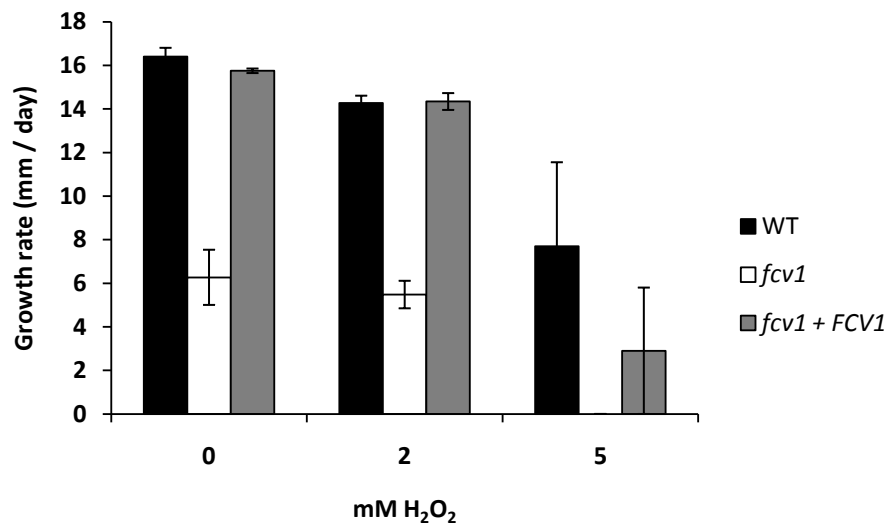
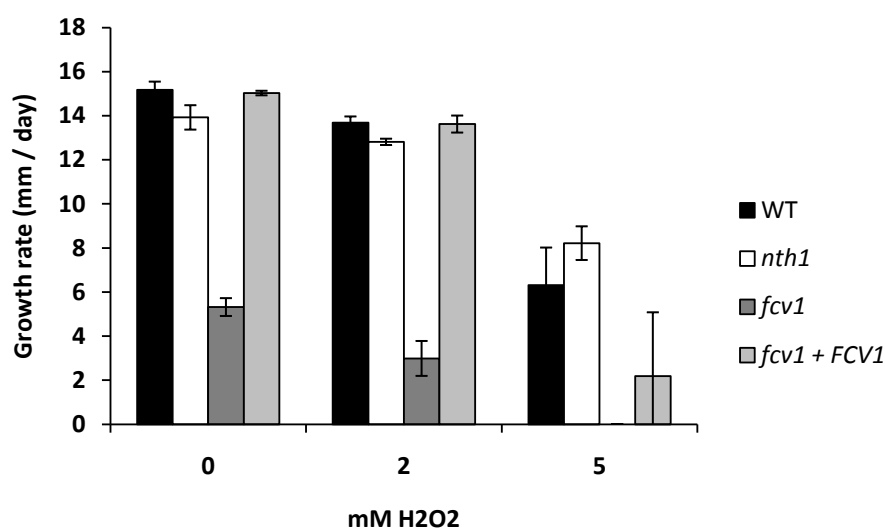


Figure 6.12. H_2O_2 oxidative stress experiment 2.

Combining datasets for H₂O₂ stress experiments using the same concentrations of reagents as above plus an experiment for *nth1*, was used to obtain an overall result for the effect of these reagents on the growth rate of the *F. graminearum* strains.

A



B

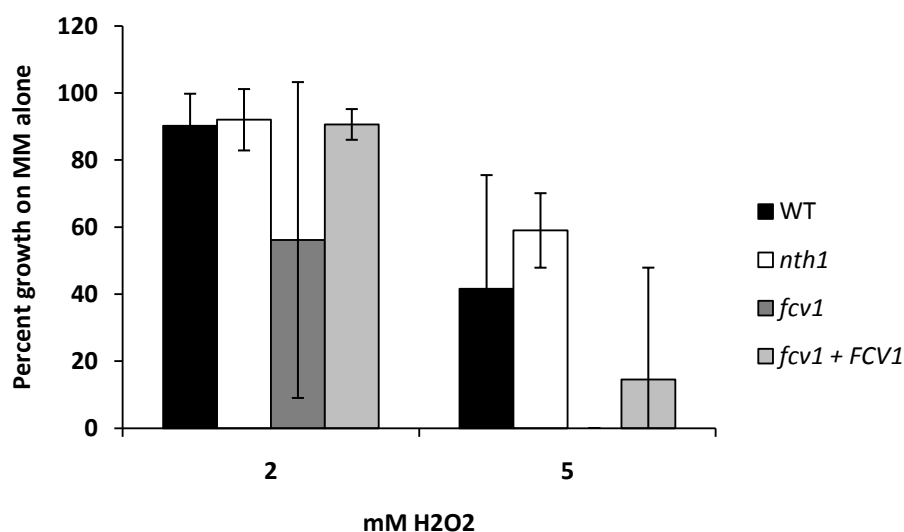


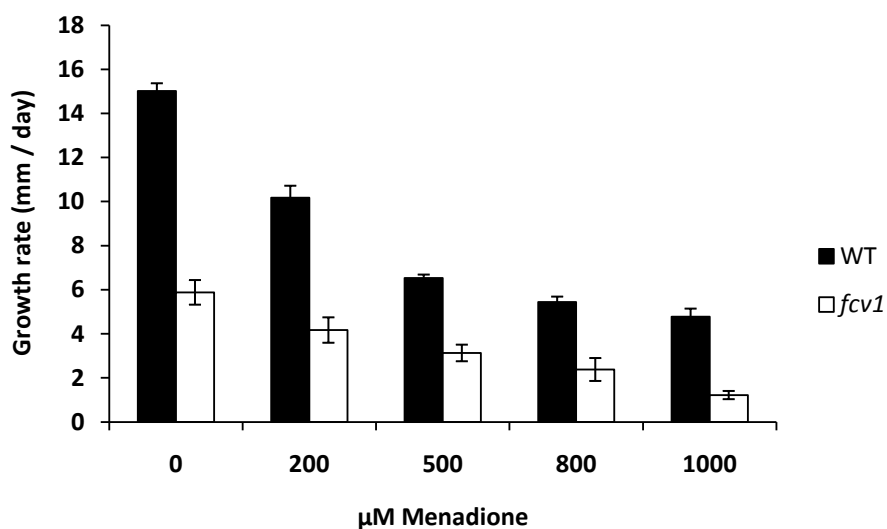
Figure 6.13. A. Means plot and **B.** Fraction of growth on minimal medium alone for the combined H₂O₂ oxidative stress analysis.

The combined H₂O₂ analysis (**Figure 6.13A and B**) indicated that the response of the different strains to H₂O₂ was significantly different ($p < 0.001$). The addition of 5 mM H₂O₂ was sufficient to stop growth of *fcv1* but not of the wild-type, *fcv1* + *FCV1* or *nth1* strains, suggesting increased H₂O₂ sensitivity for *fcv1* compared to the other strains. For these other three strains, sensitivity to 5 mM H₂O₂ appears highest for *fcv1* + *FCV1* (82% reduction in growth rate), followed by the wild-type (58% reduction) and *nth1* (41% reduction) but as a fraction of their growth rates on minimal medium alone, little difference is present between these strains (**Figure 6.13B**). At 2 mM H₂O₂, compared to minimal medium alone, the reduction in growth rate of *fcv1* (44%) is significant and much larger than that for the other strains (10%, 8% and 9% for wild-type, *nth1* and *fcv1* + *FCV1*, respectively), which is not statistically significant, however, as can be seen in **Figure 6.13B** this reduction for *fcv1* is rather variable. Therefore it appears that the *fcv1* strain may show increased sensitivity to H₂O₂ compared to the other strains over this range of concentrations. Reinsertion of the wild type *FCV1* gene however, restored this phenotype to approximately wild-type behaviour. The sensitivity of *nth1* to H₂O₂ meanwhile, appears to be comparable to the wild-type.

6.3.7.3 Oxidative Stress Provided by Menadione

In the first menadione oxidative stress experiment (**Figure 6.14A and B**), the sensitivity of the wild-type and *fcv1* strains to a range of menadione concentrations was compared. The response of both strains to menadione appeared to be similar.

A



B

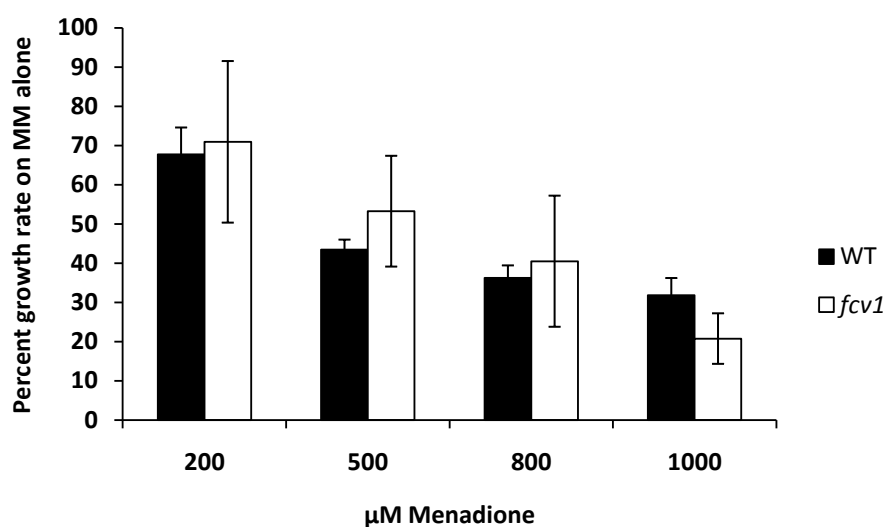
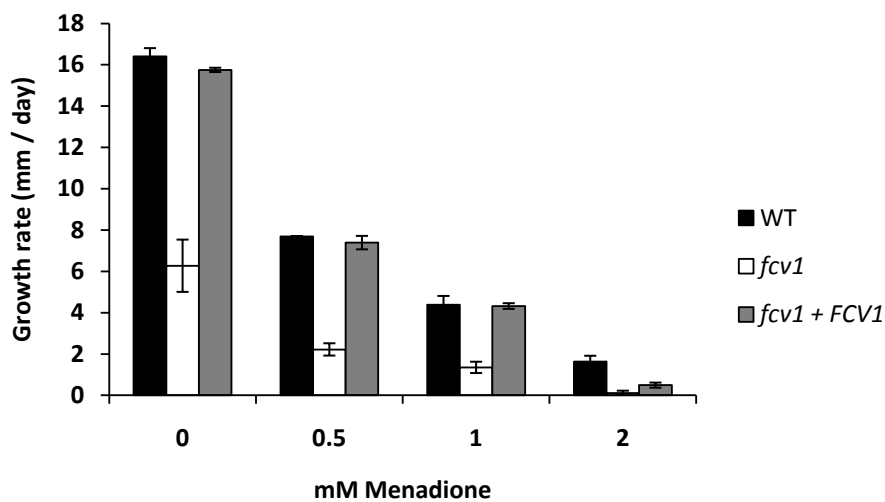


Figure 6.14. A. Means plot and B. Fraction of growth on minimal medium alone for menadione oxidative stress experiment 1.

In the second menadione experiment (**Figure 6.15A and B**), a different range of menadione concentrations were used and the *fcv1*+*FCV1* strain included. Here, the wild-type and *fcv1* + *FCV1* strains appeared to respond to menadione similarly. The *fcv1* strain exhibited a slightly larger percent decrease in growth rate upon the addition of menadione than the other strains (65% for *fcv1* compared to 53% for wild-type and *fcv1* + *FCV1* at 500 μ M menadione for example), but from the examination of growth on menadione as a fraction of growth on MM alone (**Figure 6.15B**) this does not appear to be a significant difference.

A



B

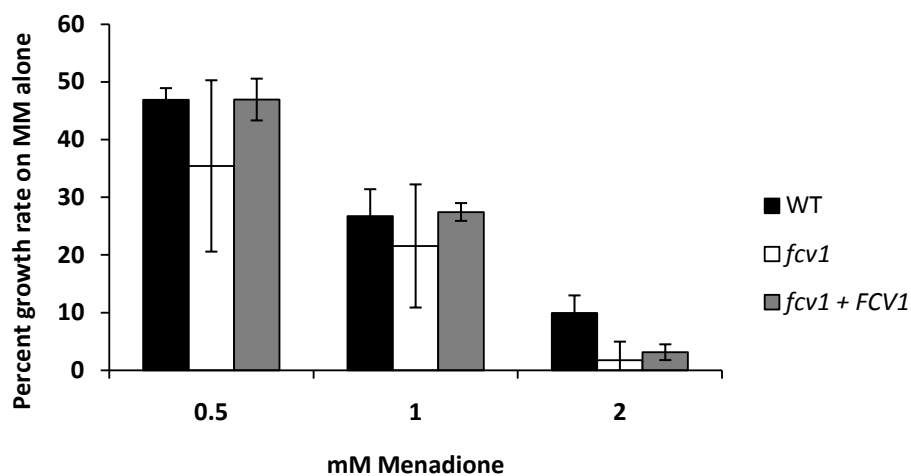


Figure 6.15. A. Means plot and **B.** Fraction of growth on minimal medium alone for menadione oxidative stress experiment 2.

Overall, these stress sensitivity tests of the *fcv1*, *fcv1 + FCV1*, *nth1* and wild-type *F. graminearum* strains have revealed the following: The *fcv1* strain appears to show reduced sensitivity to osmotic stress provided by both NaCl and glycerol compared to the other strains but increased sensitivity to oxidative stress provided by H₂O₂. The increase in sensitivity compared to the other strains to the glutathione antagonist menadione, was however only very slight for *fcv1* and not noted in all experiments. The *fcv1+FCV1* complemented strain, meanwhile, showed similar responses to the wild-type for stress provided by NaCl, H₂O₂ and menadione. Finally, the *nth1* strain, did not appear to show drastically altered sensitivity to glycerol, NaCl, H₂O₂ or menadione compared to the wild-type. The results are summarised in **Table 6.3**.

Table 6.3. Summary of stress responses of the fungal strains.

Experiment	Strain	Sensitivity compared to wild-type			
		Osmotic stress		Oxidative stress	
		<u>Glycerol</u>	<u>NaCl</u>	<u>H2O2</u>	<u>Menadione</u>
Preliminary	<i>fcv1</i>	Reduced	Reduced	Increased	Comparable/reduced
	<i>nth1</i>	Comparable	Comparable	Comparable	Comparable
NaCl 1	<i>fcv1</i>		Reduced		
	<i>nth1</i>		Comparable		
NaCl 2	<i>fcv1</i>		Reduced		
	<i>fcv1+FCV1</i>		Comparable		
H2O2 1	<i>fcv1</i>			Increased	
H2O2 2	<i>fcv1</i>			Comparable	
	<i>fcv1+FCV1</i>			Comparable	
H2O2 combined	<i>fcv1</i>			Increased	
	<i>fcv1+FCV1</i>			Comparable	
	<i>nth1</i>			Comparable	
Menadione 1	<i>fcv1</i>				Comparable/reduced
Menadione 2	<i>fcv1</i>				Comparable
	<i>fcv1+FCV1</i>				Comparable

6.3.7.4 Stress Sensitivity of the *Neurospora crassa* *fcv1* Homologue Strain

The oxidative stress sensitivity of the *N. crassa* *FCV1* homologue deletion mutant to H_2O_2 was also assayed so that oxidative stress sensitivity could be compared between the *fcv1* gene deletion strains in the two fungal species. In *N. crassa*, like in *F. graminearum*, deletion of the *fcv1* homologue also appeared to increase sensitivity to H_2O_2 . Addition of 2 mM H_2O_2 did not cause a significant change in the growth rate of the wild-type or *fcv1* strain of either mating type. However, on 5 mM H_2O_2 , the growth rate of the *N. crassa* *fcv1* strains but not the wild-type strains was significantly reduced (by 27% and 42% for the *fcv1* A and *fcv1* a strains, respectively, **Figure 6.16**).

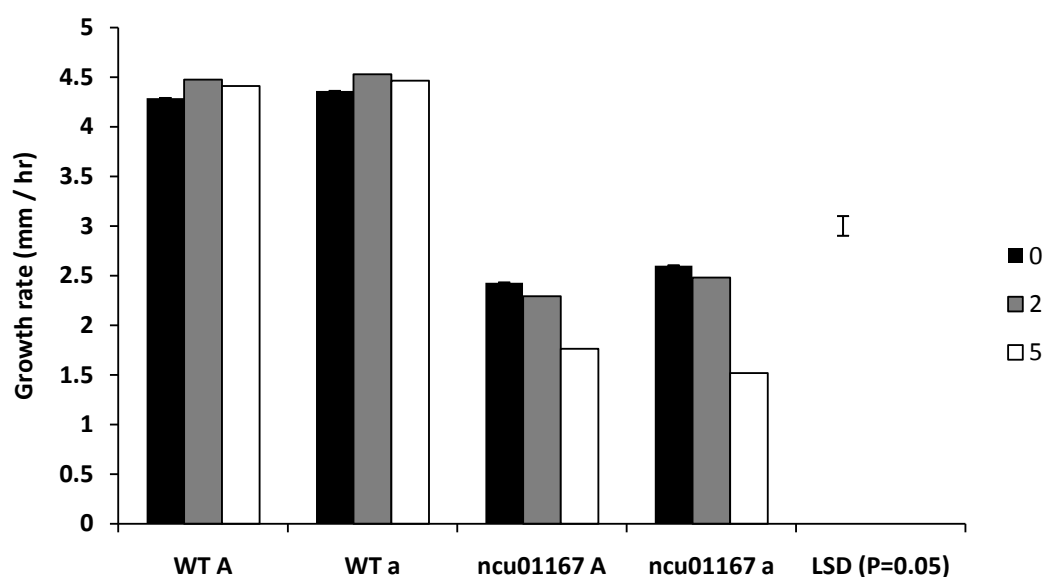


Figure 6.16. H_2O_2 oxidative stress tolerance of the *N. crassa* wild-type and *fcv1* homologue strains.

Given the apparent increased sensitivity of *F. graminearum* *fcv1* to oxidative stress, the disease progression of this strain compared to the wild-type on wounded wheat ears was somewhat unexpected. Upon wounding, plants produce an oxidative burst response at the wound site (Agrios, 1997). This response may serve to exaggerate the virulence defect of *fcv1* by combining its inherent virulence defect with a further increased reduction of growth rate compared to the wild-type due to its increased oxidative stress sensitivity. However, when inoculated onto wounded wheat ears, the spread of symptoms resulting from *fcv1* and wild-type strains was not noticeably different from intact ears.

6.4 Discussion

In this chapter the detailed biology of the *fcv1*, *nth1* and *pkar* mutants was explored in further *in planta* and *in vitro* experiments to determine the wider implications of deletion of these genes in *F. graminearum*. These experiments indicated a requirement for *fcv1* in disease progression in Arabidopsis floral tissue, of *fcv1* and *pkar* in the production of asexual spores and *pkar* in DON mycotoxin production. They also revealed the opposing roles of *fcv1* in osmotic and oxidative stress tolerance and conservation of the oxidative stress tolerance function in *N. crassa*.

6.4.1 PKAR

The *F. graminearum* *pkar* mutant was able to produce perithecia. This is in contrast to reports from several other fungal species in which the presence or proper regulation of PKA signalling has been shown to be required for sexual development. The *B. cinerea* *pkaR* mutant shows reduced production of sclerotia compared to the wild type strain (Schumacher *et al.*, 2008). The *C. neoformans* var. *grubii* *pka1* (PKAC) mutant is sterile (D'Souza *et al.*, 2001), while the *Yarrowia lipolytica* *tpk1* (the sole PKAC-encoding gene of this species) disruption mutant cannot mate (Cervantes-Chavez *et al.*, 2009). In contrast, the *Verticillium dahlia* *pkac1* disruption mutant has greater microsclerotia production than the wild type strain (Tzima *et al.*, 2010).

Production of macroconidia, the asexual spore type of *F. graminearum* abundantly produced *in vitro* was significantly reduced in the *pkar* mutant compared to the wild type strain. A role for PKAR proteins in the production of conidia has been noted in a number of species. The *rpk1* mutant of *C. lagenarium* and *pkaR1* deletion mutant of *M. circinelloides* both show reduced conidiation (Takano *et al.*, 2001; Ocampo *et al.*, 2009), while in *Aspergillus niger*, disruption of *PKAR* causes a loss of sporulation (Staudohar *et al.*, 2002). The *M. graminicola* *bcy1* mutant is unable to produce pycnidia (Mehrabi and Kema, 2006). *S. cerevisiae* diploid *BCY1* disruption (*PKAR*) homozygotes were also unable to sporulate (Temple *et al.*, 2005). In *A. fumigatus*, deletion of *PKAR* or overexpression of *PKAC1* (both of which would be expected to result

in increased PKA activity) results in reduced sporulation (Zhao, W. *et al.*, 2006; Grosse *et al.*, 2008). The *B. cinerea* *pkaR* mutant though, shows only slightly reduced conidiation (Schumacher *et al.*, 2008). These results suggest that uncontrolled PKAC activity frequently leads to an inhibition of sexual and /or asexual sporulation in fungi. This would appear to be supported by results from *A. nidulans* where *PKAA* (the primary PKAC) negatively controls conidiation (Fillinger *et al.*, 2002). In addition, while deletion of *PKAB* (the secondary PKAC) causes no apparent changes in spore production in this species (Ni *et al.*, 2005), overexpression of *PKAA* or *PKAB* reduces sporulation (Shimizu and Keller, 2001; Ni *et al.*, 2005). Mutation of *N. crassa* *PKAC-1* (the major PKAC) leads to premature conidiation on solid medium and inappropriate conidiation in liquid medium (Banno *et al.*, 2005).

However, reduced PKA activity resulting from disruption/deletion of the catalytic subunit of PKA (PKAC) has also been shown to cause reduced conidiation for *B. cinerea* (*pka1* but not *pka2*), *Fusarium verticillioides* (*fpk1*), *Verticillium dahlia* (*pkac1*) and *A. fumigatus* (*pkaC1*) (Liebmann *et al.*, 2004; Schumacher *et al.*, 2008; Pei-Bao *et al.*, 2010; Tzima *et al.*, 2010). In addition, the *M. graminicola* *tpk2* mutant is unable to produce pycnidia. The effect of PKAC activity levels on sporulation therefore appears to depend on the species under study and in the case of some species, such as *A. fumigatus*, where both deletion and overexpression of PKAC lead to reduced conidiation, tight control of PKAC activity within a specific range appears important in maintaining wild-type levels of conidia production.

The *pkar* strain also appeared to show reduced production of the DON mycotoxin *in planta*. Analysis of the diseased portion of the ear showed a significantly reduced level of DON for the *pkar* strain compared to the wild type and PKAR-e strain. Whilst the possibility cannot be eliminated that reduced fungal biomass of the *pkar* strain in each floret due to a slower growth rate is responsible for the differences in DON level recorded, the *fcv1* strain, which also shows a slower growth rate compared to the wild type strain produces DON levels comparable to the wild type strain *in planta*. Despite this, per spikelet exhibiting disease symptoms, there was less DON in the *pkar* strain infected ear compared to the wild type. Interestingly, the G protein subunits GPA1 and GPB1 appear to negatively regulate toxin production in *F.*

graminearum (Yu et al., 2008), and may act upstream of PKAC in *F. graminearum*, which also appears to positively regulate toxin production as this is reduced in the *pkar* strain.

Other examples of toxin production regulation by PKA signalling have been described in fungi. In *A. nidulans*, *PKAA* (PKAC) negatively controls production of the xanthone mycotoxin sterigmatocystin (ST). Overexpression of *PKAA* negatively regulates *AFLR*, the transcription factor controlling production of ST (Shimizu and Keller, 2001). In *M. anisopliae*, however, PKAC is not required for expression of toxin-producing genes (Fang et al., 2009). In *C. neoformans* var. *grubii*, *pka1* mutants do not produce the virulence factors melanin or capsule, while *pkr1* mutants overproduce capsule (D'Souza et al., 2001) and in *A. fumigatus*, *PKAC1* deletion reduces expression of the polyketide synthase pathogenicity determinant *PKSP* (Liebmann et al., 2004).

6.4.2 FCV1

The results of further experiments described in this chapter indicate that *FCV1* is required for the full rate of symptom development by *F. graminearum* on *Arabidopsis* in addition to wheat ears. The defect of the *fcv1* strain is therefore not limited to disease progression on wheat floral tissue, implying a role for *FCV1* in contributing to symptom spread in the infection of both monocotyledonous and dicotyledonous host species.

FCV1 also appears to be involved in the regulation of conidiogenesis, with the production of fewer conidia in the *fcv1* strain than the wild type, but has minimal effect on conidial germination efficiency, and is not required for the production of perithecia or the discharge of viable ascospores. The *fcv1* strain did not exhibit a reduction in the production of the DON mycotoxin in wheat ears compared to the wild-type strain. However, as this strain also shows a slower growth rate compared to the wild type strain it is possible that the fungal biomass per infected spikelet is lower for *fcv1* than the wild-type. If shown, this would in turn indicate that the *fcv1* strain shows an elevated DON per unit biomass production compared to the wild-type. Clarification of this matter will require further experimentation.

Interestingly, the *fcv1* strain appeared to be more tolerant of osmotic stress than the wild type or complemented strains. The *fcv1* strain was less sensitive to a reduction in water activity by glycerol or NaCl addition than the wild-type strain. The reason for this increase in osmotic stress tolerance is unknown but could result from altered cell wall structure or transmembrane transport in the *fcv1* strain compared to the wild-type. The increased tolerance to osmotic stress would be expected to aid *fcv1* proliferation in the drying tissues of the infected wheat ear, but these appear to be masked by the other defects of this strain. Interestingly, *F. graminearum gpmk1* colonies, which show a similar growth morphology to *fcv1*, do not exhibit sensitivity to high osmolarity (Jenczmionka *et al.*, 2003).

The loss of *FCV1* led to an increase in sensitivity to oxidative stress provided by H₂O₂ but not by menadione. Inoculation of wounded ears with *fcv1* did not lead to a further reduction in virulence compared to intact ears suggesting either that the oxidative burst *in planta* as a response to wounding did not significantly affect *fcv1* disease progression or its effect was masked by an easier pathogen entry to the wounded host. It is known that under high humidity conditions the oxidative burst and the wound response is often somewhat attenuated. Post-inoculation the wheat ears are placed under high humidity conditions for 4 days. Plants also produce oxidative bursts in response to pathogen attack (Agrios, 1997). If the wheat plant is producing an oxidative burst response as a consequence of infection by the pathogen then any additional burst due to wounding may only be slight. It is also possible that the *Fusarium* hyphae themselves are producing chemicals such as H₂O₂ to help attack the host, and may use a system of self-protection *in planta* that is not operational *in vitro*. Finally, the oxidative burst may actually aid symptom spread of this pathogen by causing programmed death of host cells.

The osmotic signalling pathway of *F. graminearum* is involved in the response to both osmotic and oxidative stress. Disruption of genes of the *HOG1* pathway led to increased osmotic stress sensitivity (Ramamoorthy *et al.*, 2007, Ochiai *et al.*, 2007). The *os2* (*hog1*), *os4* (MAPKKK) and *os5* (MAPKK) strains showed some increase in hydrogen peroxide and t-butyl hydroperoxide (t-BOOH) sensitivity compared to the wild-type strain. However, *os1* (histidine kinase) showed reduced and *os4* increased diamide sensitivity compared to the wild-

type (Ochiai *et al.*, 2007). The analogous signalling pathways of *S. cerevisiae* and *A. nidulans* are activated in response to both osmotic and oxidative stress (reviewed in Duran *et al.*, 2010). It is possible that targeted deletion of *FCV1* alters stress sensitivity in *F. graminearum* via the osmotic signalling cascade but a link remains to be determined. The nonribosomal peptide synthetase *NPS6*, which is involved in siderophore-mediated iron metabolism, also regulates oxidative stress sensitivity in *F. graminearum*. The *nps6* mutant shows increased sensitivity to both H₂O₂ and the superoxide radical-generating agent KO₂ and is required for full virulence (Oide *et al.*, 2006).

In *N. crassa*, *OS-2* (*Hog1*) has been implicated in the regulation of catalases, genes encoding enzymes for glycerol synthesis and gluconeogenesis and the clock-controlled gene *ccg-1* (Noguchi *et al.*, 2007; Watanabe *et al.*, 2007; Yamashita *et al.*, 2007). *OS-2* or *OS-5* mutation increases sensitivity to NaCl and sorbitol but reduces sensitivity to iprodione, in addition to *OS-2* mutation leading to increased sensitivity to 0.1 mM t-BOOH (Banno *et al.*, 2007). The *os-1*, *os-2*, *os-4* and *os-5* mutants are hypersensitive to osmotic stress yet show increased resistance to iprodione and fludioxonil (Grindle and Temple, 1982; Fujimura *et al.*, 2000a and b; Zhang *et al.*, 2002). The *os-4* and *os-5* mutants appear to represent changes to the *SSK22* and *PBS2* genes respectively (Fujimura *et al.*, 2003). In *Cochliobolus heterostrophus*, *hog1* mutants show increased pigmentation, smaller appressoria and reduced virulence (Igbaria *et al.*, 2008). In mutants of the *Cryphonectria parasitica* *CPMK1* (the homologue of *HOG1*), pigmentation is reduced, sensitivity to osmotic stress is increased, and conidiation and virulence are reduced (Park *et al.*, 2004). *Magnaporthe oryzae* *HOG1* homologue *osm1* mutants show increased osmotic stress sensitivity and morphological defects but not alteration in virulence (Dixon *et al.*, 1999). The hypersensitivity to osmotic stress noted in these mutants suggests possible opposing roles of *FCV1* and the *Os* cascade in osmosensitivity.

Knockout mutants of the *SSKA* and *SRRA* osmotic signalling response regulators of *A. nidulans* (homologous to the yeast *SSK1* and *SKN7* respectively) show increased sensitivity to osmotic stress, hydrogen peroxide and t-BOOH (hydroxyl radical producers) but interestingly not to menadione or diamide (produce the superoxide and free-thiol radicals respectively) (Hagiwara *et al.*, 2007; Vargas-Perez *et al.*, 2007), although *sskA* has also been reported

to be insensitive to hydrogen peroxide (Vargas-Perez *et al.*, 2007). The *F. graminearum fcv1* strain, similarly appeared to have an increase in sensitivity to H₂O₂ but not to menadione, and contrastingly had reduced osmotic sensitivity. This suggests differing responses to the different radical species in these strains.

The osmosensing system has also been linked to morphology, sporulation and germination in other fungal species. For example, mutation of *MA21*, the *A. fumigatus* homologue of the yeast *SHO* osmosensing sensor kinase reduces growth and leads to altered hyphal morphology with shorter hyperbranching filaments and reduced conidiation (Ma *et al.*, 2008). Deletion of the MAPKK *STEC* in *A. nidulans*, meanwhile, results in slower growth increased branching and altered conidiophore morphology (Wei *et al.*, 2003). The stress-activated kinase *SPC1* of *S. pombe* also alters cell size at division (Millar *et al.*, 1995; Shiozaki and Russell, 1995).

The *F. graminearum os2*, *os4* and *os5* mutants also exhibit reduced trichothecene production (Ochiai *et al.*, 2007). By contrast, the production of DON by the *fcv1* strain *in planta* was comparable to wild-type.

6.4.3 *NTH1*

In contrast to *FCV1*, *NTH1* only shows a reduction in symptom spread rate on wheat ears and not in disease progression on Arabidopsis floral tissue. This could reflect a differing requirement for trehalose metabolism in *F. graminearum* during infection of monocotyledonous and dicotyledonous hosts. However, the symptom spread defect of *nth1* on wheat ears is subtle and may not be easily observed on Arabidopsis floral tissue due to the different host morphologies and disease development stages on each species.

In *F. graminearum*, loss of *NTH1* did not appear to increase sensitivity to osmotic or oxidative stress. In yeast, *NTH1* is induced in response to heat, osmotic and oxidative stress, while deletion of *NTH1* leads to reduced thermotolerance (Nwaka *et al.*, 1995a, 1995b; Zähringer *et al.*, 1997, 2000). In *B. cinerea*, disruption of the trehalase-6-phosphate synthase *TPS1* reduces heat tolerance while neutral trehalase (*TRE1*) disruption slightly increases heat

tolerance, although neither gene appears to be important in sensitivity to oxidative or osmotic stress (Doehlemann *et al.*, 2006). In *C. albicans* and *Stagonospora nodorum*, the *tps1* strain shows reduced tolerance to oxidative stress (Alvarez-Peral *et al.*, 2002; Lowe *et al.*, 2009). In *Cryptococcus neoformans*, the trehalose-6-phosphate synthase *TPS1* and trehalose-6-phosphate phosphatase *TPS2* are required for high temperature growth on YEPD medium containing glucose, although this phenotype can be alleviated by the presence of galactose or sorbitol (Petzold *et al.*, 2006). In *C. gattii* serotype B, a similar phenotype is noted for *tps1* and *tps2* strains although only the growth of the *tps2* strain is recovered by galactose or sorbitol (Ngamskulrungrroj *et al.*, 2009). In *C. neoformans*, *tps1* but not *tps2* or *nth1* showed an increased sensitivity to oxidative and osmotic stress (Petzold *et al.*, 2006). In addition, the disruption of *NTH1* in *Leptosphaeria maculans* does not affect growth under stress conditions, although *nth1* expression increases in *L. maculans* in response to stress from hydrogen peroxide or hygromycin (Idnurm *et al.*, 2003; Petzold *et al.*, 2006). Similarly, *NTH1* expression in *M. oryzae* increases in response to osmotic stress (Foster *et al.*, 2003). Trehalose synthesis as opposed to degradation therefore appears to be of primary importance in growth under stress conditions, with the possible exception of thermotolerance. That *NTH1* does not appear to be required for growth under most stress conditions, yet exhibits elevated expression under such conditions, suggest perhaps that *NTH1* may play a more important role in recovery from stress, as noted in yeast (De Virgilio *et al.*, 1994) and that its expression under stress conditions is made in preparation for subsequent stress recovery. With the *nth1* strain of *F. graminearum* showing no altered sensitivity to oxidative or osmotic stress, a role in stress recovery rather than tolerance may be present in this species as well. The second trehalase described in Chapter 4 may play a more significant role during stress conditions.

The *F. graminearum nth1* deletion strain also did not exhibit a reduction in conidia or perithecia production. In *M. oryzae*, *tps1* exhibited poor sporulation (Foster *et al.*, 2003), while in *S. nodorum*, heat stress leads to reduced germination of the *tps1* strain (Lowe *et al.*, 2009). It therefore appears that the degradation of trehalose by *Nth1* is not required for the production of conidiospores by *F. graminearum*. There remains the possibility that the ability

to synthesise trehalose will impact on sporulation as noted in *M. oryzae* and *S. nodorum*.

F. graminearum nth1 is also unaffected in the ability to produce the trichothecene mycotoxin deoxynivalenol (DON). The production of other virulence-associated small molecules has also been investigated in trehalose catabolism/metabolism mutants of *Cryptococcus* species. Formation of the polysaccharide capsule and production of the pigment melanin is unaffected in *tps1*, *tps2* and *nth1* strains of *C. neoformans* (Petzold *et al.*, 2006), yet in *C. gattii*, *tps1* and *tps2* show defective melanin production and capsule formation (Ngamskulrungrroj *et al.*, 2009). Successful trehalose degradation does not therefore appear to influence DON production by *F. graminearum in planta* under the conditions tested.

6.4.4 Analysing the Sexual Reproduction Potential of All the Single-Gene Deletion Strains Generated

The five additional gene deletion strains analysed for perithecia production, namely *fgsg_09891*, *fgsg_09893*, *fgsg_09900*, *fgsg_09905* and *fgsg_09906* were all competent to produce perithecia containing ascospores. Interestingly, in an earlier study using transposon-tagging of *F. graminearum* strain *Fg820*, insertion of the *mimp1* transposable element 127 bp 5' of the *FGSG_09905* ORF halted perithecia production at an early stage, leading to the production of only initial structures that did not develop into mature perithecia (Dufresne *et al.*, 2008) This is in contrast to the results found with deletion of the *FGSG_09905* gene in strain PH-1 in this study. This difference could be due to the different strains of *F. graminearum* used in the two studies or because the effect on perithecia production resulting from transposon insertion was not due to prevention of *FGSG_09905* expression. Alternatively, transposon insertion could have lead to altered expression of *FGSG_09905* or other genes

Deletion of *GET3* (the homologue of *FGSG_09891*) in yeast led to up-regulation of sporulation and stress-associated genes but not to sporulation defects, although *get1* and *get2* strains showed defective sexual spore production which could be suppressed by *GET3* deletion (Auld *et al.*, 2006). *Get3* may therefore

function in a negative manner in protein sorting for degradation and in regulation of stress and sporulation-associated genes. *F. graminearum* *fgsg_09891* is able to produce perithecia carrying ascospores, suggesting the *F. graminearum* gene, unlike *Get3*, may not play a role in sporulation.

While the PH-1 *icl1* mutant described here retains the ability to produce perithecia and ascospores, the previously published Z03643 *ICL1* mutant (Lee *et al.*, 2009a) was able to form very few perithecia. This difference in the role of *ICL1* in sexual reproduction of *F. graminearum* is likely to be due to differences between the strains used in the two studies rather than to experimental differences. Interestingly, the Z03643 study observed a rapid reduction in the expression of *ICL1* under perithecia-inducing conditions. That the *ICL1* gene appears to be inactivated during perithecia formation is intriguing given its apparent role in sexual reproduction in strain Z03643. As discussed in the publication, Z03643 *ICL1* expression is apparent in the vegetative growth phase on carrot agar prior to perithecia formation induction and its activity may be important in the production of precursor molecules important in perithecia formation. Such molecules however, would appear to be either not required or produced by a different route in PH-1.

It is interesting to note that quite dramatic differences in phenotype can be found when the same gene is disrupted in two varieties of the same fungal species. In *C. neoformans* var. *grubii* (serotype A), *PKR1* disruption leads to overproduction of capsule and hypervirulence (D'Souza *et al.*, 2001), whereas in serotype D, *PKR1* does not appear to be important for virulence (Hicks *et al.*, 2004). Disruption of *PKA1* causes loss of mating ability, melanin production and pathogenicity in serotype A but not serotype D. *PKA2*, meanwhile, is involved in virulence, mating and haploid fruiting in serotype D but not in serotype A (D'Souza *et al.*, 2001; Hicks *et al.*, 2004).

Factors affecting *F. graminearum* stress tolerance appear to be numerous and their relationships complex. We have successfully added *FCV1* to this group of genes, however, determining the precise links between this gene and others in stress signalling will require further investigation.

Chapter 7. General Discussion

7.1 The Constant Fight Against Plant Pathogens

The production of global food supplies is now under intense pressure to match the rapidly increasing world population. With advances in healthcare providing longer life expectancy and higher survival rates, attention must now also turn to providing sufficient nutritious food for the resulting larger body of humanity. In addition, global natural disasters, such as flooding, most recently seen in Pakistan and earthquakes, such as that in Haiti can result in severe food shortages in many countries and especially in rural regions. Also agricultural land is submerged under water or transport infrastructures are damaged meaning produce cannot be delivered to market or is destroyed post-harvest. Severe heat, such as experiences in Russia in 2010, can lead to massive crop failure and then the introduction of an extended period when no grain is exported to feed other parts of the world.

Through breeding schemes, or, more controversially by genetic engineering, food crop traits can be selected for or altered to provide increased yield, tolerance of environmental factors such as drought, salinity and heat, or resistance to pests and disease. Despite the advances provided by such efforts, plant pathogens and the diseases they cause remain a heavy constraint on obtainable yield. Application of chemicals to crops is commonly used in an attempt to control disease; however, the emergence of resistance to such substances and tightening legislation on permissible substances provides a constant battle to create ever new chemistries to alleviate the disease problem. In addition, pathogens can evolve to overcome resistance bred or engineered into crops and entirely new diseases can emerge from species previously unknown to cause such conditions (Hollomon and Brent, 2009). Studies of the *F. graminearum* clade alone have indicated that a large number of distinct species are now able to be identified (O'Donnell et al., 2004; Starkey et al., 2007). New species have also recently been identified, for example, in Ethiopia (O'Donnell et al., 2008) and Russia (Yli-Mattila et al., 2009). In addition there are also reports of selection driving the spread of more toxigenic isolates across

North America (Ward et al., 2008). The development of new disease control measures is therefore paramount.

7.2 A Bioinformatics-Led Approach to Virulence-Associated Gene Discovery

A large proportion of plant diseases are caused by fungi and as such, the provision of new fungicides is of great importance in the battle against resistance. New targets for such fungicides must be sought, however traditional large scale forward genetics-based approaches to screening gene function in virulence using restriction enzyme mediated integration (REMI), *Agrobacterium tumefaciens*-mediated transformation (ATMT) or transposon-tagging, which can be used to provide large collections of mutants for the investigation of gene function, are laborious and time consuming with low rates of success, typically 0.5%.

Targeted approaches may also be used, in some cases in a *ku70 / ku80* genetic background that prevents non-homologous end joining and so improves gene targeting (Ninomiya et al., 2004; Villalba et al., 2008). RNA silencing meanwhile, allows the analysis of essential genes and the reduction of gene expression by differing degrees (Xu, 2000). However, such reverse genetics approaches to date have tended to focus on genes encoding proteins of particular classes, such as protein kinases. Apart from a few labour-intensive genome-scale deletion programmes, for example in *N. crassa* (Neurospora Genome Project), these methods are rarely used to characterise gene classes with no previous known role in virulence, or those lacking annotation or homology to other characterised genes. This may primarily be due to the large proportion of such genes in fungal genomes (Xu, 2000), rendering a reverse-genetics approach unfeasible unless some level of pre-filtering is applied to these genes to highlight more promising targets and remove those less likely to play a role in virulence.

This project has used a form of 'positive pre-filtering' to highlighting small genomic regions that may be expected to show an increased probability of

containing genes contributing to virulence. By locating homologues of known virulence-associated genes in the genome of a sequenced pathogen, the distribution of such genes was analysed to locate hotspots where statistically significant clustering of such genes occur. It was hypothesised that, if such homologues were proven to contribute to virulence of a chosen species, these virulence-contributing hotspots may then be likely to contain further virulence-associated genes that may not have initially represented obvious choices for targeted deletion as they lack annotation or belong to classes not previously associated with virulence.

The study described here applies this approach to the filamentous ascomycete *Fusarium graminearum*, a primary causal agent of Fusarium ear blight, a devastating disease of cereals. However, such an approach is applicable to any sequenced pathogen and is not limited to fungi or to plant hosts. The PHI-base database was used as a source of published virulence gene data with which to search the *F. graminearum* genome for homologous gene sequences. By displaying the results with the OmnimapFree genome visualisation software, a micro-region was identified on chromosome I that appeared to show a close grouping of virulence gene homologues. This clustering was confirmed by a Chi-square based statistical analysis (Chapter 3) indicating that the frequency of virulence genes in this locus was distinct from and significantly higher than the rest of the chromosome.

The micro-region contained five virulence gene homologues within a fifteen gene region spanning just 37.6kb. Two genes, the MAP kinase kinase-encoding *STE7* and serine/threonine kinase-encoding *SNF1* had previously been reported to contribute to the rate of FEB symptom spread (Ramamoorthy et al., 2007; Lee et al., 2009b; Beacham et al., 2010 in preparation). Targeted deletion of the remaining three homologues, revealed that two, the genes encoding a neutral trehalase, *NTH1*, and protein kinase A regulatory subunit, *PKAR*, were required for a full rate of FEB symptom spread on wheat ears, although to greatly differing extents. Loss of the *PKAR* gene restricted symptoms to the inoculated spikelets and adjacent rachis only with no further symptom spread over time. Loss of *NTH1*, however, caused only a slight reduction in rate of symptom spread compared to the wild-type progenitor. The fifth homologue,

encoding isocitrate lyase, *ICL1*, was dispensable for wild type disease progression. This indicated that the clustered virulence gene homologues did indeed contribute to the rate of *F. graminearum* spread in wheat in all but one case.

Six additional genes at the locus were selected for targeted deletion with the aim of locating novel classes of virulence determinants. One gene, *FGSG_09907*, which encodes a protein showing similarity to spliceosome components (Bcas2/Spf27/Cwf7) and to a protein involved in Arabidopsis defence signalling regulation (Mos4), was also found to contribute to disease development. This gene was named *Fusarium graminearum Contributor to Virulence 1 (FCV1)*. Targeted deletion of this gene reduced the rate of disease symptom development on both wheat ears and Arabidopsis floral tissue. In addition, this strain exhibited reduced production and germination efficiency of asexual spores and altered stress sensitivity. No gene sequence of this type appears to have been previously linked to disease symptom development in fungi or indeed any other pathogenic species. The investigation of this region therefore yielded a new class of gene required for a full rate of symptom development of a pathogenic fungus and indicated the possibility of using such a genome landscape-reverse genetics technique to locate such genes in a much more efficient manner than through forward genetics screens.

7.3 What is a Pathogenicity/Virulence Factor?

The search for genes that can potentially be targeted to reduce the virulence of a pathogen raises an interesting point of debate: what properties of a gene, or rather its protein product, constitute description as a pathogenicity/virulence factor? A pathogenicity/virulence factor is most often regarded as one whose contribution to the pathogen is solely at the point of invasion of or proliferation within a host species and which is not required for saprophytic or *in vitro* growth or reproduction. This stringent definition is academically useful in describing the precise role of different genes within the pathogen, however very few genes have so far been characterised that match these criteria. Bona fide pathogenicity/virulence factors are restricted primarily to toxin biosynthesis

enzymes and regulators, and to secreted effectors. Such examples can be found in Proctor *et al.*, 1995; Hohn *et al.*, 1997; Kamper *et al.*, 2006; Dean, 2007 and Howlett *et al.*, 2007. *In vitro* conditions can be used that best approximate the host nutrient environment, for example growth on media derived from host material or containing a mixture of compounds that aims to accurately reflect the types and concentrations thought to be present during infection. However, such *in vitro* conditions are unlikely to fully reflect those experienced by the pathogen during infection. As such, even for factors considered to represent bona fide pathogenicity/virulence factors, an inherent growth defect may be masked due to the conditions selected. The host nutrient environment may not easily be replicated *in vitro*, and so any inherent defects will likely prove hard to determine, making definition of any gene or protein as a pathogenicity/virulence factor subject to some debate. At the time of writing, forty seven *F. graminearum* genes have been published through peer review with a pathogenicity or virulence function. Of these, very few have been recorded as giving a wild type phenotype in all other biological tests and so could be regarded as bona fide virulence factors.

From a viewpoint of the practical application of this science to disease combat in the field, other genes that *contribute* to the virulence of a pathogen, whether via a role participating in growth, reproduction or other processes, are equally as important as potential targets for intervention even if not matching the description of 'classical' pathogenicity/virulence factors. One may then expect that a far higher proportion of the gene complement of a pathogen would be expected to contribute to virulence in some way, yet this appears not to be the case. A large number of genes apparently play no role in virulence, their deletion or mutation leaving a species able to infect in a manner comparable to the wild-type progenitor strain (www.phi-base.org).

In the case of *NTH1*, the slight growth defect on complete media that results from the targeted deletion of this gene is comparable to the magnitude of the disease progression defect on wheat ears. This suggests that the slightly reduced rate of disease development of the *nth1* strain derives from its growth rate defect. Such a gene may therefore not be regarded as a *bona fide*

virulence gene. For the *pkar* and *fcv1* strains, the defect in the rate of FEB symptom spread *in planta* appears to be slightly greater than that on complete media *in vitro*. This has been shown to a much greater extent for the *snf1* and *ste7* strains (M. Urban, pers. comm.; Lee *et al.*, 2009b; Ramamoorthy *et al.*, 2007). This suggests that a large proportion of the decrease in FEB symptom spread rate in *pkar* and *fcv1* is due to an inherent growth rate defect. It is possible that an additional, purely *in planta*, defect is affecting these strains within the wheat ear that cannot be attributed to their growth rate defects but further investigation may be required to confirm these additional factors. If verified, these genes could then be regarded as contributing to *in planta*-specific processes, and in which case could be considered as virulence genes.

An additional useful comparison would be to compare the infection of the wild-type and single-gene deletion strains at the same 'biological' rather than 'chronological' time points. For the wheat ear infection assays, disease extent was recorded every four days after inoculation for a twenty day period. By considering the differing *in vitro* growth rates of the strains, recording of disease extent could instead be made when each strain is expected to have grown a comparable amount. This method of disease analysis may provide further information on the relative contributions of inherent and *in planta*-specific defects to the reduced rate of symptom spread seen in the *nth1*, *pkar* and *fcv1* strains.

7.4 The Role of *FCV1*

Due to its homology to the *BCAS2/SPF27* spliceosome components it is possible to suggest a role for *FCV1* in pre-mRNA splicing in *F. graminearum*. A comparison of transcript size of selected genes in the wild-type and *fcv1* strains may indicate size differences suggesting a lack of or alternative splicing in the *fcv1* strain. However, if *FCV1* participates in splicing of pre-mRNA from only a subset of genes then choosing the appropriate genes to detect a difference in the above experiment may prove especially time-consuming. An RNA sequencing approach or genome-wide array able to differentially detect spliced

and unspliced mRNA of all *F. graminearum* genes may prove a very useful tool in this aspect.

It is interesting to note the apparent role of *MOS4* in the regulation of Arabidopsis immunity. It is currently unknown if *FCV1* plays a role in self-defence of *F. graminearum*. *mos4-1* plants show a delayed flowering time and a reduced number of seeds per silique compared to the wild type Col-0 (Palma *et al.*, 2007). In comparison, the *fcv1* strain shows a reduced growth and infection rate and a decreased level of conidiation. This suggests that both *FCV1* and *MOS4* are important for growth and development. In a new Rothamsted Research based PhD project to start in October 2010, the *mos4* mutant will be tested for its affect on the outcome of the Fusarium-Arabidopsis floral interaction.

Despite apparently participating in a core cellular process, Cwf7 (an Spf27 homologue) is not an essential protein in *S. pombe*, nor is Mos4 in Arabidopsis. While the function of Fcv1 of *F. graminearum* is not known, the protein is clearly not essential for survival of this species. The Mos4 and Bcas2/Spf27 proteins are contained within large multi-protein complexes (Neubauer *et al.*, 1998; McDonald *et al.*, 1999; Tsai *et al.*, 1999, Ajuh *et al.*, 2000; Ohi *et al.*, 2002; Chan *et al.*, 2003; Monaghan *et al.*, 2009). Functional redundancy in such complexes may be the cause of such a result, as mutation of *MOS4* together with other complex proteins is lethal (Monaghan *et al.*, 2009). This further suggests that the entire complex is required for an essential process such as splicing (Monaghan *et al.*, 2009). In human cells lacking p53, or containing a mutant p53, depletion of Bcas2 caused cell cycle arrest at the G₂/M checkpoint, while in wild type cells this depletion causes apoptosis (Kuo *et al.*, 2009). The loss of *FCV1* in *F. graminearum* slows the rate of growth and alters colony morphology. This could possibly reflect a reduced rate of cell cycle progression in the *fcv1* strain or an increase in the occurrence of cell death.

It is also possible, however, that Fcv1 contributes to *F. graminearum* growth, virulence, sporulation and stress sensitivity not via a role in splicing but by some other means, for which further characterisation of *FCV1* will be required. Tools that may assist in helping to elucidate the function of *FCV1* in *F. graminearum*

include His-tagged Fcv1 or a yeast-2-hybrid screen to identify interaction partners, which could include spliceosome components, growth or conidiation regulatory factors, proteins of the stress sensitivity regulatory systems, such as those of the Hog1 pathway, or other factors.

The production of GFP, mCherry or dsRed-tagged Fcv1 could be used to investigate subcellular localisation and protein movement, which could be performed in real time for *in vitro* cultures of *F. graminearum*. In addition, a promoter-GFP or promoter-GUS fusion strain to observe patterns of expression of the *FCV1* gene under different conditions *in vitro* and *in planta* could be particularly informative. Further analysis of the Fcv1 sequence may reveal post-translational protein modification sites that could be subject to site-directed mutagenesis to determine their role in Fcv1 activity or localisation. Coupling mutagenesis with structural studies may help to reveal amino acid residues and structural features important for Fcv1 activity or interaction with other proteins.

7.5 Comparison of the *fcv1* Strain with its *Neurospora crassa* Counterpart

The availability of a large range of *N. crassa* single-gene deletion strains at the Fungal Genetics Stock Center due to the efforts of the *N. crassa* community provided the opportunity to compare the growth and stress sensitivity of the *F. graminearum* *fcv1* strain and that of the *N. crassa* strains harbouring a deletion of the closest *FCV1* homologue, *NCU01167*. This useful comparison revealed that in these species, deletion of *FCV1* or its homologue led to a reduction of *in vitro* growth rate and increased sensitivity to oxidative stress provided by hydrogen peroxide. This revealed the importance of *FCV1* and *NCU01167* in growth and stress tolerance in both pathogenic and non-pathogenic filamentous fungi. *FCV1* could possibly also contribute to additional factors that in the pathogenic species led to a slightly increased defect in the rate of spread *in planta* compared to complete medium *in vitro*. Further investigation of the response of *N. crassa* strains to osmotic stress and menadione would provide further clues about the conservation of *FCV1* and *NCU01167* roles in these species.

7.6 The Morphological Defects of the *pkar* Strain

The *F. graminearum* *pkar* deletion strain exhibited hyphae containing a series of short, almost cuboidal cells. The hyphae of all filamentous fungi species are separated into a series of compartments by the presence of septa. Such septa each contain a small pore to allow transfer of signals or material between compartments. This phenotype observed in the *pkar* mutant could represent an increase in or alteration in the regulation of septation in hyphae. A broadly similar phenotype has been recorded in a temperature-sensitive mutant of the *N. crassa* PKAR-encoding gene *MCB* when grown below the restrictive temperature (Bruno et al., 2006, Chapter 4). This indicates a possible role for PKAR in the septation process in filamentous fungi

Septum formation is coordinated with mitosis and requires formation of a contractile actin ring (CAR) at the septum site (Balasubramanian et al., 2004), a process which is tightly coupled to septal wall material deposition (Momany and Hamer, 1997). The CAR may act to guide the recruitment of septum formation factors (Si et al., 2010).

A number of genes have been shown to have a role in septum formation in filamentous fungi. The majority of studies of this process have focussed on the species *Aspergillus nidulans*. In *A. nidulans*, a septation initiation network (SIN), together with both septins and a formin have been shown to be required for septum formation (discussed in Harris 2001). The SIN is comprised of the small GTPase-activated SepH-SepL-SidB protein kinase cascade, together with the SepM and MobA cofactors that regulate SepL and SidB, respectively (Kim et al., 2006; Krapp and Simanis, 2008; Kim et al., 2009). The SIN is required for CAR assembly (Bruno et al., 2001; Kim et al., 2006). Components that act up- and downstream of the SIN are, however, yet to be identified (Si et al., 2010). The septin AspB and formin SepA require SepH for localisation to the septation site (Sharpless and Harris 2002; Westfall and Momany, 2002). SepA but not AspB is required for CAR formation (Sharpless and Harris 2002; Westfall and Momany, 2002). The AspA and AspC septins are required for normal germ tube and branch emergence in *A. nidulans* and may interact with each other to

control new growth in *A. nidulans* (Lindsey *et al.*, 2010). Deletion of *ASPA* or *ASPC* also reduced septation and conidiation (Lindsey *et al.*, 2010).

The SIN first appears at the spindle pole body (SPB) (Simanis, 2003). The coiled-coil protein Snad, which is located at the SPB also participates in septation and is required for conidiation (Liu and Morris, 2000). Snad, together with SepK is required for SidB and MobA localisation to the SPB but not to the septation site (Kim *et al.*, 2009). SPB localisation of the SIN appears to be required for timely septation in *A. nidulans* (Kim *et al.*, 2009). Class I and II chitin synthases have also been shown to be involved in septum formation in *A. nidulans*. In the *chsA:chsC* double mutant, septa were unusually thick with a large pore and some were located abnormally close to each other (Ichinomiya *et al.*, 2005). In *U. maydis* all eight chitin synthases localise to septa, while the class IV chitin synthases *chs7* and *chs5* are important in maintaining correct yeast cell and hyphal shape and make a large contribution to virulence (Weber *et al.*, 2006). Dynein may play a role in septum positioning in *A. nidulans* (Liu *et al.*, 2003). Deletion of the *A. nidulans* putative cell end marker gene *TEAC* caused hyphal growth in a zig-zag pattern (Higashitsuji *et al.*, 2009). The *teaC* strain also showed an increased number of septa and branches compared to the wild-type and the presence of some anucleate compartments. This was thought to be due to the reduced growth rate of this strain and not specific for the *teaC* strain. However, *TEAC* overexpression resulted in a repressed septation. TeaC was also reported to interact with SepA at hyphal tips and septa. The most recently identified septum formation factor in this species is *Bud3*, a homologue of the *S. cerevisiae* axial bud site marker (Si *et al.*, 2010). *Bud3* is required for septum and CAR formation but not for polarised growth per se and appears to act as a guanine nucleotide exchange factor (GEF) for the GTPase Rho4 downstream of the SIN to recruit additional factors required for the formation of the septum. Rho4 is required for actin ring formation and septation in *N. crassa* (Rasmussen and Glass 2005).

In the related species *A. fumigatus*, the α -glucosidase 1 gene *CWH41* is required for normal conidia production. The deletion strain of this gene is reduced in conidiation and septation and shows altered growth polarity (Zhang *et al.*, 2008). In addition, the *msdS* 1,2- α -mannosidase gene deletion strain also

shows reduced conidiation and reduced/random septum formation (Li *et al.*, 2008).

Further investigation is required to determine if the deletion of *PKAR* in *F. graminearum* leads to altered regulation of septation. It is currently unknown if septum formation is still coordinated with mitosis in the *pkar* strain. Alternatively, as suggested for the *A. nidulans teaC* strain, the hyperseptate appearance may in fact be due to a reduced hyphal elongation rate that causes septa to be positioned closer together in hyphae. That the *pkar* strain exhibits a drastically reduced growth rate compared to the wild-type on both complete and minimal medium means this is a strong possibility for this strain. To further characterise the *pkar* strain, microscopic investigation to determine the presence of anucleate compartments or altered cell wall composition will help to reveal more about the morphological defects of this strain. Comparison of the *pkar* strain with adenylate cyclase and PKA catalytic subunit over-expression and deletion strains will also prove very useful.

7.7 A Novel Type of Fungal Gene Cluster

As discussed in chapter 3, the micro-region identified on chromosome I of *F. graminearum*, and investigated in this study, appears to represent a novel type of fungal gene cluster. The genes of the micro-region do not exhibit co-ordinated regulation or encode enzymes for the production of a particular secondary metabolite, as found, for example, with the *TRI* trichothecene mycotoxin biosynthetic gene cluster (Proctor *et al.*, 1995; Hohn *et al.*, 1999). The proteins encoded by the genes of the micro-region are not predicted to be secreted, as found for clusters identified in the genome of *U. maydis* (Kamper *et al.*, 2006; Dean, 2007; Howlett *et al.*, 2007) nor are these genes located on supernumerary chromosomes like the *PEP* cluster of *F. solani* (Han *et al.*, 2001).

Unlike bacterial pathogenicity islands and the *PEP* cluster (Hacker *et al.*, 1997; Han *et al.*, 2001; Hentschel and Hacker, 2001; Liu *et al.*, 2003), the micro-region does not possess a GC content distinct from the rest of the *F. graminearum* genome nor is it rich in repetitive sequence. Recent characterisation of genomic islands in the genome of the animal pathogen

Aspergillus fumigatus has revealed that these islands, unlike the *F. graminearum* micro-region presented in this study are enriched for transposons, pseudogenes and repetitive elements (Fedorova *et al.*, 2008). The genomic islands of *A. fumigatus* are now thought not to have arisen by horizontal transfer, but instead by a process of gene duplication, diversification and differential gene loss (Fedorova *et al.*, 2008). Clustered in such islands are species-specific genes, which are smaller and contain fewer exons than core genes. These genes are predominantly found at subtelomeric locations (Nierman *et al.*, 2005; Fedorova *et al.*, 2008) as was noted for species-specific and *in planta*-specific genes and SNPs in *F. graminearum* (Cuomo *et al.*, 2007). Interestingly, by contrast and unlike the *in planta*-specific genes proposed to contribute to *F. graminearum* virulence (Cuomo *et al.*, 2007), the genes of the micro-region of this study are located in a site that is neither subtelomeric nor is SNP-rich. In fact, many of the verified virulence contributors in *F. graminearum* are found in low recombination frequency regions of the genome and are not subtelomeric (**Figure 7.1**).

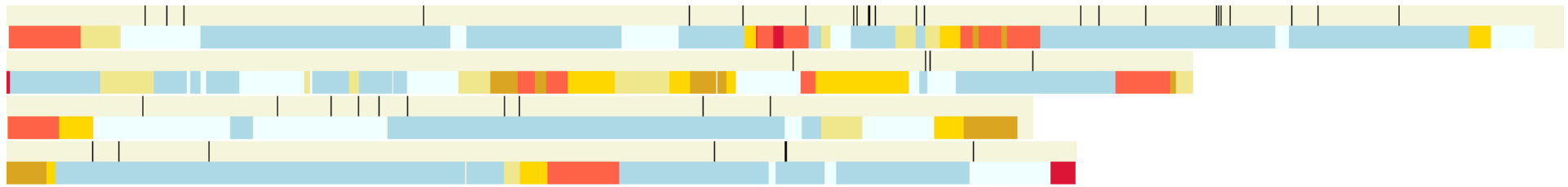


Figure 7.1 Distribution of verified virulence contributors in *F. graminearum* (black vertical bars) compared to recombination frequency (coloured blue to red, low to high) on the four chromosomes (horizontal beige bars).

7.8 The Use of Comparative Genomics

The increasing availability of genome sequences for plant pathogenic fungi, such as *M. oryzae* and *F. graminearum*, allows these organisms to be investigated in detail and comparisons to be made between different plant pathogens. Comparative genomics is allowing greater understanding of the host-pathogen interaction through analysis of conserved genes, gene arrangement and the distribution of features such as repetitive sequences and single nucleotide polymorphisms (SNPs).

By comparing genomic features such as the predicted gene complement of different species, insights can be made into their modes of pathogenicity. For example, by comparing pathogenic and non-pathogenic species, genes potentially important for virulence can be highlighted. For example, 145 *F. graminearum* genes have homologues in the pathogen *M. oryzae* but not in the non-pathogenic saprophytes *N. crassa* and *A. nidulans* (Xu, 2006). However, for most *F. graminearum* genes that have been shown to contribute to virulence, it appears that homologues are found in all 3 of the above species, so simple comparisons of gene content may not reveal all pathogenicity/virulence factors. (Xu, 2006). Regulatory differences of such genes may be important in conferring the different lifestyles of these species and so gene expression datasets can prove highly useful in such cases. Recent work by Lavoie and others has highlighted the flexibility of transcriptional regulatory networks in ascomycetes and has shown that changes in transcriptional control in regulons controlling a wide range of processes such as carbohydrate and lipid metabolism and the production of components of the ribosome is common (Lavoie *et al.*, 2009). This may help to explain why the micro-region described in this study, which is highly important for virulence in *F. graminearum* is well-conserved in the non-pathogenic fungus *T. reesei* as well as the pathogens *F. verticillioides*, *F. oxysporum* and *F. solani*. In another example, it has been found that the yeast *S. cerevisiae* contains all the genes required for filamentous growth in *Ashbya gossypii*, such that gene non-functionalisation or differences in gene expression must explain the different growth behaviour of the two species (Philippson *et al.*, 2005, Xu, 2006).

Expanded gene families can reveal cell functions that are important to the disease-causing ability of a particular pathogen. For example, the genome sequence of *F. graminearum* contains a larger number of genes predicted to encode proteins belonging to virulence-related protein families than non-pathogenic fungi, including predicted hydrolytic enzymes and transmembrane transporters (Cuomo et al., 2007). This may appear surprising as this species possesses a system known as repeat-induced point mutation (RIP) (Selker et al., 1987) which introduces mutation in duplicated sequences during the sexual cycle (Watters, et al., 1999; Cuomo et al., 2007). This may suggest that such gene families arise by means other than gene duplication. The *M. oryzae* genome contains over 700 predicted secreted proteins, a much greater number than for *N. crassa* or *A. nidulans* (Dean et al., 2005). RIP may also contribute to the very low percentage of repetitive sequence in the *F. graminearum* genome compared to other species (Selker et al., 1987; Cuomo et al., 2007).

Comparison of the genomes of the four currently sequenced *Fusarium* species has already revealed interesting insights into their differing host ranges and modes of pathogenicity (Ma et al., 2010). The genome of *F. oxysporum* f sp. *lycopersici* (*Fol*) is significantly larger than that of *F. verticillioides* (*Fv*) and *F. graminearum* (*Fg*) (by about 44% and 65%, respectively) and contains a greater number of predicted protein-encoding genes. This additional sequence in *Fol*, referred to as 'lineage-specific' (LS) regions, is present predominantly on extra chromosomes and is distinct from the 'core' conserved genome sequence. These LS regions account for nearly all of the extra genomic sequence in *Fol* compared to the other two species. They are highly enriched for repetitive sequence (28%), transposable elements and many genes predicted to encode secreted effectors, virulence factors, transcription factors, signalling proteins and secreted enzymes known to be up-regulated during infection of the tomato host but contain few house-keeping genes. Around half of the genes in *Fol* LS regions appear to be specific to this species and are not found in *Fg* or *Fv*. Such regions appear to have been acquired by horizontal transfer from other *Fusarium* species and possess distinct codon usage compared to other genomic regions. The *Fol* LS regions differ considerably in sequence among *Fo* strains with different host specificities and are absent in non-pathogenic strains.

It has been demonstrated that transfer of LS chromosomes can convert a non-pathogenic *Fo* strain into a pathogenic one (Ma et al., 2010). *Fol* LS regions therefore appear to be key to host specificity and the acquirement of pathogenicity in *F. oxysporum*. The *Fol* LS regions but not the 'core' genomic regions are also absent in *F. solani* (*Fs*). However, *Fs* itself possesses three LS chromosomes distinct from the genomes of the other three sequenced *Fusarium* species. Dispensable chromosomes that confer host-specific virulence have already been reported in this species (Han et al., 2001). In addition, small dispensable (supernumerary) chromosomes in *Alternaria alternata* contain genes that are involved in the biosynthesis of the AM, AF and AAL-toxins in apple, strawberry and tomato pathotypes (Akamatsu et al., 1999; Johnson et al., 2000, 2001; Hattta et al., 2002; Harimoto et al., 2007; Akagi et al., 2009) and which may be able to transfer horizontally (Akagi et al., 2009).

Dispensable chromosomes appear not to be present in *F. graminearum*, meaning that all genes important for causing disease must be located on the four core, and indeed only, chromosomes. The micro-region described here is located on chromosome I in *F. graminearum* but is also found in *F. oxysporum* f sp. *lycopersici* and is located on chromosome VII, one of the core chromosomes of this species. This suggests that if the micro-region functions in contributing to *F. oxysporum* virulence, as may be expected in such a closely related species, it does so in a host non-specific manner and such genes would maintain a 'core' role in virulence-associated processes that are common to all host species.

Further investigation of the four sequenced *Fusarium* species has allowed the identification of conserved motifs in promoter, intron and downstream regions of genes (Kumar et al., 2010). By comparison to the genomes of *S. cerevisiae* and *Schizosaccharomyces pombe*, many motifs were able to be assigned to specific cellular processes or transcription factor binding sites. In *F. graminearum*, a genome-wide analysis of transcription in a deletion strain of the trichothecene biosynthetic cluster transcription factor *TRI6* has revealed that the *TRI6* regulon encompasses not only the trichothecene biosynthesis *TRI* cluster but also genes that form part of the upstream isoprenoid biosynthesis pathway and a large number of additional genes (Seong et al., 2009). For example, within the

micro-region the upstream 1 kb of the *STE7* gene contains two *TRI6* binding site sequences (data not shown).

Comparisons between pathogens with different modes of infection, for example biotrophs versus necrotrophs or those that produce specialised infection structures versus those that do not, could help to highlight different genes required for disease formation in pathogens of different infection types. Fungi of different host types such as animal and plant pathogens or dicotyledonous and monocotyledonous phytopathogens can also be compared and contrasted. Examples include Rispaill *et al.* (2009), who investigated the conservation of the MAP kinase and calcium-calmodulin signalling pathways in human and plant-pathogenic fungi, finding that most but not all *S. cerevisiae* pathway components appear to be widely conserved in a range of fungal pathogen species.

The secreted enzymes of saprophytic and phytopathogenic fungi can also provide clues as to their major sources of nutrition, for example growth on decaying wood versus cereal leaves or ears. In principle, the effector and avirulence (*avr*) gene complement of different strains of a pathogenic species can help reveal insights into host choice and specificity, however, the identification of *avr* genes in the genomes of fungi is difficult because of their lack of conserved features. For *F. graminearum* infecting wheat and barley, no differential host genotype responses to different fungal isolates have so far been noted by either plant breeders or the researcher community. Therefore, currently, the gene-for-gene hypothesis developed by Flor in the 1940s (Flor, 1942, 1947), which was subsequently been used for much pathogen *avr* and host *R* gene discovery, appears not to apply to these *Fusarium* – cereal species interactions.

Comparison of genomes reveals which regions are highly conserved between species, and which are more diverse. This may be related to the recombination frequency of these different regions of the genome and can help to elucidate evolutionary relationships between species and highlight the occurrence of horizontal gene transfer. For example, Richards *et al.* (2009) compared the genomes of plants with a large number of prokaryotic and eukaryotic species

and found evidence for horizontal gene transfer events between plants and fungi and between plants and oomycetes. When orthologous pairs of genes in *M. oryzae*, *F. graminearum*, and *N. crassa* were analysed, small syntenic regions of 3 to 20 genes were identified. *F. graminearum*, based on the MIPS analysis, contains 359, 258 and 86 regions with four or more genes colinear between itself and *N. crassa*, *M. oryzae*, and *A. nidulans*, respectively (Xu, 2006). The degree of microsynteny appeared highest between *F. graminearum* and *N. crassa*. However, only the quinate/shikimate (Qa) metabolic pathway gene cluster exhibits more than seven conserved colinear genes. While some chromosomal fragments appear conserved, no clear relationship was identified between the chromosomes of the different species (Xu, 2006).

The *F. graminearum* micro-region is present in a region of low recombination in this species which may be expected to help in maintenance of the region as a whole entity. However, in more distantly-related species the region begins to break down into small syntenic units of only two to three genes. This may indicate a higher level of recombination in these species compared to *F. graminearum* or a sufficiently large evolutionary distance for breakdown to occur even with an inherently low level of recombination. In addition, the low level of recombination of this region in *F. graminearum* may help to maintain the clustering of this important group of genes, which may prove to be important in their functioning in disease-related or other cellular processes.

Genome comparisons can also be used to help improve gene prediction. A comparison of the nucleotide sequence identity of predicted *F. oxysporum*, *F. graminearum* and *F. verticillioides* orthologues indicated 91% nucleotide sequence identity between *F. oxysporum* and *F. verticillioides*, both of which show 85% identity with their *F. graminearum* orthologues. Such conserved genes numbered over 9,000 and were enriched for predicted transcription factors, lytic enzymes, and transmembrane transporters (Ma et al., 2010). In this study, the homologues of *F. graminearum* genes found in the other *Fusarium* species showed a high degree of sequence conservation to the corresponding *F. graminearum* gene. The comparative genomics analysis also helped to indicate possible discrepancies in the gene calls of the different species' genomes, whereby genes called in one species were absent in others

despite a high degree of similarity at the nucleotide sequence level. This likely indicates either non-functionalisation of the gene in one or more species such that it is not called or differences in the selectivity of the algorithms used for gene identification in the different genomes.

However, comparative genomics faces difficulties, such as may be encountered when the genomes of sequenced fungal pathogens contain numerous genes that do not show either any homologues at all or any annotated homologues in other organisms to help predict gene function. Approximately 30% of the annotated genes of *F. graminearum* and *M. oryzae* have no known homologues in other organisms (Xu, 2006). A similar situation is found with *N. crassa* and *A. nidulans*. The approach used in this study can also be applied to highlight genes of this type that may be important in disease progression.

By combining comparative genomics with transcriptomics to study gene expression under different conditions and proteomic approaches to examine, for example, the phosphoproteome and indicate the signalling state of the organism, these tools can become very powerful and reveal a wealth of data about different organism species, the relationships between such species and the way in which they complete their different modes of life. Proteomics has also been suggested as an important tool for genome annotation, in a manner similar to the mapping of ESTs or entire cDNA sequences onto the genomic sequence (Wright et al., 2009). This approach has been used for *Aspergillus niger*, where tandem mass spectrometry was used to sequence several hundred peptides separated by electrophoresis. The sequences obtained were then compared to the sequences of predicted genes in the *A. niger* genome sequence and in this way a large number of peptides could be mapped to different predicted gene loci (Wright et al., 2009). Such an approach could help to determine the success of gene calls in related *Fusarium* species that appear to lack some of the genes of the micro-region. Whole-genome microarrays are also available for species such as *F. graminearum* and *M. oryzae* to study differential expression patterns. Such microarrays have proved useful in studying transcript accumulation in different nutrient conditions and during plant infection (Güldener et al., 2006). These studies revealed the lack of co-ordinate

gene expression in the micro-region *in planta*, which contrasts with that of the *TRI* trichothecene biosynthesis gene cluster.

There are now an even greater number of fungal genome sequences available for use in comparative genomics. Sequences now available, but not yet published, include three species of the saprobe *Trichoderma*, *Fusarium circinatum*, a pathogen of pine trees, *F. pseudograminearum*, an important cereal pathogen and cause of crown rot, *F. culmorum*, a causal agent of ear blight in Europe and other areas and three species of the grass endophyte *Epichlöe*. These resources will allow an even greater in-depth analysis of the conservation and micro-synteny of this and any additional micro-regions identified in fungi across a larger number of species than possible at the start of this project. This will help to further reveal the evolutionary origins and functions of such regions.

7.9 Next-Generation Sequencing

The advent of next generation sequencing technologies is opening up potential new methodologies for the investigation of fungi and potentially can expand massively the possibilities of genomic and transcriptomic approaches. Using the latest technology, cDNA, genomic or even direct RNA sequencing (Ozsolak et al., 2009) can be performed on a larger scale than ever before. Sequencing the genomes of many closely-related fungal species or strains of the same species will help elucidate the evolutionary relationships between them and genomic differences that confer phenotypic variability. Also, biological time-course experiments can be explored in depth and will most probably reveal greater insights than the currently available microarray based datasets. Likewise, with fully sequenced fungal genomes now available, the *in planta* interaction can be explored, and the data de-convoluted to explore the pathogen and host responses in tandem.

Le Crom et al (2009) used a next-generation approach to perform the sequencing of different strains of the industrially important fungus *Trichoderma reesei*. This species is commonly used for the large scale production of

cellulases and hemicellulases (Bouws et al., 2008; Kumar et al., 2008; Stricker et al., 2008). Mutagenesis approaches have been used to increase the enzyme yield of this species (Reese, 1976). By sequencing and comparing the original *T. reesei* isolate and modern enzyme production strains, the study was able to identify the series of mutations in the strains that provide an increased yield of cellulases. The number of mutations present in the cellular hyper-producing strains was surprisingly high. Many of the detected changes were present in genes involved in nuclear transport, mRNA stability, transcription and other processes and affected the carbon assimilation patterns of the different strains. For pathogenic fungi such as *F. graminearum*, mutations that may confer fungicide resistance or increased virulence can also be screened for in this manner, allowing rapid detection of genetic changes that can lead to increasingly virulent strains. The approach is being applied in combination with the generation of mapping populations between resistant and sensitive strains, to pin-point the genomic region and subsequently the exact genetic change conferring the altered phenotype (M. Csukai, Syngenta, Jealott's Hill, UK, unpublished).

7.10 Distribution and Origins of the *F. graminearum* Micro-Region

In this study, conservation of the micro-region was compared in a number of different species. Four *Fusarium* species: *F. graminearum*, *F. verticillioides*, *F. oxysporum* and *F. solani* have so far been sequenced and published. The degree of conservation of the micro-region was extremely high across these four closely related pathogenic species, with only *F. solani* exhibiting a difference from *F. graminearum* by more than one gene. Addition of the closely related saprophyte *Trichoderma reesei* to the comparison indicated a very high level of conservation of the micro-region in this species as well. This suggests that this grouping of genes alone is not sufficient to render the fungus pathogenic and that many additional factors are required for such a lifestyle in its *Fusarium* relatives. Indeed, differential regulation of the cluster genes may contribute to explaining the differing modes of survival of *T. reesei* and the four *Fusaria*. By extending the comparative genomics analysis to other more

distantly related pathogenic and non-pathogenic species (*N. crassa*, *M. oryzae*, *M. graminicola* and *U. maydis*), breakdown of the cluster could be observed, which appeared to reflect the taxonomic relationships between the species. It therefore appears that this specific grouping of genes may only be important for *Fusarium* and closely related species. In 2010, the genomes of several additional fungal species described above have become available. In the future, it will be interesting to inter-compare these species and also to determine how far 5' and 3' of the micro-region this high degree of interspecies micro-synteny extends.

The origins and complete distribution of the micro-region cannot be fully determined from the comparative genomics analysis performed here, yet it has been shown that this micro-region is present in all sequenced *Fusarium* species to date and at least in *T. reesei* as well. The study of two different *F. graminearum* strains collected from different states in the USA in the late 1990s, also revealed that both strains contained the entire micro-region and no SNPs were present (data not shown). The micro-region may have been present in a progenitor of the species investigated here and subsequently become fragmented in species such as *M. oryzae* and *U. maydis*. Alternatively, the micro-region may be fragmented in the progenitor and been formed during the evolution of the *Fusarium* species and *T. reesei* but not in the other species above. Another possibility is acquisition via horizontal transfer. However the properties of the micro-region such as GC content and lack of flanking repetitive sequences appear to make this a less likely route. A more detailed phylogenetic analysis and investigation of micro-region properties such as codon usage and dinucleotide frequencies will be required to establish which of these situations is the most likely path of evolution for the micro-region. Interestingly, a recent study of the *GAL* galactose utilisation cluster found that the three genes of this cluster have become grouped together via different processes in three unrelated yeast lineages (Slot and Rokas, 2010). In *Saccharomyces* and *Candida*, the genes were originally unclustered and have become grouped together by relocation, whereas in *Schizosaccharomyces*, the cluster was acquired by horizontal transfer from *Candida*. In *Cryptococcus*, the genes became grouped together but independently from the other species.

Cryptococcus also contains ungrouped paralogues of the *GAL* genes in addition to the cluster. In addition, the six-gene *DAL* allantoin utilisation cluster, the largest metabolic gene cluster of yeast, also appears to have been formed by the clustering of originally dispersed genes at a single genomic locus (Wong and Wolfe, 2005). This study indicates the possibility of genes that are originally dispersed becoming collected together at a single genomic locus by relocation, a process that could have contributed to the formation of the cluster in this study. Such clustering, in the case of metabolic pathway genes, is thought to originate and be maintained because it either confers an advantage via allowing more precise coordination of regulation of the genes involved (Hurst et al., 2004), the clustered state being horizontally transferred more readily (Lawrence and Roth, 1996; Walton, 2000) or both (Slot and Rokas, 2010). In the case of the micro-region discovered in this study, the driving forces for its origination and maintenance are currently unknown but the genes are not subject to co-ordinated regulation so this appears not to be a factor in formation. Clustering of these genes into a region of low recombination in the genome may help to 'protect' these genes which are important to virulence by reducing the chance of mutation or deletion via recombination events. In addition, several of the genes may be linked to each other via regulatory pathways (see below) and so maintenance of the micro-region may serve an important role in gene regulation and signalling. It has recently been found that in some *Fusarium* species, such as *F. equiseti*, the core trichothecene (*TRI*) gene cluster has become expanded by the addition of two further *TRI* genes that in other *Fusarium* species are present at separate loci and even on separate chromosomes in some cases (Proctor et al, 2009), indicating the possibility of cluster formation and expansion in the *Fusaria*.

7.11 Micro-Region 'Intralinks'

Interestingly, connections may exist between the different genes of the micro-region at the level of regulation, metabolism and molecular interplay in the fungal cell (referred to here as micro-region 'intralinks'). Firstly, yeast neutral trehalases can be activated by Pka-mediated cAMP-dependent phosphorylation

(Thevelein 1984, 1988; App and Holzer, 1989; Carrillo et al., 1995) and both the cAMP-dependent protein kinase (Pka) regulatory subunit gene (*PKAR*) and the neutral trehalase gene *NTH1* are found in the micro-region. In yeast, Pka can also repress the expression of stress-related genes by phosphorylating the transcription factor Msn2, which causes its retention in the cytosol (Gorner et al., 1998; Rolland et al., 2002). However, in *N. crassa*, while trehalose mobilisation at the onset of germination appears to be activated by Pka signalling, mobilisation under other conditions, such as heat stress or carbon starvation appears to be independent of Pka signalling (de Pinho et al., 2001). Deletion of the *SNF1* gene, which is also located in the cluster, was found to reduce trehalose levels in the fungal cell (M. Urban, J. Ward and M. Beale unpubl.), suggesting a role for *SNF1* in negative regulation of trehalase activity. During glucose depletion, Snf1, similarly, phosphorylates Msn2 and prevents its nuclear accumulation (Mayordomo et al., 2002; De Wever et al., 2005).

The deletion of *SNF1* reduces cellular trehalose, while deletion of the *NTH1* gene would be expected to result in elevated trehalose levels in the cell. Both the *snf1* and *nth1* strains genes show a reduced rate of FEB symptom spread in wheat, albeit to differing extents (this study, M.Urban unpubl., Lee et al., 2009b). This may indicate that maintaining trehalose levels within a specific range may be critical for wild-type infection. In *M. oryzae*, the T6PS gene *TPS1* is required for appressorium function and cuticle penetration, while *NTH1* is required for post-invasive growth (Foster et al., 2003). The effect of trehalose metabolism on pathogenicity in *M.oryzae* has been suggested to be mediated via a broad regulatory activity of trehalose on metabolism and gene expression (Foster et al., 2003; Wilson et al., 2007). In addition, *SNF1* regulates the expression of the isocitrate lyase gene *ICL1* in yeast (Ordiz et al., 1998, Umemura et al., 1997). It is interesting to note that *NTH1*, *ICL1* and *SNF1* form a contiguous arrangement of genes within the cluster in the *Fusarium* species and *T. reesei* but not more distantly related species. Potential links are therefore present between four of the cluster genes (*PKAR*, *NTH1*, *SNF1* and *ICL1*).

In yeast, both Hog1 and Pka regulate the activity of the Msn2/4 stress response transcription factors (Gorner et al., 1998; Moskvina et al., 1998; Causton et al., 2001; O'Rourke et al., 2002; Gasch et al., 2007). Further investigation will be

required to determine if Fcv1, which appears to regulate osmosensitivity in *F. graminearum*, is linked to the Hog1 osmosensing pathway and other stress sensing pathways.

These close regulatory relationships could help to explain the close grouping of these genes in this hotspot, however, the genes of the micro-region do not show co-ordinated regulation so their clustering appears not to serve the purpose of their simultaneous expression. The location of the micro-region in an *F. graminearum* genomic region with little or no recombination may assist in the maintenance of this arrangement. However, in other *Fusarium* species, such as *F. oxysporum* f. sp. *lycopersici*, where vast numbers of transposons are present and presumably active, the continuing maintenance of this arrangement is remarkable.

7.12 Other Micro-Regions in *F. graminearum*

As more genes are shown to contribute to virulence in other pathogenic fungi, so more homologues and therefore more hotspots can potentially be revealed in the genome of the pathogen of choice. For example, **Figure 7.2** indicates that by plotting the homologues of the latest collection of virulence contributors (www.phi-base.org) onto the *F. graminearum* genome, so new hotspots are highlighted.



Figure 7.2 Distribution of homologues of verified pathogenicity/virulence contributors in the *F. graminearum* genome as at 2010. Homologues are highlighted as blue vertical bars on the four horizontal beige chromosomes (1 top to 4 bottom). A second hotspot is shown on chromosome 3 (black box).

7.13 Further Investigation

In addition to the possible experiments described elsewhere in this chapter there are a number of additional techniques that would likely prove interesting to use to investigate further and help to reveal more about the genes of this micro-region. Examining the role of *NTH1* and the second *F. graminearum* trehalase gene in recovery from stress and during spore germination, in addition to a role during growth under stress conditions would help to reveal more about the role of trehalose degradation during different life stages and under different conditions. As standard genetic complementation of the *pkar* strain by transformation of protoplasts derived from germlings grown from fresh conidia with the wild-type *PKAR* gene was not possible for this strain due to a lack of asexual sporulation an alternative approach could be used. For example, an antisense sequence of the *PKAR* gene under the control of an inducible promoter could help to further confirm the role of tight regulation of Pka activity in *F. graminearum*. Comparing the sequence of the micro-region in a greater number of species may also help to reveal more about the evolutionary origins and distribution of this genomic feature, for example by including the newly sequenced *Fusarium* strains in the comparative genomics analysis, using a wider range of species for comparison or by using next generation sequencing to investigate the micro-region in a large number of closely related *Fusarium* strains to help link sequence variation in the micro-region to phenotypic differences. Exploring the sequence of this micro-region in various *F. graminearum* strains collected from different crops, in different geographical regions and in different decades may also be highly informative.

7.14 In Conclusion

The need to study plant pathogenic microorganisms is paramount and becoming ever more important as food production and disease take a higher priority in global affairs. Pathogen evolution and political legislation dictate that new targets for intervention must be sought. To ensure greater success to these ends novel approaches must be used in addition to the labour-intensive forward genetics techniques and gene function biased reverse genetics approaches

with methods designed to help rapidly investigate the unannotated and non-virulence/pathogenicity gene homologue gene complement of pathogens.

This study has investigated one possible approach to deliver a more rapid identification of gene classes previously unknown to contribute to disease symptom development. By highlighting genomic regions significantly enriched for virulence-associated factors, the technique has been used to rapidly pinpoint novel classes of such genes at these sites. The success of this methodology has been demonstrated by the identification of a novel class of gene, namely *FCV1*, which is required for a full rate of disease development on wheat and *Arabidopsis*.

This approach is applicable to any sequenced pathogen, which with the advent of next-generation sequencing techniques, is a daily expanding number of organisms. It is hoped that such an approach, which is designed to complement existing forward and reverse genetic approaches, the results of which are required for the application of this technique, will prove highly useful in novel target site identification in the fight against global plant disease.

Appendix 1

List of accession numbers. Accession numbers are shown for the protein sequences of genes described in the thesis. Where possible, the accession number is the one associated with the reference found in the text. In cases where this is not possible, the accession number that is available and reference for this accession number is shown. In some cases an accession number is not available and is indicated as such. Accession numbers were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank).

Protein	Species	Accession	Reference
FG09891.1 (Asna1/Get3)	<i>Fusarium graminearum</i>	XP_390067.1	Cuomo <i>et al.</i> , 2007
FG09892.1	<i>Fusarium graminearum</i>	XP_390068.1	Cuomo <i>et al.</i> , 2007
FG09893.1	<i>Fusarium graminearum</i>	XP_390069.1	Cuomo <i>et al.</i> , 2007
FG09894.1	<i>Fusarium graminearum</i>	XP_390070.1	Cuomo <i>et al.</i> , 2007
FG09895.1 (Nth1)	<i>Fusarium graminearum</i>	XP_390071.1	Cuomo <i>et al.</i> , 2007
FG09896.1 (Icl1)	<i>Fusarium graminearum</i>	XP_390072.1	Cuomo <i>et al.</i> , 2007
FG09897.1 (Snf1)	<i>Fusarium graminearum</i>	XP_390073.1	Cuomo <i>et al.</i> , 2007
FG09898.1	<i>Fusarium graminearum</i>	XP_390074.1	Cuomo <i>et al.</i> , 2007
FG09899.1	<i>Fusarium graminearum</i>	XP_390075.1	Cuomo <i>et al.</i> , 2007
FG09900.1	<i>Fusarium graminearum</i>	XP_390076.1	Cuomo <i>et al.</i> , 2007
FG15564.1	<i>Fusarium graminearum</i>	Not Available	Cuomo <i>et al.</i> , 2007
FG09901.1	<i>Fusarium graminearum</i>	XP_390077.1	Cuomo <i>et al.</i> , 2007
FG09902.1	<i>Fusarium graminearum</i>	XP_390078.1	Cuomo <i>et al.</i> , 2007
FG09903.1 (Ste7)	<i>Fusarium graminearum</i>	XP_390079.1	Cuomo <i>et al.</i> , 2007
FG09904.1	<i>Fusarium graminearum</i>	XP_390080.1	Cuomo <i>et al.</i> , 2007
FG09905.1	<i>Fusarium graminearum</i>	XP_390081.1	Cuomo <i>et al.</i> , 2007
FG09906.1	<i>Fusarium graminearum</i>	XP_390082.1	Cuomo <i>et al.</i> , 2007
FG09907.1 (Fcv1)	<i>Fusarium graminearum</i>	XP_390083.1	Cuomo <i>et al.</i> , 2007
FG09908.1 (Pkar)	<i>Fusarium graminearum</i>	XP_390084.1	Cuomo <i>et al.</i> , 2007
Pda1	<i>Fusarium solani</i>	XP_003044225.1	Han <i>et al.</i> , 2001
Get3	<i>Saccharomyces cerevisiae</i>	Q12154	Jacq <i>et al.</i> , 1997
Mpe1	<i>Saccharomyces cerevisiae</i>	P35728.1	Dujon <i>et al.</i> , 1994
FG08077.1	<i>Fusarium graminearum</i>	XP_388253.1	Cuomo <i>et al.</i> , 2007
FG08078.1	<i>Fusarium graminearum</i>	XP_388254.1	Cuomo <i>et al.</i> , 2007
FG08079.1	<i>Fusarium graminearum</i>	XP_388255.1	Cuomo <i>et al.</i> , 2007
FG08080.1	<i>Fusarium graminearum</i>	XP_388256.1	Cuomo <i>et al.</i> , 2007
FG08081.1	<i>Fusarium graminearum</i>	XP_388257.1	Cuomo <i>et al.</i> , 2007
FG08082.1	<i>Fusarium graminearum</i>	XP_388258.1	Cuomo <i>et al.</i> , 2007
FG08083.1	<i>Fusarium graminearum</i>	XP_388259.1	Cuomo <i>et al.</i> , 2007
FG08084.1	<i>Fusarium graminearum</i>	XP_388260.1	Cuomo <i>et al.</i> , 2007
FG09910.1	<i>Fusarium graminearum</i>	XP_390086.1	Cuomo <i>et al.</i> , 2007

FG09911.1	<i>Fusarium graminearum</i>	XP_390087.1	Cuomo <i>et al.</i> , 2007
FG09912.1	<i>Fusarium graminearum</i>	XP_390088.1	Cuomo <i>et al.</i> , 2007
FG09913.1	<i>Fusarium graminearum</i>	XP_390089.1	Cuomo <i>et al.</i> , 2007
FG09914.1	<i>Fusarium graminearum</i>	XP_390090.1	Cuomo <i>et al.</i> , 2007
Icl1	<i>Cryptococcus neoformans</i>	AAL56614.1	Rude <i>et al.</i> , 2002
Icl1	<i>Leptosphaeria maculans</i>	AAM89498.1	Idnurm and Howlett, 2002
Icl1	<i>Aspergillus nidulans</i>	Not Available	Bowyer <i>et al.</i> , 1994
Icl1	<i>Magnaporthe oryzae</i>	AAN28719.1	Wang <i>et al.</i> , 2003
Icl1	<i>Colletotrichum lagenarium</i>	BAE75842.1	Asakura <i>et al.</i> , 2006
Icl1	<i>Candida albicans</i>	AAF34690.1	Eschrich <i>et al.</i> , 2002
FG07251.1	<i>Fusarium graminearum</i>	XP_387427.1	Cuomo <i>et al.</i> , 2007
FG08729.1	<i>Fusarium graminearum</i>	XP_388905.1	Cuomo <i>et al.</i> , 2007
Pkar1	<i>Mucor circinelloides</i>	Not Available	Ocampo <i>et al.</i> , 2009
Pkar2	<i>Mucor circinelloides</i>	Not Available	Ocampo <i>et al.</i> , 2009
Pkar3	<i>Mucor circinelloides</i>	Not Available	Ocampo <i>et al.</i> , 2009
Pkar4	<i>Mucor circinelloides</i>	Not Available	Ocampo <i>et al.</i> , 2009
Tps1	<i>Magnaporthe oryzae</i>	AAN46744.1	Foster <i>et al.</i> , 2003
Nth1	<i>Magnaporthe oryzae</i>	AAN46743.1	Foster <i>et al.</i> , 2003
Tps1	<i>Stagonospora nodorum</i>	Not Available	Lowe <i>et al.</i> , 2009
Tps1	<i>Botrytis cinerea</i>	ABG25558.1	Doehlemann <i>et al.</i> , 2006
Tre1	<i>Botrytis cinerea</i>	ABG25559.1	Doehlemann <i>et al.</i> , 2006
Nth1	<i>Leptosphaeria maculans</i>	AAM92143.1	Idnurm <i>et al.</i> , 2003
Tps1	<i>Cryptococcus neoformans</i>	AAT40476.1	Petzold <i>et al.</i> , 2006
Tps1	<i>Cryptococcus gattii</i>	ACB46525.1	Ngamskulrungrroj <i>et al.</i> , 2009
Tps2	<i>Cryptococcus gattii</i>	ACB46526.1	Ngamskulrungrroj <i>et al.</i> , 2009
Tps2	<i>Candida albicans</i>	CAC17748.1	De Virgilio, 1999/2000
Ntc1	<i>Candida albicans</i>	CAA64476.1	Eck <i>et al.</i> , 1997
Atm1	<i>Metarhizium anisopliae</i>	Not Available	Zhao, H. <i>et al.</i> , 2006; Xia <i>et al.</i> , 2002
Tre1	<i>Magnaporthe oryzae</i>	AAN38003.1	Foster <i>et al.</i> , 2003
FVEG_02610	<i>Fusarium verticillioides</i>	Not Available	
FVEG_13500	<i>Fusarium verticillioides</i>	Not Available	
FVEG_06865	<i>Fusarium verticillioides</i>	Not Available	
FOXG_05530	<i>Fusarium oxysporum</i>	Not Available	
FOXG_14683	<i>Fusarium oxysporum</i>	Not Available	
FOXG_09264	<i>Fusarium oxysporum</i>	Not Available	
TreB	<i>Aspergillus nidulans</i>	AAB99831.1	d'Enfert <i>et al.</i> , 1999
FG05622.1	<i>Fusarium graminearum</i>	XP_385798.1	Cuomo <i>et al.</i> , 2007
Icl1	<i>Aspergillus fumigatus</i>	Q6T267.2	Nierman <i>et al.</i> , 2005
Icl1	<i>Saccharomyces cerevisiae</i>	CAA43575.1	Fernandez <i>et al.</i> , 1992
FG00176.1 (Mcl1)	<i>Fusarium graminearum</i>	XP_380352.1	Cuomo <i>et al.</i> , 2007
Pkr1	<i>Crptococcus neoformans</i> <i>var. grubii</i>	AAG30146.1	D'Souza <i>et al.</i> , 2001
Pkr1	<i>Crptococcus neoformans</i> <i>var. neoformans</i>	AAM74046.1	Hicks <i>et al.</i> , 2004

pkaR	<i>Aspergillus fumigatus</i>	AAL09588.1	Oliver <i>et al.</i> , 2002
pkaR	<i>Botrytis cinerea</i>	CAQ30275.1	Schumacher <i>et al.</i> , 2008
Bcy1	<i>Candida albicans</i>	Q9HEW1.1	Cassola <i>et al.</i> , 2004
Rpk1	<i>Colletotrichum lagenarium</i>	AAK31209.1	Takano <i>et al.</i> , 2001
Bcy1	<i>Mycosphaerella graminicola</i>	ABD92792.1	Mehrabi and Kema, 2006
Ubc1	<i>Ustilago maydis</i>	AAA57470.1	Gold <i>et al.</i> , 1994
Pka1	<i>Crptococcus neoformans</i> var. <i>grubii</i>	AAG30145.1	D'Souza <i>et al.</i> , 2001
Pka1	<i>Crptococcus neoformans</i> var. <i>neoformans</i>	AAM74045.1	Hicks <i>et al.</i> , 2004
PkaC1	<i>Aspergillus fumigatus</i>	CAC82611.1	Liebmann <i>et al.</i> , 2004
Tpk2	<i>Candida albicans</i>	AAG38600.1	Cloutier <i>et al.</i> , 2003
Uka1	<i>Ustilago maydis</i>	AAC24243.1	Durrenberger <i>et al.</i> , 1998
Adr1	<i>Ustilago maydis</i>	Not Available	Durrenberger <i>et al.</i> , 1998
Fpk1	<i>Fusarium verticillioides</i>	Not Available	Pei-Bao <i>et al.</i> , 2010
Cpka	<i>Magnaporthe oryzae</i>	AAA93199.1	Mitchell and Dean, 1995
Pka1	<i>Metarhizium anisopliae</i>	Not Available	Fang <i>et al.</i> , 2009
Pkac1	<i>Verticillium dahliae</i>	Not Available	Tzima <i>et al.</i> , 2010
Tpk2	<i>Mycosphaerella graminicola</i>	ABD92791.1	Mehrabi and Kema, 2006
Pka1	<i>Botrytis cinerea</i>	CAQ30273.1	Schumacher <i>et al.</i> , 2008
Pka2	<i>Botrytis cinerea</i>	CAQ30274.1	Schumacher <i>et al.</i> , 2008
PkaR	<i>Aspergillus niger</i>	CAC36308.1	Staudohar <i>et al.</i> , 2002
PkaB	<i>Aspergillus nidulans</i>	Not Available	Ni <i>et al.</i> , 2005
PkaA	<i>Aspergillus nidulans</i>	CBF69742.1	Wortman <i>et al.</i> , 2009
Pkac-1	<i>Neurospora crassa</i>	ACA48490.1	Huang <i>et al.</i> , 2007
Tpk1	<i>Candida albicans</i>	Not Available	Bockmuhl <i>et al.</i> , 2001
Tpk1	<i>Yarrowia lipolytica</i>	CAR95794.1	Cervantes-Chavez <i>et al.</i> , 2009
Mcb	<i>Neurospora crassa</i>	AAB00121.1	Bruno <i>et al.</i> , 2006
Mos4	<i>Arabidopsis thaliana</i>	ABS20115.1	Palma <i>et al.</i> , 2007
Spf27/Cwf7	<i>Schizosaccharomyces pombe</i>	Q9USV3.1	McDonald <i>et al.</i> , 1999
Bcas2/Spf27	<i>Homo sapiens</i>	NP_005863.1	Kuo <i>et al.</i> , 2009
Bcas2	<i>Aspergillus fumigatus</i>	XP_750395.1	Nierman <i>et al.</i> , 2005
Bcas2	<i>Talaromyces stipitatus</i>	XP_002341279.1	Fedorova <i>et al.</i> , 2007
Bcas2	<i>Penicillium marneffeii</i>	XP_002144265.1	Fedorova <i>et al.</i> , 2007
Spf27	<i>Lepeophtheira salmonis</i>	ADD38570.1	Yasuike <i>et al.</i> , 2010
Bcas2	<i>Xenopus laevis</i>	NP_001088952.1	Klein <i>et al.</i> , 2002
Cdc5	<i>Schizosaccharomyces pombe</i>	AAA17515.1	Ohi <i>et al.</i> , 1994
Cef1	<i>Saccharomyces cerevisiae</i>	Q03654.1	Tsai <i>et al.</i> , 1999
Cdc5	<i>Homo sapiens</i>	NP_001244.1	Neubauer <i>et al.</i> , 1998
Prp19	<i>Saccharomyces cerevisiae</i>	CAA97487.1	Duesterhoeft <i>et al.</i> , 1996
Cwf8	<i>Schizosaccharomyces pombe</i>	AAF67750.1	McDonald <i>et al.</i> , 1999

Snt309	<i>Saccharomyces cerevisiae</i>	Q06091.1	Chen <i>et al.</i> , 1998
Cdc5	<i>Arabidopsis thaliana</i>	P92948.2	Monaghan <i>et al.</i> , 2009
Prl1	<i>Arabidopsis thaliana</i>	CAA58031.1	Nemeth <i>et al.</i> , 1998
Prlg1	<i>Homo sapiens</i>	AAH20786.1	Strausberg <i>et al.</i> , 2002
Mac3B	<i>Arabidopsis thaliana</i>	Q94BR4.1	Monaghan <i>et al.</i> , 2009
Mac3A	<i>Arabidopsis thaliana</i>	ACO38702.1	Monaghan <i>et al.</i> , 2009
Gef1	<i>Saccharomyces cerevisiae</i>	CAA80663.1	Greene <i>et al.</i> , 1993
Get1	<i>Saccharomyces cerevisiae</i>	P53192.1	Schuldiner <i>et al.</i> , 2005
Get2	<i>Saccharomyces cerevisiae</i>	P40056.2	Schuldiner <i>et al.</i> , 2005
Ncu01167	<i>Neurospora crassa</i>	XP_961533.1	Galagan <i>et al.</i> , 2003
OS-2 (Hog1)	<i>Neurospora crassa</i>	AAK83125.1	Zhang <i>et al.</i> , 2002
OS-1	<i>Neurospora crassa</i>	AAB01979.1	Schumacher <i>et al.</i> , 1997
OS-4	<i>Neurospora crassa</i>	BAC56234.1	Fujimura <i>et al.</i> , 2003
OS-5	<i>Neurospora crassa</i>	BAC56235.1	Fujimura <i>et al.</i> , 2003
Hog1	<i>Cochliobolus heterostrophus</i>	BAD99295.1	Yoshimi <i>et al.</i> , 2005
Cpmk1	<i>Cryphonectria parasitica</i>	AAO27796.1	Park <i>et al.</i> , 2004
Osm1	<i>Magnaporthe oryzae</i>	AAF09475.1	Dixon <i>et al.</i> , 1999
SskA	<i>Aspergillus nidulans</i>	CBF79927.1	Wortman <i>et al.</i> , 2009
SrrA	<i>Aspergillus nidulans</i>	AAN75016.3	Vargas-Perez <i>et al.</i> , 2007
MA21	<i>Aspergillus fumigatus</i>	Not Available	
Sho	<i>Saccharomyces cerevisiae</i>	Not Available	
SteC	<i>Aspergillus nidulans</i>	CAD44493.2	Wei <i>et al.</i> , 2003
Spc1	<i>Schizosaccharomyces pombe</i>	AAA91020.1	Shiozaki and Russell, 1995
Nth1	<i>Saccharomyces cerevisiae</i>	CAA46718.1	Kopp <i>et al.</i> , 1994
Glk1	<i>Arabidopsis thaliana</i>	AAK20120.1	Fitter <i>et al.</i> , 2001
Pdc1	<i>Zea mays</i>	ABG78829.1	Liu, WZ <i>et al.</i> , 2006
Ste20	<i>Saccharomyces cerevisiae</i>	Q03497.1	Mosch <i>et al.</i> , 1996
Ste11	<i>Saccharomyces cerevisiae</i>	Q03497.1	Mosch <i>et al.</i> , 1996
Ste7	<i>Saccharomyces cerevisiae</i>	P06784.1	Roberts <i>et al.</i> , 1997
Fus3/Kss1	<i>Saccharomyces cerevisiae</i>	CAA84835.1	Feldmann <i>et al.</i> , 1994
Ras2	<i>Saccharomyces cerevisiae</i>	CAA95974.1	Saiz <i>et al.</i> , 1996
Cdc42	<i>Saccharomyces cerevisiae</i>	AAB67416.1	Johnston <i>et al.</i> , 1997
Bck1	<i>Saccharomyces cerevisiae</i>	CAA89389.1	Miosga <i>et al.</i> , 1995
Mkk1	<i>Saccharomyces cerevisiae</i>	CAA99451.1	Boyer <i>et al.</i> , 1996
Mkk2	<i>Saccharomyces cerevisiae</i>	AAB68220.1	Bussey <i>et al.</i> , 1997
Slf2	<i>Saccharomyces cerevisiae</i>	CAA41954.1	Torres <i>et al.</i> , 1991

Ssk2	<i>Saccharomyces cerevisiae</i>	AAC41665.1	Maeda <i>et al.</i> , 1995
Ssk22	<i>Saccharomyces cerevisiae</i>	P25390.2	Oliver <i>et al.</i> , 1992
Pbs2	<i>Saccharomyces cerevisiae</i>	CAA89423.1	Cziepluch <i>et al.</i> , 1996
Hog1	<i>Saccharomyces cerevisiae</i>	P32485.2	Johnston <i>et al.</i> , 1997
Cst20	<i>Candida albicans</i>	Q92212.2	Kohler and Fink, 1996
Hst7	<i>Candida albicans</i>	XP_717333.1	Jones <i>et al.</i> , 2004
Cek1	<i>Candida albicans</i>	CAX42463.1	Jackson <i>et al.</i> , 2009
Mst11	<i>Magnaporthe oryzae</i>	Not Available	
Mst7	<i>Magnaporthe oryzae</i>	Not Available	
Pmk1	<i>Magnaporthe oryzae</i>	AAC49521.2	Xu and Hamer, 1996
Ras1	<i>Magnaporthe oryzae</i>	Not Available	
Ras2	<i>Magnaporthe oryzae</i>	EDJ99125.1	Dean <i>et al.</i> , 2005
Cdc42	<i>Magnaporthe oryzae</i>	AAF73431.1	Zhang <i>et al.</i> , 2009
Mst50	<i>Magnaporthe oryzae</i>	Not Available	
Mkc1	<i>Candida albicans</i>	CAA54129.1	Navarro-Garcia <i>et al.</i> , 1995
Mps1	<i>Magnaporthe oryzae</i>	AAC63682.1	Xu <i>et al.</i> , 1998
Hog1	<i>Candida albicans</i>	Q92207.2	Alonso-Monge <i>et al.</i> , 1999
Osm1	<i>Magnaporthe oryzae</i>	AAF09475.1	Dixon <i>et al.</i> , 1999
Mck1	<i>Magnaporthe oryzae</i>	Not Available	
Ypd1	<i>Candida albicans</i>	AAG01679.1	Calera <i>et al.</i> , 2000
Ssk1	<i>Candida albicans</i>	AAD55813.1	Calera and Calderone, 1999
Ssk2	<i>Candida albicans</i>	XP_717181.1	Jones <i>et al.</i> , 2004
Pbs2	<i>Candida albicans</i>	XP_716629.1	Jones <i>et al.</i> , 2004
MagA	<i>Magnaporthe oryzae</i>	AAB65425.1	Liu and Dean, 1997
MagB	<i>Magnaporthe oryzae</i>	AAB65426.1	Liu and Dean, 1997
MagC	<i>Magnaporthe oryzae</i>	AAB65427.1	Liu and Dean, 1997
Mgb1	<i>Magnaporthe oryzae</i>	BAC01165.1	Nishimura <i>et al.</i> , 2003
Mgb2	<i>Magnaporthe oryzae</i>	Not Available	
Ric8	<i>Magnaporthe oryzae</i>	XP_001405357.1	Dean <i>et al.</i> , 2005
Mac1	<i>Magnaporthe oryzae</i>	AAB66482.1	Choi and Dean, 1997
Cdc35	<i>Candida albicans</i>	AAG18428.1	Rocha <i>et al.</i> , 2000
Ras1	<i>Candida albicans</i>	AAD52662.1	Feng <i>et al.</i> , 1999
Ras2	<i>Candida albicans</i>	XP_722969.1	Jones <i>et al.</i> , 2004
Flo11	<i>Saccharomyces cerevisiae</i>	P08640.2	Churcher <i>et al.</i> , 1997
Pkc1	<i>Candida albicans</i>	XP_722968.1	Jones <i>et al.</i> , 2004
Gpb1	<i>Cryptococcus neoformans</i>	AAD03596.1	Wang <i>et al.</i> , 2000
Gpa1	<i>Cryptococcus neoformans</i>	AAD46575.1	Allen <i>et al.</i> , 1999
Fga1	<i>Fusarium oxysporum</i>	BAB69488.1	Jain <i>et al.</i> , 2002
Gna1	<i>Stagonospora nodorum</i>	Not Available	
Sln1	<i>Saccharomyces cerevisiae</i>	P39928.1	Posas <i>et al.</i> , 1996
Ypd1	<i>Saccharomyces cerevisiae</i>	Q07688.1	Li <i>et al.</i> , 1998

Ssk1	<i>Saccharomyces cerevisiae</i>	Q07084.1	Li <i>et al.</i> , 1998
Skn7	<i>Saccharomyces cerevisiae</i>	AAB69734.1	Johnston <i>et al.</i> , 1994
Nik1	<i>Candida albicans</i>	AAC72284.1	Srikantha <i>et al.</i> , 1998
Chk1	<i>Candida albicans</i>	XP_721017.1	Jones <i>et al.</i> , 2004
Fos1	<i>Aspergillus fumigatus</i>	Not Available	
App1	<i>Cryptococcus neoformans</i>	AAX77221.2	Mare <i>et al.</i> , 2005
Nox1	<i>Podospora anserina</i>	Not Available	
NoxA	<i>Aspergillus nidulans</i>	AAN75017.1	Lara-Ortiz <i>et al.</i> , 2003
Nox1	<i>Magnaporthe oryzae</i>	ABS01490.1	Egan <i>et al.</i> , 2007
Nox2	<i>Magnaporthe oryzae</i>	ABS01491.1	Egan <i>et al.</i> , 2007
Sod1	<i>Botrytis cinerea</i>	CAD88591.1	Rolke <i>et al.</i> , 2004
Fum1	<i>Fusarium verticillioides</i>	AAD43562.2	Desjardins <i>et al.</i> , 2002
NoxA	<i>Epichloe festucae</i>	BAE72680.1	Tanaka <i>et al.</i> , 2006
NoxR	<i>Epichloe festucae</i>	BAF36501.1	Tanaka <i>et al.</i> , 2006
RacA	<i>Epichloe festucae</i>	BAF36499.1	Tanaka <i>et al.</i> , 2006
SepH	<i>Aspergillus nidulans</i>	Q5B4Z3.2	Wortman <i>et al.</i> , 2009
SepL	<i>Aspergillus nidulans</i>	Not Available	
SidB	<i>Aspergillus nidulans</i>	Not Available	
SepM	<i>Aspergillus nidulans</i>	Not Available	
MobA	<i>Aspergillus nidulans</i>	Not Available	
AspB	<i>Aspergillus nidulans</i>	AAB41233.1	Momany and Hamer, 1997
SepA	<i>Aspergillus nidulans</i>	CBF70912.1	Wortman <i>et al.</i> , 2009
AspA	<i>Aspergillus nidulans</i>	AAK21867.1	Momany <i>et al.</i> , 2001
AspC	<i>Aspergillus nidulans</i>	AAK21000.2	Momany <i>et al.</i> , 2001
Snad	<i>Aspergillus nidulans</i>	AAC23686.1	Liu and Morris, 2000
SepK	<i>Aspergillus nidulans</i>	Not Available	
ChsA	<i>Aspergillus nidulans</i>	BAA04806.1	Yanai <i>et al.</i> , 1994
ChsC	<i>Aspergillus nidulans</i>	BAA75501.1	Motoyama <i>et al.</i> , 1994
Chs5	<i>Ustilago maydis</i>	O13394.2	Weber <i>et al.</i> , 2006
Chs7	<i>Ustilago maydis</i>	Q4P333.1	Weber <i>et al.</i> , 2006
TeaC	<i>Aspergillus nidulans</i>	CBF88145.1	Wortman <i>et al.</i> , 2009
Bud3	<i>Aspergillus nidulans</i>	Not Available	
Rho4	<i>Neurospora crassa</i>	Not Available	
Cwh41	<i>Aspergillus fumigatus</i>	Not Available	
MsdS	<i>Aspergillus fumigatus</i>	AAS77884.1	Li <i>et al.</i> , 2008
Msn2	<i>Saccharomyces cerevisiae</i>	P33748.1	Martinez-Pastor <i>et al.</i> , 1996
Msn4	<i>Saccharomyces cerevisiae</i>	P33749.1	Martinez-Pastor <i>et al.</i> , 1996
Tps1	<i>Saccharomyces cerevisiae</i>	CAA85083.1	Feldman <i>et al.</i> , 1994
Tps2	<i>Saccharomyces cerevisiae</i>	CAA98893.1	Foury <i>et al.</i> , 1996
FG06871.1	<i>Fusarium graminearum</i>	XP_387047.1	Cuomo <i>et al.</i> , 2007
Snf1	<i>Saccharomyces cerevisiae</i>	P32578.2	Hedbacker and Carlson, 2008

Icl1	<i>Saccharomyces cerevisiae</i>	P28240.1	Ordiz <i>et al.</i> , 1998
Hog1	<i>Saccharomyces cerevisiae</i>	P32485.2	O'Rourke <i>et al.</i> , 2002
MsnA	<i>Aspergillus nidulans</i>	Not Available	
SskA	<i>Aspergillus nidulans</i>	CBF79927.1	Wortman <i>et al.</i> , 2009
TpsA	<i>Aspergillus nidulans</i>	AAC18060.1	d'Enfert <i>et al.</i> , 1998
OrlA	<i>Aspergillus nidulans</i>	CBF82703.1	Wortman <i>et al.</i> , 2009
Rrr1	<i>Fusarium graminearum</i>	Not Available	
Abc2	<i>Fusarium graminearum</i>	Not Available	
Lyp1	<i>Fusarium graminearum</i>	Not Available	
Tri8	<i>Fusarium graminearum</i>	AAK33086.1	Brown <i>et al.</i> , 2001
Tri3	<i>Fusarium graminearum</i>	AAK33082.2	Brown <i>et al.</i> , 2001
Tri4	<i>Fusarium graminearum</i>	AAK33083.1	Brown <i>et al.</i> , 2001
Tri9	<i>Fusarium graminearum</i>	AAK33087.1	Brown <i>et al.</i> , 2001
Tri11	<i>Fusarium graminearum</i>	AAK33080.1	Brown <i>et al.</i> , 2001
Tri12	<i>Fusarium graminearum</i>	AAK33081.1	Brown <i>et al.</i> , 2001
Tri13	<i>Fusarium graminearum</i>	AAL29524.1	Brown <i>et al.</i> , 2001
Tri7	<i>Fusarium sporotrichioides</i>	AAK33076.1	Brown <i>et al.</i> , 2001
Tri	<i>Fusarium sporotrichioides</i>	AAK77224.1	Meek <i>et al.</i> , 2003
Tri16	<i>Fusarium sporotrichioides</i>	AAO31979.1	Peplow <i>et al.</i> , 2003b
Tri101	<i>Fusarium sporotrichioides</i>	AAD19745.1	McCormick <i>et al.</i> , 1999
Tri7	<i>Fusarium graminearum</i>	AAK53575.1	Lee <i>et al.</i> , 2001
Tri1	<i>Fusarium graminearum</i>	AAQ02672.1	McCormick <i>et al.</i> , 2004
Tri16	<i>Fusarium graminearum</i>	Not Available	
Tri101	<i>Fusarium graminearum</i>	BAA29037.1	Kimura <i>et al.</i> , 1998
Tri15	<i>Fusarium graminearum</i>	AAQ55290.1	Alexander <i>et al.</i> , 2004
Zea1	<i>Fusarium graminearum</i>	Not Available	
Zea2	<i>Fusarium graminearum</i>	Not Available	
Ecp6	<i>Cladosporium fulvum</i>	ACF19427.1	Bolton <i>et al.</i> , 2008
Tri4	<i>Fusarium sporotrichioides</i>	AAK33073.1	Brown <i>et al.</i> , 2001
Rht1	<i>Triticum aestivum</i>	Q9ST59.1	Peng <i>et al.</i> , 1999

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