Responses of wheat to infection by *Mycosphaerella graminicola*

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Abstract

The necrotrophic fungal pathogen *Mycosphaerella graminicola* causes septoria tritici blotch, the most important foliar disease of wheat in Europe. During a compatible interaction, host responses to infection allow the development of necrosis as a form of programmed cell death which appears to aid infection rather than hindering it. Aspects of the response of wheat to infection with *M. graminicola* were studied.

Blumeria graminis f. sp. *tritici* causes powdery mildew disease of wheat. A method for dual inoculation of wheat with both *M. graminicola* and mildew was developed. Leaves preinoculated with virulent *M. graminicola* and subsequently inoculated with a normally virulent isolate of *B.graminis* had substantially reduced formation of mildew colonies, conidiophores and conidia. This was not the result of reduced success of infection or early development of *B. graminis* spores on leaves inoculated with virulent *M. graminicola*.

Expression of 11 plant defence- and senescence-related genes was tested in eight variety/isolate combinations which involved *Stb6* and *Stb15,* the two most common resistance genes in European wheat. Inoculation with *M. graminicola* caused a large effect on plant gene expression with seven genes showing differential expression compared to mock inoculated controls. Patterns of gene expression were largely characteristic of varieties rather than compatible or incompatible interactions in general. TaMPK3 protein accumulated in all the compatible interactions tested, above the level of the mock-inoculated controls, but also accumulated in some incompatible interactions, although not to greater levels than the mock-inoculated controls, implying that this is a consistent feature of the wheat *M. graminicola*-wheat interaction.

Three common microscopy stains were tested to develop a reliable method of investigating *M. graminicola* growth and development during wheat infection, especially visualisation in the apoplast. None of the stains tested were suitable for this purpose, implying that the fungal hyphal wall is modified or protected within the plant

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Introduction

A substantial proportion of this chapter was included in a review paper, '*Mycosphaerella graminicola*: from genomics to disease control' by E.S. Orton, S. Deller and J.K.M. Brown in *Molecular Plant Pathology* **12**(5),413-424 (2011).

1.1 Septoria tritici blotch disease and its importance

1.1.1 The pathogen

Mycosphaerella graminicola (Fuckel) Schröter in Cohn is the teleomorph of *Septoria tritici* Roberge in Desmaz.. It is an ascomycete fungus in the Order of Dothideales. This pathogen is the causal agent of septoria tritici blotch (STB) on bread and durum wheat (*Triticum aestivum* L. and *T. turgidum* ssp. *durum* L.). It is a well characterised filamentous fungal pathogen propagated by both sexual ascospores and asexual pycnidiospores and spread by wind-dispersal and rain splash respectively (Cohen & Eyal, 1993; Duncan & Howard, 2000; Kema *et al.*, 1996).

The *M. graminicola* genome was sequenced by the USA Department of Energy's Joint Genome Institute and has been of enormous importance for research on *M. graminicola* and for phytopathology in general. The *M. graminicola* sequence has revealed that the Dutch field isolate IPO323 has a total genome size of 39.7 Mb, and 21 chromosomes ranging in size from ~0.3Mb to ~6Mb. Thirteen chromosomes are considered core chromosomes, being apparently essential, while the other 8 are known to be independently dispensable despite containing approximately 12% of the genome (Mehrabi *et al.*, 2007; Wittenberg *et al.*, 2009). Of ~10,900 genes in the genome that have been functionally annotated approximately 59% of the genes on the core chromosome have annotations (including automatic and manual curation) while this is only the case for approximately 10% of genes on the dispensable chromosomes (Goodwin *et al.*, 2011). The genome browser maintained by the Joint Genome Institute provides the genome sequence, organisation, automatic and manual

annotations and large amounts of other information on the genes and intervening regions at http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html.

In addition to loss of dispensable chromosomes, there are other features of genomic plasticity in *M. graminicola*. Translocation of chromosome sections, chromosome length polymorphisms and chromosome copy number polymorphisms including disomy, the presence of two copies of a chromosome, have all been detected between progeny and parent isolates (Wittenberg *et al.*, 2009). The high genome plasticity could be among the strategies that enable the pathogen population to quickly overcome adverse biotic and abiotic conditions in wheat fields.

1.1.2 The disease

STB is of global economic importance, but the disease thrives especially in climates with rain during the development of the wheat until flag leaf emergence. STB is currently the most important foliar disease of wheat in Europe, including the UK, and many other temperate parts of the world. In the UK, 52% of wheat leaf samples surveyed by the UK's Home Grown Cereals Authority (HGCA) in 2010 were infected by STB (www.cropmonitor.co.uk). That was a reduction on 2009 levels but it remained the most important foliar disease of wheat in the UK.

Symptoms of STB are characterised by necrotic blotches that contain pycnidia, the asexual fruiting-bodies containing pycnidiospores (figure 1.1). The anamorph mainly contributes to disease development during the growing season, whereas the teleomorph is the primary source of inoculum for emerging wheat in the autumn (Eriksen & Munk, 2003; Shaw & Royle, 1993). The heterothallic mating system recently determined by Kema *et al.* (1996) provides an explanation for the wide genetic variation in *M. graminicola* and its ability to evolve rapidly under selection.

1.2 The infection cycle

The infection cycle is completed when a compatible interaction occurs between the pathogen and a susceptible host.

Figure 1.1 Pycnidia of *M. graminicola* on a wheat leaf.

1.2.1 Germination

Germination of conidia occurs within hours of a spore landing on a wheat leaf. Conidia of both compatible and incompatible isolates produce germ tubes. Differences have been seen in the percentage of conidia germinating; Cohen and Eyal (1993) stated that 85-90% of all conidia germinated regardless of cultivar. Kema *et al.* (1996) agreed with this but Shetty *et al.* (2003) noticed a low germination rate, although importantly this was not dependent on cultivar. Many factors apart from cultivar are likely to have an impact on germination rate; environmental conditions are especially likely to play a large role.

Hyphal growth has also differed between studies; Duncan and Howard (2000) noticed that germ tubes grew towards stomata, others noted that most germ tubes actually grew away from the stomata (Shetty *et al.*, 2003). Cohen and Eyal (1993) stated that hyphal ramification was perpendicular to the leaf axis. An exogenous nutrient supply has been demonstrated to be important for germling morphogenesis *in vitro* by comparing water with yeast peptone dextrose agar (Duncan & Howard, 2000).

1.2.2 Penetration

Once the spore has germinated it must penetrate the host leaf, this occurs within 24 hours after inoculation (Kema *et al.*, 1996). The general consensus is that penetration occurs solely through the stomata (Duncan & Howard, 2000; Kema *et al.*, 1996; Shetty *et al.*, 2003) although direct penetration has occasionally been observed between through the cuticle (Hilu & Bever, 1957) either between the guard cells (Cohen & Eyal, 1993) or through host epidermal cells (Rohel *et al.*, 2001). The requirement for an appressorium-like structure remains unclear; appressorium-like structures (swellings) have been observed before the occurrence of penetration through the stomata (Cohen & Eyal, 1993; Kema *et al.*, 1996; Shetty *et al.*, 2003). As *M. graminicola* entry appears to be (almost) entirely through the stomata it may not to require appressorial development (although it would be required for direct penetration).

1.2.3 Colonisation

The hyphae colonise the substomatal cavity of the leaf after penetration. This occurs within 48 hours after inoculation with the pathogen (Duncan and Howard, 2000). The fungus colonises the mesophyll intercellular space approximately two days post inoculation (dpi) (Cohen & Eyal, 1993; Kema *et al.*, 1996). The rate of increase in fungal growth during symptomless colonisation of *M. graminicola* is relatively low (Keon *et al.* 2007). Fungal growth is usually in close contact with the cell walls (Cohen & Eyal, 1993), although the reason for this has not been elucidated. The hyphae form an increasingly dense network from 9 to 15 days post inoculation (Shetty *et al.*, 2003). This correlates with the appearance of macroscopic symptoms. Kema *et al.* (1996) noted that 8 dpi mesophyll cell walls had a wrinkled appearance and sometimes collapsed, increasing at 10 dpi. The pathogen remains entirely intercellular during the infection process. No specialised feeding structures such as haustoria are formed during the infection process, leading to the question of how the fungus acquires its nutrients.

1.2.4 Growth and Pycnidia Formation

On a susceptible host, STB symptoms appear coincident with the activation of host programmed cell death (PCD), with similarity to a hypersensitive response (HR) (Keon *et al.*, 2007). This response is normally associated with a resistance reaction particularly towards biotrophs, but is not seen in the resistance response to *M. graminicola* by wheat. The collapse of plant cells during this time releases nutrients into the apoplast due to a loss of membrane integrity. This is a clear difference from natural senescence, where nutrients are gradually redistributed to an alternative part of the plant without the loss of membrane integrity. The increase in the availability of nutrients is suggested to allow the fungus to grow and form pycnidia in stomatal cavities, thereby completing its infection cycle within the host (Keon *et al.*, 2007).

1.2.5 The incompatible interaction

No differences are seen between incompatible and compatible interactions during germination or penetration. The resistance must therefore occur once the pathogen has entered the host.

Very little hyphal growth was seen after penetration of the *M. graminicola* isolate IPO323 on the resistant cultivar Stakado studied by Shetty *et al.* (2003). Kema *et al*. (1996) reported similar findings, in the interaction between Kavkaz/K4500 1.6.a.4 and isolate IPO87016, only occasionally observing hyphae between mesophyll cells. Cohen and Eyal (1993) reportedly saw immature pycnidia of the Israeli isolates, ISR 398A1 and ISR 8036, in an arrested state in the resistant cultivar Kavkaz/K4500 L.6.A.4. The authors reported that these isolates differ in their virulence on Kavkaz/K4500 L.6.A.4. but did not explain how they differed. Immature pycnidia on a resistant variety has not been reported since. These observations suggest that resistance genes may act to stop or slow hyphal growth or development of the pathogen.

In an incompatible interaction there is no measurable increase in biomass, indicating that the fungus is not an effective biotroph and ultimately requires host cell death to complete infection (Keon *et al.*, 2007) There is no hypersensitive response. No macroscopic visible symptoms have been reported in incompatible interactions, although pycnidia in an arrested state of development have been seen microscopically (Cohen and Eyal, 1993).

1.3 Comparisons between pathogens with differing nutritional lifestyles

The long latent period of *M. graminicola* is an unusual infection strategy for what is ultimately a necrotrophic pathogen. The genome sequence of *M. graminicola* reveals distinct differences in its ability to infect and obtain nutrients compared with necrotrophic fungal pathogens and biotrophic pathogens. Comparing the recently published genomes of two pathogens, *Blumeria graminis* and *Botrytis cinerea,* differing in nutritional lifestyle, with *M. graminicola* gives some clues as to how *M. graminicola* has developed as a pathogen.

B. graminis is a specialist pathogen, infecting only cereals. The species is divided into several *formae speciales* adapted to specific cereal hosts; i.e. wheat, barley, oats and rye (Inuma *et al.,* 2007). Biotrophic pathogens are obligate feeders, requiring a living host to grow and develop; they cannot generally be grown in axenic culture. *B. graminis* reproduces both asexually through conidia borne on conidiophores and sexually producing asci contained in chasmothecia (Braun *et al.,* 2002). The asexual cycle is completed in a strictly programmed way (Both *et al.,* 2005a). When a spore comes into contact with a leaf, tiny projections that cover the surface of the conidium, provide a conduit for the release of an extracellular matrix (ECM) (Carver *et al.,* 1999; Zhang *et al.*, 2005). It appears that the ECM is used as a means for signal exchange between the conidium and the host (Zhang *et al.,* 2005). A primary germ tube (PGT) is rapidly produced and then an appressorial germ tube (AGT) is formed, during which time the germling uses stored glycogen as an energy

source (Both *et al.,* 2005b). The germinated spore produces an appressorium with an infection peg, allowing penetration into an epidermal cell. Penetration of the host cell wall occurs by a combination of turgor pressure and enzymatic means. *B. graminis* germlings can produce cutinases and glucanses which assist with hydrolysing plant cell walls (Zhang *et al.* 2005). Once inside the cell an haustorium is formed, this generally happens within 48 hours after inoculation (Zhang *et al.,* 2005). Haustoria allow the uptake of glucose from the plant which is used to fuel glycolysis and feed the elongating secondary hyphae which develop on the surface of the epidermal cells (Both *et al.,* 2005b). The hyphae rapidly proliferate, synthesising proteins and nucleic acids. Approximately 4-7 days after initial inoculation, conidiophores form on the surface of the leaf.

Necrotrophic pathogens are generalist feeders and can be grown in axenic culture. *B. cinerea* rapidly adopts a necrotrophic lifestyle once it has entered a host plant. *Botrytis* produces an appressorium to enter host cells. In conditions conducive to the pathogen, infection occurs soon after inoculation and lesions rapidly form (Benito *et al.* 1998). The pathogen creates an acidic environment and produces toxins which kill host cells, allowing the pathogen to obtain nutrients from the host and complete its lifecycle. It reproduces by both asexually and sexually, although mostly by conidiophore production.

The infection strategy of *M. graminicola* is different from either of the pathogens described above. By comparing it with the infection strategies of both *B. graminis* and *B. cinerea*, both similarities and differences can be perceived. *M. graminicola* is a specialist feeder, infecting only bread and durum wheat, as is *B. graminis*, but is not obligate and is easily grown in axenic culture. Unlike *B. graminis* and *B. cinerea* it produces no specialist infection structures; the genome of *M. graminicola* contains no predicted genes linked to appressorial production (Goodwin *et al.,* 2011). The long latent period where it remains intercellular is unlike either of the other pathogens described here and appears to be more indicative of an endophytic lifestyle. *B. cinerea* and *M. graminicola* both ultimately adopt a necrotrophic lifestyle.

B. cinerea achieves this by bombarding the host with chemicals that alter the leaf environment to its advantage and ultimately kill host cells, whereas, to date, it is unknown if any effectors or toxins are produced which enable the *M. graminicola* to kill host cells. The means by which host cells die during infection with *M. graminicola* is, as yet, unknown.

Comparing the genome of *M. graminicola* (Goodwin *et al.*, 2011) with recently published data on both *B. graminis* (Spanu *et al.,* 2010) and *B. cinerea* (Amselem *et al.* 2011) reveals distinct differences, probably due to the differing nutritional lifestyles of these pathogens. The most striking difference is in genome size; the genome of *B. graminis* is ~120 Mb, whilst *B. cinerea* is 38-39 Mb and *M. graminicola* ~40 Mb, the latter two being more in line with other ascomycetes. The number of genes in *B. graminis* is comparatively small, ~6000 whereas *B. cinerea* has ~16,500 and *M. graminicola* ~11,000.

The genome expansion of *B. graminis* is due to the large number of transposable elements (TEs) (Parlange *et al.,* 2011; Spanu *et al.,* 2010). Parlange *et al.* (2011) found that 85% of the *B. graminis* f. sp. *tritici* genome was made up of TEs. TEs generate high levels of genetic variation and provide a way for the genome to be flexible and adaptable. It has been shown that effector genes can be associated with TEs and coevolve. Sacristan *et al.* (2011) showed that the effector encoding gene family, *AVRk1*, lies closely associated with a retrotransposon, TE1a, and has coevolved with it providing a mechanism for amplifying and diversifying these effector alleles. Very little is known about the abundance or function of effectors proteins encoded in the *M. graminicola* genome. Three LysM-containing domain effector proteins have been identified and functionally tested that are homologous to Ecp6 from *C. fulvum* and have been shown to interfere with chitin-triggered immunity (Marshall *et al.* 2011). Motteram *et al.* (2009) identified MgNLP1, which is possibly able to induce cell death, but is not a major virulence factor. It is not known if MgNLP1 functions as an effector protein. Three putative effectors, homologous to the *C. fulvum* Ecp2, have been identified, but these have not been functionally tested (Stergiopoulous *et al.*

2010). It appears that there are far fewer effectors in the genome of *M. graminicola* than in *B. graminis.*

In terms of nutritional lifestyle, the biggest differences are seen in the numbers of carbohydrate active enzymes (CAZymes) predicted in each of the three genomes. *B. graminis* has no enzymes for degrading cellulose, xylan or pectin, whereas *B. cinerea* has many enzymes for degrading plant cell walls, particularly those which degrade pectin, which is found particularly in soft fruits. *M. graminicola* has a reduced number of genes encoding CAZymes, compared with other cereal pathogens (*S. nodurum* and *M. oryzae*), with few for cellulases, xylanases or xyloglucanses, which is in accordance with its intercellular lifestyle. The relatively small number of cell wall degrading enzymes (CWDEs) seen in *M. graminicola* is comparable to the reduced number of CWDEs found in the ectomycorrhizal fungus, *Laccarria bicolor* (Goodwin *et al.,* 2011), indicating that *M. graminicola* perhaps has a similar nutritional lifestyle to endophytes and has perhaps only recently evolved a pathogenic lifestyle. *B. cinerea* has a large number of proteases, which may benefit the parasite by reducing the activity of antifungal proteins produced as a host defence response and also by providing nutrients for the pathogen. *M. graminicola* has an expanded repertoire of protease genes compared with other cereal pathogens and also of amylase genes. It has been proposed that utilising alternative nutrition sources, such as proteins and starch, may reflect the stealthy mode of pathogenesis of this of this fungal pathogen (Goodwin *et al.,* 2011).

1.4 Methodologies

A well tested toolbox of methods is now in place for studying the *M graminicola*-wheat interaction. The fungus can be detected and measured in infected leaves and seeds by quantitative PCR (Bearchell *et al.*, 2005; Consolo *et al.*, 2009; Guo *et al.*, 2006; Shetty *et al.*, 2007), which has been used to great effect in studying historical wheat samples in the Rothamsted Research Broadbalk archive from 1844 until 2003 (Bearchell *et al.*,

2005). Studying the interaction has been aided by the use of young plants, requiring a short growth time and no vernalisation, and by the use of both attached and detached leaf assays (Arraiano *et al.*, 2001a; Keon *et al.*, 2007). Samples of *M. graminicola* isolates from around the world representing populations from many different spatial scales over time and in conditions with varying plant husbandry have been used in research on fungicide sensitivity, virulence and the genetic structure of the global *M. graminicola* population. Data on disease severity provides the basis for epidemiological studies such as the correlation between STB severity and weather (Pietravalle *et al.*, 2003), and predictive models for use by agronomists and farmers (Beest *et al.*, 2009).

1.5 Plant disease resistance and control

Plants are subjected to a constant attack of potential pathogens from which they cannot escape. They have developed mechanisms that allow them to resist infection. The many components of defence overlap to provide resistance against most pathogens. Resistance (or incompatibility) can be broadly defined as the ability of a plant to prevent infection or growth of the pathogen. Tolerance is also an important concept in plant disease and can be defined as the ability of the host to compensate for damage caused by the pathogen (Brown & Handley, 2006). Three types of resistance are broadly defined in the literature: non-host (Heath, 2000; Thordal-Christensen, 2003); partial (Johnson, 1984) and gene-for-gene (Flor, 1971). There is an overlap between these defence systems which make studying these mechanisms difficult.

1.5.1 Non-host resistance

Most plants are resistant to most pathogens. Plants have both constitutive and inducible defences not requiring known *R* genes for specific pathogen recognition by the plant (Heath, 2000). Passive defences are the first obstacle which potential pathogens must overcome. These defences are usually physical or chemical barriers

that provide a broad spectrum resistance to pathogens. Plants have structural barriers to pathogens which the pathogen must circumvent to infect the plant. Wax and cuticle layers form a hydrophobic barrier that can inhibit pathogen spores from attaching to the surface of the plants, especially if spread by water. The size and location of stomata can be regulated, for example there may be a narrow entrance, or guard cells may protrude from the stomata, preventing pathogen germtube entry. Various preformed chemical defences are employed; phenolic compounds including tannins and dienes are potent inhibitors of hydrolytic enzymes, saponins are antifungal and act to exclude pathogens, glucanases and chitinases degrade fungal cell walls. These various peptides, proteins and other secondary metabolites are a major component of non-host resistance (Heath, 2000).

Inducible non-host responses involve the recognition of a pathogen leading to the activation of defence signalling. This type of recognition involves the detection of MAMPs (microbe associated molecular patterns); which are proteins and other molecules that are thought to be indispensable, abundant elements of pathogens that are not present in plants, making them capable of acting as non-self recognition determinants. MAMPs enable the plant to recognise an attack by a potential pathogen and initiate an appropriate response. Transmembrane pattern recognition receptors (PRRs) recognise MAMPs (Jones & Dangl, 2006), and, when associated with a pathogen, lead to PAMP (pathogen associated molecular pattern)-triggered immunity (PTI), halting further colonization by an incompatible pathogen. A bacterial flagellin protein has been shown to induce this kind of response in *Arabidopsi*s via a mitogen-activated protein kinase (MAPK) cascade (Asai *et al.*, 2002). Other MAMPs which initiate this cascade are elf-18 from bacteria (Kunze *et al.*, 2004) and Pep-13 from *Phytophthora spp.* (Brunner *et al*., 2002). MAPKs form part of a highly conserved signalling cascade that is able to respond to extracellular stimuli and regulate various cellular responses such as gene expression, mitosis, differentiation and cell survival or PCD during responses to pathogen infection. Structural barriers can also be inducible, potentially activated by MAMPs. One example of this is papilla formation. It has been

shown that *Blumeria graminis* f. sp. *hordei* cannot infect the nonhost *Arabidopsis* as the pathogen induces papilla formation (Thordal-Christensen, 2003). It has been indicated that virulence factors from pathogenic bacteria, such as *P. syringae* pv. *tomato*, suppress expression of genes induced by MAMPs and target the MAPK signalling cascade (Nurnberger & Lipka, 2005) indicating that establishment of infection is associated with suppression of plant non-host resistance.

1.5.2 Fungal effectors and plant recognition

Pathogens need to suppress or evade the plant's basic lines of defence and the MAMP/PAMP based surveillance system to adopt a pathogenic lifestyle within the plant. A successful pathogen can deliver effectors that interfere with PTI and promote virulence within the host. Effectors may be recognised by the host in a gene-for-gene manner, by specific nucleotide binding and leucine rich repeat (NB-LRR) domain proteins, encoded by *R* genes, resulting in effector triggered immunity (ETI). ETI results in disease resistance and usually a hypersensitive cell death response (Jones & Dangl, 2006), which is effective against biotrophic pathogens. Pathogens must evolve to evade this recognition if they are to successfully infect the plant. The initiation of further plant defence responses to pathogen attack impedes the pathogen's progress, exerting a selective pressure for the pathogen to become ever more specialised. Recognition of the increasing levels of specialisation has led to the development of a 'Zigzag Model' (Jones & Dangl, 2006) in which natural selection favours new plant *R* gene alleles to recognize new fungal effectors as the pathogen overcomes ETI after evading PTI. The evolution of this gene-for-gene relationship between the pathogen and the host is an isolate-specific response where a pathogen avirulence gene is recognised by a specific *R*-gene in the plant. Although resistance to *M. graminicola* follows a gene-for-gene relationship (Brading *et al.* 2002) the pathosystem does not fit the standard zigzag model. An HR is not seen as part of ETI, although the *R*-genes do recognise as yet uncharacterised *Avr* genes which result in plant immunity to the

pathogen. It is unknown how the plant resistance mechanism responds to *M. graminicola.*

1.5.3 Resistance genes and breeding to control the disease

Resistant cultivars are an effective means of controlling STB, but until recently breeders relied on uncharacterised genetic resistance in breeding programmes (Chartrain *et al.*, 2005b). Resistance to STB has been broadly divided into two classes; specific and quantitative. Specific resistance is near complete and oligogenic. Partial, or quantitative, resistance is incomplete and polygenic (Jlibene *et al.*, 1994; Zhang *et al.*, 2001).

Specific resistance interactions between wheat cultivars and specific *M. graminicola* isolates occur in detached leaf tests, seedling tests and tests under field conditions (Brading *et al.*, 2002). Fourteen major genes in wheat for resistance to STB have so far been identified, mapped and published *Stb1-Stb12, Stb15* and *Stb18,* with another four that are, as yet, unpublished (table 1.1). The resistance mechanisms by which these genes confer resistance to specific pathogen genotypes is currently unknown and none of the *Stb* genes have been cloned. A gene-for gene relationship has been demonstrated between wheat *Stb6,* the best understood of these genes, and *M. graminicola* (Brading *et al.*, 2002). The gene-for-gene relationship is the most studied and yet is the least durable in the field, because pathogen populations can adapt to the selection pressure placed on them by the presence of a major resistance gene; for example commercial use of the cultivar Gene led to selective changes in the pathogen population to strains adapted to overcome the resistance gene (Cowger *et al.*, 2000).

Partial resistance is generally much more durable than gene-for-gene, race-specific resistance but is harder to select and less well studied than specific resistance. Chartrain *et al.* (2004b) used a doubled-haploid population produced from a cross between susceptible and resistant varieties, Riband and Arina respectively, to attempt to locate quantitative trait loci (QTL) and determine the genetics of this partial

resistance. No QTL controlled a significant fraction of variation in the resistant parent, Arina, in which partial resistance, therefore, is most probably controlled by several dispersed genes. Chartrain *et al.* (2004b) also showed that partial resistance is isolate non-specific and therefore likely to be durable. Investigating quantitative resistance is complicated by the fact that resistance at the seedling and adult stage are sometimes controlled by different genes. Chartrain *et al*. (2004b) reported no correlation between disease levels on seedlings and adult plants in the Arina x Riband population while Eriksen *et al.* (2003) detected QTLs for resistance at the adult stage in a population of Senat x Savannah which were not present at the seedling stage. This has implications for breeding, because resistance at all growth stages is desirable, although resistance is most important when the weather is most conducive to symptom development and pathogen spread, namely in the later adult stages.

The emergence of strains of *M. graminicola* resistant ot quinine outside inhibitor (QoI) fungicides and more recently triazole based fungicides has increased the need to develop resistant varieties of wheat as a cost effective means of controlling the disease. There has been substantial progress in breeding resistant wheat varieties in the last 15 years, largely relying on partial resistance which is broadly effective against all known fungal genotypes and therefore durable (Angus & Fenwick, 2008). Knowledge about the distribution of resistance genes in wheat varieties has advanced considerably. The presence of specific resistance genes (Arraiano & Brown, 2006) and partial resistance (Arraiano *et al.*, 2009) in 238 European wheat varieties has been ascertained. Chartrain *et al.* (2004a) screened 24 varieties with 12 isolates of *M. graminicola* for isolate-specific resistance and identified new sources of resistance that could be utilised in breeding. Some varieties, such as TE911, could be used in crossing programmes to provide both partial and specific resistance (Chartrain *et al.*, 2005c). It appears that the major resistance gene *Stb6* had entered wheat breeding

Gene	Chromosome location	Isolate	Variety	Reference
Stb1	5B L	IN95-Lafayette-1196-ww 1-4	Bulgaria 88	(Adhikari *et al.*, 2004c)
Stb2	3BS	Paskeville	Veranopolis	(Adhikari *et al.*, 2004b)
Stb3	6DS	Paskeville	Israel 493	(Adhikari *et al.*, 2004b)
Stb4	7D	IN95-Lafayette-1196-ww 1-4	Tadinia	(Adhikari *et al.*, 2004a)
Stb5	7D	IPO92469	Synthetic 6X	(Arraiano *et al.*, 2001b)
Stb6	3AS	IPO323	Flame	(Brading *et al.*, 2002)
Stb7	4AL	MG2	ST6	(McCartney *et al.*, 2003)
Stb8	7BL	IN95-Lafayette-1196-WW 1-4	W7984 synthetic	(Adhikari *et al.*, 2003)
Stb9	2BL	IPO89011	Courtot	(Chartrain *et al.*, 2009)
Stb10	1D	IPO94269 and ISR8036	Kavkaz-K4500 L.6.A.4	(Chartrain *et al.*, 2005a)
Stb11	1BS	IPO90012	TE911	(Chartrain *et al.*, 2005c)
Stb12	4AL	ISR398	Kavkaz-K4500 L.6.A.4	(Chartrain *et al.*, 2005a)
Stb13	7BL	MG2 and MG96-13	Salamouni	USDA Wheat Newsletter 53
Stb14	3DS	MG2	Salamouni	USDA Wheat Newsletter 53
Stb15	6AS	IPO88004	Arina	(Arraiano *et al.*, 2007)
Stb16	3DL	Various	M3 synthetic	(Ghaffary *et al.*, 2011a)
Stb17	5AL	Various	M3 synthetic	(Ghaffary *et al.*, 2011a)
Stb18	6DS	IPO98, IPO022,IPO323 and IPO89011	Balance	(Ghaffary *et al.*, 2011b)

Table 1.1 Location of known *Stb* genes and the cultivar and isolates used for mapping them.

programmes on numerous occasions and been used world-wide as a source of STB resistance (Chartrain *et al.*, 2005b). *Stb6* explains a significant level of variability in susceptibility to STB in the field (Arraiano *et al.*, 2009), which may explain why it was present in many well-known sources of resistance. The mechanism behind this resistance is not known; *Stb6* may confer partial resistance to STB itself or may be linked to a gene conferring partial resistance. Studies have shown that pyramiding genes for resistance may also help with breeding efforts for more durably resistant wheat. The identification of varieties with more than one resistance gene, such as the breeding lines Kavkaz-K4500 and TE9000, that have good resistance to STB suggests that pyramiding of R genes might achieve high levels of field resistance (Chartrain *et al.*, 2004a). In field trials, several cultivars were identified with especially high levels of partial resistance, some with no known specific resistance genes. These may be useful sources of resistance in breeding for STB resistance (Arraiano *et al.*, 2009). In summary, this research has shown that breeders, at least in Europe, have sufficient genetic variation in their germplasm and they increasingly have the information available to make more informed choices about specific parents to use in crosses.

1.5.4 The use of fungicides

While fungicides have been successfully used against *M. graminicola*, the effectiveness of the two main groups of chemicals has declined as insensitivity to triazoles and QoIs, also known as strobilurin, has evolved in the fungal population (Fraaije *et al.*, 2005b; Fraaije *et al.*, 2007). Sustained application has led to evolution of fungicide insensitivity, for example, isolates from Europe show resistance to QoI fungicides and there is widespread failure of these fungicides to control STB (Fraaije *et al.*, 2005b). QoIs act against mitochondrial protein cytochrome *b* but a mutation from glycine to alanine at residue 143 (G143A) in the *cytochrome b* protein sequence, has caused apparently total loss of efficacy of these fungicides (Fraaije *et al.*, 2003). Both breeding of cultivars with improved genetic resistance and development of effective

fungicides are slow and demanding processes. Fungicide use remains the main control practice, although fungicides are expensive and not entirely reliable.

1.6 Septoria tritici blotch disease

1.6.1 Host responses to pathogen recognition

In most of the current 'model' systems where non-host defences have been evaded, further responses must be activated to attempt to suppress or kill the invading pathogen. These can be triggered during inducible non-host resistance and during *R*-gene mediated recognition by both nonspecific and specific pathogen signals (Heath, 2000). There is often a large degree of similarity in the responses triggered. These defence strategies can include: generation of reactive oxygen species (ROS) (oxidative burst); cell wall modifications; induction of pathogenesis-related (PR) proteins and an HR. The inter-relationships between these events has also been studied.

There have been significant advances in knowledge of how *M. graminicola* interacts with host cells to result in host cell death and completion of the fungus' life cycle. Firstly, the cell death pathway induced in the wheat cells during compatible interactions resembles the apoptosis-like pathway (Keon *et al.*, 2007). Secondly, features of the plant-pathogen interface between wheat and *M. graminicola* have been examined, including glucans which may act as pathogen associated molecular patterns, and the interchange of ROS (Shetty *et al.*, 2003; Shetty *et al.*, 2007).

The defence mechanisms conveying resistance in wheat to STB do not currently appear to follow the pattern of response to either of the common models for biotrophic fungi or necrotrophic fungi. How plant defence responses contribute to wheat resistance to *M. graminicola* is largely unknown, but the resistance mechanisms are beginning to be elucidated by work studying the compatible interactions. Specific interactions can now be exploited to determine the mechanisms of resistance in relation to the presence or absence of major resistance genes.

1.6.2 Living in a biotrophic phase and the switch to necrotrophy

Plant pathogens often trigger host PCD. The HR is a form of PCD which is characteristically associated with disease resistance. HR can feature in inducible non-host reactions or this resistance response can be triggered either directly or indirectly when the product from a dominant host *R* gene corresponds with the product of the dominant pathogen *avr* gene (Greenberg & Yao, 2004). Although HR is not always necessary, the *Arabidopsis thaliana* mutant, *dnd1* exhibits resistance to virulent pathogens in the absence of HR (Yu *et al.*, 1998). Biotrophs and necrotrophs react differently to PCD; many biotrophs are inhibited by the HR, whereas necrotrophic pathogens are able to utilise it (Glazebrook, 2005; Govrin and Levine, 2002).

Some pathogens are adapted to utilising nutrients that are available in the apoplast, or to manipulating the plant to gain a nutrient supply. The hemibiotrophic pathogen *Cladosporium fulvum* is closely related to *M. graminicola* (Goodwin *et al.*, 2001). Soloman and Oliver (2001) found that available nitrogen in the apoplast greatly increases during a compatible interaction between tomato and *C. fulvum* adding evidence to the hypothesis that the apoplast is not rich in nutrients and that biotrophic fungi must manipulate this to support infection. Soluble carbohydrate in the apoplast represents a very small fraction, less than 1 %, of the total soluble carbohydrate of the leaf (Tetlow & Farrar, 1993). Strains of the hemibiotrophic bacteria, *P. syringae,* are specifically adapted to utilizing sugars and amino acids that are found within the apoplast, to multiply within the plant and further infect plant tissues before entering the necrotrophic phase (Rico & Preston, 2008). This gives weight to the hypothesis that there may be enough availability of nutrients in the apoplast to support *M. graminicola* during its latent phase of development.

Nutrition of *M. graminicola* is thought to be greatly influenced by host cell death. It has been stated that nutrients in the apoplast are sufficient to support the growth of intercellular fungi such as *M. graminicola* (Spencer-Phillips, 1997). However, qPCR measurements showed little increase in fungal biomass before host cell death (Keon *et al.*, 2007; Shetty *et al.*, 2007), so it is unclear how much *M.*

graminicola growth is supported by apoplastic-derived nutrients in the latent or biotrophic phase. Keon *et al.* (2007) examined apoplast metabolite levels using ^{1}H-nuclear magnetic resonance spectroscopy and metabolomic analysis during symptomless and necrotic periods of infection. They found very little difference in metabolite levels between infected and uninfected leaves during the symptomless phase of growth (days 6-9). This could be because the plant is constantly replacing lost nutrients providing a renewable source of nutrient supply, although other studies have found quantitative differences. Later in infection, day 13, they found an increase in the quantity of plant-derived compounds available in the apoplast. Current evidence therefore suggests that there is little biotrophic feeding, perhaps even none, in the asymptomatic phase which precedes the host cell death.

The start of fungal growth coincides with nutrient release at the time of host cell death as reflected in the induction of a number of genes related to energy production at this time. Keon *et al.* (2007) showed that cellular features consistent with apoptosis were present in wheat cells dying following leaf infection by a compatible strain of *M. graminicola* including leakage of cytochrome *c* from the mitochondria into the cytoplasm, and the characteristic DNA laddering seen on agarose gels which indicates internucleosomal cleavage along with degradation of RNA. It was shown that cell contents leak into the apoplastic spaces during cell death, but only after the initial asympomatic phase.

The activation of PCD in the host appears to aid infection rather that restrict it. No form of PCD takes place in the incompatible interaction. The trigger and mechanism by which PCD is activated is as yet unknown, but the host plant must be able to recognise that it is undergoing pathogen attack. MAP kinases have been shown to initiate cell death during HR-mediated resistance in tomato infected with *C. fulvum* after a signal is received (Stulemeijer *et al.*, 2007). AtMPK3 and AtMPK6 have been implicated in stress and pathogen responses in tobacco and *Arabidopsis* (Asai *et al.*, 2002). In *Arabidopsis* both of these MPKs are activated downstream of a pathway induced by H_2O_2 but have also been suggested to trigger oxidative burst in tobacco

suggesting induced a positive feedback pathway. The wheat mitogen-activated protein kinase TaMPK3 has been implicated in the induction of the cell death pathway that produces STB symptoms (Rudd *et al.*, 2008). *TaMPK3* gene expression was induced during asymptomic colonisation by a compatible *M. graminicola* isolate, possibly indicating either non-specific PAMP recognition or highly specific manipulation of the host responses to initiate cell death. The TaMPK3 protein was post-translationally activated during the infection period coincident with the first appearance of disease symptoms and the initial commitment of wheat cells to programmed cell death (PCD). Finally, the TaMPK3 protein was found in increased concentrations from the time of macroscopic appearance of disease symptoms onwards. This pathway was only activated in a compatible *M. graminicola*-wheat interaction, not in incompatible interactions. This is the opposite of the pattern reported previously in interactions of plants with biotrophic pathogens. This is the first study to implicate mitogen-activated protein kinases in the STB-wheat pathosystem and in disease susceptibility towards necrotrophic pathogens. It highlights the emerging similarities between resistance signalling towards biotrophs with 'susceptibility' signaling towards necrotrophs.

It is not yet known how the MPK3 pathway is activated, but one model proposes stage-specific production of fungal toxins or elicitors which initiate MAP kinase activity and trigger host cell death (Kema *et al.*, 1996; Keon *et al.*, 2007). The dothideomycete wheat pathogens *Pyrenophora tritici-repentis* and *Stagonospora nodorum* generate necrosis-inducing toxins as components of their virulence arsenal (Ciufetti *et al.*, 2010; Deller *et al.*, 2011). Effectors have not yet been isolated from *M. graminicola* and their activity confirmed but three homologues of the *C. fulvum* effector gene *Ecp2* have been identified in the *M. graminicola* genome (Stergiopoulos *et al.*, 2010). The results of research on PCD and the MAPK pathway provides further support for the hypothesis that resistance may result from an interaction between, as yet unidentified AVR-R proteins while the aggressive host response seen in a compatible interaction may be the result of the fungus hijacking disease resistance signalling pathways (Hammond-Kossack & Rudd, 2008).

1.6.3 The role of reactive oxygen species in the resistant and susceptible

One of the first reactions to occur in defence is the accumulation of cytosolic calcium ions (Grant *et al.*, 2000). Cytosolic calcium ions have been strongly implicated as a post-recognition molecular switch, capable of producing responses in multiple downstream processes. One of the effects of this is to lead to an oxidative burst (Bolwell, 1999). The oxidative burst is critical to a highly complex signaling system that is activated upon recognition of pathogen avirulence signals (Lamb & Dixon, 1997). The oxidative burst rapidly generates and releases reactive oxygen species (ROS) which include superoxide, the hydroxyl radical and hydrogen peroxide (H_2O_2).

ROS, in particular hydrogen peroxide, H_2O_2, are proving to be an important factor in the mechanisms regulating STB. H_2O_2 accumulated in a resistant cultivar early in infection. This initial response was expected as a precursor to a hypersensitive response but no necrotic symptoms were seen in a resistant interaction. In a susceptible cultivar, much larger quantities of H_2O_2 accumulated later in infection than in earlier stages, when the pathogen started to increase its rate of hyphal growth (Shetty *et al.*, 2003). It is possible that the accumulation of H_2O_2 aids pathogen growth but H_2O_2 may only be indirectly necessary for pathogenicity; ROS have been shown to have antimicrobial effects. Treating resistant tomato plants with H_2O_2 scavengers after infection with virulent *Cladosporium fulvum* increased fungal growth, showing a critical role for ROS in limiting colonisation (Borden & Higgins, 2002). A direct antimicrobial effect on *M. graminicola* has been demonstrated by treating cultures with H_2O_2. Shetty *et al.* (2007) demonstrated that H_2O_2 inhibited the growth of *M. graminicola in vitro*, and hindered pathogen growth *in planta* at both early and late stages of infection. Genes encoding some ROS scavenging enzymes are highly induced in *M. graminicola* during symptomatic infection stages (Keon *et al.*, 2007). Studies of other necrotroph-plant interactions suggested ROS can aid development of the pathogen (Govrin & Levine, 2000) whilst ROS were required for limiting the growth of an endophyte (Tanaka *et al.*, 2006). This indicates a potential regulation role for ROS in the stealthy growth of *M. graminicola* during the early stages of

infection. It remains unclear as to whether the H_2O_2 observed is host or pathogen generated. If it is generated from the pathogen, there may be a role in inducing or regulating cell death which aids pathogen development. Kovtun *et al.* (2000) demonstrated that H_2O_2 activates a MAPK kinase cascade, which ultimately ends in a stress response. H_2O_2 can also act as a signalling molecule regulating PCD, although little is known about how the molecule is perceived (Apel & Hirt, 2004).

1.6.4 Induction of pathogenesis-related proteins

Pathogenesis-related proteins (PRs) are made by the host plant and are only induced after an interaction by a pathogen or related situation eg. mechanical wounding. The HR triggered during infection with a biotrophic pathogen causes localised necrosis. Within a few hours of this necrosis developing the plant will express defence genes both locally and systemically (Van Loon, 1985). PR proteins can be induced by H_2O_2 which also triggers HR. β-1,3-glucanases (PR2) and chitinases (PR3) are PR proteins that act against fungal cell walls. These enzymes act to degrade the cell wall which produce monomers of chitin and glucans, which can in turn act as PAMPs triggering further defence responses perhaps involving PR proteins. Although the many PR proteins are well characterised, such as the chitinases and β-1,3-glucanases, many still have an unknown function, PR1 being an example. PR1s are induced to high levels in many pathogen interactions often acting as a marker of systemic acquired resistance (SAR). PR1s role in inducing salicylic acid mediated resistance is well recognised in dicotyledenous plants, but the function of the proteins are unclear. In interactions with *M. graminicola* wheat *PR1, PR2, PR3* and *PR9* genes have all been induced (Adhikari *et al.*, 2007; Ray *et al.*, 2003; Shetty *et al.*, 2009). Different studies show differing levels of expression in incompatible and compatible interactions. The expression of *PR* genes and their role in the interaction between wheat and *M. graminicola* to host resistance will be returned to in chapter 4.

The *M. graminicola*-wheat interaction is not related closely to any other model pathosystem and therefore defining the differences between incompatible and

compatible interactions is important to assist with the selection of STB resistant material. As yet, few studies have been carried out that attempt to identify host genes involved in the resistance response but those that have have indicated that defence responses in wheat are activated before the fungus has even penetrated the host, 12 hours after inoculation (Adhikari *et al.*, 2007; Ray *et al.*, 2003). Most of the genes identified as differentially expressed during infection are PR genes, although a few others, possibly involved with signalling or regulatory pathways, have been investigated (Adhikari *et al.*, 2007).

In plant diseases in general, *PR* gene transcript accumulate during both incompatible and compatible interactions, but earlier and more strongly in incompatible responses (Boyd *et al.*, 1994b; Ray *et al.*, 2003). During the interaction with *M. graminicola*, wheat defence-related genes such as *chitinase* and *PR1,* were strongly up-regulated at an early stage (Ray *et al.*, 2003). Adhikari *et al.* (2007) proposed that the expression level of *PR1,* 12 hours after inoculation could distinguish resistant and susceptible lines in segregating mapping populations, as there was little change in expression of any of the defence-related genes tested in two susceptible cultivars. However, later timepoints (after 6 dai), revealed no differences in *PR1* levels between susceptible and resistant cultivars. Shetty *et al.* (2009) showed that although *β-1,3-glucanase* and *chitinase* are slightly but significantly upregulated early in an incompatible interaction, they are strongly upregulated in a compatible interaction from 9 dai. The results of these studies have evidently been variable and although the number of such studies is still limited, they indicate that resistance to *M. graminicola* may be complex.

There may be two stages of defence at early and later stages of infection (Adhikari *et al.*, 2007) once during initial recognition and again later, when the fungus starts to grow within the leaf. Some studies suggest that genes other than known PR genes are upregulated during the wheat-*M. graminicola* interaction (Adhikari *et al.*, 2007; Ray *et al.*, 2003) but the mechanism leading to resistance or susceptibility is as yet unknown. Rudd *et al.* (2008) proposed that, in compatible interactions, there

appears to be an active response to *M. graminicola* involving the wheat TaMPK3 as described previously. The molecular basis of resistance has an absence of the MPK3 activation that is seen in other fungal pathogen-host incompatible interactions e.g. *Cladosporium fulvum* on *Cf-9* tomato where the MPK3 homologue is activated (Romeis *et al.*, 1999).

In some experiments, the pattern of gene expression in wheat in response to *M. graminicola* has contrasted sharply with that expected from prior studies of plant infection by biotrophic pathogens, while the lack of consistency between experiments points to significant genotype-by-environment interaction in defence mechanisms. Further studies of gene expression in both incompatible and compatible interactions using different variety and isolate combinations may elucidate what appears to be a complicated pattern. The decrease in the cost of global gene expression studies may enable this useful tool to assist in the identification of quantifiable markers and perhaps novel genes specific to compatible or incompatible interactions.

1.6.5 Host physical modifications in resistant and susceptible interactions.

In some systems, modifications may be made to the cell wall that inhibits the growth of the pathogen. These defences include the formation of papillae, callose deposition, accumulation of phenolic compounds and cell wall cross linking. Cross-linking of cell walls has not been observed in *M. graminicola* interactions, but has been observed in tomato plants infected with *C. fulvum* in both incompatible and compatible interactions (although at a significantly greater level in the incompatible), especially in areas showing H_2O_2 accumulation (Borden & Higgins, 2002). It is hypothesised that cell wall responses may restrict the flow of nutrients from host cells suppressing growth, although this is most likely to be an important defence against cell penetrating pathogens. Callose deposition and the cross-linking of cell walls may restrict the flow of nutrients to the apoplast and perhaps confine the pathogen to a limited area (Borden and Higgins, 2002).

An increase in autofluorescence is an intrinsic property of cells, indicative of a defence mechanism (Christiansen & Smedegaard, 1990). Autofluorescence of wheat cells has been seen in incompatible interactions with *M. graminicola* (Cohen & Eyal, 1993; Duncan & Howard, 2000; Shetty *et al.*, 2003). It can be seen in epidermal cell walls and subsequently the mesophyll, accumulating approximately 48 hours post inoculation (Cohen & Eyal, 1993). Duncan and Howard (2000) also detected autofluorescence in these areas although only after a period of 6 dpi. They noted that after 11 dpi these autofluorescing cells became necrotic. Autofluorescent compounds also strongly accumulate in the compatible interaction as lesions develop (pers. comm., Jason Rudd, Rothamsted Research, Harpenden, UK).

Cohen and Eyal (1993) proposed that suppression of hyphal growth can partly be explained by the production of fluorescing materials, indicative of a defence mechanism, early in infection. If the fungus is retarded in its ability to accumulate hyphal biomass in the substomatal cavity, it cannot produce pycnidia. Shetty *et al*. (2003) also suggest that the accumulation of biomass in the initial phase of development is essential for initiating its reproductive stage. This would fit with the hypothesis that the fungus is not a successful biotroph and during an incompatible interaction the fungus cannot obtain enough nutrients to grow within the plant.

1.7 Objectives of this research

The objectives of this research were to gain an understanding of how the host responds to infection by *M. graminicola*. The specific aims were:

- To investigate how the presence of *M. graminicola* on the host affects the host's interactions with a biotrophic pathogen (Chapter 3).

Crop species are attacked by more than one pathogen in the field, which may be both biotrophic and necrotrophic. Because of their different lifestyles, a trade-off may occur that affects the ability of the pathogens to cause disease. The biotrophic

pathogen, *Blumeria graminis* f.sp. *tritici,* was used as it is a comparatively tractable biotrophic pathogen.

- To test how selected wheat defence-related and other genes are involved in resistance towards *M. graminicola* and if any could be used as a marker for resistance during the early stages of selection in a breeding programme. In particular, to investigate if the TaMPK3 protein is a marker for susceptibility in different variety/isolate interactions (Chapter 4).

Breeding for resistance to STB is an increasingly important target. As yet, there is no fast way to select for resistant lines early in the breeding cycle. These experiments aimed to identify a defined wheat set of genes that are associated with either a resistant or a susceptible interaction. The presence of TaMPK3 has been investigated by Rudd *et al.* (2008), who demonstrated that the protein accumulates during a compatible interaction in two variety/isolate combinations. Here, other varieties and isolates were studied to test if this protein is consistently associated with a compatible interaction.

- To develop a method for staining *M. graminicola* reliably when it is in the apoplast to enable the timing of the resistance response to be identified using microscopic techniques (Chapter 5).

There is not yet a reliable way to stain *M. graminicola* once it has entered the apoplast. This has meant that although the compatible interaction has been studied in depth using scanning electron microscopy, less is known about the incompatible interaction. It was hoped that, by developing a straightforward method to reliably stain the pathogen during its latent phase, the timing and mode of resistance of a gene-for-gene interaction could be identified.

2

Materials and methods

2.1 Fungal Material

2.1.1 *Mycosphaerella graminicola*

The *M. graminicola* isolates used throughout the experiments were IPO323 (Netherlands) and IPO88004 (Ethiopia). IPO323 is avirulent on lines containing the *Stb6* (Brading *et al.*, 2002) resistance gene and IPO88004 is avirulent in lines with *Stb15* (Arraiano & Brown, 2006). The isolates were stored at -80 °C. Spores for plant inoculation were grown on YPD+ agar plates (2% bacto agar, 2% peptone, 1% yeast extract, 2% glucose at pH 5.8) for 4-7 days at 18 °C with blacklight blue UV light at 350nm. The leaves were inoculated evenly with a fungal spore solution at a density of 10^7 spores per mL of water containing 0.1% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, MO,USA) using a swab stick with a cotton sterile tip (Fisher Scientific, Loughborough, Leicestershire, UK). Spore concentration was assessed using a Mod Fuchs Rosenthal counting chamber (Hawksley, Lancing, UK). The number of spores in four of the smallest squares were counted and the average number taken. This was multiplied to give an estimate of spore concentration using the equation:

$$\frac{\text{Quantity of inoculum required} \times 10^7}{(\text{Average number of spores}) \times 16 \times 5000}$$

Control leaves were mock-inoculated with water containing 0.1% Tween 20.

2.1.2 *Blumeria graminis* f.sp. *tritici*

The *B. graminis* f.sp. *tritici* (*Bgt*) isolates used were JIW11 and JIW48. Isolates were kept on the susceptible wheat variety Cerco. For inoculations, fresh spores were produced by tapping spores off the host leaves two days before the isolate was needed so new spores were ready for the inoculation. Inoculations were carried out using

aluminium inoculation towers (Boyd *et al.*, 1994b) by blowing the spores over the plants and leaving them to settle.

2.2 Plant material

For plant infection the second leaf of 17 day old seedlings at growth stage 12 on the Zadok *et al.* (1974) decimal code, was attached adaxial side up to Perspex sheets using double-sided tape (Keon *et al.*, 2007). Plants inoculated with *M. graminicola* were placed in high relative humidity in the dark for 48 hours. The plants were kept at 18 °C in the light for 16 h and 12 °C in the dark for up to 21 days.

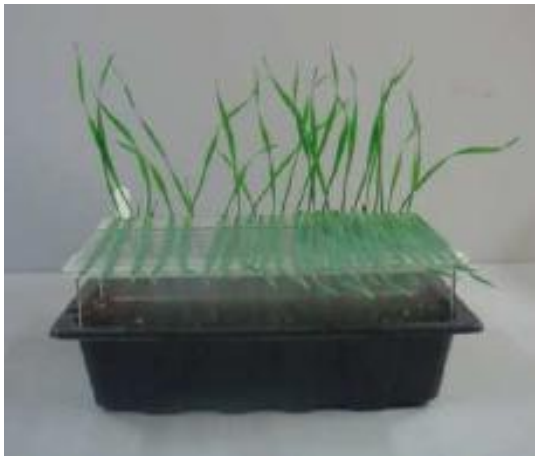


Figure 2.1 Perspex sheets used to attach second leaves of seedlings for inoculation.

Inoculated leaves used for RNA, DNA or protein extraction were cut off the plants and placed immediately into liquid nitrogen and then stored at -80°C before further processing.

The varieties used throughout experiments were Longbow, Flame, Arina, Poros, Cadenza, Avalon and Courtot. The known resistance genes that each variety has are shown in Table 2.1. *Stb6* and *Stb15* were chosen as they are the two most common STB resistance genes in the current European germplasm (Arraiano & Brown, 2006). Also IPO323, which is avirulent on *Stb6* is the isolate that has been sequenced (Goodwin *et al.*, 2011).

The varieties Longbow and Flame were used for experiments with *Bgt*. Longbow has the resistance gene *Pm2* (Bennett & Van Kints, 1982) and Flame has *Pm4b* (Slater & Mitchell, 1995). The *Bgt* isolates used were JIW11 and JIW48. JIW11 is avirulent on both varieties and JIW48 is virulent on both varieties.

Variety	***Stb6***	***Stb15***
Arina	Yes	Yes
Avalon	No	Yes
Cadenza	Yes	No
Courtot	No	No
Flame	Yes	No
Longbow	No	Yes
Poros	Yes	Yes

Table 2.1. List of wheat varieties and whether or not they have *Stb6* and *Stb15* (Arraiano and Brown 2006).

2.3 Detached leaf boxes

The bottom of rectangular clear polystyrene boxes were filled with 50 mL 1% water agar with 10% (v/v) benzimidazaol (from 1 g L^{-1} stock solution). The leaves were placed in the box so the cut ends were held in place by the agar (Arraiano *et al.*, 2001a).

2.4 Interactions between *B. graminis* f.sp. *tritici* and *M. graminicola* and the host.

Plants were attached to Perspex sheets as described in section 2.2 and inoculated with isolate IPO323 with the modification that the inoculum did not contain Tween 20. Tween 20 was found to inhibit the growth and development of mildew. Control leaves were mock inoculated with water only. Leaves were then placed into detached leaf boxes as described in section 2.3. The leaves were inoculated with mildew spores under a settling tower as described in section 2.1.

Details of the experimental designs of experiments involving interactions between *Bgt* and *M. graminicola* are described in section 3.2.

2.4.1 Staining mildew with Aniline Blue

Aniline Blue 0.1 % made up in lactoglycerol (1:1:1 solution of lactic acid, glycerol and water) was used to visualise fungal spores. Leaves were cleared using 75 % ethanol until all chlorophyll was visibly removed. The leaves were then rinsed in water and placed in a storage solution of lactoglycerol. The leaves were placed on a glass slide and the stain pipetted onto them before visualisation.

2.4.2 qPCR determination of mildew biomass on infected leaves

Three boxes of Longbow leaves, two replicates in each box, were inoculated with and without *M. graminicola* and then dual inoculated after 6 days with the virulent *Bgt* isolate, JIW48. 10 days after *Bgt* inoculation, DNA was extracted and the amount of mildew DNA quantified using a Taqman probe assay (Fraaije *et al.*, 2006).

To test that isolate JIW48 contained the same cytochrome *b* gene fragment that is amplified by the primers, the fragment was cloned and sequenced. DNA was extracted from leaves with visible sporulating mildew colonies of isolate JIW48 using a Qiagen DNeasy kit (Qiagen, Valencia, CA, USA). A 136bp fragment of the cytochrome *b* gene was amplified with primers PMR1 (5'-TTACTGCATTCCTGGGTTATGTATTG-3') and PMS1 (5'ACAGAGAAACCTCCTCAAAGGAACT-3') (Fraaije *et al.*, 2006). The fragments amplified were cloned into pGEM-T easy vector (Promega, Madison, Wi, USA) following the manufacturers protocol. The vector was transformed into OneShot TOP10/P3 competent cells (Invitrogen, Carlsbad, CA, USA) following the manufacturers protocol using a heatshock transformation procedure. Blue/white colony selection was used to select for transformed cells, which were purified using Qiagen MinElute plasmid purification kit (Qiagen, Valencia, CA, USA) and sent for sequencing at The Genome Analysis Centre, Norwich, UK. Sequences were aligned using WebPrank using the default settings (http://www.ebi.ac.uk/goldman-srv/webprank/) to four known *Bgt* cytochrome *b* sequences from different isolates available on the NCBI database (http://www.ncbi.nlm.nih.gov): Fel08 (AF343442.1), Fel12 (AF343441.1), JAS501 (AJ293567.1) and W26 (AJ293566.1).

DNA was extracted from the leaf samples as above. Total DNA was quantified on a picodrop and diluted so each sample contained 50 ng/ µL. For reaction mixture for qPCR contained 0.5 uM foward primer, 0.3 uM reverse, 0.1 µM of 5'-CY5/3'-BHQ2 - labelled probe (5'-CTTGTCCTATTCATGGTATAGCGCTCATTAGG-3') and 50 ng of DNA sample and 10 µL iQ supermix (Bio-Rad, Hemel Hempstead, Herts, UK) to a volume of 20 µL. A standard curve was produced by plotting known amounts of DNA against Cq values. Reactions were cycles were for 2 min at 50 °C, 2 min 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The increase in fluorescence from the probe was recorded at 60 °C during every cycle.

2.4.3 Gene expression of TaMPK3 in leaves inoculated with a compatible and incompatible mildew isolate.

17 day old seedlings of Longbow were attached to Perspex trays (section 2.2) and inoculated with either the compatible isolate JIW48, or with the incompatible isolate, JIW11, under settling towers. Control seedlings had no inoculation. Plants were placed in a growth cabinet at 17 C light/ 12 C dark cycle. Three leaves were excised at 1, 3, 7, 10 and 14 days after inoculation. Two replicates of the experiment were carried out. RNA extracted using Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) as described in 2.5.1 and cDNA was synthesised as described in section 2.5.2. The TaMPK3 primer set used was foward 5'-TACATGAGGCACCTGCCGCAGT-3' and reverse 5'-GGTTCAACTCCAGGGCTTCGTTG-3' (described in chapter 4, table 1). qPCR was carried out as described in 2.5.3, with the exception that Sigma SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma-Aldrich, St. Louis, MO,USA) was used instead. The PCR reaction cycle was 2 minutes 90 °C followed by 40 cycles of denaturation at 95 °C for 30 sec; annealing at 56 °C for 30 sec and extension at 72 °C for 30 sec.

2.5 Expression Analysis of wheat genes

The varieties Longbow, Flame, Avalon, Cadenza, Arina and Poros were inoculated with the isolate IPO323. The varieties Longbow and Courtot were inoculated with isolate

IPO88004. Three leaves were sampled for each interaction on the following days after inoculation: 0.5, 1, 3, 7, 10 and 14. Control leaves were also sampled at each timepoint. Three biologically replicated experiments were carried out.

2.5.1 RNA isolation

Total RNA was isolated from frozen leaf tissue using either the Tri-reagent procedure (Sigma-Aldrich, St. Louis, MO,USA), following the supplier's protocol and using the additional suggested step for polysaccharide-containing tissues or Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), following the suppliers instructions.

2.5.2 cDNA synthesis

A DNase treatment was carried out on the extracted RNA using Turbo DNA-free (Ambion, Austin, TX, USA), following the supplier's Rigourous procedure, which is designed to remove DNA from samples containing >2 µg DNA/50 µL RNA To test that all genomic DNA had been removed from the RNA sample, each sample was subjected to real-time PCR analysis using a reference gene set of primers designed for cDNA (table 1.2). The total quantity of RNA was quantified using a Picodrop100 (Picodrop Ltd, Saffron Walden, UK). 1000 ng of total RNA was converted to cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol using random hexamers.

2.5.3 Quantitative Real-time PCR

Each cDNA sample was diluted 1:20 in nuclease-free water. Quantitative Real-time–PCR (qRT-PCR) was performed using a CFX96 detection system (Bio-Rad, Hemel Hempstead, Herts, UK), in white plates with optically clear seals (both Thermo Scientific, Waltham, MA, USA). Each reaction contained 5 µL of the diluted cDNA and 12.5 µL Brilliant II SYBR® Green master mix (Agilent Technologies, Edinburgh, UK), with 500 nM each of the left and right primers (unless otherwise specified) to a total volume of 25 µL. All PCR reactions were carried out using the following cycle: 95 °C

for 10 minutes; followed by 40 cycles of denaturation at 95 °C for 30 sec; annealing at 56 °C for 30 sec and extension at 72 °C for 30 sec. Immediately after this a melt curve analysis was carried out by ramping from 65 °C to 90 °C. All samples had two technical repetitions.

Primer efficiencies for the genes of interest were tested for each primer pair using a dilution series from 1:10 to 1:10000 made from a mixture of cDNA samples. Amplification values ranged from 1.89 to 2.15 giving efficiencies of between 90 and 115 %. The efficiency was assessed using the equation: $-1+10^{(-1/slope)}$ available on the agilent technologies website (http://www.genomics.agilent.com).

Reference Gene	Left Primer (5'-3')	Right Primer (5'-3')	Reference
Ta Elongation factor	TGGTGTCATCAAGCCTGGTATGGT	ACTCATGGTGCATCTCAACGGACT	Coram *et al.* 2008
Hv GapDH	CCTTCCGTGTTCCCACTGTTG	ATGCCCTTGAGGTTTCCCTC	McGrann *et al*. 2009
Ta Ubiquitin	CCTTCACTTGGTTCTCCGTCT	AACGACCAGGACGACAGACACA	van Riet *et al.* 2006

Table 2.2. Reference genes used for normalising Cq values

Quantification cycle (Cq) values of three reference genes (table 2.2) were checked for stability using the geNORM software (Vandesompele *et al.*, 2002) (http://medgen.ugent.be/ ~jvdesomp/genorm/). The reference genes in all experiments were found to be stable.

2.5.4 Meta-analysis of genes of interest

Predicted nucleotide sequences of the genes of interest were retrieved from NCBI (http://www.ncbi.nlm.nih.gov). Probe sets representing the genes of interest on the Affymetrix Wheat GeneChip were retrieved by using BlastN and the coding sequences of the proteins used to query the microarray (Wise *et al.*, 2007). Wheat Affymetrix data sets for experiments investigating wheat-pathogen interactions were downloaded from PlexDB under the accessions TA9 (Coram *et al.*, 2008b), TA11 (Coram *et al.*, 2008a), TA24 (Tufan *et al.*, 2009), TA25 (Bozkurt *et al.*, 2010), TA31 (Desmond *et al.*,

2008) and TA32 (Bolton *et al.*, 2008). Analysis of the data sets was carried out by Tufan *et al.* (2012) in R using the package AffylmGUI (Wettenhall *et al.*, 2006). Differential expression was calculated using linear models and an Empirical Bayes moderated *t*-statistic (Smyth, 2004). In all cases contrasts were made between pathogen-inoculated and mock-inoculated control samples. Differential regulation of probe sets were assessed based on expression levels and the data exported in to a tab delimited file. The comparisons between treatments and probe sets were analysed in Cluster 3.0 (Eisen *et al.*, 1998) using a Euclidean distance matrix and complete linkage clustering. The results were viewed in Treeview v.1.0.13.

2.6 Wheat mitogen-activated protein kinase 3 analysis

As for gene expression analysis, the varieties Longbow, Flame, Avalon, Cadenza, Arina and Poros were inoculated with the isolate IPO323. The varieties Longbow and Courtot were inoculated with isolate IPO88004. Three leaves were collected for protein analysis on the following days after inoculation: 1, 3, 7, 10, 11, 14, 15, 16 and 17. Control leaves were taken at 1, 10 and 17 days after inoculation.

2.6.1 Protein Extractions

Three leaves were collected on various days after inoculation into liquid nitrogen and stored at -80 °C until extraction. Protein was extracted by grinding frozen cells in extraction buffer (37.5 mM Tris-HCl pH 7.4, 112.5 mM NaCl, 22.5 mM EGTA, 0.15% v/v Tween 20 , 1.5 mM NaF, 0.75 mM Na-Molybdate, 1.5 mM DTT, 0.75 mM PMSF, 15 µg /ml Leupeptin, 15 µg/ml Aprotinin, 22.5 mM β-glycerophosphate) followed by centrifugation at 23000 *g* for 20 min at 4 °C.

A Bradford assay was performed to quantify the concentration of protein in each sample using Biorad Bradford protein assay reagent (Bio-Rad, Hemel Hempstead, Herts, UK) and comparing with a Bovine Serum Albumin standard of 2 mg mL^{-1} (Sigma-Aldrich, St. Louis, MO,USA). Readings were taken using a Biophometer (Eppendorf, Hamburg, Germany) at 595 nm, the wavelength at which the bound

formed of the reagent is absorbed. Samples were mixed with a loading dye consisting of 5 % v/v 2-Mercaptoethanol, 250 mM Tris-HCl pH 6.8, 10% w/v SDS, 30% v/v glycerol, and bromophenol blue, so that all samples contained an equal amount of protein. Samples were heated to 90 °C for at least five minutes to solubilise the protein and then spun for 5 minutes at maximum speed before use. Samples were stored at -20 °C.

2.6.2 MAPK-Specific Antisera

Antisera used to detect TaMPK3 were those reported by Rudd *et al*. (2008)

2.6.3 Western Blotting

Approximately 120 µg of protein was separated on 10 % SDS-PAGE gels and wet blotted onto Hybond ECL nitro cellulose membrane (GE healthcare Life Sciences, Little Chalfont, Bucks, UK). Membranes were blocked overnight at 4 °C in TBS-Tween pH 7.3 (20mM Tris-HCl, 137mM NaCl, 0.1 % V/V Tween 20). The MAPK-specific antibody TaMPK3-N (affinity purified) (Rudd *et al*. 2008) at 1:500 dilution was used against leaf extracts at room temperature for 90 minutes. After the membranes were washed five times, chemiluminescent detection using Amersham ECL Plus Western Blotting Detection Reagents was carried out in accordance with manufacturer's instructions (GE Healthcare Life Sciences, Little Chalfont, Bucks, UK).

3

Interactions between pathogens and the host

3.1 Introduction

Wheat crops are attacked by many different pathogens. Two lines of defence are available to control them, fungicide use and breeding for resistance. Powdery mildew is well controlled in the UK with a combination of resistance genes and fungicides, particularly carboximide and specific anti-mildew compounds (HGCA, 2011), so although mildew is the second most common disease in the UK, disease severity remains low (www.cropmonitor.co.uk). Septoria tritici blotch (STB) is the most common disease of wheat in the UK, with 53% of samples showing the disease in 2010 (www.cropmonitor.co.uk). Efficacy of fungicides is declining for control of this disease as the population develops insensitivity to both triazoles and strobulurins (Fraaije *et al.*, 2005a; Fraaije *et al.*, 2007). Breeding is improving the level of resistance to disease, but not to the extent that fungicides can be dispensed with, while carboximides and modern triazoles still give good control of STB. As two of the major pathogens of wheat in the UK which often infect the same plants, how they interact with each other is of great interest. It is of little value to breeders if a variety has good resistance to one disease but the resistance against another is poor. Investigating how varieties respond to infection with more than one pathogen will assist efforts to breed varieties resistant to multiple pathogens.

Classical *R*-gene mediated resistance allows rapid recognition of the pathogen and a hypersensitive response (HR), which includes an oxidative burst and programmed cell death (PCD) of surrounding cells, limiting the pathogen's access to nutrients and thereby killing it. Most pathogens are adapted to cause disease only on a limited number of plant species, or sometimes even just one species. Each plant usually has several well-adapted pathogens that are capable of initiating effector-

triggered immunity (ETI). The HR is very effective against biotrophic pathogens, as it specifically kills the cells under attack by the pathogen, rendering them unable to obtain nutrients. The HR is not effective against necrotrophic pathogens, as they are able to exploit the HR by obtaining nutrients after the loss of cellular membrane integrity, enabling them to cause disease (Govrin & Levine, 2000). No HR response is seen during an incompatible interaction between wheat and *M. graminicola*, although during a compatible interaction a PCD response occurs which aids infection (Keon *et al.* 2007).

It has been proposed that there is a biological cost to resistance (Agrawal *et al.*, 1999; Baldwin, 1998), which when breeding crop plants such as wheat must be taken into consideration. Smedegaard-Petersen and Stølen (1981) showed that infection of barley with an avirulent *B. graminis* f.sp. *hordei* reduced the yield of the plants. Felton and Korth (2000) reviewed how trade-offs occur between pathogen and herbivore resistance and how this is mediated by the salicylic acid (SA) and jasmonate (JA) pathways. Biotrophic pathogens often induce SA signalling whereas wounding responses to herbivory induce a JA response. Cross-talk between SA and JA pathways can lead to a trade-off between responses, for example when tobacco plants are infected with tobacco mosaic virus production of systemic SA is induced and the plants are unable to produce a normal wound response because JA production is inhibited (Preston et al 1999). These pathways also mediate resistance between pathogens with different modes of infection (Spoel *et al.*, 2007) and trade-offs affect plant defence responses controlling these resistances. The biotrophic pathogen, *Pseudomonas syringae* pv. t*omato (Pst)*, induces a SA-mediated defence response which suppresses JA-mediated signalling. Leaves that were inoculated with *Pst* and a normally avirulent *Alternaria brassicola*, a necrotrophic pathogen, showed susceptibility towards the necrotroph because the SA suppressed the JA-dependent defence (Spoel *et al.*, 2007). This provides evidence that interactions between biotrophs and necrotrophs are antagonistic.

The research presented here investigated levels of *TaMPK3* gene transcript accumulation to test the hypothesis that a rise in TaMPK3 due to a compatible interaction with a necrotrophic pathogen will result in a loss of susceptibility to a normally virulent biotrophic pathogen, such as *Blumeria graminis* causing it to become avirulent. It is known that a compatible interaction between wheat and *M. graminicola* increases the level of TaMPK3 (Rudd *et al*., 2008 and chapter 4), a protein which has previously been shown to accumulate during *R*-gene-mediated resistance responses in tomato (Stulemeijer *et al.*, 2007), tobacco expressing tomato *Cf9* (Romeis *et al.*, 1999) and tobacco infected with Tobacco Mosaic Virus (Zhang & Klessig, 1998) for an overview see Pedley and Martin (2005). Therefore, it can be hypothesised that an increase of TaMPK3 during a compatible interaction between wheat and *M. graminicola* is the same response as seen during an incompatible interaction between wheat and *B. graminis.* The rise may be enough to change the response of a normally virulent *B. graminis* isolate to an incompatible one.

Few studies have investigated how infection by one pathogen has an effect on infection by another pathogen, although the question has been posed for a number of years. The first recorded interaction between *S. tritici* and *B. graminis* f.sp. *tritici* was by Brokenshire (1974) under glasshouse conditions who described an increase in *S. tritici* in the presence of mildew. Cooper *et al.* (2008) found that infection of *Arabidopsis* with a virulent *Albugo candida*, a biotroph, can suppress a plant's resistance to avirulent, biotrophic *Hyaloperonospora arabidopsis*. It appears to be linked to the ability of *A. candida* to suppress cell death of the *Arabidopsis* host cells, thereby inhibiting HR in response to a normally avirulent biotroph. In contrast, Aghoum and Niks (2011) investigating interactions between a virulent *Puccinia hordei* and *B. graminis* f.sp. *hordei* (*Bgh*) isolates on barley, found that pre-inoculation with the rust isolate induced an increased resistance to both avirulent and virulent isolates of *Bgh* by preventing haustorium formation. Virulent and avirulent isolates of the same pathogen can also induce either accessibility or inaccessibility. Lyngkjaer and Carver

(1999) found that successful penetration of an inducer appressorium of *B. graminis* f.sp. *hordei* into barley epidermal cells rendered the cells highly accessible to future attacks by other isolates. Conversely, failed attacks by an inducer led to subsequent failed attacks by secondary inoculations. Other studies have investigated whether the use of mycelial extracts of various fungi and oomycetes could act as a form of biological control against a fungal pathogen (Haugaard *et al.*, 2001). Colony formation of mildew could be reduced using some extracts as a pretreatment, but the mechanism behind this is yet to be fully understood; it appears that there are different modes of action, both a direct antifungal and an induced resistance response (Haugaard *et al.*, 2002). There are many possible interactions between pathogens inoculated onto the same leaf; for interactions on crop plants, knowing how the pathogens behave in the presence of each other may assist with crop protection and breeding.

The aim of this research was to investigate how infection of wheat with a necrotrophic pathogen affects the plant's response to inoculation with a biotrophic pathogen. *M. graminicola* has a long latent period before symptoms appear on the leaf. It enters the leaf through the stomata and remains within the substomatal cavity for at least 7 days after inoculation (dai) before the onset of host cell collapse and growth of the fungus in the mesophyll layer between 7 and 14 dai. Pycnidia are formed through the stoma after at least 14 days. *Blumeria graminis* f.sp. *tritici* (*Bgt*) grows on the epidermis infecting cells by forming appressoria by approximately 12 hours after inoculation (hai). Haustoria are formed from 24 hai onwards within host cells, enabling the fungus to feed. Except for the haustoria, which occupy the epidermal cells, the fungus grows on the surface of the leaf throughout its lifecycle. Conidiophores are produced on the surface of the leaf from 5-10 dai (figure 3.1). The objective of this study was to investigate whether *M. graminicola* can inhibit *Bgt* from infecting. The hypotheses are that in a compatible interaction between *M. graminicola* and the wheat host, cell death caused by a virulent *M. graminicola* isolate will inhibit a

virulent *Bgt* isolate from growing on the same leaf and that the avirulent isolate will

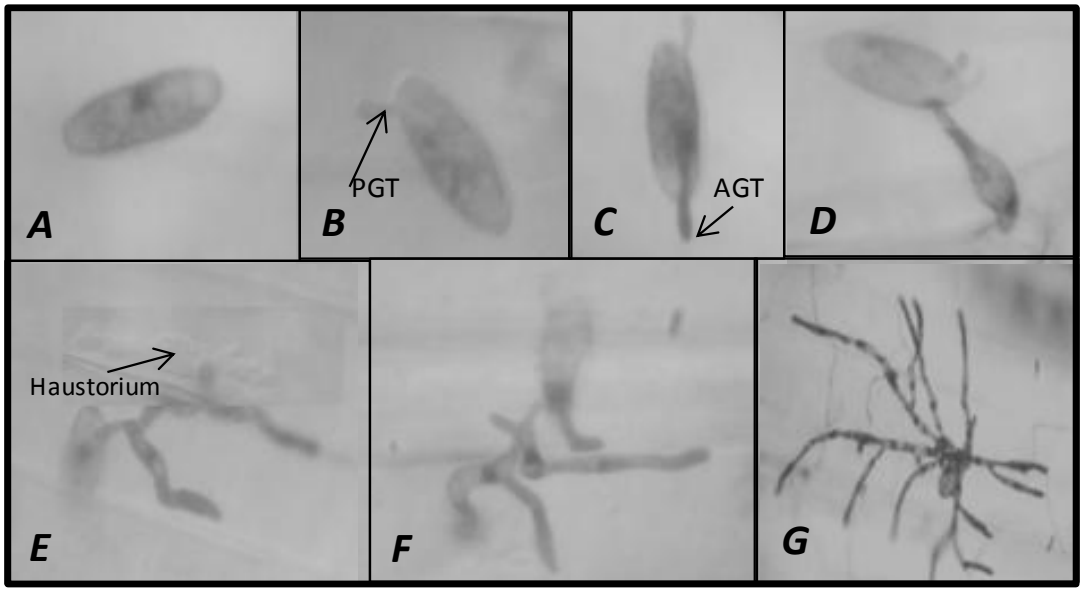


Figure 3.1 Stages of mildew spore growth and development: A.ungerminated spore B. primary germ tube C. appressorial germ tube D. appressorium E. haustorium F. elongating secondary hyphae G. colony

remain avirulent. During an incompatible interaction between *M. graminicola* and the wheat it is predicted there will be an increase in the susceptibility of the leaf to an avirulent *Bgt* isolate but virulent *Bgt* will remain equally virulent.

3.2 Experimental Design and Methods

A system was designed to test infection of two pathogens of wheat that could be carried out repeatably under experimental conditions. It was not feasible to do this with whole plants as it was difficult to get the conditions conducive for both pathogens to infect the leaves. Additionally, powdery mildew disease is easily spread causing cross contamination to be a problem. A system of using detached leaf boxes (Arraiano *et al.*, 2001a) was investigated and found to allow manipulation of many leaves without risk of mildew contamination, whilst allowing both pathogens to grow.

Leaves were inoculated with the required *M. graminicola* isolate and placed into detached leaf boxes. Each box contained two replicates of each variety plus isolate or

control combination. Boxes were subsequently inoculated with the required *Bgt* isolate using settling towers (Boyd *et al.*, 1994b).

Details of materials and methods used throughout the experiments can be found in chapter 2, including details of inoculations, culture maintenance and growing conditions for plant material.

3.2.1 Assessment of the effect of *M. gramnicola* on the development of avirulent or virulent isolates of *B. graminis* f.sp. *tritici*

Leaves of both Longbow and Flame, each inoculated with and without *M. graminicola,* were placed in detached leaf boxes with the mildew susceptible variety, Cerco. Flame has the resistance gene *Pm4b* and Longbow carries *Pm2.* The *Bgt* isolate, JIW11 is avirulent towards both of these genes and JIW48 is virulent on both of these genes. Both interactions were also tested to ensure that the isolate virulence was as expected before any experimental tests were carried out. The leaves were then inoculated with JIW11 or JIW48 at 2, 5, 7, or 10 days after inoculation with *M. graminicola.* Photographs were taken to compare the development of colonies on leaves inoculated with and without *M. graminicola.*

3.2.2 Dosage effect of *M. graminicola* spores on the number of mildew colonies formed by virulent *B. graminis* f.sp. *tritici*

Leaves of Longbow were inoculated on Perspex trays, with decreasing concentrations of the *M. graminicola* isolate, IPO323. The dilution series was the same as in Keon *et al.* (2007) starting at 10^7 spores per mL and diluting the inoculum by 2.5 times for each new dilution to give the following dilution series: 10^7, $4x10^6$, $1.6x10^6$, $6.4x10^5$, $2.6x10^5$, $1x10^5$, $4.1x10^4$, $1.6x10^4$, $6.6x10^3$ and $2.6x10^3$ spores per mL. The leaves were placed into detached leaf boxes. Each detached leaf box contained a leaf of Cerco and 10 leaves encompassing the whole dilution series to give four individual boxes. Three of the replicate boxes were inoculated individually but on the same

date, with the virulent *Bgt* isolate JIW48 under settling towers, 4 days after inoculation with *M. graminicola*. One box was left uninoculated as a control. Colony formation was assessed by counting visible colonies under a 2x magnifying lens after 8-10 days.

3.2.3 The effect of non-viable *M. graminicola* spores on the growth of *B. graminis* f.sp. *tritici*

A spore suspension of *M. graminicola* isolate IPO323 was autoclaved at 121 °C at 15 psi for 15 minutes. The spore suspension was then inoculated onto leaves of Flame and Longbow and the leaves placed into detached leaf boxes. Two leaves of each treatment were included in each box and the experiment was carried out a total of three times. Leaves inoculated only with autoclaved water were included as controls. After initial inoculation with *M. graminicola* the leaves were inoculated with either *Bgt* isolate JIW11 or JIW48 after 1 day or 10 days. Leaves were assessed at 21 days after inoculation with *M. graminicola*.

3.2.4 Development of *B. graminis* f.sp. *tritici* spores at the early stages of development on leaves preinoculated with *M. graminicola*

Three leaves each of Longbow inoculated with IPO323, Flame inoculated with IPO323 and mock-inoculated Longbow, were placed in detached leaf boxes with the susceptible variety Cerco. Leaves were inoculated with the mildew isolate JIW48, 1 day or 6 days after *M. graminicola* inoculation. One box was left as a control to assess STB disease. One box was left inoculated with mildew but not sampled to ensure that mildew colonies formed on Cerco as expected. The whole experiment was replicated three times.

Leaves were destructively sampled at 8 hours, 24 or 32 h, 48 h, and 72 h after infection with mildew. After the first replicate was assessed at 24 h, it was decided that 32 h would be a better timepoint to sample at as more development of the spores had taken place. This is valid because in terms of mildew development, the time

points are fairly close and during statistical analysis each replicate is treated as a block effect. The sampled leaves were placed onto filter paper soaked in 3:1 ethanol: acetic acid until the leaves had cleared and were stored in lactoglycerol until assessment by microscopy. For assessment the leaves were placed on a glass slide and stained with Aniline Blue. For each leaf 30 spores were assessed for growth development at the following stages; no germination, primary germ tube, appressorial germ tube, appressorium, balloon haustorium, digitate haustorium or elongating secondary hyphae (ESH). Only spores that were isolated, undamaged and not infecting the same cell were assessed. Observations were made using a Nikon Microphot-SA (2) general light microscope. Haustoria were visualised under differential interference contrast (DIC) microscopy where necessary.

The data were statistically analysed separately for each timepoint. The categories were formed into groups. At 8 hours the number of spores that had germinated with either a primary germ tube or an appressorium, was studied as a proportion of the total spores counted. The 24/32 h timepoints were combined and the proportion of spores that had infected the host as a proportion of all germinated spores was analysed. The spores scored as having infected had at least formed an appressorium. At 48 and 72 h the categories were grouped to analyse spores that had developed secondary hyphae as a proportion of the total number of infecting spores. A logistic regression model was fitted with a binomial distribution. The model analysed at each sampling time was Replicate+ Day*Treatment, where the * operator indicates that both the main effects and the interaction of the factors were estimated. Treatment was either: Longbow inoculated with IPO323, Flame inoculated with IPO323 or Longbow mock inoculated. Standard errors were calculated on a logit scale and backtransformed predicted means were calculated for the purposes of presentation.

3.2.5 Pre-inoculation with virulent *M. graminicola* and its effect on the later stages of mildew colony development

Two leaves of Longbow were inoculated either with *M. graminicola* isolate IPO323 or mock inoculated in the same way as the early stage development inoculations. Leaves were subsequently inoculated after 6 dai with the *Bgt* isolate JIW48. Leaves were sampled at 5, 6, 7, 8, and 9 dai with *Bgt*. One box was left as the control box so that mildew colony formation could be assessed and one to check for good STB disease levels. This experiment was carried out three times.

Leaves were cleared using 75 % ethanol until all visible chlorophyll was removed. The leaves were then stored and stained as in 3.2.4. All mildew colonies on the leaves were measured using a graticule on 10 x eyepieces under 10 x magnification. The area of the colony was calculated by assessing the area as that of an ellipse $\pi(ab)$ where a and b are half the ellipse's major and minor axes respectively. The number of conidiophores was assessed on a scale of 0-4: (0 = zero, 1= <5, 2= 5-10, 3= 11-30 and 4= 30+ conidiophores). The data were analysed using linear modelling using the model Treatment*Day, where the Treatment factor was indicated whether the leaves were inoculated with IPO323 or mock-inoculated. Colony sizes were transformed to square roots for statistical analysis. This normalised the variance and made it independent of fitted values. In addition, this procedure reflects the constant radial growth rate of mildew colonies. Least significant differences of predicted means were calculated at the 5 % level.

3.2.6 qPCR determination of mildew biomass on infected leaves

Two boxes of Longbow leaves, two leaves of each treatment in each box, were inoculated with and without virulent *M. graminicola*, IPO323 and then inoculated after 1 or 6 days with the virulent *Bgt* isolate, JIW48. 5 or 12 days after *Bgt* inoculation, DNA was extracted and the amount of mildew DNA quantified using a Taqman probe assay (Fraaije et al 2006). Further details are in section 2.4.2.

3.2.7 Gene expression of TaMPK3 in leaves inoculated with a compatible and incompatible *B. graminis* f.sp. *tritici* isolate

17 day old seedlings of Longbow were attached to Perspex trays (section 2.2) and inoculated with either the compatible isolate JIW48, or with the incompatible isolate, JIW11, under settling towers. Control seedlings had no inoculation. Plants were placed in a growth cabinet in a 17 °C light for 16 h/ 12 °C dark cycle. Three leaves were excised at 1, 3, 7, 10 and 14 days after inoculation. Two replicates of the experiment were carried out. Details of laboratory methods are in section 2.4.3.

The data were analysed by restricted maximum likelihood (REML) using a linear mixed model, with the fixed model term: Time*Treatment*Type, where Treatment was either inoculation with the mildew isolate or not inoculated with mildew and Type was either a reference gene or the TaMPK3 gene. Reference genes were standardised to 0 (section 4.2.1). The random model was the interaction term Rep.Time.Treatment.Gene, where Gene was the individual reference genes and the gene of interest. 'Rep' was initially included in the random model but was then removed as it did not have a significant effect.

3.3 Results

3.3.1 Pre-inoculation with *M. graminicola* and its effect on the development of *B. graminis* f.sp*. tritici*

When the variety Longbow was inoculated first with the compatible *M. graminicola* isolate, IPO323, and subsequently inoculated with a virulent *Bgt* isolate, JIW48, fewer or no colonies were macroscopically visible on the leaf (figure 3.2 E-H) than on the mock inoculated controls. This result was consistent, regardless of whether the *Bgt* inoculation was carried out 2, 5, 7 or 10 dai with *M. graminicola*. When both Flame and Longbow were pre-inoculated with *M .graminicola* and subsequently inoculated with an avirulent *Bgt* isolate, JIW11, no colonies of mildew formed (figure 3.2 A-D).

When Flame was preinoculated with *M. graminicola* and subsequently inoculated with a virulent *Bgt* isolate, JIW48, the appearance of the pre-inoculated leaves was the similar in terms of mildew colony numbers to that of the mock inoculated leaves (figure 3.2 E-H).

3.3.2 Dosage effect of *M. graminicola* spores on the number of colonies formed by virulent *B. graminis* f.sp*. tritici*

Higher concentrations of *M. graminicola* spores hindered the formation of *Bgt* colonies more than lower concentrations (figure 3.3). An increase in the concentration of *M. graminicola* spores reduces the number of mildew colonies formed by a normally virulent *Bgt* isolate (P=0.02); for every 10-fold reduction in *M. graminicola* spores there were 18 more mildew colonies on average.

3.3.3 The effect of non-viable spores on the growth of *B. graminis* f.sp*. tritici*

When Flame and Longbow were inoculated with a *M. graminicola* spore suspension that had been autoclaved to kill the spores, the appearance of mildew colonies on the leaves was similar to that on the mock inoculated leaves (figure 3.4). The leaves were either inoculated after 1 day or 10 dai with the non-viable *M. graminicola* spores; at 10 days, less mildew developed on all the leaves inoculated with the virulent mildew.

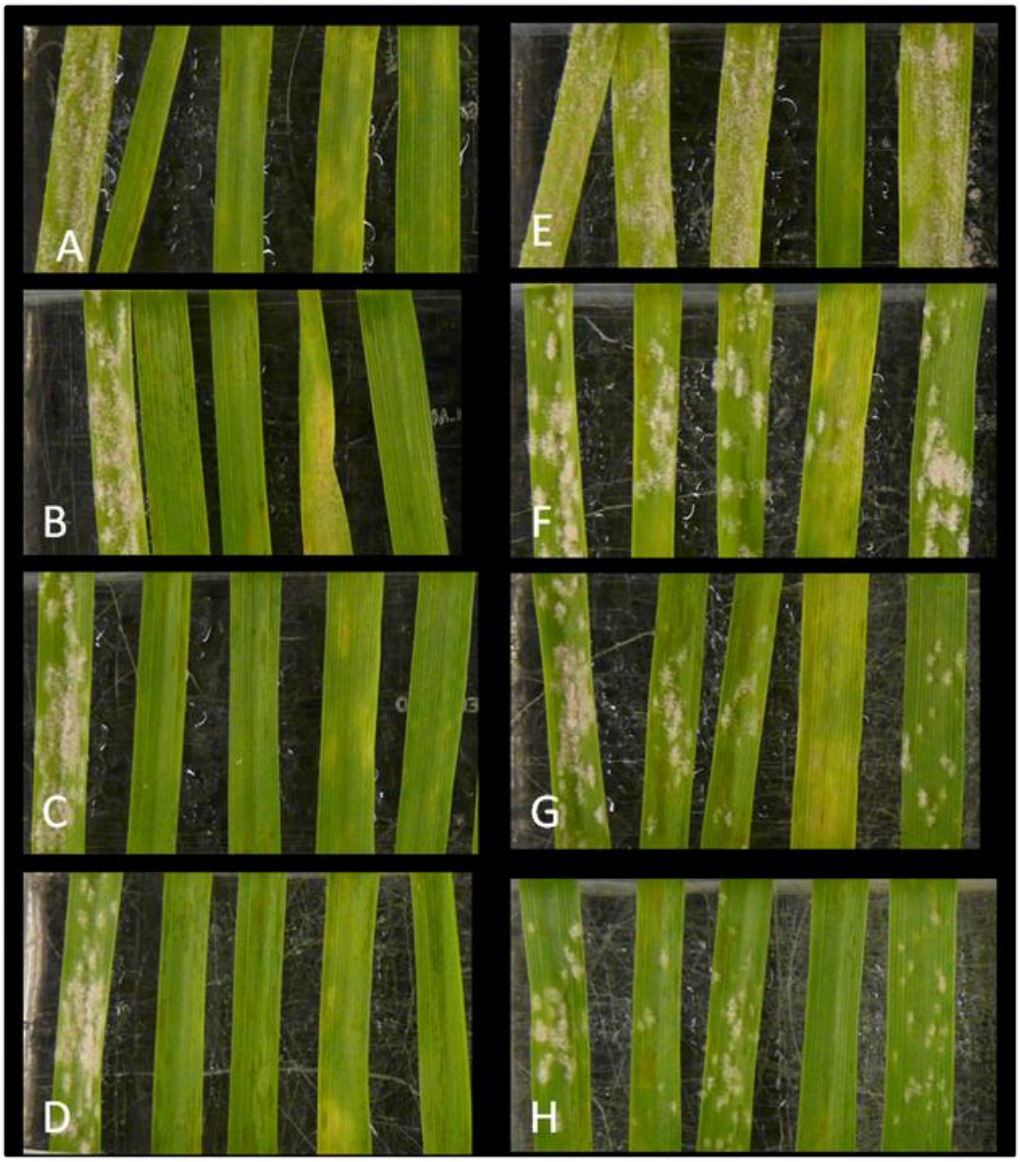


Figure 3.2. The effect of prior inoculation with *M. graminicola* on the development of mildew on the varieties, Flame and Longbow. *M. graminicola* isolate IPO323 was used which is virulent on Longbow and avirulent on Flame. Order of leaves in each photograph, from left: Cerco, Flame IPO323, Flame Mock, Longbow IPO323, Longbow Mock. **A-D** were inoculated with avirulent *Bgt* isolate JIW11. **E-H** were inoculated with virulent *Bgt* isolate JIW48. *Bgt* was inoculated 2 days (**A,E**) 5 days (**B, F**) 7 days (**C, G**) and 10 days (**D, H**) after inoculation with *M. graminicola.*

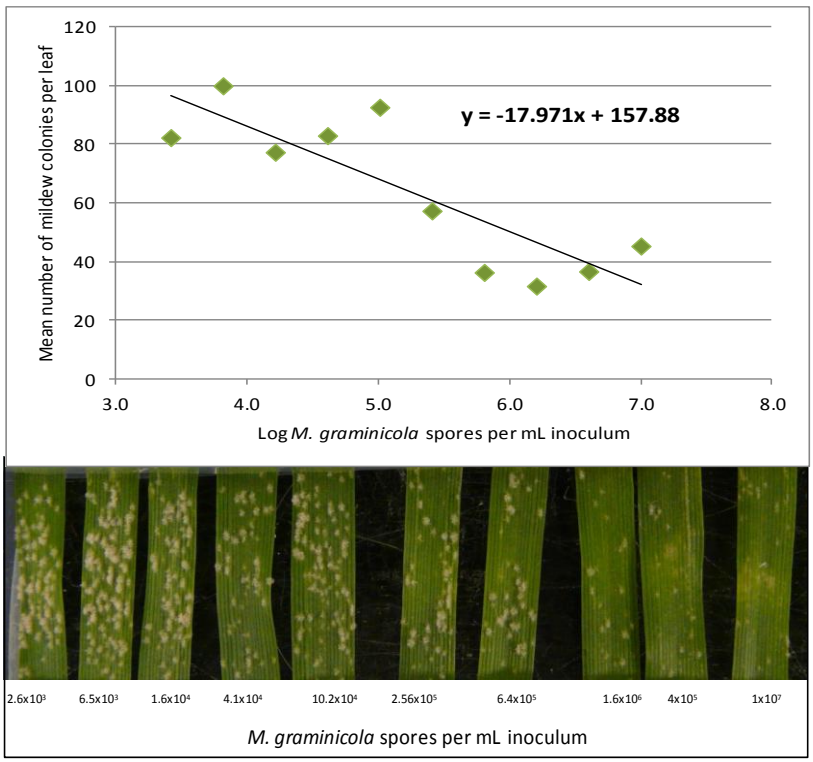


Figure 3.3 The effect of different concentrations of virulent *M. graminicola* inoculum on the number of visible colonies of virulent *Bgt.* A reduction of 18 mildew colonies is seen for every 10 fold increase in *M. graminicola* spores (P=0.002 for linear regression).

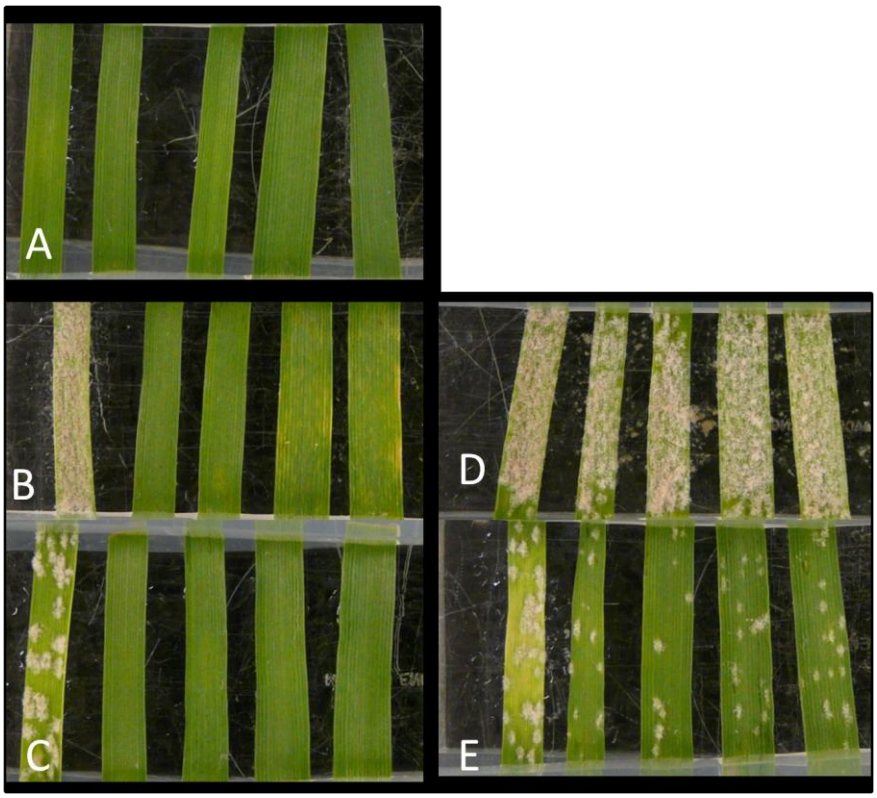


Figure 3.4 The effect of non-viable *M. graminicola* spores on Flame and Longbow. Order of leaves in each photograph, from left: Cerco, Flame IPO323, Flame Mock, Longbow IPO323, Longbow Mock. All leaves were inoculated with non-viable *M. graminicola* at the same time. **A** shows leaves inoculated only with unviable *M. graminicola* spores. **B** and **C** were inoculated with the avirulent *Bgt* isolate JIW11. D and E were inoculated with virulent *Bgt* isolate JIW48. **B** and **D** inoculated with *Bgt* 1 day after *M. graminicola* inoculation **C** and **E** inoculated with *Bgt* 10 days after *M. graminicola* inoculation.

3.3.4 Development of *B. graminis* f.sp*. tritici* spores at the early stages of development on leaves preinoculated with *M. graminicola*

At 8 hai, *Bgt* germination rates (germlings scored as having at least a PGT) on all the leaves ranged from 60 % to 74.8% for leaves inoculated at 1 dai with *M. graminicola* and 62.2 % to 77.8% for leaves inoculated 6 dai with *M. graminicola* (figure 3.5 A). There were no significant differences in the proportion of spores that had germinated between the treatments: Longbow with virulent *M. graminicola* IPO323, Longbow with a mock inoculation, Flame with avirulent *M. graminicola*, IPO323. There were also no significant differences between the leaves inoculated with *Bgt* at 1 or 6 dai with *M. graminicola* (table 3.1).

At 24 and 32 hai, a proportion of *Bgt* spores had attempted or succeeded in infecting the host, producing appressoria and occasionally haustoria and hyphae. The percentage of germinated spores that had formed at least an appressorium ranged from 45.6 % to 59 % in leaves inoculated with *Bgt* 1 dai with *M. graminicola* and from 41.9 % to 52.7 % in leaves inoculated with *Bgt,* 6 dai with *M. graminicola* (figure 3.5 B). No significant differences in the proportion of germinated spores which had infected the plant were seen either between treatments or between days (table 3.2).

At 48 hai and 72 hai many spores produced ESH. At 48 hai the percentage of *Bgt* spores which had formed an appresorium that had gone on to form ESH ranged from 26.1 % to 42.8 % at 1 dai with *M. graminicola* and 8.4 % to 21.0 % at 6 dai with *M. graminicola.* At 72 hai the percentage of spores forming ESH ranged from 32.0 % to 49.9 % at 1 dai with *M. graminicola* and 9.5 % to 13.8 % at 6 dai with *M. graminicola* (figure 3.5 C and D). There were no significant differences were seen between treatments, but there was a significant effect of day (tables 3.3 and 3.4). Consistently fewer *Bgt* spores produced ESH when infected with *Bgt* 6 dai with *M. graminicola* than when infected 1 dai with *M. graminicola.* This was seen in the Longbow mock-inoculated samples, as well as those treated with *M. graminicola,* indicating that there was an overall change in susceptibility.

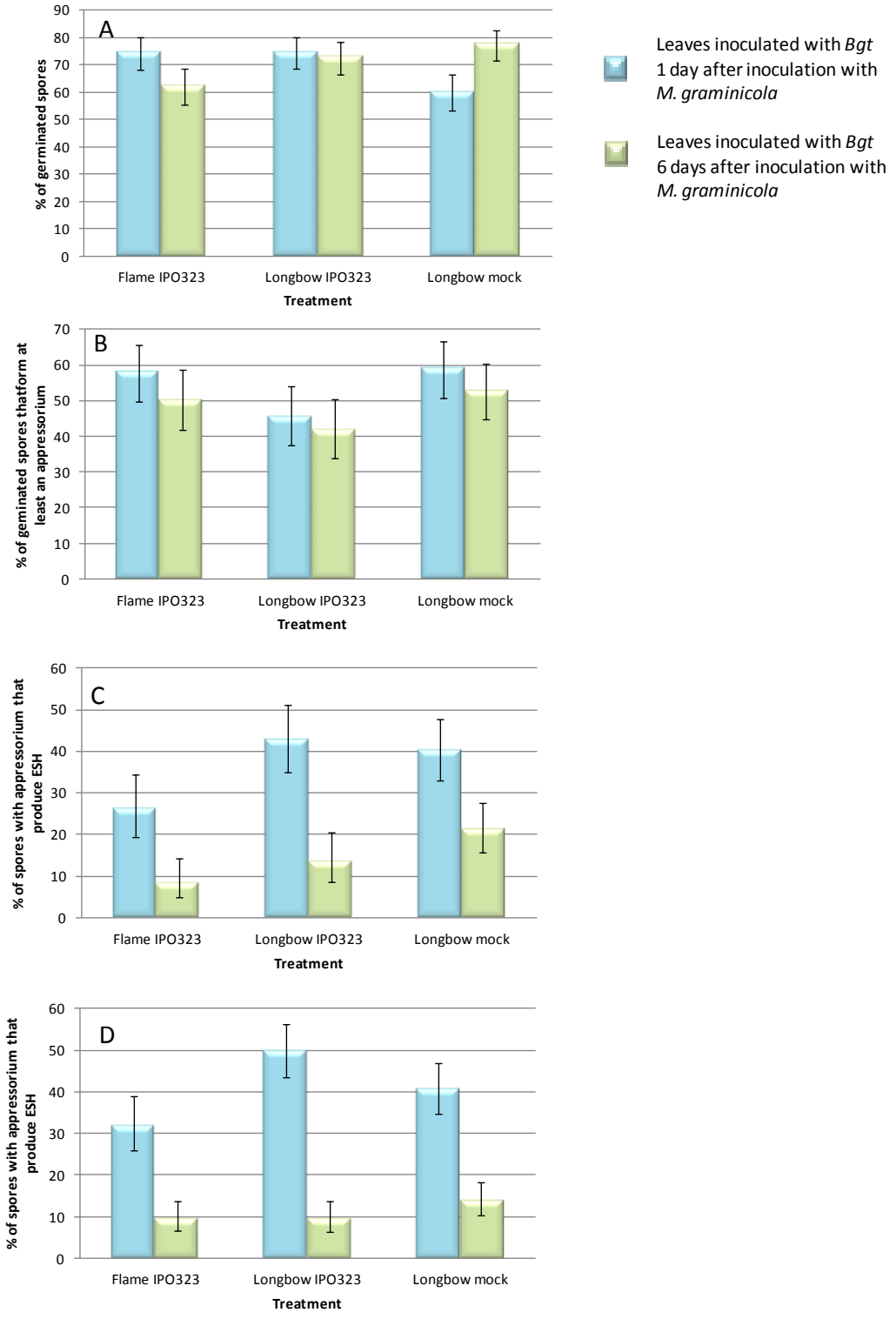


Figure 3.5 Progression of *Bgt* spore development
A % of germinated spores 8 hours after inoculation (hai) with *M. graminicola*. **B** % of germinated spores that form at least an appressorium up to 24/32 hai. **C** % of spores which form an appressorium that produce elongated secondary hyphae (ESH) at 48 hai. **D** % of spores which form an appressorium that produce ESH at 72 hai. Error bars indicate ±1 s. e. of predicted mean.

	d.f.	deviance	F pr.
Rep	1	0.578	0.7
Day	1	0.159	0.8
Trt	2	3.12	0.6
Day.Trt	2	19.89	0.06
Residual	29	92.8	

3.1.Proportion of *Bgt* spores which had germinated at 8 hai

	d.f.	deviance	F pr.
Rep	1	11.427	0.1
Day	1	3.194	0.4
Trt	2	9.402	0.3
Day.Trt	2	0.271	1
Residual	29	114.041	

3.2 Proportion of germinated spores that have formed at least an appressorium by 24/32 hai

	d.f.	deviance	F pr.
Rep	1	0.189	0.8
Day	1	39.475	<0.001
Trt	2	11.675	0.1
Day.Trt	2	2.03	0.7
Residual	29	75.495	

3.3 Proporation of spores with an appressorium that produce ESH at 48 hai

	d.f.	deviance	F pr.
Rep	1	1.404	0.4
Day	1	87.999	<0.001
Trt	2	4.865	0.3
Day.Trt	2	3.298	0.4
Residual	29	49.635	

3.4 Proporation of spores with an appressorium that produce ESH at 72 hai

Tables 3.1-3.4. Analysis of deviance tables showing effect of replicate, day of inoculation with *Bgt* after inoculation with *M. graminicola* and pre-inoculation treatment (Trt): Flame with IPO323, Longbow with 323 or Longbow mock inoculation.

3.3.5 Pre-inoculation with *M. graminicola* and its effect on the later stages of mildew colony development

The area of each mildew colony formed was measured from 5-9 days after inoculation with *Bgt.* At 5 and 6 days, pre-inoculation with virulent *M. graminicola* had no effect on the area of the colonies produced. By 7 days, the colonies formed on the leaves infected with *M. graminicola* were significantly smaller than the mock inoculated control leaves. At 9 days, the gap between the two treatments was wider, with a difference of 20.6 μm^2 between them (figure 3.6).

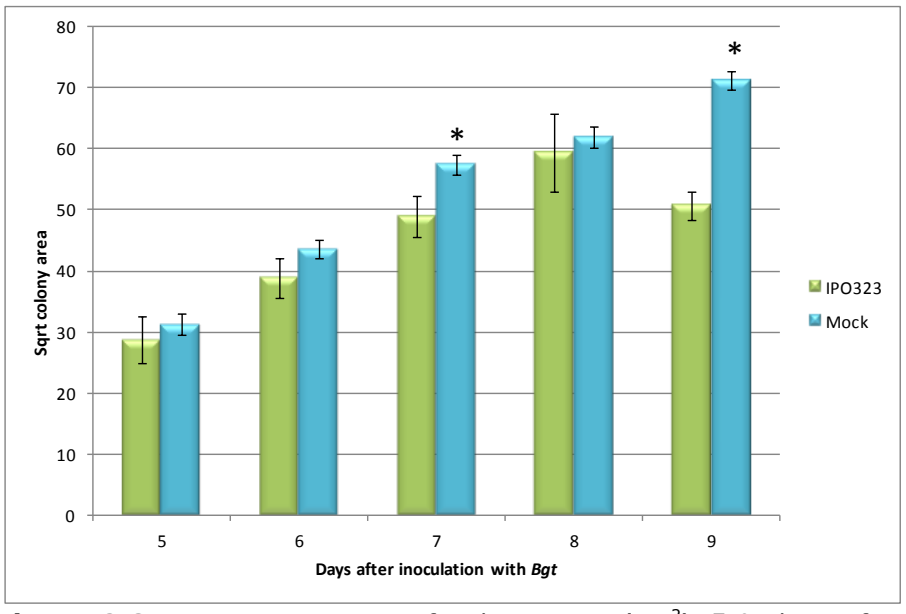


Figure 3.6 Mean square root of colony area (μm^2) 5-9 days after inoculation with *Bgt* after pre-inoculation with either *M. graminicola* isolate IPO323 or water (mock). The square root of the area is proportional to the length of the axis of the ellipse formed by the colony. Error bars are ± 1 s.e of predicted means. * P<0.05 (Fisher's protected least significant difference).

The number of conidiophores produced by mildew colonies on leaves preinoculated with *M. graminicola* was greater than on leaves mock inoculated at 7 days. After 8 and 9 days the number of conidiophores produced by the colonies on mock inoculated leaves was greater than those on the preinoculated leaves (figure 3.7). At 8 days the mock inoculated leaves produced on average over 10 conidiophores per colony, whereas the leaves preinoculated with *M. graminicola* produced no more than 10 conidiophores. At 9 days the mock inoculated leaves were

producing between 10 and 30 conidiophores per colony, while the preinoculated leaves were still only producing up to 10 condiophores per colony. The leaves assessed were the same as those used in figure 3.6.

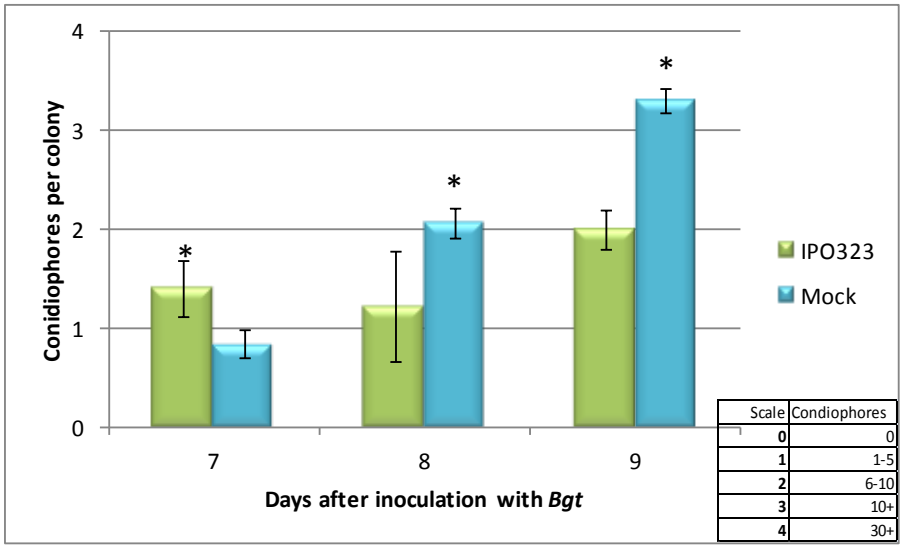


Figure 3.7 Mean number of conidiophores per colony at 7-9 days after inoculation with *Bgt* after preinouclation with *M. graminicola* isolate IPO323 or water (mock). Error bars are ± 1 s.e of predicted means. *0.05>P≥0.01

3.3.6 qPCR determination of mildew biomass on infected leaves

Primers used to amplify a cytochrome *b* gene fragment from Bgt have been designed by Fraaije *et al.* (2006). The primers were used to amplify a fragment from isolate JIW48. This was cloned, sequenced and aligned to known cytochrome *b* sequences from four other *Bgt* isolates to ensure that JIW48 has the same cytochrome *b* sequence. The fragment aligned with the known cytochrome *b* sequences (figure 3.8) and therefore the primers used were suitable for use in quantifying the amount of mildew in the leaves.

A Taqman probe assay was used to determine the quantity of mildew on the leaves of Longbow, both with and without pre-inoculation with *M. graminicola*. Leaves preinoculated with *M. graminicola* spores at 1 day before inoculation with *Bgt* had less *Bgt* DNA in both the 5 and 12 day samples than the mock-inoculated leaves

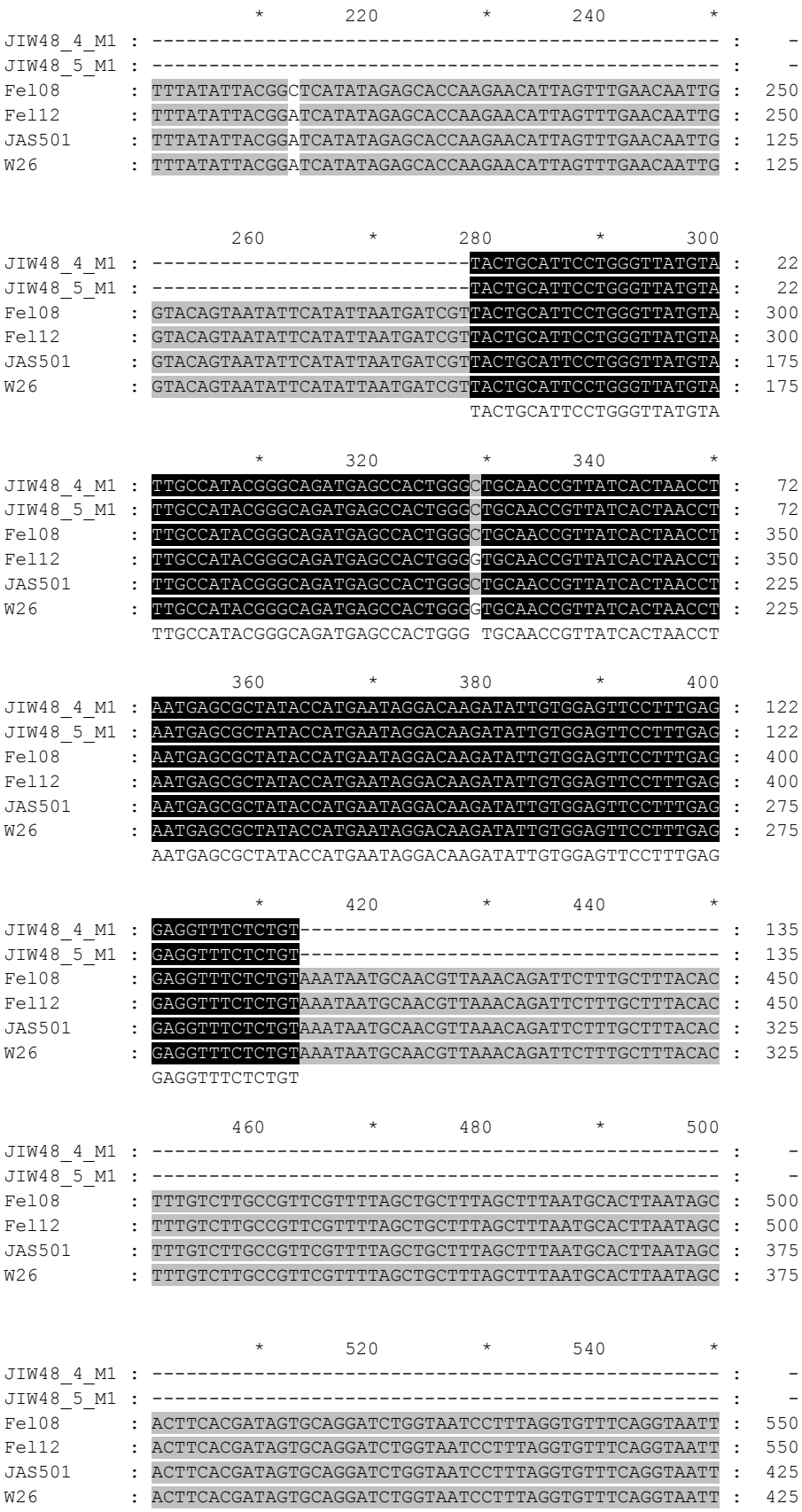
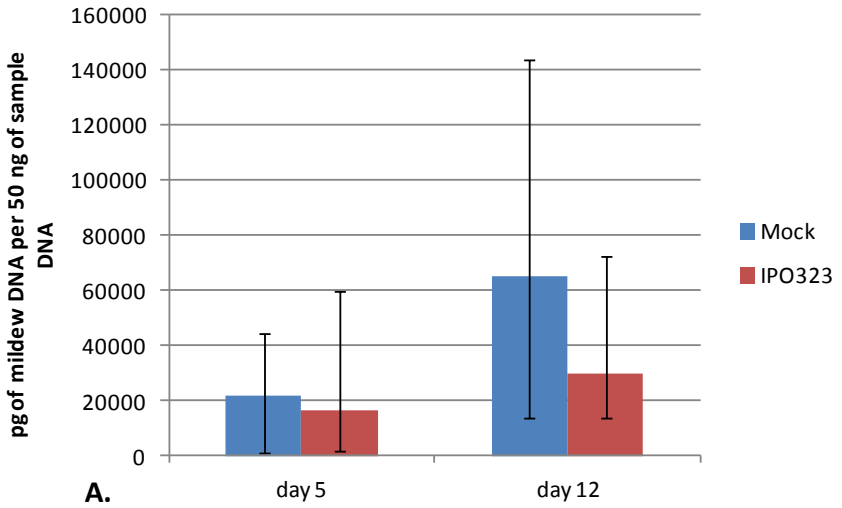
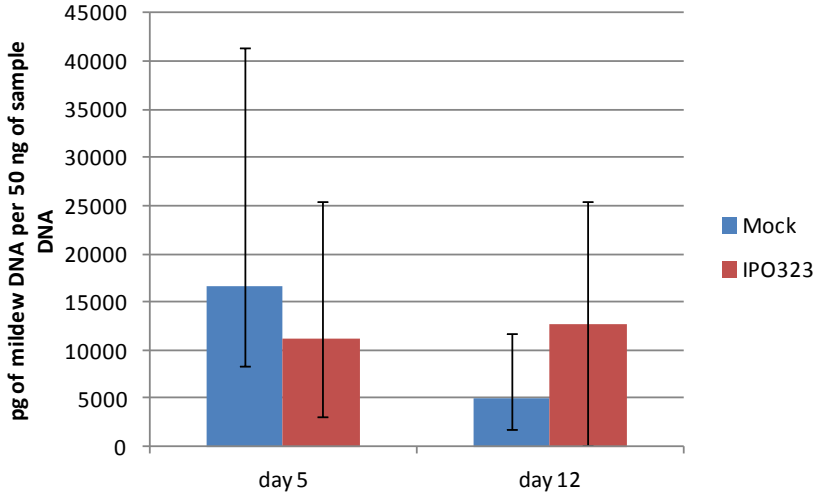


Figure 3.8 Sequence alignment of two clones from isolate JIW48 to a fragment of the cytochrome *b* gene from four known *Bgt* isolates.

(figure 3.9A). The leaves inoculated with virulent *M. graminicola* 6 days before *Bgt* inoculation had less *Bgt* DNA in the leaves sampled 5 days after inoculation by *Bgt* samples than the mock-inoculated samples but had more *Bgt* DNA in the 12 day samples. However, both the 5 and 12 day samples that were inoculated with *Bgt* 6 days after inoculation with *M. graminicola*, had less *Bgt* DNA overall than the samples inoculated after 1 day(figure 3.9B). These results are based on only 2 replicates and more replication is needed to make statistical comparisons.



A.



B.

Figure 3.9 Mean quantity of *Bgt* DNA per 50 ng of total DNA extracted from leaf tissue and determined by qPCR. Leaves of the susceptible variety Longbow were either inoculated with *M. graminicola* IPO323 or mock inoculated before inoculation with virulent *Bgt* isolate JIW48. Samples were taken at 5 and 12 days after mildew inoculation. **A.** *Bgt* inoculated onto leaves 1 day after leaves were inoculated with *M. graminicola.* **B.** *Bgt* inoculated onto leaves 6 days after were inoculated with *M. graminicola.* Error bars =±1 s.e.m. Data are based on two observations so no statistical comparisons have been made.

3.3.7 Gene expression of TaMPK3 in leaves inoculated with virulent or avirulent *B. graminis* f.sp. *tritici*

The level of TaMPK3 transcript accumulation was assessed in both a compatible and incompatible interaction with mildew over time. The term Type used in the fitted model describes whether the gene was the reference gene or the target gene. A significant Treatment.Type effect implies that there is a significant difference between treatments in the expression of the target gene in relation to that of the reference genes, the analysis showed that the most significant interactions involved differences

Fixed term	Wald statistic	n.d.f	F pr
Time	51.16	4	<0.001
Treatment	10.52	2	0.007
Type	21836.51	1	<0.001
Time.Treatment	36.97	8	<0.001
Time.Type	2.6	4	0.6
Treatment.Type	15.12	2	<0.001
Time.Treatment.Type	5.73	8	0.7

Table 3.5 Level of TaMPK3 transcript accumulation in leaves of Longbow inoculated with virulent, JIW48, or avirulent, JIW11, *B. graminis* f.sp. *tritici.* Leaves were inoculated at 1, 3, 7, 10 and 14 dai. Tests for fixed effects sequentially adding terms to the model Time*Treatment*Type.

between expression of TaMPK3 and the reference genes (Type) (table 3.5), indicating that the level of TaMPK3 accumulation is strongly affected in these experiments.

The effect of the interaction between Treatment and Type demonstrates that the effect of mildew isolate was significant (figure 3.10). Over time, more transcript accumulated in leaves inoculated with virulent *Bgt* than with the avirulent isolate. When all the timepoints were combined leaves inoculated with JIW48 showed significantly higher expression of TaMPK3 than leaves inoculated with JIW11 (P=<0.001). Neither of the terms Time.Type or Treatment.Time.Type were significant indicating that there was no temporal effect of *TaMPK3* accumulation.

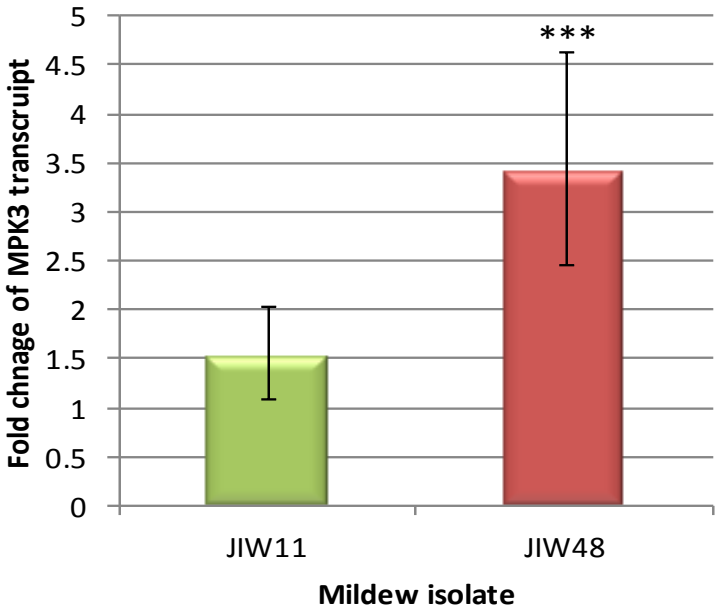


Figure 3.10. Accumulation of *TaMPK3* in Longbow inoculated with avirulent JIW11 or virulent JIW48. Figures show fold change of gene expression in relation to the control plants. TaMPK3 accumulated more transcript in Longbow inoculated with *Bgt* isolate JIW48 than JIW11. (P=<0.001). Error bars indicate ±1 s.e.of the mean

3.4 Discussion

When environmental conditions are conducive to more than one pathogen, wheat plants must defend themselves against different pathogens, often with different lifestyles. This research investigated the biotroph, *B. graminis* f.sp. *tritici* and the necrotroph, *M. graminicola* and how they interact on the host leaf. The main findings were that a compatible interaction between *M. graminicola* and the wheat leaf reduced the number and size and reproduction of mildew colonies that a normally virulent *Bgt* isolate produced. An incompatible interaction between *M. graminicola* and the wheat leaf had no apparent effect on the ability of the virulent *Bgt* isolate to form mildew colonies. An incompatible interaction between *M. graminicola* and the wheat leaf did not detectably alter the susceptibility of the leaf towards the avirulent *Bgt* isolate. The effect of a compatible interaction with *M. graminicola* was an active process, as

inoculation with dead, unviable spores did not reduce the appearance of mildew colonies. The effect is also dosage dependent, as higher inoculum levels of *M. graminicola* reduced the number of mildew colonies formed on the leaf.

Few studies have looked at the effect of the interactions between two pathogens of differing lifestyles on crop plants. The interaction between *Puccinia striiformis* and *M. graminicola* (Madariaga & Scharen, 1986) showed that *M. graminicola* has a negative effect on the ability of the biotrophic rust pathogen to colonise the leaves the whole plants grown in glasshouse conditions. The amount of rust infection was reduced in the presence of *M. graminicola* as it could only colonise areas of the leaf that had not been infected by *M. graminicola.* This was different to what was seen in the experiments here where *M. graminicola* spores covered the leaves and therefore mildew was developing in the presence of *M. graminicola* spores.

Weber *et al.* (1994) showed that the necrotrophic pathogen *Stagonospora nodorum* reduced the disease severity of mildew in both field trials and under glasshouse conditions. The presence of mildew also increased the final accumulated disease of *S. nodurum* in field trials. The effects found in glasshouse conditions were confined to the inoculated leaves demonstrating a lack of systemic effect. These papers provide evidence that necrotrophic pathogens have a negative effect on the ability of biotrophic pathogens to cause disease on the host leaf, supporting the research presented here, where mildew is reduced in the presence of *M. graminicola*. The implication of these findings is that there is a trade-off between biotrophs and necrotrophs and the defence systems that regulate them. The research presented here builds on these studies by starting to investigate the mechanisms that cause these interactions.

M. graminicola is a necrotrophic pathogen with a long latent period. Host cell death is essential for the compatible interaction to take place, but this takes place only after at least 7 days (Keon *et al.*, 2007). *Bgt* infects the cells and actively inhibits cell death to produce the 'green island' effect in order to feed from the intact leaf cell

(Walters *et al.*, 2008). In these experiments the mildew spores started to form haustoria by 32 hai. The development of the *Bgt* spores at early stages was not seen to be significantly altered by pre-inoculation with *M. graminicola,* independent of whether the mildew spores had been inoculated after 1 or 6 dai with *M. graminicola*. This contrasts with what has been shown for *P. striiformis* where germination of uredinospores is reduced by the presence of *M. graminicola* spores and rust development was restricted to areas of the leaf not infected by *M. graminicola* (Madariaga & Scharen, 1986*)*. During the time when *Bgt* is inhibited, *M. graminicola* is still in its latent phase, with little or no growth (Kema *et al.,* 1996); it is unknown how it obtains nutrients at this time. There are no differences seen in the penetration ability of avirulent and virulent spores in the early stages of infection of *M. graminicola* (Shetty *et al.*, 2003). The pathogen may be suppressing plant defence responses in order for it to colonise the substomatal cavity. The virulent *Bgt* is able to develop as normal during these early stages of the infection process of *M. graminicola*.

Bgt spore development was observed during the time when *M. graminicola* was still in its initial and latent stages of infection, 2-9 dai. This is the time before PCD occurs during a *M. graminicola* infection. There are two types of resistance mechanism against mildew, one of which involves an HR, which occurs during *R*-gene mediated resistance following penetration of an epidermal cell by a haustorium (Boyd *et al.*, 1995). In the other, known as partial resistance, infection of epidermal cells by appressoria is inhibited (Carver, 1986). During *R*-gene mediated resistance, *Bgt* spores may develop haustoria or go on to develop ESH. In the experiments reported here, development of haustoria and ESH on leaves preinoculated with *M. graminicola* was not significantly different from those preinoculated with an avirulent isolate or mock-inoculated. During partial resistance, fewer spores form haustoria and failed penetration attempts are caused by papilla formation (Carver, 1986). This type of resistance was not seen here as there was no reduction in the number of spores forming haustoria on leaves inoculated with the virulent *M. graminicola* isolate.

During the later stages of mildew spore development studied here the *M. graminicola* hyphae are starting to interact with the leaf mesophyll cells, eventually causing cell death and nutrient release (Keon *et al.*, 2007). The reduction in the number of mildew colonies and the quantity of mildew at the late stages of development shows growth is limited after they have formed ESH indicating that the response that occurs is vital for the survival of the biotroph. During infection with *M. graminicola,* although there is some formation of hydrogen peroxide in cells during both compatible and incompatible interactions, during a compatible interaction from 11 dai onwards, there is a massive accumulation of hydrogen peroxide which is seen to aid the susceptible response of the leaf (Shetty *et al.*, 2003). In the experiments presented here, this would coincide with the timepoints where the mildew colony size on Longbow inoculated with the virulent *M. graminicola* is significantly smaller than the mock-inoculated leaves. Hydrogen peroxide accumulation in barley has been demonstrated as a response to infection by avirulent *B. graminis* f. sp. h*ordei* which is linked to HR (Thordal-Christensen *et al.*, 1997), therefore it is feasible that the growth of *Bgt* would be stopped by the presence of an influx of hydrogen peroxide produced as a consequence of the compatible interaction between the virulent *M. graminicola* and the wheat leaf. Staining leaves with 3,3'-diaminobenzidine (DAB) would show when and where H_2O_2 was present and if it coincided with the timing of restricted growth of *Bgt.* In addition, hyphal penetration and attempted penetration at the edges of the mildew colonies could be examined to assess if *M. graminicola* has an effect on the later stages of the growth of *Bgt*

The effect of non-viable *M. graminicola* had on mildew colony formation was investigated to test if the interaction was mediated by recognition of PAMPs by the leaf surface. PAMPs such as chitosan, a deacetylated derivative of chitin, act as general elicitors of basal defences through pathogen recognition receptor (PRR)–mediated responses (Iriti & Faoro, 2009). Chitosan directly inhibited the growth of *Botrytis cinerea* on grapes (Munoz & Moret, 2010) and chitosan also activated defence gene

expression in *Oryza sativa* seedlings (Agrawal *et al.*, 2002). Purified β-1,3-glucans gave protection against *M. graminicola* in wheat (Shetty *et al.*, 2009). Inoculation with non-viable *M. graminicola* spores did not produce the reduction in mildew colony formation seen with viable *M. graminicola* spores. This suggests that the effect is not mediated by the recognition of PAMPs in the absence of a viable pathogen. Recognition of *M. graminicola* is more likely to take place following stomatal penetration when hyphal components are recognised by the plant.

To investigate if TaMPK3 has a role in the interaction between *M. graminicola* and mildew, transcript levels of *TaMPK3* were assessed in wheat infected with both virulent and avirulent Bgt. Over time, the plants inoculated with the virulent isolate accumulated more transcript than those inoculated with the avirulent isolate. Previous reports have shown that MPK3 orthologues accumulate during incompatible interactions involving biotrophic pathogens or non-host responses in tomato, tobacco and *Arabidopsis* (Asai *et al.*, 2002; Romeis *et al.*, 1999; Stulemeijer *et al.*, 2007), although there are few reports of compatible interactions with which these results can be compared. Many studies focus on the very early stages of infection, whereas here the experiments covered 1-14 dai. MPK3 expression and protein accumulation has not been studied in response to powdery mildew in cereals. In rice, a MPK3 orthologue accumulates both during an incompatible and compatible interaction with *M. grisea* (Xiong & Yang, 2003). Rudd *et al.* (2008) demonstrated that TaMPK3 protein accumulated in wheat during a compatible interaction with *M. graminicola,* and chapter 4 of this thesis indicates that the protein accumulated to a greater extent in the compatible interaction, but also accumulated during resistant interactions in some varieties. The experiments presented here suggest that TaMPK3 may have a role in enabling the compatible interaction with *Bgt.* This result does not conclusively show that TaMPK3 is involved in the interaction between the two pathogens on the host, but it indicates it may not be important. It also suggests the possibility that, in wheat at least, MPK3 may be involved in compatible rather than incompatible responses to

infection by fungal pathogens. To test this further, accumulation of the TaMPK3 protein could be tested in both interactions with virulent and avirulent *Bgt* isolates. It may be that post-translational activation of the protein is more important than transcription of the gene. Protein accumulation could also be investigated in the presence and absence of virulent *M. graminicola,* to discern if any changes take place during the interaction from what is seen in response to single pathogen inoculation.

This chapter establishes that two pathogens of wheat with differing lifestyles do have an effect on each other, although the mechanism behind this remains unclear. Cross-talk between the SA and JA signalling pathways may be the underlying mechanism for resistance trade-offs between pathogens of different lifestyles (Schenk *et al.*, 2000; Spoel *et al.*, 2007). Resistance to necrotrophs such as *Botrytis cinerea* require JA signalling and camalexin production (Glazebrook, 2005). Ethylene (ET) signalling also plays an important role in regulating disease development of necrotrophic fungi, often in conjunction with JA (Glazebrook, 2005). Little is known about ET signalling in monocotyledonous species, but its role is beginning to be elucidated (Chen *et al.*, 2009). ET is required for disease symptom development of *Fusarium graminearum*; enhancing ET levels of wheat and barley increased conidial production of *F. graminearum* and conversely reducing ET perception reduced conidial production (Chen *et al.*, 2009). The effect of ET signalling and perception on *M. graminicola* disease development is unknown. It is known that ET plays a role in senescence and therefore it could be predicted that ET levels will increase as necrosis of the leaf develops and disease symptoms progress. In tomato infected with the actinomycete, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), genes involved in ethylene biosynthesis and response are upregulated. In plants with impaired ethylene perception, the appearance of wilt symptoms is delayed in comparison with WT (Balaji *et al.*, 2008). The effect of ethylene on wheat has not been studied for disease interactions with powdery mildew. In *Arabidopsis*, *Erysiphe* spp. do not trigger the JA-/ET- signalling pathways which can induce resistance against them; this

is mediated by a mutation, *cev1*, which regulates the ethylene signalling pathways (Ellis & Turner, 2001). It could be predicted that if ET accumulates in wheat during a compatible interaction with *M. graminicola* this could induce resistance to the normally virulent *Bgt* isolate. (In experiments I conducted using silver thiosulphate, an inhibitor of ethylene perception (Chen *et al.*, 2009), the silver thiosulphate was found to be fungistatic towards the *M. graminicola* so it was not possible to tell if ethylene was having an effect on disease development (data not shown)).

Breeding for mildew resistance has taken place for many years, whereas breeding for resistance to STB has been relatively recent. In the 1960s and 1970s stagonospora nodorum blotch (SNB) was the major facultative disease in the UK (Bearchell *et al.*, 2005). In the 1970s a shift occurred, as a result of which STB became the major facultative disease of wheat in the UK from the 1980's onwards (Arraiano *et al.*, 2009; Bearchell *et al.*, 2005). There are varieties available which provide good STB resistance for breeding (Arraiano *et al.*, 2009; Chartrain *et al.*, 2004a), but it can take seven years to get a variety onto the commercial market (Brown, 2011) and disease resistance is not as important as yield and quality characteristics. Breeders and farmers require moderate resistance to all diseases, and all the varieties on the recommended list in the UK in the last 2 years have been rated 5 or higher for STB indicating a moderate level of resistance to this disease (http://www.hgca.com/document.aspx?fn=load&media_id=6707&publicationId=481). More information on how different diseases interact with each other is desirable when breeding new varieties, especially in the face of growing government and public concern over fungicides (Haynes *et al.*, 2010). Pesticide availability in the UK and Europe will decline over the next decade due to changes in pesticide approvals legislation and insensitivity (Clarke *et al.*, 2011). There is little value in having good resistance to one disease if its resistance to another is poor as this will not reduce demand for pesticides. The results here indicate that because susceptibility to *M. graminicola* inhibits powdery mildew, breeding efforts should focus on increasing

resistance to STB whilst maintaining the existing current level of moderately good partial resistances to powdery mildew. Resistance to *M. graminicola* had no effect on *Bgt,* so focusing attention on breeding resistance to *M. graminicola* should not have a detrimental effect on *Bgt* resistance. Speculatively, long term breeding for resistance to powdery mildew may have meant that wheat is now well adapted to defence against biotrophic pathogens, but less well adapted to defence against necrotrophic pathogens. Further insights into the mechanism controlling these defences in wheat are needed to breed plants that are able to withstand disease pressure from pathogens of differing infection mechanisms.

4

Gene expression and TaMPK3 protein accumulation

4.1 Introduction

The goal of identifying specific host responses to *Mycosphaerella graminicola* inoculation is of interest to understand how disease symptoms are caused and how resistance works. For example, there is no means of identifying specific resistances in wheat varieties or avirulences in *M. graminicola* isolates other than a rather complicated statistical analysis of quantitative disease symptoms (see Arraiano & Brown (2006) for an example). It would be greatly preferable to have a test in which an incompatible interaction could be recognised by its phenotype, as with powdery mildew and rust diseases of cereals (Ma & Singh, 1996; Moseman *et al.*, 1965; Stubbs *et al.*, 1986). In addition, it would benefit breeding programmes where time and money could be saved through the early identification of resistant lines.

Specific resistant interactions involve recognition of the pathogen by the host. The resistance response towards a biotrophic pathogen frequently involves a hypersensitive response (HR). Gene expression of host defence-related and other genes during interactions between biotrophic pathogens and their host have been extensively studied, particularly for both rust pathogens and powdery mildew (Boyd *et al.*, 1994b; Bozkurt *et al.*, 2010; Coram *et al.*, 2008b). These tend to show that defence-related genes are upregulated rapidly after a resistant cultivar is inoculated with a pathogen. The response of barley to *Blumeria graminis* f.sp. *hordei* (powdery mildew), the pathogenesis-related genes, *PR1, chitinase, peroxidase* and *PAL* were upregulated in both compatible and incompatible interactions, but the response was seen later and was not as strong in a compatible interaction (Boyd *et al.*, 1994b).

Many gene expression studies have now been done using microarray transcription profiling, which shows whole sets of genes that are differentially expressed. During the interaction with wheat and *Puccinia striiformis* 54 transcripts

that were upregulated in both incompatible and compatible interactions were considered to be basal defence transcripts including some *chitinases*, *β-1,3-glucanases*, *PAL*s, *peroxidase* and *protein kinases*. Different *chitinases*, *β-1,3-glucanases* and *peroxidase* were specifically upregulated in response to the HR. Bozkurt *et al.* (2010) found that only four wheat genes, encoding two *chitinases,* a *β-1,3-glucanase* and a *peroxidase* were upregulated during interactions with *P. striiformis* isolates either virulent or avirulent on *Yr1*. These genes consistently appear as differentially regulated between incompatible and compatible interactions in studies of different pathogens indicating a strong involvement in defence responses following recognition of pathogens (Boyd *et al.* 1994b; Coram *et al.* 2008b; Coram *et al.* 2008a; Bozkurt *et al.*2010).

Few studies have compared transcriptional responses of the cereal hosts during incompatible/compatible interactions with either hemibiotrophic or necrotropic pathogens. Transcriptional changes during compatible and incompatible interactions with pathogens such as *Magnaporthe* species and *Pyrenophora tritici-repentis* have been investigated with the use of microarray studies. These studies demonstrate that the transcription of traditionally defined defence genes is not as clear in necrotrophic interactions as is demonstrated during biotrophic interactions. During interactions between wheat and *M. oryzae* (adapted) or *M. grisea* (non-adapted) isolates there was a subset of genes that were upregulated in all interactions. The genes included *β-1,3-glucanases*, *chitinases*, cell wall defence-related genes and *phenylalanine ammonia-lyase* (*PAL*) (Tufan *et al.* 2009). The ToxA-*Tsn1* toxin receptor interaction between wheat and *P. tritici-repentis* induces host recognition that is as specific as an *Avr-R* type disease interaction but results in susceptibility rather than resistance. Recognition of ToxA by *Tsn1* in wheat triggers transcription of numerous defence-related genes including *chitinase* and *β-1,3-glucanase*, genes involved in the phenylpropanoid pathway and the production of reactive oxygen species (ROS) which play a role in avirulent interactions (Adhikari *et al.*, 2009). These genes were often more strongly

upregulated in the compatible than the incompatible interaction once cell death was triggered. The transcriptional changes seen during ToxA induced cell death are consistent with responses usually associated with defence (Pandelova *et al.*, 2009). *Tsn1* is encoded by a NBS-LRR disease resistance protein (Faris *et al.*, 2010).

There is emerging evidence that successful infection by a necrotrophic pathogen, such as *M. graminicola*, activates the same signalling pathways that are triggered during a resistance response towards a biotrophic pathogen (Deller *et al.*, 2011; Hammond-Kossack & Rudd, 2008). The TaMPK3 protein and transcript accumulate in wheat leaves after infection by a compatible *M. graminicola* isolate (Rudd *et al.,* 2008) in two tested isolate-variety interactions. The gene transcript of TaMPK3 accumulated during a compatible *B. graminis* isolate (chapter 3 of this thesis). Orthologues of this protein had previously been shown to accumulate in *Avr-R* mediated resistance responses in tobacco and tomato (Romeis *et al.*, 1999; Stulemeijer *et al.*, 2007; Zhang & Klessig, 1998). This evidence goes some way to support the view that non-biotrophic pathogens have a more sophisticated mechanism of infection than previously thought. For example, instead of simply using toxins and enzymes to cause cell death which it can then ustilise as a nutrient source, *M. graminicola* appears to appropriate host defence signalling (Hammond-Kossack and Rudd, 2008). Understanding how host responses work in specific *M. graminicola*-wheat interactions may enable the development of a phenotypic method of identifying resistance as well as an understanding of how this non-biotrophic pathogen causes infection.

Host responses to *M. graminicola* have begun to be elucidated, producing an emerging picture of what still remains a highly variable response. The pathogen is unusual in several respects, first that it remains extracellular for its entire lifecycle, and second because it has a long latent period between inoculation and symptom development suggesting that responses do not have highly regulated timings. The published data on this pathosystem have focused on a few selected defence-related

genes and others found to be differentially expressed (Adhikari *et al.*, 2007; Ray *et al.*, 2003).

The aim of the experiments reported in this thesiswas to find a potential marker for resistance or susceptibility that could be used to phenotype whether a wheat line was resistance or susceptible using an easily identifiable gene or protein. This aim was similar to that of Ray *et al.* (2003) and Adhikari *et al.* (2007). The hypothesis here is that genes normally upregulated in resistant responses towards biotrophic pathogens will be upregulated during a susceptibility response to *M. graminicola*, as it appears that a response similar to the resistance response against biotrophs is activated during susceptibility towards *M. graminicola* (Deller *et al.*, 2011). It was expected that genes associated with cell death and senescence would be differentially regulated at later time points of a compatible interaction when the plant cells start to necrose as the pathogen colonises the leaf and the tissue dies. It was also predicted that strongly resistant varieties would show a lesser response than strongly susceptible varieties, and weakly resistant varieties would respond somewhere in the middle.

The accumulation of TaMPK3 was analysed in different variety/isolate combinations to test if the same pattern emerged as that found by Rudd *et al.* (2008) in which TaMPK3 accumulated during two compatible interactions but not in an incompatible interaction. It was predicted that TaMPK3 would not accumulate during an incompatible interaction, but would accumulate to an intermediate level in the varieties with intermediate levels of resistance.

The varieties chosen for study have known resistances conferred by specific *Stb* genes. Arraiano and Brown (2006) tested 238 European wheat lines in detached leaf box tests to ascertain which specific resistances they have. *Stb15* was found to be the most common with 142 displaying specific resistance to isolate IPO88004. This was followed by *Stb6* which was found in 43 lines tested. Resistance to septoria tritici blotch (STB) is a quantitative trait although the difference between responses to

virulent and avirulent isolate are highly distinct in the cases of *Stb6*, *Stb15* and a few other genes (Arraiano *et al.*, 2007; Brading *et al.*, 2002). In these experiments, four varieties have the *Stb6* gene, Arina, Flame, Poros and Cadenza. Longbow and Avalon, both of which have *Stb15* but not *Stb6,* were chosen as highly susceptible varieties to the isolate IPO323. The varieties with *Stb6* show varying levels of resistance to IPO323, Flame and Arina are strongly resistant whereas Poros and Cadenza show a more weakly resistant response (Arraiano and Brown, 2006). The varieties Courtot and Longbow were chosen to test the interaction with IPO88004. Courtot has *Stb9* but not *Stb15* and is therefore susceptible to IPO88004, whereas Longbow has *Stb15* and is resistant to IPO88004. In field trials of UK varieties, the presence of the *Stb6* gene explains a significant proportion of the variation in levels of STB; it may confer a partial resistance, although the mechanism by which this could operate is unknown (Arraiano *et al.*, 2009).

4.2 Experimental design

Full details of materials and methods are described in chapter 2.

4.2.1 Selection of genes of interest

A list of 11 candidate wheat gene targets that are hypothesised to play a role in the infection process of *M. graminicola* were selected from previous studies of *M. graminicola* and from studies of biotrophic pathogens (table 4.2). Meta-analysis of these genes is fully described in section 2.5.4 (Tufan *et al.* 2012*)*.

4.2.2 Gene expression analysis of wheat genes

The varieties Longbow, Flame, Avalon, Cadenza, Arina and Poros were inoculated with the isolate IPO323. The varieties Longbow and Courtot were inoculated with isolate IPO88004. Mock inoculations were also carried out in which the leaves were inoculated with the water and Tween 20 solution only (section 2.2). Three leaves were

sampled for each interaction at 0.5, 1, 3, 7, 10 and 14 days after inoculation according to the linear infection assay described by Keon *et al.* (2007). Mock-inoculated leaves were also sampled at each timepoint. The leaves sampled at each timepoint were pooled. Three biologically replicated experiments were carried out at different times with each isolate. In total, 12 replicates were set up at different times. The replicates chosen for analysis all showed macroscopic necrosis at 14 dai. RNA was extracted using the Trizol (Invitrogen, Carlsbad, CA, USA) method for experiments with IPO323 and Qiagen RNeasy kits (Qiagen, Valencia, USA) for experiments using IPO88004 following the manufacturer's protocol (section 2.5.1). cDNA synthesis was carried out using Invitrogen Superscript III (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol (section 2.5.2).

qPCR reactions were carried out using the following cycle: 95 °C for 10 minutes; followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 30 sec. Immediately after this a melt curve analysis was carried out to check that all samples had a similar melting temperature by raising the temperature slowly from 65 °C to 90 °C and measuring the fluorescence. All samples had two technical repetitions. Quantification cycle (Cq) values were normalised to three reference genes (table 2.2). Primer efficiencies for the genes of interest were tested for each primer pair and were between 90 and 115 %, equivalent to amplification values from 1.89 to 2.04 (table 4.1).

Gene of interest	Amplification value
β-1,3-glucanase	1.93
Chitinase	1.90
Chorophyll a/b	2.05
Cysteine protease (Sag12)	2.04
LOX	2.04
mlo3	1.98
TaMPK3	2.01
PAL	1.99
PDI	1.94
Peroxidase	1.90
PR1	1.89

Table 4.1 Primer amplification values for genes of interest.

Data were analysed using restricted maximum likelihood (REML) to fit a linear mixed model. The mean amount of RNA of the reference genes was standardised to 0 to avoid large but irrelevant differences between the mean level of transcription of each gene. The model fitted was Treatment.Type.Time.Compatibility.Variety where Type was either the reference genes or the gene of interest and Treatment was either inoculation with *M. graminicola* or mock inoculation. Compatibility was either the resistant or susceptible response of the variety/isolate combination. Predicted means were calculated for each combination of Treatment with Variety and Type at each Time.

The effect of infection by *M. graminicola* on gene expression was calculated from the Cq values. For each gene, Cq is equal to the logarithm of the quantity of cDNA in the sample to the base of the amplification value (table 4.1). First, Cq for the target gene in an inoculated sample (Cq_{ti}) was standardised by comparing it to the mean Cq for the reference genes in that sample ($Cq_{ri)}$. Likewise, Cq for the target (Cq_{tm}) and reference genes (Cq_{rm}) were calculated for the relevant mock-inoculated sample. The four Cq values were estimated separately and the logarithm of the fold increase in gene expression was proportional to $(Cq_{ti} - Cq_{tm)} - (Cq_{ri} + Cq_{rm}) = \Delta Cq$. The standard error of ΔCq was calculated from the variance-covariance matrix of the predicted means as the square root of the sum of the four variances and six covariances of the Cq estimates. Calculation of ΔCq by the method described here is based on the assumption that all genes have the same amplification values. This is approximately correct because the amplification values for the 11 genes varied within a narrow range from 1.89-2.05. While comparisons between treatments of ΔCq for the same target gene are exact, comparisons involving different target genes are approximate.

4.2.3 Wheat mitogen-activated protein kinase 3 analysis

Amounts of the TaMPK3 protein were quantified in the varieties Longbow, Flame, Avalon, Cadenza, Arina and Poros inoculated with the isolate IPO323. The varieties Longbow and Courtot were inoculated with isolate IPO88004 in separate experiments. Three leaves were collected for protein analysis on the following days after inoculation: 1, 3, 7, 10, 11, 14, 15, 16 and 17. Mock inoculated leaves were taken at 1, 10 and 17 days after inoculation, to cover the beginning, middle and end of the infection process.

Protein was extracted by grinding frozen tissue in extraction buffer as described in Rudd *et al.* (2008). A Bradford assay was used to quantify the amount of protein in each sample. Samples were mixed with a loading dye so that all samples contained an equal amount of protein and stored at -20 °C. Approximately 120 µg of protein was separated on 10 % SDS-PAGE gels and wet blotted onto Hybond ECL nitro cellulose membrane (GE Healthcare Life Sciences, Little Chalfont, Bucks, UK). Membranes were blocked overnight at 4 °C in TBS-Tween pH 7.3 with 5% skimmed milk powder. The MAPK-specific antibody TaMPK3-N at 1:500 dilution was used against leaf extracts at room temperature for 90 minutes. The membranes were washed and chemiluminescent detection using Amersham ECL Plus Western Blotting Detection Reagents was carried out in accordance with the manufacturer's instructions (GE Healthcare Life Sciences, Little Chalfont, Bucks, UK) (see section 2.6.3).

4.3 Results

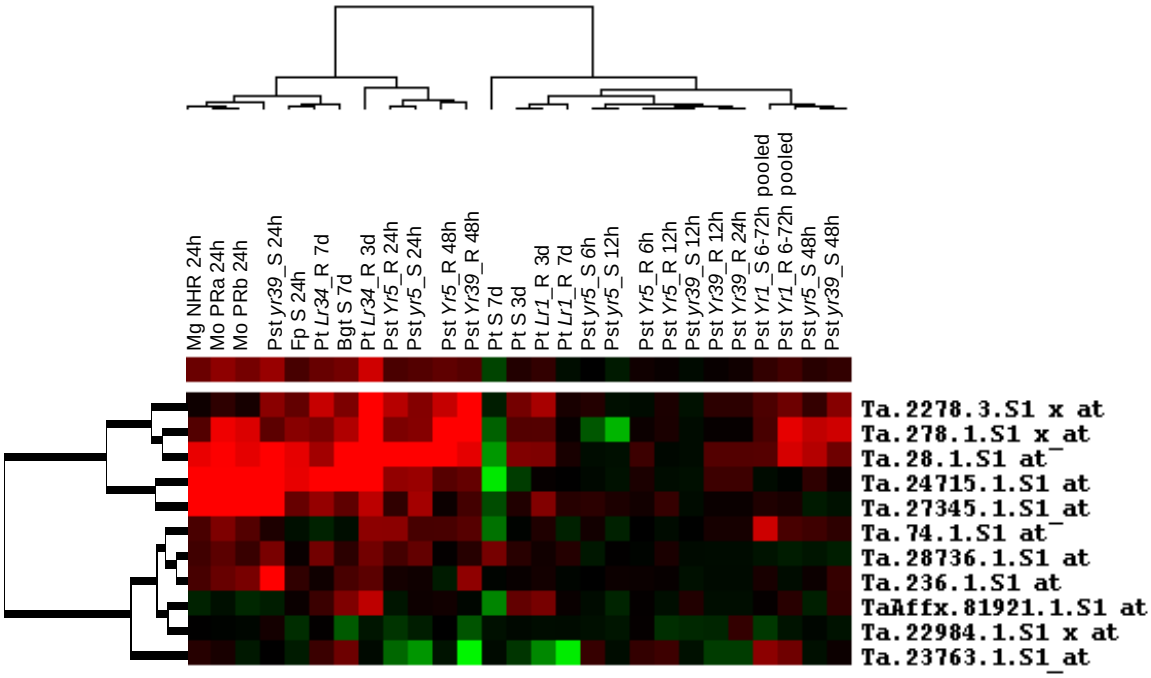
4.3.1 Selection of genes to be investigated

A list of 11 candidate wheat gene targets that are hypothesised to play a role in the infection process of *M. graminicola* were selected from previous studies of *M. graminicola* and from studies of biotrophic pathogens (Adhikari *et al.*, 2007; Bolton *et al.*, 2008; Ray *et al.*, 2003; Rudd *et al.*, 2008; Shetty *et al.*, 2009) (table 4.2). A hierarchical cluster analysis was performed to identify how these genes performed during other host pathogen interactions. In general, the analysis supports the list of

chosen candidates as genes that have shown differential expression in host-pathogen interactions (figure 4.1). The data sets analysed were mostly from biotrophic interactions, but also included early stages of infection with *M. grisea, M. oryzae*, and *Fusarium pseudograminearum*, all of which are non-biotrophic pathogens. The data show strong support for a basal defence response in many of the interactions involving *chitinase*, *PR1 β-1,3-glucanase*, *peroxidase* and *mlo* genes. Genes involved in senescence were not strongly differentially expressed in most of these pathosystems, but the predicted genes for the *cysteine protease* and *chlorophyll a/b binding precursor* group together suggesting that their transcription responds similarly to environmental stimuli. The interactions involving very early timepoints (6 hours and 12 hours) grouped together and showed very little change in gene expression of any of the genes analysed. The 7 dai timepoint of a compatible rust interaction had a lineage that stands slightly apart from the other data sets, showing downregulation of many defence related genes and the cysteine protease. Overall this analysis provides support for testing the selected genes, as they showed differential expression between different interactions and over time.

Gene of Interest	GenBank Accession Number	Wheat Affymetrix code	Expect value	Reference	Left Primer 5'-3'	Right Primer 5'-3'
PR-1	AY258615.1	Ta.278.1.S1_x_at Ta.278.1.S1_at	0 0	Ray *et al.,* 2003	ACGTACGCCAACCAGAGGATCA	GCATGCGATTAGGGACGAAAGAC
Chitinase 2	CD490414	Ta.2278.3.S1_x_at Ta.2278.2.S1_at	0 0	Bolton *et al*., 2008	GAGCAGCCTCACTTGCTAGG	ATACGCATGCCGAACGTTTA
Inducible *PAL*	AY005474	Ta.28736.1.S1_at	0	Adhikari *et al.,* 2007	GTGTCTCCATGGACAACACCCG	TCAATGGCCTGGCACAGAGC
Protein disulfide isomerase	AF262980	Ta.74.1.S1_at Ta.796.1.S1_x_at	0 0	Ray *et al., 2003*	TTATGACTTTGGCCACACCG	CGAGCTCATCAAATGGCTTG
Peroxidase	X85229	Ta.24715.1.S1_at Ta.22564.1.S1_at	0 1.00E-168	Adhikari *et al.,* 2007	CCAGCACGACACGTGAATG	CATGATTTGCTGCTGCTCGTA
TaMPK3	AF079318.1	Ta.236.1.S1_at	0	Rudd *et al., 2008*	TACATGAGGCACCTGCCGCAGT	GGTTCAACTCCAGGGCTTCGTTG
Lipoxygenase	AY253443	Ta.23763.1.S1_at Ta.28171.1.S1_at	0 1.00E-18	Ray *et al., 2003*	GGGCACCAAGGAGTACAAGGA	CGATCACCGACACTCCAATG
Cysteine protease (*Sag12* homology)	CA680100	TaAffx.81921.1.S1_at	0	Rudd (pers. comm.)	GTTCTCGGACCTCACCAGCGAA	ACGCCCACCAACAACCGCAT
mlo	CA745732	Ta.27345.1.S1_at TaAffx.50046.1.S1_at	0 1.00E-158	Rudd (pers. comm.)	CCTACCACTATACGCCGTCGTCTCC	CACCGACGAGTTTGCCCGTGTAT
β-1,3-glucanase	Y18212.1	Ta.28.1.S1_at Ta.8584.1.S1_at	0 0	Shetty *et al.,* 2009	AACGACCAGCTCTCCAACAT	GTATGGCCGGACATTGTTCT
Chlorophyll a/b binding precursor	U73218.1	Ta.22984.1.S1_x_at	0	Rudd (pers. comm.)	CCTTGGTGAGGCCCGAGTCACTAT	TTGGCAAAGGTCTCGGGGTC

Table 4.2 List of the genes of interest and their GenBank accession number and most likely wheat Affymetrix code and primer sequences. (Pers. comm., Dr Jason Rudd, Rothamsted Research, Harpenden, UK)



Key:

Abbreviation	Pathogen and interaction studied
Mg NHR	*Magnaporthe grisea* nonhost
Mo PRa	*M. oryzae* Partially resistant a
Mo PRb	*M. oryzae* Partially resistant b
Fp	*Fusarium pseudograminearum* Compatible
Pst yr39_S	*Puccinia striformis* _Yr39 compatible
Pst Yr39_R	*P. striformis* _Yr39 incompatible
Pst yr5_S	*P. striformis* _Yr5 compatible
Pst Yr5_R	*P. striformis* _Yr5 incompatible
Pt S	*P. triticiana* compatible
Pt Lr34_R	*P. triticiana* _Lr34 incomaptible
Pt Lr1_R	*P. triticiana_Lr1* incompatible
Pst Yr1_R	*P. striformis* _Yr1 incompatible
Pst Yr1_S	*P. striformis* _Yr1 compatible
Bgt S	*Blumeria graminis* f.sp. *tritici* compatible

NB. Timepoints are specified on the diagram

Figure 4.1 Hierarchical cluster analysis of the eleven chosen genes of interest. Red squares indicate upregulation and green squares indicate downregulation in the specific interaction. Accessions for experiments used for analysis were: TA9 (Coram *et al.*, 2008b), TA11 (Coram *et al.*, 2008a), TA24 (Tufan *et al.*, 2009), TA25 (Bozkurt *et al.*, 2010), TA31 (Desmond *et al.*, 2008) and TA32 (Bolton *et al.*, 2008).

4.3.2 Gene expression analysis of wheat genes.

The timepoints at which the samples were taken were chosen to cover the period from inoculation to when necrosis first starts. Once necrosis begins, the RNA in the leaf degrades and is not suitable for analysis. Figure 4.2 shows symptoms when the final samples were collected on day 14 after inoculation. The resistant varieties Arina, Cadenza, Flame and Poros remained green throughout the course of the experiment. The resistant variety Longbow inoculated with IPO88004 showed some necrosis at 14 dai, but no pycnidium development, even by 21 dai.

When the Cq data for plants inoculated with either of the isolates were analysed

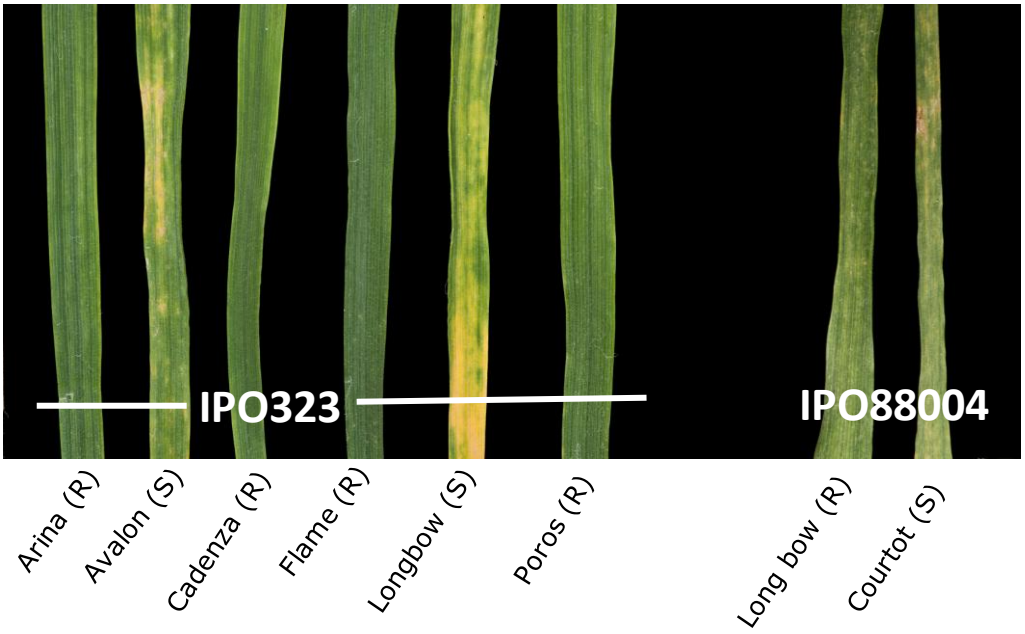


Figure 4.2 Example of symptoms on selected varieties 14 days after inoculation with either IPO323 or IPO88004 R=Resistant S=susceptible.

as a set, taking into account the compatibility between the isolate and the variety, it was found that isolate had little effect on the outcome and therefore, all the data, regardless of the isolate used for inoculation, was analysed together. Inoculation with *M. graminicola* had a highly significant effect on gene expression (table 4.3). The Treatment.Type term identifies whether or not inoculation with *M. graminicola* affects the gene of interest. If Treatment.Type is significant the gene of interest is

upregulated (or downregulated) relative to the reference genes in the inoculated plants compared to the mock inoculated plants, as indicated by a large ΔCq value. The Treatment.Type effect is related to the average value of ΔCq across Varieties and Times. The genes that were significantly affected by inoculation with *M. graminicola,* as indicated by a significant Treatment.Type effect in table 4.3 were; *chitinase*, *LOX*, *mlo*, *PAL*, *peroxidase*, *PR1* and *β-1,3-glucanase*.

Gene	Wald statistic	d.f.	P	
Chitinase	63.12	1	<0.001	***
Chlorophyll a/b precursor	2.03	1	0.2	ns
Cysteine protease	1.19	1	0.3	ns
LOX	5.22	1	0.02	*
mlo	56.86	1	<0.001	***
PAL	31.65	1	<0.001	***
PDI	0.6	1	0.4	ns
Peroxidase	174.1	1	<0.001	***
PR1	135.96	1	<0.001	***
TaMPK3	3.21	1	0.07	ns
β-1,3-glucanase	106.26	1	<0.001	***

Table 4.3 The effect of inoculation with *M. graminicola* on the genes tested: statistical significance of Trt.Type effect, where Treatment (Trt) is either *M. graminicola* or mock inoculation and Type is either the reference gene or the gene of interest. This indicates the size of the effect of inoculation with *M*. *graminicola* on the expression of the gene of interest relative to a set of reference genes. P=chi-squared probability *0.05>P≥0.01, **P0.01>P≥0.001, ***P<0.001. *Lox= Lipoxygenase, PAL= Phenylalanine lyase, PDI= Protein disulfide isomaerase.*

For five of these genes more complex interactions which include Treatment and Type as fixed terms had a significant effect on Cq values (table 4.4). There was a significant effect of compatibility on expression of *chitinase* (Trt.Type.Comp effect in table 4.4; P=0.04)) because on average more transcript accumulated in compatible interactions than in incompatible ones across varieties and time points (figure 4.3). There was a significant difference in the temporal pattern of expression of *mlo* in compatible and incompatible interactions (Trt.Type.Time.Comp effect in table 4.4;

P=0.009) with *mlo* more upregulated in the susceptible varieties at 7 and 14 dai than in the resistant varieties (figure 4.4).

Interaction	**d.f.**	***Chitinase***	***mlo***	***Peroxidase***	***PR1***	***β-1,3-glucanase***
Trt.Type.Comp	1	4.2**	1.5	0.1	4.8*	2.3
Trt.Type.Time	5	15.6**	9.2	23.3***	60.5***	25.8***
Trt.Type.Time.Comp	5	8.6	15.4***	6.3	4.7	18.1**
Trt.Type.Variety.Comp	6	4.1	3.8	19.0**	22.2***	3.4
Trt.Type.Time.Variety.Comp	30	37.1	28.2	64.2***	69.2***	49.4*

Table 4.4 Wald statistics and their statistical significance for effects which include the Trt.Type interaction indicating a difference between inoculated and mock-inoculated plants in the expression of the genes of interest relative to a set of reference genes. Trt=Treatment Comp=compatibility. Significance level of chi-squared probability: *0.05>P≥0.01, **P0.01>P≥0.001, ***P<0.001.

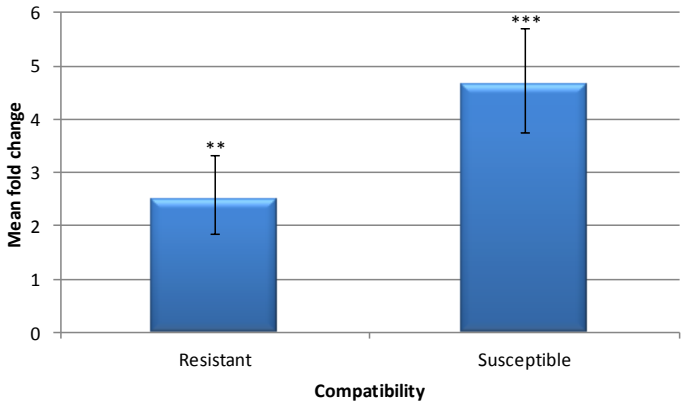


Figure 4.3 Expression of *chitinase* in either resistant or susceptible varieties inoculated with *M. graminicola*, averaged over time and variety. Significant differences compared to the mock inoculated control: **P0.01>P≥0.001, ***P<0.001.Error bars =±1 s.e of predicted means.

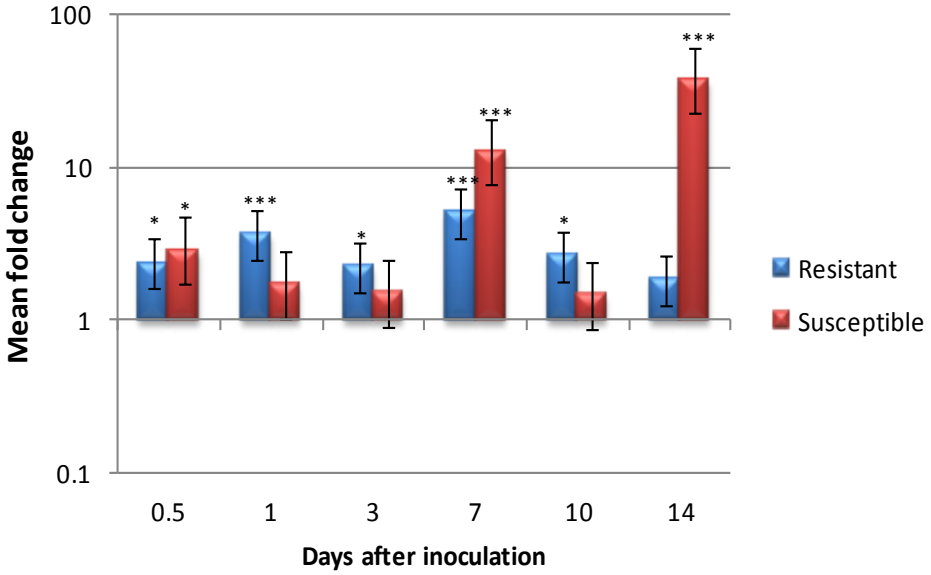


Figure 4.4 Expression of *mlo* in either resistant or susceptible varieties inoculated with M. *graminicola* over time determined by qPCR. Significant differences compared to the mock inoculated control: *0.05>P≥0.01, **P0.01>P≥0.001, ***P<0.001. Error bars =±1 s.e of predicted means.

Peroxidase, *PR1* and *β-1,3-glucanase* have several significant interaction terms, but all show a significant interaction between all the factors Time, Treatment, Variety, Type and Compatibility (table 4.4) demonstrating that there is significant variation in the expression of these genes, over time and varieties and dependent on whether the interaction was compatible or incompatible, independent of the isolate used for inoculation.

β-1,3-glucanase (figures 4.5) showed significant differential regulation between susceptible and resistant varieties at all timepoints. The relative expression of *β-1,3-glucanase* in inoculated plants changes significantly over the time course (Treatment.Type.Time in table 4.4; P=<0.001). When the effect of resistance or susceptibility to the isolate is introduced (Treatment. Type.Time.Compatibility in table 4.4; P=0.003 and figure 4.5) the effect remains significant. Susceptible varieties accumulated more of the transcript at 7 and 14 dai than the resistant varieties (figure 4.5).

Peroxidase (figures 4.6) was significantly differentially regulated at all timepoints, although there was no effect of compatibility indicating that *peroxidase*

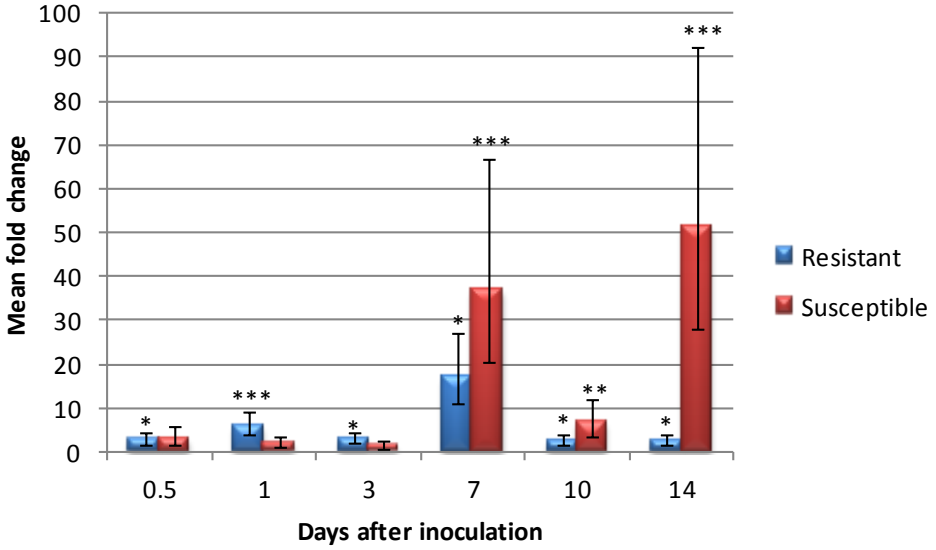


Figure 4.5 Expression of *β-1,3 glucanase* in either resistant or susceptible varieties inoculated with *M. graminicola* over time determined by qPCR. Significant differences compared to the mock inoculated control: *0.05>P≥0.01, **P0.01>P≥0.001, ***P<0.001. Error bars =±1 s.e of predicted means

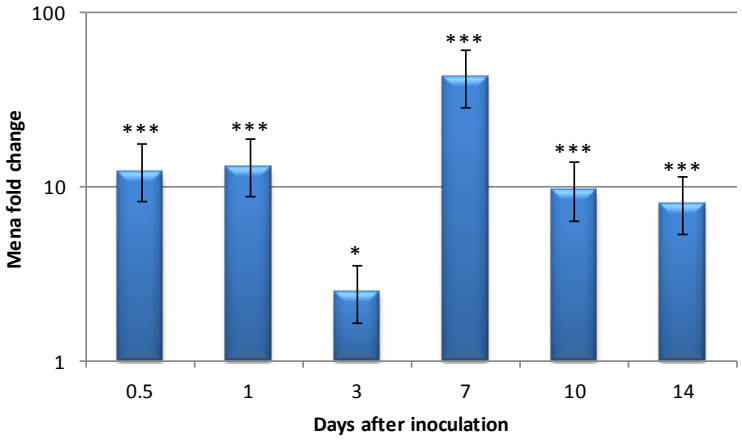


Figure 4.6 Expression of *peroxidase* in leaves inoculated with *M. graminicola,* irrespective of the compatibility of the isolate used determined by qPCR. Significant differences compared to the averaged mock inoculated controls **0.01>P≥0.001, ***P<0.001. Error bars =±1

was produced in response to inoculation rather than as a resistance or susceptibility response. There was substantial variation in the level of expression in inoculated

plants over time (Trt.Type.Time in table 4.4; P=<0.001) with the highest levels of transcript accumulation at 7 dai (figure 4.6).

Levels of *PR1* were differentially regulated at all timepoints (figure 4.7). The

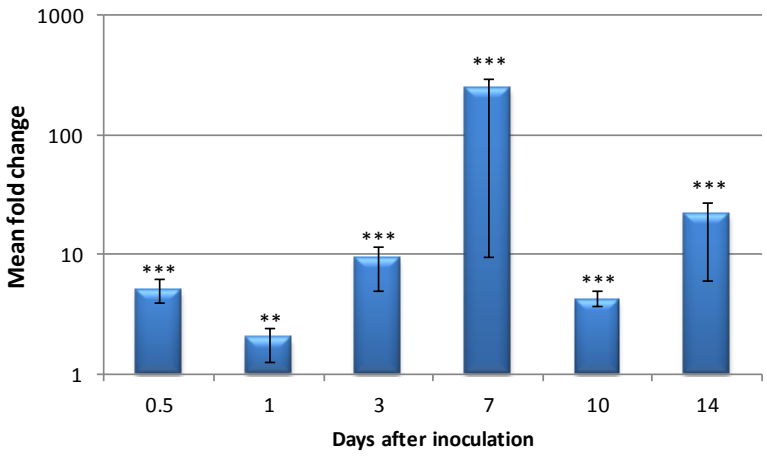


Figure 4.7 Expression of *PR1* in leaves inoculated with *M. graminicola,* (predicted mean over varieties and isolates) determined by qPCR. *0.05>P≥0.01, **P0.01>P≥0.001 Error bars =±1 s.e of predicted means.

relative expression of *PR1* in inoculated plants changes significantly over the time course (Trt.Type.Time in table 4.4; P=<0.001) with more transcript accumulating at 7 dai (figure 4.7).

Figure 4.8 shows the fold change over the mock inoculated samples for *peroxidase*, *PR1* and *β-1,3-glucanase* for the most resistant (Flame inoculated with IPO323) and most susceptible (Longbow inoculated with IPO323) interactions. Current evidence suggests that Flame only has *Stb6* and Longbow only *Stb15* (Arraiano and Brown, 2006). There is a clear differential in the gene expression between a resistant and susceptible variety. The expression of all three genes is consistently higher from 7 dai onwards in the susceptible variety Longbow than in the resistant one, Flame. At 7 dai all three genes are significantly upregulated in Longbow but not in Flame, suggesting that this timepoint may have some biological significance. Expression of *peroxidase*, *PR1* and *β-1,3-glucanase* in Flame shows very little change over the mock

inoculated samples at the 7, 10 and 14 dai timepoints, but the *peroxidase* gene is significantly upregulated at 0.5 dai and 1 dai.

Figures 4.9-4.11 compare the expression levels of *peroxidase, PR1* and *β-1,3-glucanase* over the eight interactions over time. There was a great deal of variation in the transcript accumulation for all genes between varieties and at all timepoints. Moreover, gene expression was variable between replicates, giving large standard errors. Overall, the expression of the genes is strong at 7 dai followed by 14 dai.

Expression of *β-1,3 glucanase* was strongest at 7 dai followed by 14 dai (figure 4.9). Although the average value of relative expression of *β-1,3-glucanase* over the time-course did not vary significantly between varieties (Treatment.Type.Comp Variety in table 4.4; P=0.753), there were marked differences in the change in expression of *β-1,3-glucanase* between timepoints in different varieties (Treatment.Type.Time.Comp.Variety in table 4.4; P=0.014 and figure 4.9). Across the varieties tested with both isolates over the time course, there was no clear pattern to differentiate between resistant and susceptible varieties using *β-1,3 glucanase* as a marker. This does not mean that the levels of *β-1,3 glucanase* accumulation are not biologically significant.

Peroxidase was most strongly upregulated at 7 dai followed by 10 dai (figure 4.10). The temporal pattern of peroxidase expression was strongly influenced by the variety (Trt.Type,Time.Comp.Variety in table 4.4; P=<0.001 and figure 4.10), implying that *peroxidase* gene expression does not differ consistently between resistant and susceptible varieties.

Expression levels of *PR1* were highest at 7 dai, followed 14 dai (figure 4.11). More transcript accumulated in the susceptible varieties averaged over time and varieties (Trt.Type.Comp in table 4.4; P=0.028), but there was no clear differential between susceptible and resistant varieties once separated into independent varieties at either early or late timepoints (figure 4.11).

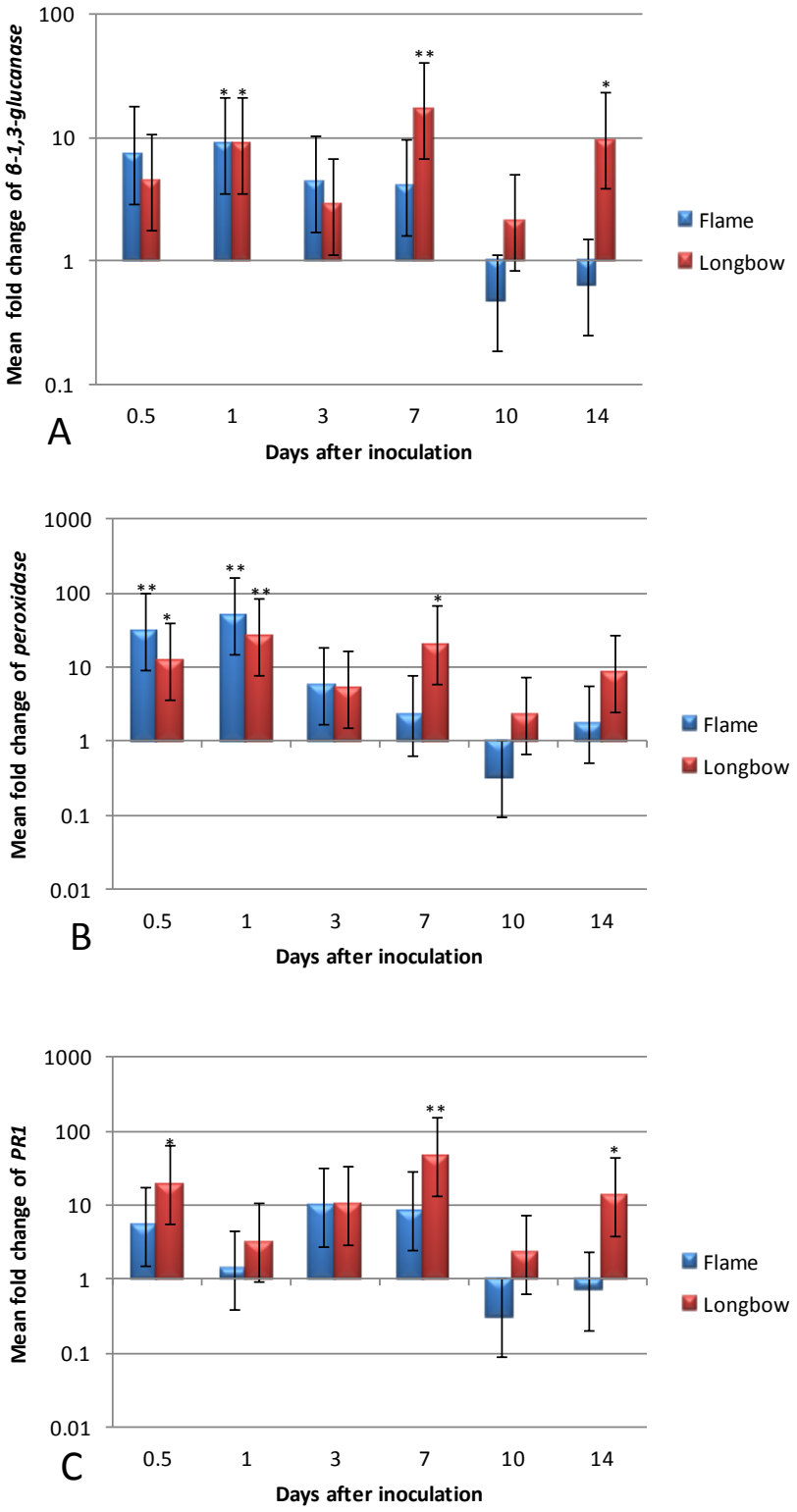


Figure 4.8 Expression levels (ΔCq) of **A** *β-1,3-glucanase*, **B** *Peroxidase* and **C** *PR1* in the varieties Flame (highly resistant) and Longbow (highly susceptible) inoculated with IPO323 as determined by qPCR. Error bars are ±1 s.e. of the predicted mean (explained in section 4.2.2) Significant differences from mock-inoculated control: *0.05>P≥0.01, **P0.01>P≥0.001, ***P<0.001. Fitted model: Treatment.Type Time.Variety.Compatibility.

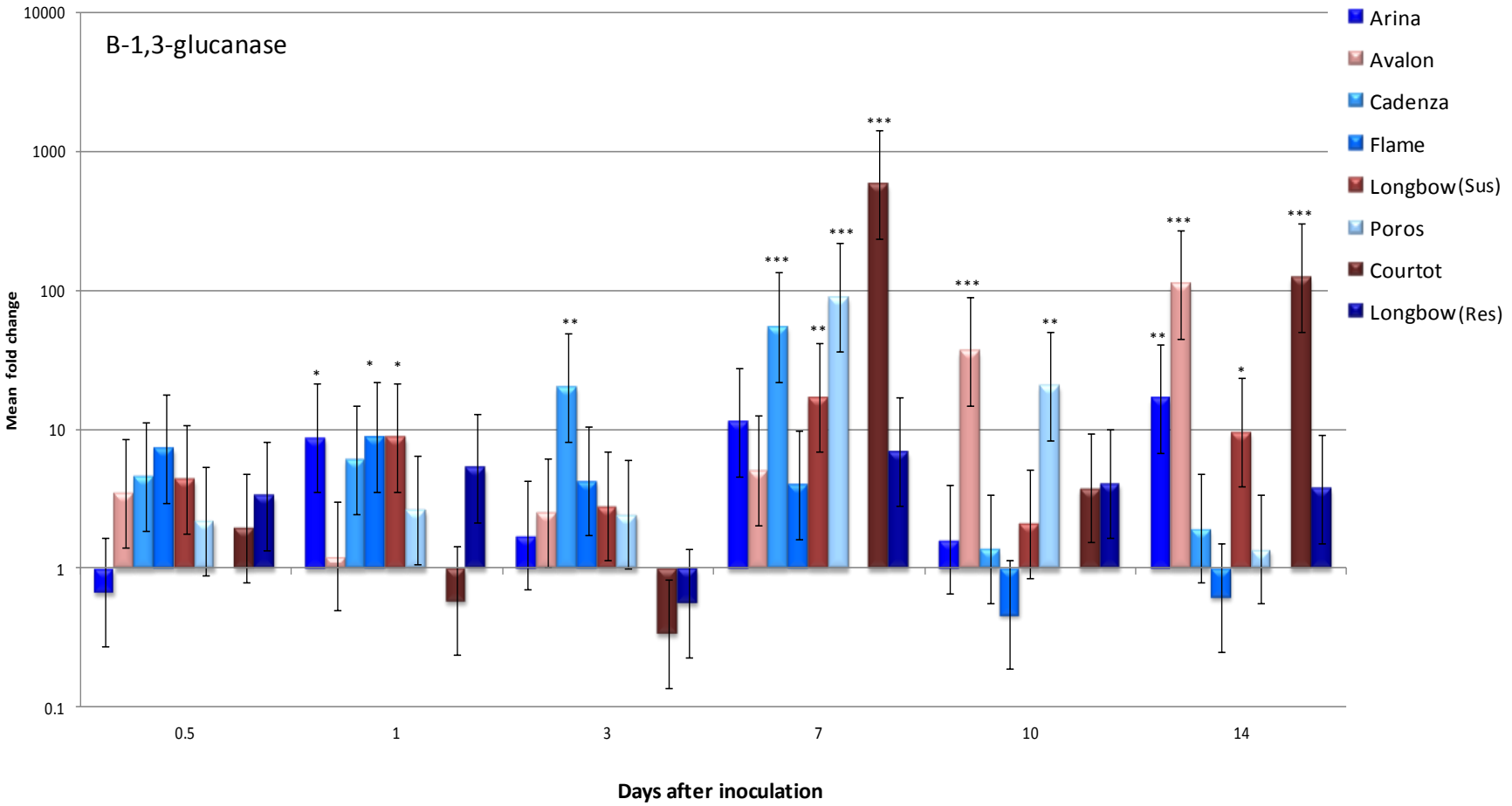


Figure 4.9 Expression (ΔCq) of *β-1,3-glucanase* over time. Incompatible interactions are coloured blue and compatible interactions are red with *M. graminicola* isolate IPO323 or IPO88004. Error bars are ±1 s.e. of the predicted mean (explained in section 4.2.2) Significant differences from mock-inoculated control: *0.05>P≥0.01, **P0.01>P≥0.001, ***P<0.001.Fitted model: Treatment.Type Time.Variety.Compatibility.

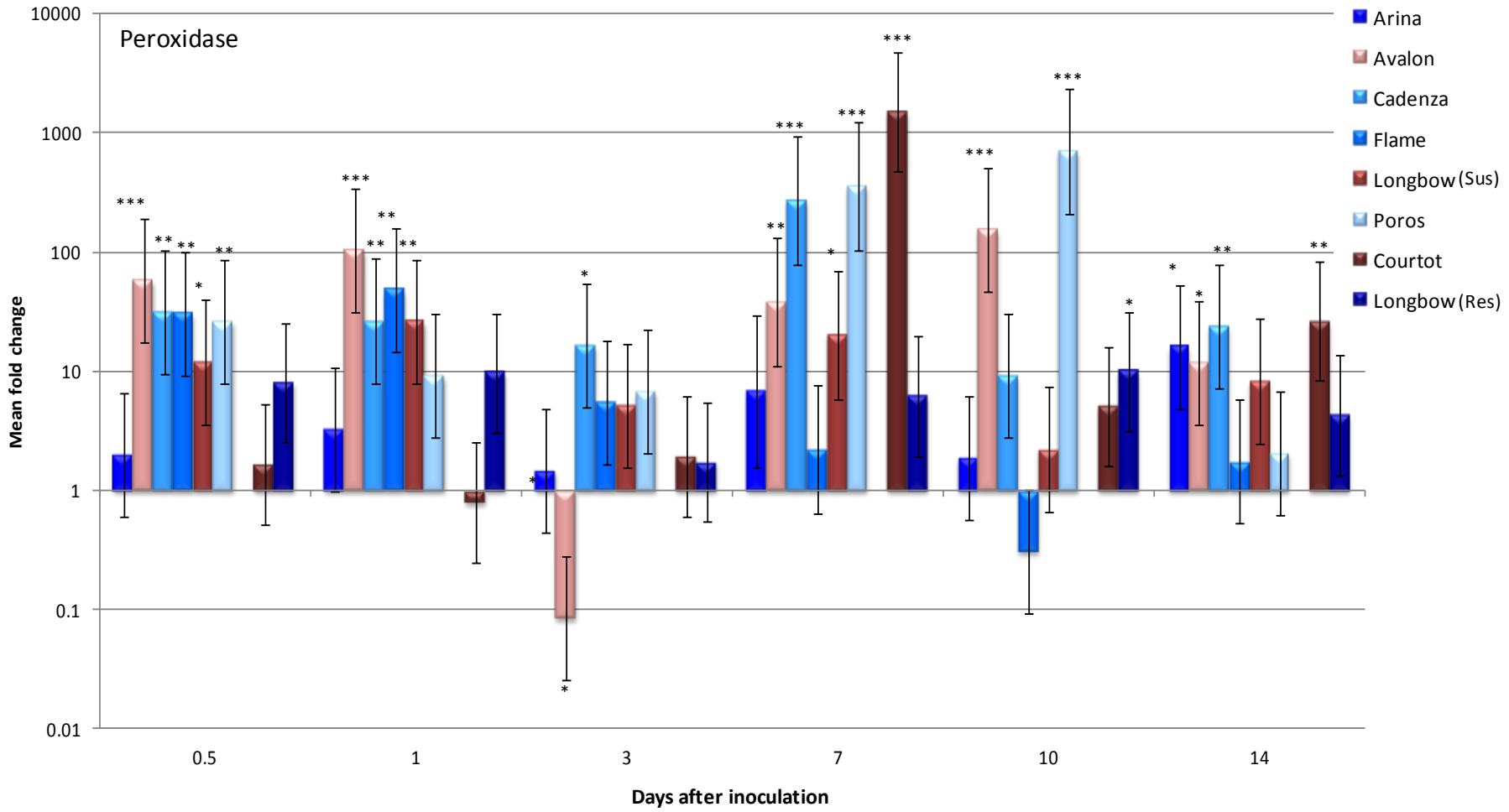


Figure 4.10 Expression (ΔCq) of *peroxidase* over time. Incompatible interactions are coloured blue and compatible interactions are red with *M. graminicola* isolate IPO323 or IPO88004. Error bars are ±1 s.e. of the predicted mean (explained in section 4.2.2) Significant differences from mock-inoculated control: *0.05>P≥0.01, **P0.01>P≥0.001, ***P<0.001. Fitted model: Treatment.Type Time.Variety.Compatibility.

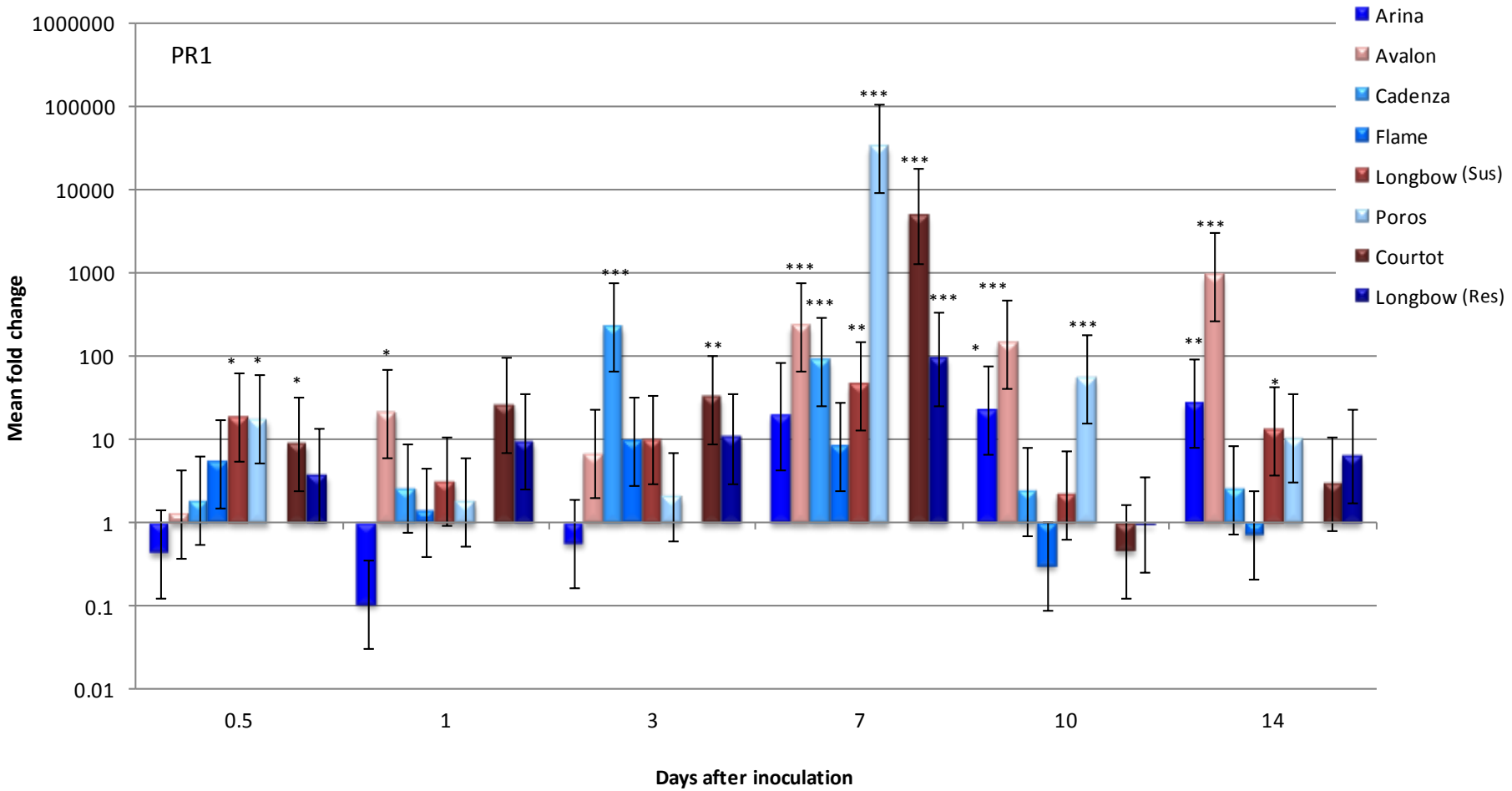


Figure 4.11 Expression (ΔCq) of for *PR1* over time. Incompatible interactions are coloured blue and compatible interactions are red with *M. graminicola* isolate IPO323 or IPO88004. Error bars are ±1 s.e. of the predicted mean (explained in section 4.2.2) Significant differences from mock-inoculated control: *0.05>P≥0.01, **P0.01>P≥0.001, ***P<0.001.Fitted model: Treatment.Type Time.Variety.Compatibility.

4.3.2 Accumulation of the wheat mitogen activated protein kinase 3 protein

Changes in the levels of TaMPK3 gene expression were not significant in the experiments in 4.3.1, although it was previously found to be upregulated in a compatible interaction involving the variety Avalon (Rudd *et al.,* 2008). TaMPK3 expression was also upregulated in the resistant variety Cadenza, although to a lesser extent than in the compatible interaction (Rudd *et al.,* 2008).

Western blots were used to assess the amount of the TaMPK3 protein that accumulated in each variety from 1 to 17 dai (figure 4.11 and 4.12). The whole timecourse of the experiment was run over two gels; days 1-10 and 11-16 and run at the same time; because of this, levels across varieties cannot be directly compared. All reasonable steps were taken to ensure that each set of gels were treated in exactly the same way. The two gels were processed together in the same gel tank and then probed with the antibody in separate vials, which may account for the apparent rapid increase in TaMPK3 levels between days 10 and 11. The two gels are shown side by side in figures 4.11 and 4.12. The inset shows days 7-14 run on the same gel (from a second replicate) to give a more realistic indication of the change from days 10-11. The loading levels are indicated by the 60-kD region. Very little difference in TaMPK3 levels was seen between 1 and 10 dai in all varieties independent of the isolate used for inoculation (figures 4.11 and 4.12). All varieties with the exception of Avalon showed little accumulation of TaMPK3 at 1 dai. At 3 dai some TaMPK3 accumulated in both the treated samples and in the mock inoculated samples, indicating a response to either the experimental set up or environmental conditions. In all treatments, levels of TaMPK3 remained fairly constant up to 10 dai. Mock inoculated samples at 10 dai had the same levels of TaMPK3 as in inoculated leaves or slightly less (Avalon and Courtot).

The varieties inoculated with isolate IPO323 (figure 4.11) started to show differences in the accumulation of TaMPK3 from 11 dai onwards. The effect can be seen most strongly by comparing the 16 dai sample with the mock-inoculated sample

at the same timepoint. In Avalon and Longbow, which are susceptible to IPO323 much more of the protein had accumulated in the treated sample than in the mock inoculated sample at 16 dai. Avalon had a particularly high level of accumulation. The level of TaMPK3 did not increase in the variety Longbow after 11 dai but still had much higher levels than in the mock inoculated sample at 16 dai. The weakly resistant varieties, Poros and Cadenza, did show some accumulation of TaMPK3, especially Poros, which appeared to show an increase at 14 and 15 dai, before the level reduced again by 16 dai. The resistant varieties, Flame and Arina, showed little accumulation of TaMPK3 through the timecourse; although, from the second replicate of Flame shown in figure 4.11, there is an accumulation of the protein at 14 dai, by 16 dai there is no increase over the mock inoculated sample, In the two varieties inoculated with IPO88004 the susceptible variety Courtot (figure 4.12), accumulated the TaMPK3 protein gradually from 11 dai. While the resistant variety Longbow did accumulate the protein there was no increase in comparison with the mock inoculated sample.

These results show that TaMPK3 protein accumulated during both compatible and incompatible interactions between *M. graminicola* and wheat in response to different isolates on different varieties. In the susceptible varieties TaMPK3 accumulated to greater levels than in the resistant and weakly resistant varieties and at the late stages of the interaction showed greater levels than in the mock inoculated samples. The resistant and weakly resistant varieties also accumulated TaMPK3 to differing levels, although by 16 dai none of these varieties showed greater accumulation than in the mock inoculated samples. The accumulation appears to have been independent of the level of gene expression in these experiments because TaMPK3 expression levels were not significantly different between varieties (tables 4.3 and 4.4).

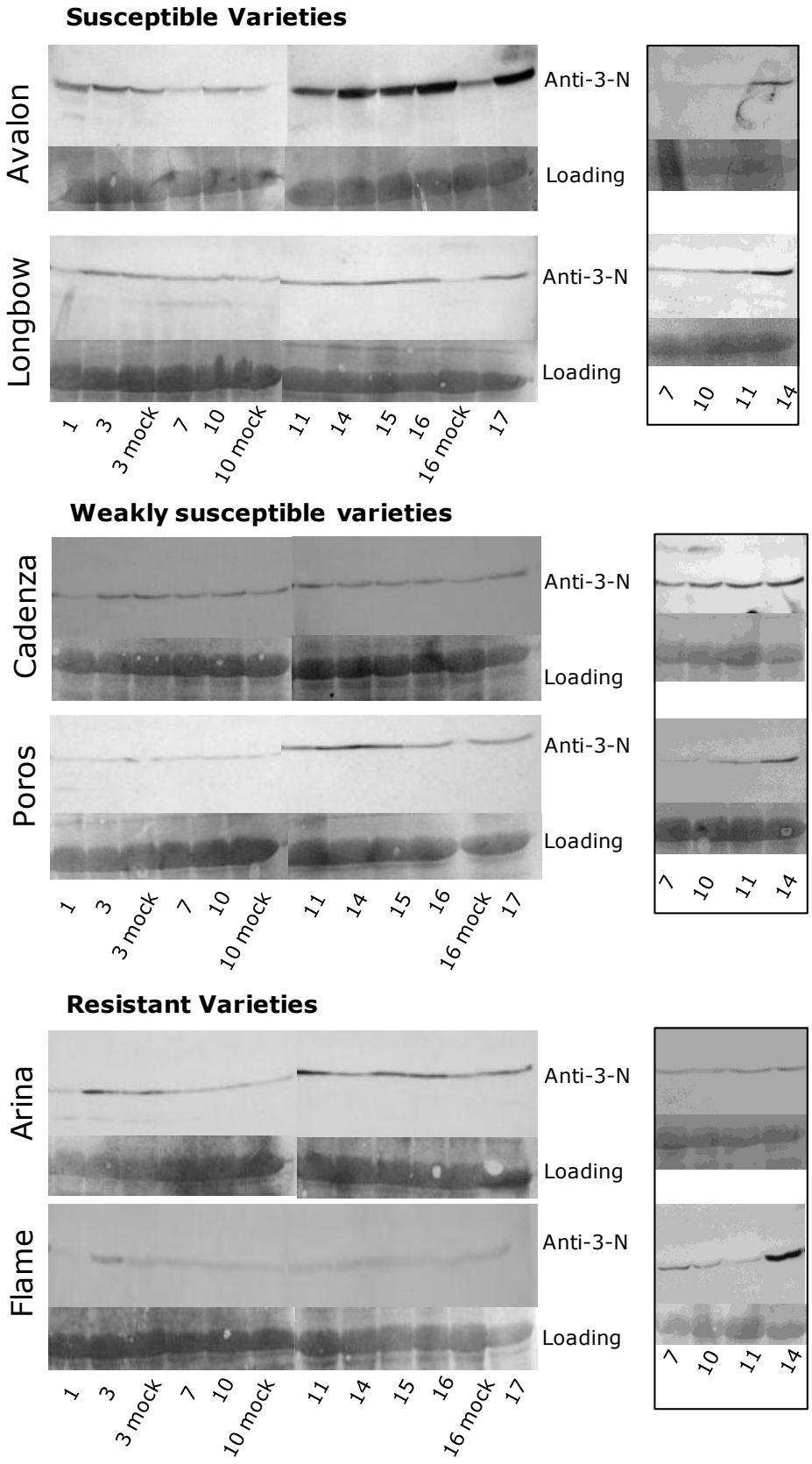


Figure 4.12 Western blots showing TaMPK3 accumulation in Avalon, Longbow, Cadenza, Poros. Arina and Flame inoculated with IPO323 over a timecourse of 17 days. Samples were loaded over two gels. The loading control was stained with Coomassie blue in the 60-kD region. X-axis = dai. Anti-3-N=kinase specific antibody to TaMPK3 recognising the N terminus of the protein. Boxes to the right show samples from days 7-14 after inoculation from a second replicate loaded on the same gel.

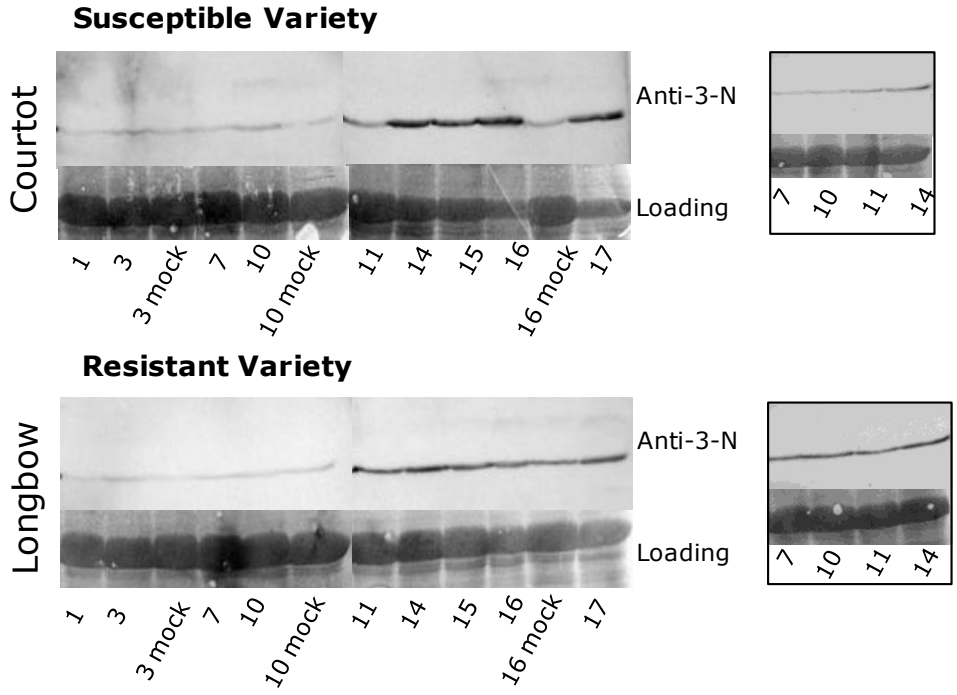


Figure 4.13 Western blots showing TaMPK3 accumulation in Longbow and Courtot inoculated with IPO88004 over a timecourse of 17 days. Samples were loaded over two gels. The loading control wa stained with Coomassie blue in the 60-kD region. X-axis = dai. Anti-3-N=kinase specific antibody to TaMPK3 recognising the N terminus of the protein. Boxes to the right show samples from days 7-14 after inoculation from a second replicate loaded on the same gel.

4.4 Discussion

Currently, there is no reliable method for identifying specific resistances by phenotype in plants inoculated with *M. graminicola;* by contrast resistance to rust is well correlated with infection type and latent period (Ma and Singh, 1995) and resistance to mildew is well defined on an infection type scale (Moseman, 1965). This is important for understanding how disease is caused, but also in breeding programmes where the early, quick and reliable identification of resistant lines is of commercial importance. The experiments described here investigated wheat gene expression, using several genes which are known to be differentially regulated in both compatible and incompatible interactions with other pathogens. The accumulation of TaMPK3 protein was studied to test the pattern of accumulation in the eight variety by isolate

interactions used for gene expression analysis and to test that this response is not specific to infection by IPO323 (Rudd *et al.*, 2008).

The gene expression analysis presented here used a linear mixed model to analyse the Cq values in the samples in comparison with the reference genes. Normalisation of the Cq value to the reference genes took place after the statistical analysis, to allow the accurate calculation of the fold change in the expression level. This method improves the accuracy of statistical analysis, using a widely available method of calculation without the need for specialist software, and allows standard errors to be calculated by conventional means.

Study of the compatible interactions between two *M. graminicola* isolates and two different gene-for-gene interactions using several varieties allows comparisons to be made between the expression of the 11 genes chosen for analysis in the different interactions over time. The biggest effect in these experiments was that of the *M. graminicola* inoculum; simply inoculating the plants with *M. graminicola* caused differential regulation in 7 out of the 11 genes tested compared with the mock inoculated control plants. The isolates used were not found to have an effect on gene expression, rather it was the compatibility of the interaction that affected gene expression and was therefore used as a factor in the model. There was no evidence that there was a distinct response specifically to either a virulent or an avirulent isolate. Across the varieties, the response to inoculation was varied and overall the expression levels of the genes tested in the resistant varieties were not less than those of the susceptible varieties.

β-1,3-glucanase was differentially expressed across the timecourse in all varieties and differences were seen between compatible and incompatible interactions. Shetty *et al.* (2009) found that in wheat inoculated with *M. graminicola,* β-1,3-glucans induced the expression of a wheat apoplastically located *endo-1,3-beta glucanase* gene and induced callose deposition. It was proposed that these responses inhibited STB symptom development of a virulent isolate at 15 dai. This recognition of β-1,3-

glucans resembles a PAMP-pathogen recognition receptor interaction (Zipfel & Felix, 2005). β-1,3-glucans from *M. graminicola* must be recognised by the plant, presumably by a receptor which has yet to be discovered in wheat (Orton *et al*., 2011). If *β-1,3-glucanase* has a role in the interaction between wheat and *M. graminicola*, it is as yet unclear what this might be. From my experiments and the evidence from previous investigations, it appears that using *β-1,3-glucanase* as a marker for either resistance or susceptibility of wheat lines would be ineffective. Upregulation of the gene occurs in both compatible and incompatible interactions with *M. graminicola* so it may be a general PAMP defence response.

The results here show that the susceptible varieties expressed *β-1,3-glucanase* more than the resistant varieties, but the pattern seen was highly variable and no pattern emerged by separating the data by variety. Large differences between replicate tests have been seen in other experiments investigating the expression of various defence-related genes in interactions with *M. graminicola*. Ray *et al.* (2003) showed that although trends were similar between replicates, induction of various *PR* genes, including *β-1,3-glucanase*, varied between replicates indicating that the timing of the interaction between *M. graminicola* and the host is inherently variable. An experiment testing *β-1,3-glucanase* transcription up to 15 dai only investigated one compatible and one incompatible interaction (Shetty *et al.,* 2009); this renders it difficult to make general conclusions about the role of *Stb* genes because it is not possible to tell if it is the reaction of a single variety or more generally of the gene. In the experiments presented here, using several varieties with two isolates, the isolate used does not have an effect on gene expression and it is the compatibility of the interaction which is significantly associated with the response of the wheat variety.

In the experiments reported here, *chitinase* was more upregulated in the susceptible than the resistant varieties, although it accumulated significantly in both. This is consistent with other experiments (Shetty *et al.,* 2009), although in some experiments, very little transcript accumulated during compatible interactions

(Adhikari *et al.,* 2007). The role of *chitinase* during the interaction between wheat and *M. graminicola* is unclear; it may acting in a similar way to *β-1,3-glucanase* as these genes often show transcription levels that follow the same pattern (as shown by the hierarchical cluster analysis in figure 4.1). Marshall *et al.* (2011) reported an *M. graminicola* secreted protein, Mg3LysM, that has chitin binding properties. This protein also appears to protect the fungus from hydrolysing enzymes. *Mg3LysM* is only expressed during the symptomless phase of growth, so it could be hypothesised that the increase in chitinase seen in susceptible varieties is mostly due to an increase in the presence of chitin during macroscopic symptom development, although this would not take into account why there is no effect of time on this interaction.

Peroxidase has been reported to be associated with resistance to pathogens (Hiraga *et al.*, 2001; Thordal-Christensen *et al.*, 1997). Here, expression of *peroxidase* was differentially regulated over time and between the varieties and compatibilities over time. There was high variability between replicates with no clear pattern of expression. The variability between varieties has been noted in previous investigations; Adhikari *et al.* (2007) showed that *peroxidase* transcript accumulated to high levels in one of the resistant varieties investigated at 1-3 dai, but in another resistant variety and two susceptible varieties investigated, the transcript accumulation was low at all timepoints. Shetty *et al.* (2003) found that H_2O_2 accumulation was not well correlated with accumulation of host peroxidase, suggesting that the H_2O_2 may be partly or wholly produced by the pathogen, not the plant, perhaps to advance the host cell death needed for susceptibility; in the experiments reported here the *peroxidase* gene used was specific to the plant. Peroxidase can be involved with both the production and scavenging of H_2O_2. It has also been proposed to aid lignification and cross-linking of cell wall components during pathogen resistance reposnses (Hiraga *et al.*, 2001). To date these effects have not been reported in the interaction with *M. graminicola;* the nature of its involvement in the wheat-*M. graminicola* interaction is unclear. More investigation would be needed to determine if

expression of this *peroxidase* gene is a marker for resistance at 7 dai. Other peroxidases could be investigated and further investigation of the staining patterns of H_2O_2 may allow resistant or susceptible varieties to be differentiated early on in the infection process.

Shetty *et al.* (2003) demonstrated that different varieties constitutively produce different levels of peroxidase; the resistant variety Stakado produced more than the susceptible Stevin. This may have an effect on the amount of H_2O_2 produced *in planta*. Comparisons between the two varieties inoculated with the same isolate showed that although *peroxidase* transcript accumulation was higher in the resistant variety compared to the susceptible variety, the change in the level of *peroxidase* was greater in the susceptible variety. There may be a biological threshold for transcript accumulation which is different in different varieties. These experiments and the experiment presented here show that the production of *peroxidase per se* may not be directly related to susceptibility. Comparing Longbow with Flame inoculated with IPO323, however, shows that Flame accumulates higher levels of peroxidase between 1-3 dai and Longbow accumulates more at 7-14 dai, implying that temporal variation in the expression of peroxidase may be related to susceptibility. Even in the resistant varieties, Flame and Arina, there was a quantitative difference in the amount of peroxidase transcript, which means that if *peroxidase* transcription plays a role in the host response to *M. graminicola*, the amount of transcript produced may be important.

PR1 is often used as a marker for a disease resistance. It is upregulated in both incompatible and compatible interactions, although the response tends to be faster and stronger response in incompatible interactions (HammondKosack & Jones, 1996). The experiments here showed that there was differential regulation between virulent and avirulent isolates and between different varieties. As with the other genes tested the variability was high and there was no clear pattern to differentiate between resistant and susceptible varieties. Ray *et al.* (2003) showed that *PR1* was upregulated strongly in both resistant and susceptible interactions with *M. graminicola*

at 12 hai (the study did not include timepoints at the start of macroscopic symptom development). Here, *PR1* was not upregulated strongly at the early timepoints in any of the varieties, but showed much greater upregulation at 7 dai. Adhikari *et al.* (2007) demonstrated strong induction of *PR1* in incompatible interactions but little *PR1* response in susceptible cultivars. The experiments here showed that the susceptible cultivars do accumulate *PR1*. *PR1* does not appear to be a good indicator for either resistance or susceptibility as it shows high variability both within and between experiments.

Of the other genes tested only *chitinase*, *LOX*, *mlo* and *PAL* were found to be significantly affected by treatment with *M. graminicola* inoculum. The evidence did not support the hypothesis that resistant varieties accumulate less transcript of these genes than susceptible varieties, with the weakly resistant varieties showing an intermediate response. However, overall *chitinase* was more upregulated in the susceptible varieties than resistant varieties and *mlo* showed significant upregulation in susceptible varieties at 7 and 14 dai, indicating that there is some effect of compatibility. The pattern of *mlo* transcript accumulation was similar to that of *β-1,3-glucanase* over time and separated by compatibility, indicating that the expression patterns of these genes may be biologically significant, and that these defence genes may be regulated by the same mechanisms to cause resistance or susceptibility within the plant. Two of the genes tested, *β-1,3-glucanase* and *peroxidase*, showed similar patterns of expression in response to both the tested isolates, suggesting that *Stb6* and *Stb15* may function in a similar way with regards to resistance. It also suggests that a compatible interaction may induce expression of *β-1,3-glucanase* and *peroxidase* in a similar way.

The transcription profiles of the three genes with the most significant results shows a large upregulation in gene transcript at 7 dai, before macroscopic symptoms appear on the leaves. It is possible that at 7 dai the plant responds to infection by the fungus as its biomass starts to increase. This could be the point at which resistance

and susceptibility begin to be differentiated; more investigation would be needed to investigate this further using more isolates and varieties with different *Stb* genes. It has been proposed that a contributing factor to resistance may be the failure of the fungus to trigger host cell death that is a feature of the compatible interaction (Rudd et al 2008; Hammond-Koasack and Rudd 2008); this could arise from an active suppression of the cell death element of the resistance response. Incompatibility would require an *R-Avr* interaction to directly or indirectly prevent the 'susceptibility' events from happening (Hammond-Koasack and Rudd, 2008). Wolpert *et al.* (2002) reviewed several pathosystems involving host selective toxins and suggested that avirulence factors and toxins essentially trigger the same pathways. Lorang *et al.* (2007) demonstrated that the host-specific Victorin toxin produced by the necrotrophic pathogen, *Cochliobolus victoriae,* can exploit the host PCD signalling response to gain virulence by inducing HR-like cell death. In *Arabidopsis* this has been shown to be mediated by LOV1 a 'resistance' protein belonging to the NBS-LRR resistance gene family. During the wheat-*M. graminicola* interaction this type of *R*-gene mediated 'susceptibility' is not seen as the genetics follow the gene-for-gene interaction, but a toxin could potentially mediate susceptibility which manipulates the host resistance reponse.

In these experiments it has been assumed that resistance against IPO323 is dependent on the same gene, *Stb6* (and similarly *Stb15* for IPO88004). No *Stb* genes have been cloned therefore it is not known what function they may have; only the effects of inoculations with isolates known to have corresponding *Avr* genes can be studied (Arraiano and Brown, 2006). Whereas it has been demonstrated by genetic analysis that many wheat varieties have the *Stb6* gene effective against isolate IPO323 (Chartrain *et al*. 2005b), the presence of *Stb15* has only been inferred from observations of resistance to IPO88004. A factor that may be the cause of some of the variation seen between resistant varieties is that there is likely to be genetic variation in the expression of *Stb6* (Arraiano and Brown, 2006).

Stb6 and *Stb15* may not cause resistance through the same mechanism. It remains to be discovered how *Stb* genes confer resistance and if the different genes cause this through the same mechanism. It has been assumed that they do because varieties with different resistance genes all present resistance in the same way; no macroscopic symptoms are seen and there is no HR.

The accumulation of the TaMPK3 protein in all of the three compatible interactions tested shows that this is a consistent feature of the *M. graminicola*-wheat interaction, irrespective of the variety or isolate. In contrast to Rudd *et al.* (2008), the accumulation of the gene transcript did not show significant differential regulation in the experiments reported here, but there were differences seen in the accumulation of the protein. In the susceptible varieties, accumulation of the protein started from 11 dai in these experiments, at the time when the levels of nutrients in the apoplast are increasing (Keon *et al.,* 2007). Two of the resistant varieties, Poros and Flame, also showed some accumulation of TaMPK3, whereas three of the resistant varieties showed no increase in levels of TaMPK3 over the timecourse. The data suggest that TaMPK3 protein accumulation is a consequence of inoculation with *M. graminicola* and that during compatible interactions, TaMPK3 always accumulates during the later stages of infection. During incompatible interactions TaMPK3 accumulation appears to be variety dependent. The mock inoculated samples also accumulate a small amount of TaMPK3, perhaps due to a senescence response to experimental conditions. The resistance genes *Stb6* and *Stb15* may both either directly or indirectly reduce the strength of the signalling cascade that results in accumulation of TaMPK3 and differences in the expression of the resistance genes contributing to differences in the accumulation levels of TaMPK3.

To date, the function of the TaMPK3 protein or any of its orthologues remains unknown. It appears to be related to cell collapse or disruption or the development of HR (in response to biotrophic pathogens) or PCD. MPK3 and MPK6 have been implicated in stress and pathogen responses in *Arabidopsis* (Asai *et al*., 2002) and

orthologues have been shown to initiate cell death during HR-mediated resistance in tomato infected with *C. fulvum* after a signal is received (Stuhlemeijer *et al.,* 2007). In *Arabidopsis* both of these MPKs are phosphorylated downstream of a pathway induced by H_2O_2 (Kovtun *et al.*, 2000). There are few studies that have investigated MAPK involvement of pathogen infection of monocots; an orthologue of AtMPK3, OsMAPK5 in rice, accumulates during an incompatible interaction with *Magnaporthe oryzae* (syn. *M. grisea* (Couch & Kohn, 2002)) before an HR is seen, but accumulation accompanies the onset of lesion formation during a compatible interaction (Xiong & Yang, 2003). All these studies suggest that the accumulation of the MPK3 orthologues across several species is involved with HR or PCD, either during the HR to an avirulent biotroph or lesion formation during a compatible interaction with a non-biotroph. As no HR is seen during an incompatible interaction with *M. graminicola* (Cohen & Eyal, 1993; Kema *et al.*, 1996), it is unsurprising that there is no accumulation of TaMPK3 above the mock inoculated samples. During PCD and cell collapse in a compatible interaction, accumulation of the TaMPK3 is seen which may be related to the increased levels of H_2O_2 that accumulate in the late stages of the pathogen development (Shetty *et al.*, 2007). H_2O_2 has been shown to initiate a MAPK cascade that results in the induction of AtMPK3 (Kovtun *et al.,* 2000), but it may also be part of a positive feedback loop to induce more H_2O_2. It is known that *M. graminicola* can tolerate H_2O_2 but growth is inhibited by its presence (Shetty *et al.,* 2007), unlike in other non-biotrophic interactions, such as *B. cinerea* and *Drechslera* spp., where it appears to aid infection (Govrin & Levine, 2000; von Gonner & Schlosser, 1993). H_2O_2 accumulates in both resistant and susceptible varieties when infected with *M. graminicola* (Shetty *et al.* 2003) and the experiments presented here show that *peroxidase* transcript accumulation was also variable between varieties. Varying levels of H_2O_2 during the interaction with *M. graminicola* may therefore be influencing the level of TaMPK3 accumulation in the different varieties. If the plant produces more H_2O_2 in response to the increasing biomass of the pathogen, that could explain the increase in the

accumulation of TaMPK3. It is possible that the *Stb* resistance genestops this positive feedback loop. The results in chapter 3, where *TaMPK3* gene transcripts accumulated in response to a virulent isolate of *B. graminis* indicate that the induction of MPK3 response is not the same for all fungal pathogens. This is discussed further in chapter 6.

The high variability in the expression of the genes tested may be an inherent problem with this pathosystem. There is a long latent period in which the fungus grows very little and it is unlikely that all the infection points will start host cell death signalling at the same time. As the growth and lesion formation do not spread throughout the leaf there is unlikely to be a systemic effect of *M. graminicola* inoculation (Rudd *et al.,* 2008). This might cause gene expression to be less uniform than in a disease in which there is a systemic host response. If there are localised responses over a longer period of time, they are unlikely to be perceived in a gene expression study. The timing of resistance and/or susceptibility events may not be the same in all varieties; from figure 4.2 it can be seen that the three susceptible varieties differ in the level of macroscopic chlorosis and necrosis at 14 dai. To investigate this further an appropriate method for staining *M. graminicola* inside the mesophyll is required. A further factor which has not been considerd either here or in previous work on *M*. *graminicola* or most other pathogens is that expression of defence-related genes is affected by exposure to microbes other than the pathogen of interest (Boyd *et al.*, 1994b). Variation in plant response to saprophytic or endophytic bacteria and fungi may be an additional source of variation in these experiments, as discussed further in chapter 6.

Even in well defined race-specific interactions, such as those between *Puccinia striiformis* f.sp. *tritici* and wheat, identifying an expression pattern of a gene or genes known to be involved with incompatibility is difficult. Bozkurt *et al.* (2010) showed that for several PR genes, including a *peroxidase* and a *β-1,3-glucanase*, although these genes were upregulated to a greater level in the incompatible interactions than

the compatible interactions, there was a high level of variation seen both between different varieties and between replicates. Similar results were found in barley inoculated with *B. graminis* f.sp. *hordei* (Boyd *et al.*, 1994a; Boyd *et al.*, 1994b). Even in these well defined systems, it is difficult to identify a specific level of gene expression at a particular timepoint that could be used to clearly predict that an interaction would be an incompatible one. Genes that have previously been identified as being differentially regulated during interactions with *M. graminicola* show no correlation with known resistances (*PAL* and *LOX,* Adhikari *et al.,* 2007; *PDI*, Ray *et al.,* 2003). In the experiments presented here *β-1,3-glucanase* showed significant upregulation in susceptible varieties at 7 dai. The response was highly variable and involved statistical analysis over three replicate experiments (with three leaves pooled within each experiment) to determine the results; it is therefore unlikely that using this gene as a marker of resistance would be of more value than the current system of measuring quantitative resistance by scoring visual symptoms.

It may be that the results here could be used to develop a more appropriate method, based on either gene expression analysis or microscopy, that could identify phenotypic resistance. Powdery mildew has a well defined set of characteristics for cytological analysis that are not available for studying interactions with *M. graminicola.* Boyd *et al.,* (1995) and Kmecl *et al.* (1995) both demonstrated that cytological analysis of powdery mildew development was more effective at defining resistance than using gene expression analysis. From the results presented here, it appears that defining a set of characteristics for cytological study would be of more benefit to obtain an indication of resistance or susceptibilty soon after inoculation, than trying to identify gene induction or suppression. The lack of clear patterns in gene expression uncovered by this detailed analysis of 11 genes covering the period of infection by *M. graminicola* demonstrates that the variable nature of the results is not conducive for use of the genes studied to classify wheat varieties as resistant or susceptible to particular isolates of *M. graminicola.*

The experiments presented here on gene expression analysis, offer no evidence that any of the genes tested were consistently involved in either a resistant or susceptible responses towards *M. graminicola* in different varieties. Although several of the genes were affected by inoculation by the pathogen, this could perhaps be a basal response to recognition of an unknown entity. The genes that showed a differential response over time suggests that the plant is responding to as yet uncharacterised changes in the development of the pathogen. This is indicative that it is difficult to reduce the variability seen after inoculation with *M. graminicola* and that selection of a single timepoint that allows informative comparisons between a compatible and incompatible interaction is difficult to achieve. No microarray analysis of wheat genes in response to *M. graminicola* has yet been published.

5

Symptom development and timing of resistance

5.1 Introduction

Mycosphaerella graminicola which causes septoria tritici blotch of wheat is a pathogen with a long latent period. Susceptibility is characterised by necrosis and the formation of pycnidia which produce conidia. Both virulent and avirulent spores germinate on the leaf surface and penetrate through the stomata. Although the lifecycle of the virulent isolate is well studied (Duncan and Howard 2000; Kema *et al.,* 1996) the avirulent interaction is less well studied (Cohen and Eyal, 1993; Kema *et al.,* 1996) with specific details and timings of the incompatible interaction unclear. Details of the timing of the incompatible interaction during isolate-specific resistance are ill defined and how this resistance operates is largely unknown; different *R*-genes may confer resistance by different mechanisms.

A resistance phenotype has been identified in several cereal pathogens including rusts and powdery mildew. For powdery mildews a infection scale has been designed: 0= no signs of infection through to 4= abundant mycelial growth and no necrosis or chlorosis visible (Moseman *et al.,* 1965). For rusts infecting cereals an infection type from 0-9 is assigned and the latent period of the uredinospores is quantified (Ma and Singh, 1996). More histological observations have also been made for powdery mildews, identifying the hypersensitive response (HR) in relation to race-specific genes (Boyd *et al.,* 1995) and penetration attempts and haustoria formation in relation to partial resistance. Recently, *Rhyncosporium secealis* (Linsell *et al.*, 2011; Thirugnanasambandam *et al.*, 2011), has been studied using GFP-transfomed isolates. This has allowed infection processes to be characterised and a resistance phenotype is beginning to emerge. *M. graminicola* evidently differs in many ways from that of other well studied pathogens therefore inferences about resistance mechanisms cannot be compared directly. Currently, resistances are identified using quantitative analysis

(Arraiano and Brown, 2006; Orton *et al.,* 2011). It is unclear when the resistance response occurs; Kema *et al.* (1996) found that less colonisation of the sub-stomatal cavity takes place in an incompatible interaction and there is less hyphal growth in the surrounding intercellular space compared with a compatible interaction but this is a quantitative trait. In terms of efficiency for breeding efforts it would be of great value to have an easily identifiable resistance phenotype that could be identified soon after inoculation has taken place. Identifying a resistance phenotype would also aid investigations into how the resistance mechanism works. The existence of the long latent period offers no clues as to the timing of resistance; it is known that it is *R*-gene mediated (Brading *et al.,* 2002) but as neither the *R*-gene or the corresponding *Avr* gene have been cloned their function remains unknown.

The aim of these experiments was to develop a reliable method for visualising *M. graminicola* in the intercellular space which could be used to identify a phenotypic resistance response. The prediction is that an avirulent isolate won't grow into the intercellular space and will remain in the substomatal cavity. The hyphae will die if they cannot eventually access nutrients through host cell death. Identifying the resistance response would enable the timing of the response to be more clearly defined and should eventually lead to understanding how resistance works. The prediction is that the latent period in a compatible interaction ends at 7 dai. Therefore, any resistance phenotype could be identified from this time.

5.2 Materials and methods

Descriptions of materials and methods not included here are in Chapter 2.

5.2.1 Plant and fungal material

17 day old seedlings of Flame (resistant) and Longbow (susceptible) were inoculated with *M. graminicola* isolate IPO323 when attached to Perspex sheets. Plants were

grown at 18 °C in the light for 16 h and 12 °C in the dark. At each collection time at least 3 leaves were sampled for staining. All inoculated plants were kept for at least 21 dai to ensure that pycnidia formation was seen in the compatible interaction and that no macroscopic symptoms were seen in the incompatible interaction (section 2.2).

5.2.2 Staining Uvitex 2b

Uvitex 2b stains chitin. Leaves were fixed and cleared in chloral hydrate (80% w/v) based solution, in 95% ethanol (30%): 90% lactic acid (12.5%) made up with chloroform. After washing in water, the samples were incubated at 90 °C in 0.05M sodium hydroxide for 17 minutes. Leaves were then rinsed twice in water and soaked in 0.1M Tris-HCl buffer (pH 5.8) for 30 minutes. Subsequently, leaves were then stained in 0·1% Uvitex 2B (Ciba-Giegy, (BASF), Ludwigshafen, Germany) in 0.1M Tris-HCl buffer for 5 minutes and rinsed with water. After a rinse with 25% glycerol samples were stored in 40% glycerol (modified after Rohringer *et al.*, 1977; Tufan *et al.,* 2009).

Leaves were examined using a Nikon fluorescence microscope equipped with epifluorescence optics. A UV-1A filter (excitation filter 330–380 nm, barrier filter 420 nm) was used to visualise fungal structures by their light blue fluorescence. A confocal laser scanning microscope (Leica), was used to excite the leaves with UV-laser beams at 351 and 364 nm and scanned with filter settings at 400–500 nm for Uvitex stained fungal structures.

5.2.3 Staining with Trypan Blue

Trypan Blue is a vital stain which is commonly used for staining dead tissue. It is unknown as to what this stain actually binds to. Leaves were cleared in 3:1 ethanol:acetic acid overnight. The leaves were then washed in water for 4 hours before being fixed in lactoglycerol (1:1:1 water:lactic acid:glycerol) for 20 minutes. A 0.1 % solution of Trypan Blue made up in 1:2 lactoglycerol:ethanol was used to stain

leaves. The cleared leaves were incubated at 70 °C for 2 hours in the Trypan Blue solution. Samples are stored in lactoglycerol. A Nikon Microphot-SA general light microscope was used to visualise fungal structures. Samples were taken at 8 dai, when it would be expected that the fungus had entered the apoplast. This method followed Siah *et al.* (2010).

5.2.4 Staining with Chlorazol Black E

Chlorazol Black E is used to selectively stain chitin. Several variations of the method of Brachmann et al (2002) and Sesma and Osbourn (2004), were tried to stain *M. graminicola* hyphae in the apoplastic space. All leaves were cleared in 75 % ethanol overnight before being rinsed with water and stained with 0.03 % Chlorazol Black E stain (CBE) in lactoglycerol solution and then stored in 50 % glycerol after staining.

Method 1: Leaves were incubated in 10 % potassium hydroxide (KOH) in a waterbath at 90 °C for 3 hours. Leaves were then washed in water and treated in CBE at 60 °C overnight.

Method 2: Leaves were incubated at 60 °C in 5 % KOH for 3 hours. Leaves were then washed in water and treated with CBE at 60 °C overnight.

Method 3: Leaves were incubated at 70 °C in 5 % (KOH) for 3 hours. Leaves were then washed in water and treated with CBE at 60 °C for 2 days. The waterbath containing the samples was turned off after 3 hours.

5.3 Results

5.3.1 Uvitex 2b

Staining with Uvitex 2b (U2b) was carried out on samples collected at different timepoints, 8, 13, and 20 dai. At each timepoints staining was attempted at least three times and on three separate leaves. Infection was successful and pycnidia visible on Longbow in all replicates used for staining tests. At 8 dai after inoculation, spores but no hyphae were visible on the surface of the leaf on both varieties, Flame and

Longbow (figure 5.1). No fungal hyphae was stained. Staining was very weak against the strong autofluorescent background, the trichomes in particular strongly autofluoresced at this time. Leaves were sampled from Longbow at 13 dai to check for staining in the mesophyll layer, as it was assumed that there would be a lot of growth of the pathogen in the substomatal cavity at this stage in a susceptible variety. Some spores were visible on the surface of the leaf, but no fungal material was visualised in the mesophyll layer. To see if the conidia formed as a result of the compatible interaction would be stained by U2b, samples were taken at 20 dai when pycnidia were clearly present on the leaves. The pycnidia were stained with the uvitex, although the staining was not clear enough to differentiate individual conidia (figure 5.2). Conidia stained on the leaves and when they are produced from pycnidia but no staining of hyphae was seen.

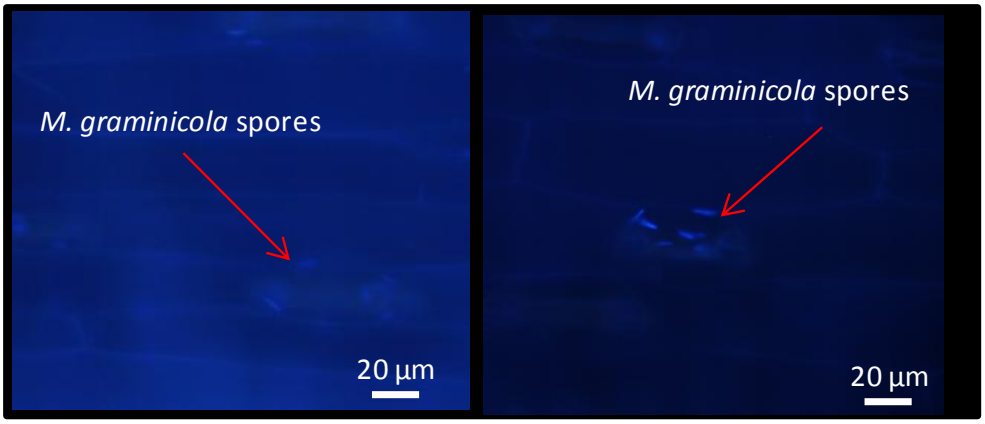


Figure 5.1 Spores of *M. graminicola* isolate IPO323 on Flame (left) and Longbow (right) stained with Uvitex 2b 8 days after inoculation.

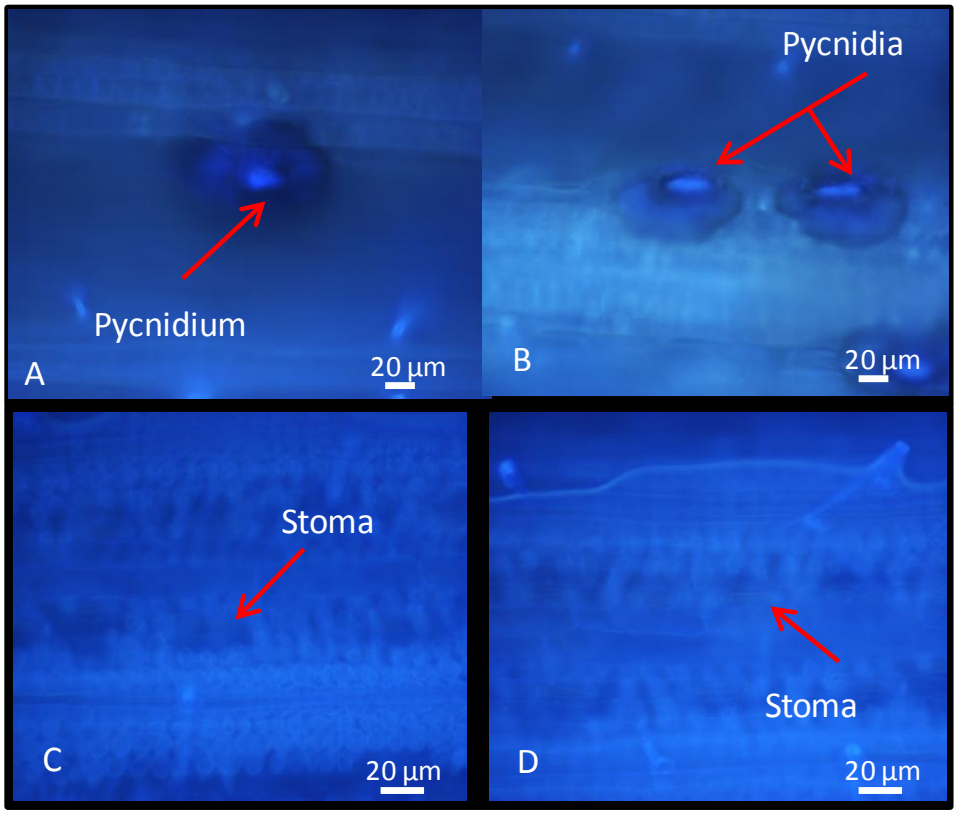


Figure 5.2 Longbow (**A, B**) and Flame (**C,D**) stained with Uvitex 2b at 20 dai inoculation with *M. graminicola isolate* (IPO323). Pycnidia have stained on the variety Longbow. No spores are visible on the variety Flame.

5.3.2 Trypan Blue

Trypan Blue has has been used to successfully stain *Blumeria graminis* f. sp. t*ritici* in wheat. It has also been used to stain *M. graminicola* in wheat during the later stages of infection (Siah *et al.*, 2010). It also differentiates necrotic host cells as it stains dead tissue. The method of Siah *et al.* (2010) was followed to stain leaves 7 dai with *M. graminicola.* The method was tried 3 times. Trypan blue stained the germinated spores on both Flame and Longbow on the surface of the leaf (figure 5.3) and germinated spores can be seen entering the stomata. No staining took place in the mesophyll layer.

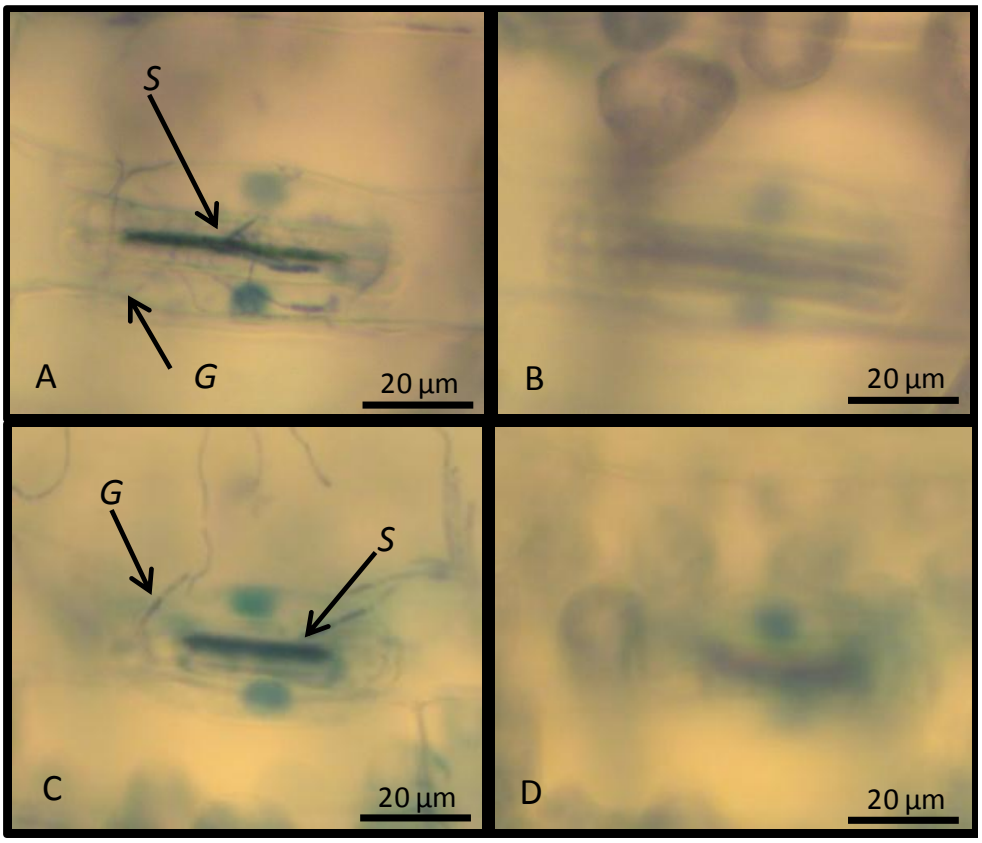


Figure 5.3 Longbow (**A,B**) and Flame (**C,D**) stained with Trypan Blue 7 dai with *M. graminicola* isolate IPO323. *S* indicates the stoma and *G* the germ tube on the surface of the leaves. **B** and **D** focus on the mesophyll layer, the stoma are just visible. No staining has taken place in the mesophyll layer.

5.3.3 Chlorazole Black E

Several methods were tried for staining with Chlorazol Black E as the seedling leaves used were very fragile after heating in potassium hydroxide. On several occasions the leaves disintegrated completely. Using a 5% concentration of KOH still meant that some of the leaf tissue disintegrated, but a fair proportion remained intact. Staining overnight proved to be ineffective, therefore staining for a longer period was tried (method 3), on three occasions. None of the methods resulted in staining *M. graminicola* (figure 5.4). Leaves were also examined under 200x magnification; the clearing process allowed all the leaf layers to be seen, no fungal material was visible in any of the layers.

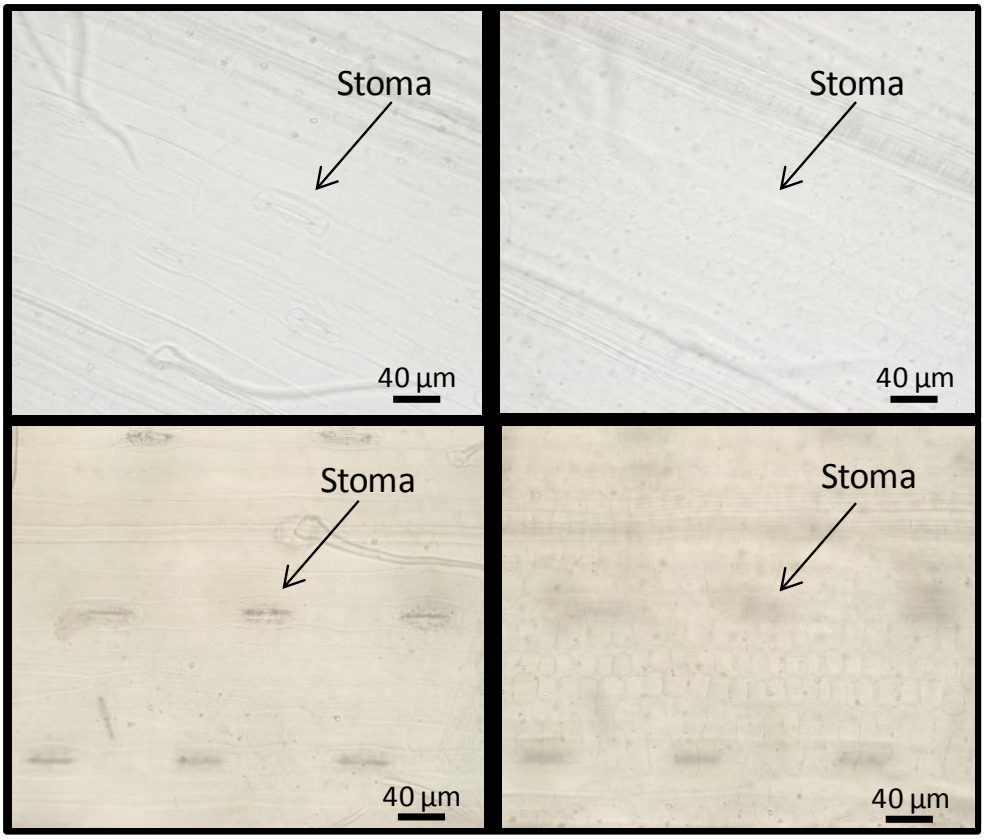


Figure 5.4 Flame (**A,B**) and Longbow (**C,D**) stained with Chlorazol Black E 8 dai with *M. graminicola* isolate IPO323. **A** and **C** show the surface of the leaves with the stoma of each variety visible. **B** and **D** show the mesophyll layer. No *M. graminicola* spores or hyphae have been stained.

5.4 Discussion

The aim of this research was to find a reliable visual method to investigate the timing of the growth and development of *M. graminicola* in relation to responses of wheat to infection. There is a lack of literature that clearly defines what is happening in a resistance response and the timing of this response. One reason for this appears to be difficulties in obtaining a reliable microscopy stain for visualising the fungus in the apoplast. Here, three commonly used microscopy stains were tested to see if they could stain *M. graminicola* in the apoplast during the latent stages of infection. However, they either did not stain the fungus at all, or did not stain the fungus within the apoplast.

U2b is a fluorescent brightener that binds to chitin. U2b has long been used in medical pathology tests involving pathogenic fungi. More recently, it has been used successfully for staining *Puccinia striiformis* f.sp. *tritici* (*Pst*) infecting wheat (Moldenhauer *et al.*, 2006). A similar stain, Calcofluor, has been used for staining *Blumeria graminis*, although only on the leaf surface (Huckelhoven *et al.*, 2003; Rohringer *et al.*, 1977). Here, spores were only visible on the surface of the leaf but not within the apoplast. Two possibilities for the inability of U2b to stain the *M. graminicola* in the apoplast is that either the conditions within the wheat mesophyll layer interact with the stain rendering it inactive, or the stain cannot pass into the mesophyll layer. Both are unlikely however because U2b has been shown to stain both *Pst* (Moldenhauer *et al.,* 2006) and *Magnaporthe grisea* and *M. oryzae* in wheat (Tufan *et al.,* 2009) which all grow into the mesophyll layer. A third possibility is that the surface of *M. graminicola* is modified in some way so as to render it inaccessible to the stain, this may be due to chemical modification of the surface, or a macromolecular barrier, such as glycoproteins or polysaccharides, preventing the stain attaching to the surface. U2b was found unsuitable for staining *Fusarium pseudograminearum* in infected wheat tissue (Knight & Sutherland, 2011). Other laboratories have sent me personal communications stating they have been unable to stain non-biotrophic fungi with U2b; Dr G. Kema (Plant Research International, Wageningen, Netherlands) was unable to stain *M. graminicola* with U2b and Prof. M. Shaw (University of Reading, Reading, UK) was unsuccessful in staining *Botrytis cinerea* with U2b. This implies that there may be a wide spread mechanism for protecting the cell walls of non-biotrophic fungi within the apoplast.

Trypan Blue is generally used for staining dead cells as it cannot pass through the membrane of a viable cell. However, this stain is frequently also used for staining powdery mildews in wheat and barley, as well as downy mildews in Arabidopsis. The method tried here has previously been used to stain *M. graminicola* at 22 dai (Siah *et al.,* 2010). In my experiments, Trypan Blue stained the spores and germinated spores

on the surface of the wheat leaves but did not stain any fungal material in the apoplast. In the experiments by Siah *et al.* (2010) the leaves were completely necrotic before staining was attempted, which may have made the pathogen more accessible to the stain. In a personal communication, Dr A. Siah (Institut Supérieur d'Agriculture, Lille, France) said that the staining technique was difficult and implied that it was not very efficient. Cohen and Eyal (1993) also stained *M. graminicola* with Trypan Blue using a similar method to that described by Siah *et al.* (2010) and noted that different cultivars have different affinities for Trypan Blue. This might speculatively explain why the fungus did not stain well in my experiments with Flame and Longbow, if these varieties did not enable good uptake of the stain. To investigate the timing of resistance and phenotypic differences between an incompatible and compatible interaction a method is needed that will stain the leaves early on in the infection process to enable the course of infection to be studied and is not limited by leaf variety. The other difficulty with Trypan Blue is that it is not entirely selective for fungi as it will also stain plant tissue if it is senescing or dead, which can lead to problems with clearly visualising fungi clearly under the microscope (Rohringer *et al.,* 1977).

Chlorazol Black E is selective for chitin. It has been used successfully to stain many fungi including *M. grisea* (Sesma and Osbourn, 2004) and *Ustilago maydis* (Brachmann *et al.,* 2003). It has been found to most successfully stain in alkaline conditions (Burke & Jones, 1984); potassium hydroxide is commonly used. In these experiments, no staining of the fungus was observed at all, not even on the surface of the leaf. One possible explanation would be that the treatment with potassium hydroxide which is needed as a buffer, dissolved or otherwise removed fungal material from the leaf.

A possible explanation for the difficulty in visualising *M. graminicola* with stains such as U2b and Chlorazol Black E, which selectively stain chitin, might be that *M. graminicola* may shield chitin once it is on the leaf. Chitin is a recognised to be a

pathogen associated molecular pattern (PAMP) (Nurnberger & Brunner, 2002) and its recognition can elicit plant defence responses (Boller, 1995). Some fungi use stealth mechanisms to avoid detection by host immune responses. These mechanisms of host immune evasion have been well studied for human pathogens. For example, *Candida albican*s has two morphological states, yeast and hyphal. When in the yeast like state, β-glucans are exposed on the cell surface, rendering it liable to detection by the pathogen recognition receptor (PRR), dectin-1. When growing in the hyphal state the β-glucans are masked from recognition by dectin-1 by an outer cell wall component, mannan (Chai *et al.*, 2009). The basiodiomycete pathogen of humans, *Cryptococcus neoformans,* masks its PAMPs by forming an extracellular capsule which is not recognised by the host innate immune system (Chai *et al*., 2009). It is possible that *M. graminicola* avoids host defence responses in a similar way to *C. albicans* or *C. neoformans.* Once on the plant, the *M. graminicola* spores grow in a hyphal state entering the apoplast through the stomata. In the substomatal cavity, the fungus grows, very slowly, in a hyphal mass (Kema *et al.*, 1996). During this time, it appears that although the plant recognises the presence of a foreign body (see chapter 4) and expresses host defence genes, these are not specific to a compatible interaction. It may be that *M. graminicola* is able to shield its PAMPs, such as chitin and β-glucans, from the host. Recently, Marshall *et al.* (2011) found two LysM domain effectors, Mg3LysM and Mg1LysM, in *M. graminicola* which were found to bind chitin. These proteins increased in expression from 4-9 dai and disappeared durng macroscopic symptom development. It can be hypothesized that Mg3LysM and Mg1LysM prevent the stains tested from accessing the chitin on the pathogen. If so, this would explain why the chitin selective stains were ineffective for visualising the pathogen in the apoplast. It may partly explain why the pathogen is able to remain latent for so long; because a pathogen which is initially shielded from host defence responses may not be disadvantaged by not completing its lifecycle rapidly. U2b was able to stain pycnidia, demonstrating that these spores do contain chitin that the stain

is able to bind to in the later stages of infection, this would also be consistent with the finding that Mg3LysM and Mg1LysM are not expressed during development of pycnidia (Marshall *et al.,* 2011).

Other stains are available which could be tested on *M. graminicola*: time did not permit a comprehensive study during my PhD research. The fluorescent stain, Solophenyl flavine 7GFE has been used to stain *F. pseudograminearum* and *Pythium* spp. inside host tissues (Knight & Sutherland, 2011; Oliver *et al.*, 2009). The fluorescein isothiocyanate (FITC)-labelled lectin, wheat germ agglutinin, was used by Duncan and Howard (2000) to stain a virulent *M. graminicola* isolate. Another possibility is to vacuum infiltrate stains such as Trypan Blue into the leaves which may facilitate uptake of the stain. To test these stains, leaves of Longbow inoculated with *M. graminicola* isolate IPO323 could be sampled at 7-10 dai, a time when there should be lots of fungal biomass in the apoplast, but no necrosis is visible.

A GFP-tagged isolate of IPO323, IPO323*gfp*, was obtained and tested during investigation into symptom development and the timing of resistance, but found to be unsuitable for use. Comparisons were made between leaves of Longbow inoculated with IPO323*gfp* and leaves inoculated with IPO323. Those inoculated with IPO323*gfp* developed chlorosis and necrosis several days before the control leaves and fewer and later pycnidia also appeared on these leaves than on the controls. The GFP-isolate also weakly fluoresced, making it indistinguishable from background autofluorescence of the leaf.

Using a GFP-tagged isolate for investigating details and timings of resistance would have substantial advantages. A GFP-tagged isolate allows confocal microscopy to be used which allows penetration and later infection events to be studied using a high imaging resolution. The ability to visualise the pathogen at different depths within the plant allows for detailed studies to take place (Linsell *et al.,* 2011). The disadvantage of using a GFP-tagged isolate appears to be that it is difficult to obtain stable expression in *M. graminicola* and that confocal laser scanning microscopy is

need for high resolution visualisation. Widespread use of GFP-isolates would also increase the risk of accidental GMO release.

There are two aims of investigating resistance to *M. graminicola*; the first is to differentiate between resistance and susceptibility using a reliable method to identify a phenotype, the second is to investigate fully the timing of resistance and how the resistance mechanism works. The first aim could be achieved by finding a stain that can reliably bind to *M. graminicola* when it is in the apoplast. The second aim is probably most likely to be achieved by transforming an isolate with GFP, although a good fluorescent stain may also achieve this outcome.

6

General discussion

The aim of the experiments presented here was to investigate the responses of wheat to infection with *Mycosphaerella graminicola.* Three investigations were carried out; one was to research the interaction between a biotrophic pathogen and *M. graminicola* when they were both present on the same leaf, the second was to analyse selected wheat gene responses to infection with either a virulent or avirulent *M. graminicola* and to assess accumulation of TaMPK3 in these interactions, the third was to develop a method to enable the study of the pathogen growth and development inside the apoplast to try and determine a resistance phenotype.

The experiments presented in this thesis answer some questions about how wheat responds to infection by *M. graminicola*, but also raise many new questions and hypotheses to be tested. This discussion addresses some of the findings and speculates on what might be happening in this pathosystem.

Many questions are raised by the finding that mildew caused by a virulent *Bgt* is inhibited by prior inoculation with a virulent *M. graminicola* isolate. These results demonstrate that there is an antagonistic interaction between *Bgt* and *M. graminicola* on wheat and begin to elucidate the mechanism by which this interaction occurs. Investigation into *Bgt* spore development and growth in leaves also infected with *M. graminicola* has not been carried out before. The findings indicate that the resistance mechanism occurs before macroscopic symptoms from infection with *M. graminicola* develop. What is not known is whether or not there is cell death on a microscopic level that limits the growth of the mildew colony. To test this the leaves could be stained with Evans Blue which can be used to stain for cell death in wheat leaves (Shetty *et al.,* 2003), indicating if there is localised host cell death and if it coincides with mildew colony development. A chlorophyll meter could be used to measure if leaf senescence occurs over many leaf cells by measuring if there is a reduction in chlorophyll content. As mildew is a biotrophic pathogen, it relies on living cells for

nutrition; any decrease in chlorophyll indicates that the host cells are dying and therefore nutrition is limited. These effects may take place before they are visible macroscopically.

An interesting question raised by these experiments is; how does a pathogen that resides in the mesophyll layer, *M. graminicola*, have an effect on a pathogen that remains solely on and in the epidermis, *Bgt*? It is likely that cell-to-cell communication events are involved. In barley, plants carrying the *Mla3* and *Mla7* resistance genes induce a resistance phenotype of an HR which is not only seen in the epidermal layer, but also in the mesophyll (Boyd *et al.,* 1995). This suggests that communication does occur between cell layers when infected by a pathogen. It is unknown if a similar effect occurs during the *M. graminicola-Bgt*-wheat interaction, but events in the mesophyll layer must be having some effect on the epidermal layer, otherwise no interaction would occur. Lyngkjaer and Carver (1999 and 2000) found that mildew spores could induce accessibility to further inoculation by avirulent mildew and that avirulent mildew could make cells inaccessible to future inoculations. This type of induction is not seen in the experiment of chapter 3, whereas pre-inoculation with *M. graminicola*, which infects the mesophyll layer, renders the leaf less susceptible to a subsequent inoculation with a normally virulent mildew.

During investigations into the interactions between *M. graminicola*, *Bgt* and wheat, the reverse inoculation procedure was not carried out during these experiments i.e. pre-inoculation with *Bgt* and then inoculating with *M. graminicola.* It can be predicted that a virulent *M. graminicola* isolate inoculated within a few days after *Bgt* would reduce mildew colony formation, because the effect would happen before macroscopic symptom development. The effect of mildew on the growth and development of pre-inoculated *M. graminicola* was also not studied in detail, although virulent *M. graminicola* was always able to produce pycnidia in the presence of mildew. *M. graminicola* fungal biomass accumulation could be measured using a qPCR based assay to test if there is any reduction (or increase) in the amount of biomass the pathogen produces in the presence of *Bgt.* It may be that the 'green island' effect

produced by a virulent *Bgt* isolate is able to inhibit the growth of virulent *M. graminicola*, this might depend on the timings of the two inoculations e.g. if *M. graminicola* was inoculated soon after *Bgt* inoculation it may be able to develop normally, but if *Bgt* has produced many elongated secondary hyphae, there may be an effect on *M. graminicola.* This effect may be a reduction in area the *M. graminicola* can colonise (as is seen between the interaction of rust and *M. graminicola* (Madariaga and Scharen, 1986)).

The experiments presented in chapter 3 only included one isolate of *M. graminicola.* Additional *M. graminicola* isolates should be tested to ensure the results apply more generally to disease caused *M. graminicola,* rather than just a single isolate. Furthermore, there is also a need to test the incompatible interaction with a different *M. graminicola* resistance gene; only S*tb6* has been tested. Longbow has *Stb15* and is resistant to isolate IPO88004 which could be used to test this without involving other wheat varieties which could introduce additional variation. Only a single virulent and avirulent isolate of mildew was tested, therefore additional mildew isolates should be investigated as well.

An assay that allows the inoculation of intact leaves needs to be developed. The method of using detached leaf boxes is a convenient way to study many leaves at one time, without the risk of cross contamination, the risk of which is a particular threat from mildew spores. Studying responses of intact leaves allows findings to be applied more readily to a field situation. Investigation could be extended from using seedling plants to adult plants that would indicate if the findings apply to plants in the field and whether or not they are specific to young plants.

How inoculation with *M. graminicola* affects other biotrophic pathogens, such as rusts, would also be of interest to see if the trade-off seen in the experiments reported here is specific to powdery mildew or applies more generally to other biotrophic fungi. Rusts infect the mesophyll layer and would potentially be in direct contact with *M. graminicola.* Pre-inoculation with *M. graminicola* may affect the rust in the early stages of development because it is occupying cells in close proximity to the

M. graminicola fungal hyphae. To develop the study by Madariaga and Scharen (1986) on an interaction between rust and *M. graminicola,* the microscopic analysis of the growth and development of rust spores, as has been done with powdery mildew (chapter 3) would enable the resistance response to be more fully characterised during the interaction.

The results presented in chapter 4 showed that there was no clear pattern of gene expression that differentiates susceptible from resistant varieties over time, involving any of the genes tested. Most of the genes tested have been linked to a role in defence. This leads to the question; what is the role of these genes in the *M. graminicola*-wheat interaction? The genes tested were not specifically differentially regulated in either incompatible or compatible interactions. This has been found in other pathosystems; rusts (Bozkurt *et al.,* 2005) and powdery mildew (Boyd *et al.,* 1994a) are two examples where defence-related genes are upregulated in both incompatible and compatible interactions. The role of genes, such as *chitinase* and *β-1,3-glucanase*, in response to *M. graminicola* infection appears not to be specific to a resistance response. They may be involved in basal recognition of microbes, which may then trigger a separate suite of genes that are involved in the resistance response aimed specifically at an infecting pathogen; if this happens, it is unknown what these other genes may be. The upregulation of these genes may be a general defence response to inoculation with the pathogen, independent of virulence, or that the level of transcript produced in response to the pathogen is variety specific.

The greatest upregulation of the genes tested was seen at 7 dai. This suggests that something biologically significant occurred at 7 dai. The target genes *β-1,3-glucanase, mlo, peroxidase* and *PR1* are all upregulated to the greatest levels at 7 dai. One hypothesis is that between 1 and 6 dai the plant perceives (or does not perceive) both virulent and avirulent isolates as non-threatening endophytic fungi that do not cause harm and therefore plant defences are not activated as there is no perception of a threat. Data from the sequenced genome of *M. graminicola* suggests that there are many similarities to an endophytic genome (Goodwin *et al.* 2011), and it is known that

the fungus has a stealthy approach to pathogenicity. At 7 dai it appears that a change occurs and this could allow the pathogen to be recognised by the plant as a threat and activate defences.

The reason for trying to find a microscopy stain which clearly stains *M. graminicola* at all stages of infection was to phenotype the pathogen in relation to gene expression data. Because of the large variability seen between data sets it is important to match the growth and development of the pathogen to the response of the plant. This is important when investigating the *M. graminicola*-wheat interaction because of the variability of gene expression data between replicates and between varieties. By matching the development of the pathogen to gene expression data, more accurate inferences could be made about the amount of growth and/or the developmental stage which affects the genes analysed. This would be of particular importance if using microarray analysis to investigate gene expression. To date, there have been no published microarray analyses of wheat's response to infection by *M. graminicola*. This is likely due to the high variability seen between replicates, as has been demonstrated in this thesis and in other studies during gene expression analysis (Ray *et al.* 2003).

The pathogen *Fusarium pseudograminearium* has been found to specifically induce genes classified as ROS-defence (e.g. *germin-like* and *peroxidase*) when compared with a biotrophic pathogen, rust (Desmond *et al.* 2008a), although *F. graminearum*, increased *peroxidase* transcript levels in both resistant and susceptible cultivars at the early stage of infection (Pritsch *et al.* 2000). Inoculation with *M. graminicola* upregulated the transcription of *peroxidase,* particularly at 7 dai, this occurred in both compatible and incompatible interactions, suggesting that peroxidase plays an, as yet unknown, role in the interaction. The data in chapter 4 indicate that *M. graminicola* has commonalities with other necrotrophic pathogens, but does not disregard similarities to biotrophic pathogens. Without a microarray analysis it cannot be predicted what classes of genes will be upregulated and to what degree.

It has been suggested that *M. graminicola* produces a toxin that either aids or causes pathogenicity (Kema *et al.* 1996) and that this toxin activates defence responses specific to a compatible interaction. No data yet published or this thesis has shown any response which is specific to a compatible interaction. Non-biotrophic pathogens such as *Fusarium spp.*, often produce toxins which cause or assist in the necrotrophic stage of the pathogen's lifecycle. Many crop pathogens produce toxins that are known to aid pathogenicity, the most notable of these is deoxynivalenol (DON), produced by several *Fusarium* species including those infecting wheat and barley. Toxins such as DON can activate defence responses in wheat that are the same as activated by the pathogen itself (Desmond *et al.* 2008b). No toxin has ever been found to be produced by *M. graminicola*, but if one does exist it is likely to enable a virulent pathogen to cause plant cells to die, enabling the pathogen to utilise the available nutrients. It can be hypothesised that the toxin triggers this response at 7 dai, which is the time when the greatest defence-related gene upregulation occurs.

The lack of a pattern in gene expression data may, speculatively, be in part due to opportunist bacteria or fungi being able to enter the leaf due to inoculation by *M. graminicola.* In barley infected with powdery mildew, stomatal 'lock-up' has been observed where stomata have lost the ability to close in darkness (Withers *et al.*, 2011) during infection with avirulent mildew isolates. It is unknown what effect *M. graminicola* has on wheat stomata, but as the pathogen penetrates the leaf through the stomata, it is likely to cause some effect. Boyd *et al.* (1994b) grew barley plants in both non-sterile and sterile conditions and, after wounding, probed for *chitinase, peroxidase* and a *pathogenesis-related gene* (*PR-R*). They showed that wounded plants grown in non-sterile conditions induced transcript accumulation of all the genes tested with no discernible pattern, although induction was greater at later timepoints. Plants grown under sterile conditions accumulated no *chitinase* or *peroxidase,* although they did accumulate *PR-R* at 0 hours after wounding, which may have been due to physiological stress. Boyd's results indicated that defence genes may be induced by opportunistic organisms accessing the leaf through wounds and I suggest

that this may explain the variation seen in the data presented in chapter 4 of this thesis. If *M. graminicola* infection keeps stomata open, it may decrease the plants ability to resist entry by opportunistic, non-pathogenic microbes and the plant response to these organisms may be to produce defence-related proteins, such as chitinases, β-1,3-glucanases and peroxidases. This would produce 'noise' seen during the gene expression studies here which hides what the specific response to *M. graminicola* is. To investigate these possibilities, measurements of stomatal conductance, using a porometer, could be carried out on plants inoculated with avirulent and virulent *M. graminicola*, to relate stomatal movements to disease development. Secondly, if wheat plants could be grown in sterile conditions and inoculated with avirulent and virulent *M. graminicola* without causing the pathogenicity of the isolate to change due to the plant being under stressful conditions, gene expression of defence-related genes could be measured. Leaves could also be surface sterilised before inoculation with *M. graminicola,* this may enable a quantitative result about the role of non-specific microbes in the *M. graminicola*–induced defence response. The results of chapter 4 were not consistent with the results obtained in other experiments on defence-related genes (Adhikari *et al*. 2007 and Ray *et al.* 2003). In particular no correlation was found between an incompatible response and the *protein disulfide isomerase* (*PDI*) gene in the experiments presented here, whereas Ray *et al.* (2003) found that *PDI* was upregulated during the incompatible interaction. If opportunist microbes are able to induce defence-related gene transcription through microbe associated molecular pattern (MAMP)- triggered immunity, *PDI* may have been elicited by a particular MAMP involved in their experimental set-up.

Another hypothesis is that the defence-response to *M. graminicola* is mounted by a specific set of plant cells, possibly the guard cells or those immediately present in the sub-stomatal cavity. As *M. graminicola* enters the leaf through stomata, guard cells might be involved in defence against the pathogen. The guard cells may produce a defence response towards *M. graminicola* in a way specific to avirulent or virulent

isolates. The apparent lack of a pattern in the gene expression study presented in chapter 4 may be caused by other plant cells, most likely the epidermal cells, responding to fungal hyphae on the leaf surface by triggering a pathogen-associated molecular pattern (PAMP) response, which is not determined by the compatibility of the pathogen. It is now possible to isolate specific cells from tissue using laser capture microdissection, enabling gene expression of the guard cells to be tested in the absence of other plant tissue to determine if they mount a specific resistance or susceptibility response on single cell levels.

It has been proposed that the accumulation of the TaMPK3 protein and its subsequent post-translational activation seen during the compatible interaction of *M. graminicola* and wheat is due to a functional relationship with the onset of leaf cell programmed cell death (PCD) (Rudd *et al.* 2008; this thesis, chapter 4). In the experiments presented in chapter 3, *TaMPK3* gene transcript was upregulated more during the compatible interaction than the incompatible interaction between wheat and powdery mildew. This is not consistent with the hypothesis that MPK3 orthologues accumulate during a *R*-gene mediated hypersensitive response (HR) during a resistance response to a biotrophic pathogen (Reyna & Yang, 2006; Stulemeijer *et al.*, 2007)

One possible explanation for this is that the accumulation of TaMPK3 behaves differently in monocotyledonous plants from what has been found from work on *Arabidopsis,* tobacco and tomato (Pedley and Martin, 2005). It may be that TaMPK3 accumulates as part of a wound response towards pathogens, no matter what the lifestyle of that pathogen. The tobacco wound-induced protein kinase (WIPK) shares high sequence similarity to AtMPK3 (Ichimura *et al.*, 2002) which is orthologous to TaMPK3 (Rudd *et al.,* 2008). WIPK is rapidly activated after wounding (Seo *et al.*, 1995) and also during resistance to infection by tobacco mosaic virus (Zhang and Klessig, 1998). Virulent *M. graminicola* causes the host cells to rupture and collapse, releasing nutrients into the apoplast; this loss of membrane integrity may, speculatively, be recognised by the plant as wounding. Virulent mildew forms

haustoria inside the cell by penetrating the cell wall using an appressorium. This results in rupture of the cell wall and disturbance to the plasma membrane as well as a possible change in turgor pressure and leakage of cell contents. Again, this may be perceived by the plant as a wound. The HR that occurs after inoculation with an avirulent isolate is localised and only occurs in the infected epidermal cell. This cell death may not be on a large enough scale to produce a detectable change in the level of TaMPK3, although there may be a change on a local scale. This could be clearly tested in barley where the phenotype of powdery mildew resistance genes has been characterised (Boyd *et al.* 1995). Mildew resistance alleles in barley which produce large necrotic lesions, often into the mesophyll, during an incompatible interaction such as *Mla3* and *Mla7* would be predicted to accumulate more MPK3 than during the incompatible interaction involving *Mla1* and *Mla6* which produce a more discrete response in the epidermal cells only (Boyd *et al.* 1995). Another, potentially overlapping, possibility is that TaMPK3 transcript and protein levels may correlate with the amount of fungal biomass produced in the plant mediated via PAMP recognition. Chitin has been shown to induce kinase activity of AtMPK3 in *Arabidopsis* (Wan *et al.*, 2004). An increase in fungal biomass may increase the amount of defence inducing chitin perceived by the plant which could trigger a MAPK cascade (Asai *et al.*, 2002; Pitzschke *et al.*, 2009), resulting in the accumulation of TaMPK3. This could apply to both *M. graminicola* and *Bgt.* The late accumulation of TaMPK3 in plants infected with *M. graminicola* may be associated with the levels of the fungal effector protein, Mg3LysM (Marshall *et al.*, 2011). This a LysM domain protein which has some similarities to the Ecp6 effector found in the biotrophic tomato pathogen, *Cladosporium fulvum* (de Jonge *et al.*, 2010). Mg3LysM has chitin binding properties and is able to protect fungal hyphae against hydrolytic plant enzymes, but significantly it is only strongly produced during the latent period of infection by *M. graminicola* when TaMPK3 protein levels remain low. During the transition to necrotrophic growth the Mg3LysM effector is downregulated. This may increase the availability of chitin to plant receptors and trigger defence signalling perhaps resulting in the protein

accumulation during this phase. The fact that Mg3LysM is necessary for full virulence of *M. graminicola*, highlights the importance of suppressing early chitin mediated recognition events and PAMP- triggered immunity during the latent period of infection (Marshall *et al.,* 2011). During infection with *Bgt* there may be a more gradual increase in biomass. There may be a threshold of chitin (or another PAMP) above which the plant response is to accumulate MPK3, more would therefore accumulate in a compatible rather than an incompatible interaction with *Bgt.* There was some accumulation of TaMPK3 during the incompatible interactions in the experiments presented in chapter 4. The presence of the pathogen appears to be enough to activate the accumulation of TaMPK3 in some wheat varieties, again this may be due to chitin levels or wounding responses specific to each variety. The function of MPK3 remains unknown; it may be involved in a more general response to pathogen inoculation or a general senescence response rather than a specific susceptibility response, this should to be investigated further.

Over recent years many plant *R*-genes have been cloned. Analysis of these genes has lead to recognition of the NBS-LRR common protein motif, suggesting that plants may share a common mechanism of resistance to a diverse range of pathogens. More specifically, cloning of *R*-genes will lead to a greater understanding of the molecular basis of disease resistance specificity. Cloning of either *Stb6* or *Stb15*, the most common *Stb* genes, could mean that the function and molecular basis of disease resistance to *M. graminicola* could be defined. Resistance to *M. graminicola* does not follow the zig-zag model of plant resistance, therefore it can be predicted that the *Stb* genes will not only function in a unique way when compared with other *R*-genes, but that it will not share the common protein motif of previously cloned *R*-genes. The cloning of genes in wheat, such as *Lr34,* has allowed defence mechanisms in a single variety to be tested with and without the resistance gene in the same genetic background, e.g. Thatcher and Thatcher-*Lr34* (Krattinger *et al.* 2009) which confers adult plant resistance to rust. Cloning of an *Stb* gene would mean that gene and protein expression relating to a defence mechanism could be identified using the same

plant genetic background, something that is important in the wheat-*M. graminicola* pathosystem due to its high variability.

M. graminicola has characteristics which more closely fall into the category of necrotroph than biotroph; it has no specific feeding structures, can be grown in axenic culture and is not killed by hypersensitive cell death. However, it remains entirely intercellular throughout its lifecycle and has no specific feeding structures and is not, as yet, known to produce any toxins or elicitors of cell death. The initial stealthy phase of this pathogen and the lack of carbohydrate enzymes, suggests that this is a pathogen that is similar to an endophyte during the initial stages of infection. There is a lack of evidence for feeding from the plant during the latent period, although Rohel *et al.* (2001) suggested that *M. graminicola* takes up soluble carbohydrates as early as 1 dpi. No evidence for carbohydrate uptake was found by Keon *et al.* (2007) using mass spectrometry. It is unclear as to whether the pathogen is feeding off of the plant or from degradation of its own spore, which could provide nutrition especially as very little growth occurs at the early stages. *M. graminicola* may have evolved necrotrophic tendencies but not be a fully developed as a necrotroph; the cell death that occurs is localised to the cells next to the infecting spore, with no movement of necrosis throughout the plant. Necrotrophs, such as *Botrytis cinerea*, tend to cause spreading necrosis to enable the pathogen to spread quickly and utilise all available nutrients. *M. graminicola* may not have fully developed as a necrotroph from an endophytic lifestyle or, as suggested by Goodwin *et al.* (2011), may be evolving towards necrotrophy.

This thesis presents new findings about how wheat responds to infection by *M. graminicola,* but it also raises new questions to be investigated, which have been outlined in this chapter. Overall, this work adds weight to the growing body of evidence that *M. graminicola* is a sophisticated pathogen, which is able to evade and manipulate host defences to eventually exploit host resources to its advantage.

Abbreviations

µg	microgram
µL	microlitre
µM	micromolar
µm	micrometre
avr	avirulence
Bgt	*Blumeria graminis* f.sp.*tritici*
Bgh	*Blumeria graminis* f. sp. *hordei*
CBE	Chlorazol Black E
Cmm	*Clavibacter michiganensis* subsp. *michiganensis*
cDNA	Complementry DNA
Comp	Compatibility
Cq	Quantitative cycle
DAB	Diaminobenzidine
Dai	Days after inoculation
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
Dpi	Days post inoculation
DTT	Dithiothreitol
EGTA	Ethylene glycol tetraacetic acid
ESH	Elongated secondary hyphae
ET	Ethylene
ETI	Effector-triggered immunity
FITC	Fluroecein isolthiocyanate
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HGCA	Home Grown Cereals Authority
HR	Hypersensitive response

Abbreviations

JA	Jasmonate
kD	kilo Dalton
KOH	Potassium hydroxide
LED	Light emitting diode
LOX	Lipoxygenase
MAPK/MPK	Mitogen activated protein kinase
mL	Millilitre
mM	Millimolar
NaCl	Sodium chloride
NaF	Sodium fluoride
NB-LRR	Nucleotide binding-leucine rich repeat
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
nM	Nanomolar
PAL	Phenylalanine lyase
PAMP	Pathogen associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
pg	Picogram
PlexDB	Plant Expression Database
PMSF	Phenylmethylsulfonyl fluoride
PR	Pathogenesis-related
PR1	Pathogenesis-related 1
PRR	Pathogen recognition receptor
Pst	*Pseudomonas syringae* f.sp*. tomato*
Pst	*Puccinia striiformis* f. sp. *tritici*
PTI	PAMP-triggered immunity

QoI	Quinine outside inhibitor
qPCR	Quantitative PCR
QTL	Quantitative trait loci
R-gene	Resistance gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicyclic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SDW	Sterile distilled water
STB	Septoria tritici blotch
TaMPK3	Wheat mitogen-activated protein kinase 3
TBS-Tween	Tris-buffered saline-Tween
Tris	Tris (hydroxymethyl) aminomethane
Trt	Treatment
U2b	Uvitex 2b
UV	Ultra violet
WGA	Wheat germ agglutinin

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