Developing a silencing tool with short antisense oligonucleotides to investigate effectors and susceptibility genes during barley powdery mildew infection

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Declaration of Authorship

I, Katherine Orman, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed:

Date:

Abstract

Barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) is a prevalent fungal pathogen of barley in the UK. As an obligate pathogen, methods for gene knockouts or silencing have been limited, with no stable transformation method available. A transient assay using a biolisitic delivery system has been previously applied for Host Induced Gene Silencing (HIGS) of *Blumeria* effector candidates (BECs). However, HIGS requires cloning of a silencing cassette and since only a small proportion of cells are transformed, it forbids downstream whole-tissue analyses. The aim of the project was to overcome these caveats by developing a new silencing strategy of genes from barley and its powdery mildew in order to investigate their roles during infection. The method required the delivery of phosphorothioate-modified, short, antisense oligonucleotides (PTOs) via the vascular tissue of excised leaves. The method was validated by silencing the known *Blumeria* effectors *BEC1011, BEC1019* and *BEC1054*, and the barley susceptibility factors *Mlo* and *Blufensin1*. While the disease phenotype was monitored microscopically by measuring the infection rate in term of successful secondary hyphae formation, relative mRNA and protein amounts of the cognate silenced gene were measured by qRT-PCR and multiple reaction monitoring mass spectrometry. Targeting the aforementioned effectors or susceptibility genes resulted in 40-60% reduction of secondary hyphae formation and up to 30% reduction in transcript. The technology was then applied to silence putative *Blumeria* Candidate Secreted Effector Proteins (CSEPs) AVRa1 and AVRa13, resulting in 22% and 31% reduction in secondary hyphae formation, respectively. A barley pathogenesis related protein 5 (PR5) was also targeted. Surprisingly, PR5 silencing led to a drastic decrease in *Blumeria* infection, suggesting that PR5 is a susceptibility factor. To investigate BEC1011 or PR5 roles in compromising plant immune responses, production of reactive oxygen species such as H_2O_2 was monitored after *Blumeria* infection in control, BEC1011, and PR5 silenced plants, showing that BEC1011 and PR5 are preventing a sustained oxidative burst response.

Contents

List of Tables

List of Figures

List of supplementary figures and tables

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Aim

The aim of this project was to develop a new gene silencing technique using short, antisense oligodeoxynucleotides to investigate fungal and plant determinants involved in disease establishment during the barley – powdery mildew interaction.

1 Introduction

1.1 Barley and *Blumeria*

Barley (*Hordeum vulgare*) is a monocot cereal crop. It has long been the second largest crop in the UK by both area and yield with 6.7 million tonnes yielded from 1.1 million hectares in 2016 (Department of Environment, Food and Rural Affairs, 2016). Although wheat production is larger in the UK, barley is more often used as a model plant in research as it has a sequenced diploid genome, compared to hexaploid wheat.

Crops are constantly under pressure from biotic and abiotic stresses including bacterial, fungal, oomycete and viral pathogens. Obligate biotrophic pathogens such as powdery mildews are difficult to investigate because of their lack of culture methods and lack of tools for functional genomics.

The aim of this study was to use barley powdery mildew as a model system to develop a new tool for functional genomics of biotrophic pathogens and to use this tool to investigate genes involved in the interaction between plant and pathogen.

1.2 Plant Immunity

Although plants are exposed to a large diversity of potential pathogens, plants are only actually susceptible to a limited number of pathogens. This suggests that the lack of susceptibility of the plant is either the result of inadequate host compatibility, or lack of components required for susceptibility. Successful infection is not wholly based on the susceptibility or resistance of the plant host, but also on the pathogenicity of the pathogen.

In most situations, the lack of host compatibility is conferred by non-host resistance mechanisms, meaning they are outside the host range of a given pathogen. Non-host resistance is a key area of research as this form of resistance is durable and effective against all isolates of a given pathogen species. It involves a combination of constitutive and inducible responses including both physical and chemical responses (Nürnberger and Lipka, 2005). However, little is understood about the molecular mechanisms which determine whether a plant species is a host for a particular pathogen.

The host range of a pathogen can vary widely from only being able to infect one species (narrow host range) or a wide range of plants (broad host range). It has been shown that the probability that two plant species can be infected by a particular pathogen reduces the more phylogenetically distant the species (Gilbert and Webb, 2007).

In terms of compatible host-pathogen interactions, immunity can be initiated at several levels. In the simplified zigzag model (Figure 1), there are two levels of plant immune responses, as described by Jones and Dangl, (2006).

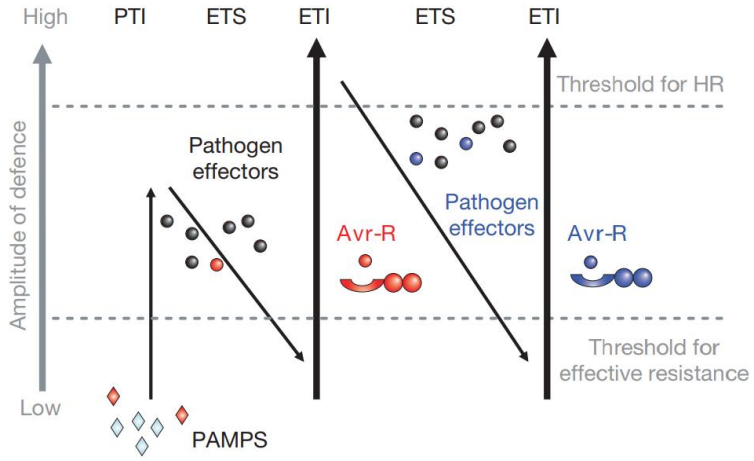


Figure 1.1 The zigzag model of plant immunity. Pathogen Associated Molecular Patterm PAMP-triggered immunity (PTI) is triggered by recognition of PAMPs by pathogen recognition receptors, pathogen avirulence genes (AVRs) or effectors overcome PTI to trigger effector triggered susceptibility (ETS). Recognition of AVRs by plant resistance (R) genes leads to effector-triggered immunity (ETI) and the hypersensitive response (HR) (Jones and Dangl, 2006).

The first is Pathogen Associated Molecular Pattern (PAMP)- Triggered Immunity (PTI). During PTI, conserved molecules from the pathogen – PAMPs such as flagellin or chitin – are detected by their cognate pattern recognition receptor (PRR) on the plant cell surface. Many PRRs are leucine rich repeat receptor kinases (LRR-RK) with an intercellular kinase domain and the LRR domain on the cell surface (Zipfel and Robatzek, 2010). Others may contain LysM motifs such as the fungal chitin receptors Chitin Elicitor Binding Protein (CEBiP) and Chitin Elicitor Receptor Kinase 1 (CERK1) (Miya *et al.*, 2007). These chitin PRRs are different to other identified PRRs because CEBiP has no intercellular kinase domain and in rice has been shown to form a complex with CERK1 in order to initiate the kinase signalling cascade (Petutschnig *et al.*, 2010), although CERK1 is known to also work independently of CEBiP (Shimizu *et al.*, 2010). Figure 2 summarises the best known PAMPS and PRRs.

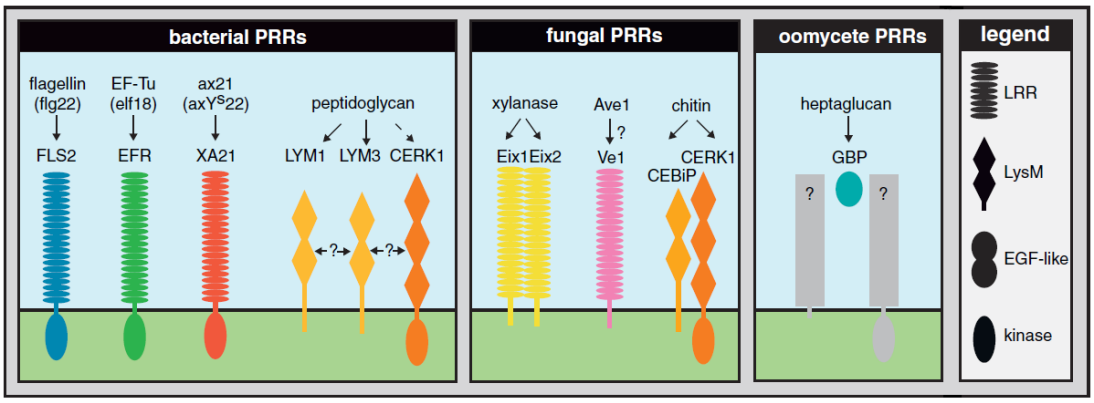


Figure 1.2 Summary of well-studied plant recognition receptors (PRRs) and the pathogen associated molecular patterns (PAMPs) recognised by these PRRs. A 22 amino acid peptide (flg22) from the bacterial flagellin is recognised by the FLS2 receptor, Elongation factor Tu (EF-Tu) is recognised by EFR and ax21 is recognised by XA21 PRRs. Bacterial peptidoglycans are recognised by several receptors which may interact with each other, including LYM1, LYM3 and CERK1. Fungal PAMPs such as xylanase and Ave1 are recognised by leucine-rich repeat (LRR) membrane receptors, including Eix1/ Eix2 and Ve1 respectively. Chitin, the main polymer of all fungal cell walls, is recognised by LysM containing receptors such as CEBiP and CERK1 PRRs which interact, initiating kinase signalling. (adapted from Monaghan and Zipfel, 2012)

Detection of the PAMP by the PRR initiates a signalling cascade leading to a number of well characterised responses including production of reactive oxygen species (ROS), MAPK activation and callose deposition (Boller and Felix, 2009), amongst others, and these can prevent further growth of the pathogen (Nicaise *et al.*, 2009).

However, pathogens have evolved systems by which PTI can be evaded or suppressed. This can involve the evolution of effector proteins which can act as virulence factors that can otherwise manipulate the host metabolism to favour the susceptibility of the plant, for example by supressing the plant cell death mechanisms (Panstruga and Dodds, 2009). Plants have therefore developed a second level of immune response called effector triggered immunity (ETI). A plant may have specific resistance (R) genes, most of which encoding proteins containing a nucleotide binding-leucine rich repeat (NB-LRR) domain (Dangl and Jones, 2001), which recognise a specific effector from a specific pathogen. This recognition initiates a stronger immune response than PTI and will often culminate in the hypersensitive response (HR).

Effectors which are recognised by R proteins are more often virulence factors and are termed avirulence (*Avr*) genes/proteins. The gene-for-gene hypothesis states that for each pathogen *Avr* gene there is a cognate R gene in the host which initiates a defence response (Flor, 1971). The functionality of an R gene is either through direct interaction of the R gene product with the *Avr* gene product or indirectly by interacting with, or "guarding", the target of the AVR protein, thereby preventing its action (Van der Biezen *et al.*, 1998). One

example of this “guard hypothesis” is the interaction of the *Arabidopsis thaliana* RPM1 protein with RIN4 (RPM1 INteracting protein 4). RIN4 is a negative regulator of defence and a target of two *Pseudomonas syringae avr* gene products – *avrRpm1* and *avrB.* RPM1 detects *avr* induced phosphorylation of RIN4 and initiates a defence response (Mackey *et al.*, 2002). Protection of the targets of effectors also enables plants to deal somewhat with the redundancy of effectors often seen in pathogens. The evolutionary pressure on a single effector means pathogens often have functional redundancy of effectors which prevents detection based on structure. *P. syringae avr* gene products *avrRpm1* and *avrB* are structurally unrelated but both target RIN4, but, as described, RPM1 mediates defence against both.

ETI can be effective in controlling a specific pathogen but there are several problems which mean that R gene mediated resistance cannot be the sole source of resistance protecting our crops. The evolution of effectors means that R gene-mediated resistance – particularly when mediated through direct interaction with the effector – is not durable and can break down in the field, causing epidemics of disease in previously resistant crops. Most notably is the development of a virulent strain of stem rust (*Puccinia graminis*) in wheat with the *Sr31* resistance gene. This virulent strain (Ug99) was identified in Uganda in 1998 and has now spread across Africa and into Asia (Singh *et al.*, 2011).

With pathogens so rapidly losing or mutating effectors to evade resistance mechanisms, studying effector biology and the mechanisms and targets of the host is of increasing importance.

Some of the characteristic responses of PTI and ETI, including Ca^{2+} influx and MAPK phosphorylation, lead to a signalling cascade and the activation of further downstream immune responses. These include the expression of pathogenesis-related (PR) proteins (van Loon *et al.*, 2006).

The signalling events following pathogen detection leading to an immune response are generally mediated by one of two hormonal signalling pathways, the salicylic acid (SA) pathway or the jasmonic acid (JA) pathway. Response to biotrophic pathogens generally leads to activation of the SA pathway and leads to upregulation of genes including *PR* genes (Fan and Dong, 2002). The JA pathway is generally triggered by necrotrophic pathogens or by insects. These two hormonal pathways have many interactions, often leading to antagonistic effects. This can result in a trade-off between resistance to biotrophic or necrotrophic pathogens (Pieterse *et al.*, 2012). For example, infection of *Arabidopsis* with

the biotrophic bacteria *Pseudomonas syringae* increased the plants' susceptibility to the necrotrophic fungus *Alternaria brassicicola* by supressing the JA-mediated pathway (Spoel *et al.*, 2007).

Induction of the SA mediated pathway at the site of local infection has also been shown to trigger immune responses in distal tissues leading to increased resistance in other parts of the plant. This is known as systemic acquired resistance (Vlot *et al.*, 2009).

1.3 Fungal pathogen lifestyles

Plant pathogens are the cause of 10-16% loss in global crop harvests annually and the majority of plant diseases are caused by fungi and oomycetes (Strange and Scott, 2005; Oerke, 2006). It has been estimated that annual crop losses due to fungi could feed 8.5% of the global population (Fisher *et al.*, 2012). In 2012, a list was produced of the top 10 fungal pathogens. This list was topped by the rice blast fungus *Magnaporthe oryzae*, *Botrytis cinerea*, the causal agent of grey mould and *Puccinia spp.* causing rust diseases on wheat (Dean *et al.*, 2012). *Blumeria graminis* came in sixth on the list. Unsurprisingly these pathogens present problems on the world's most important crops such as rice and wheat. It is also notable how diverse these pathogens are. They also represent the three pathogen lifestyles – *Puccinia spp* are biotrophs, *Botrytis cinerea* is a necrotroph and *Magnaporthe oryzae* is hemibiotrophic.

Biotrophs feed on living tissue of their host and can include symbionts such as arbuscular mycorrhizal fungi as well as pathogens (Lewis, 1973). This lifestyle involves penetration of the host cells and development of intracellular hyphae or specialised feeding structures called haustoria which are the site of nutrient exchange (O'Connell and Panstruga, 2006). *Blumeria graminis* is also thought to interfere with host cell death processes causing characteristic "green islands" around sporulating pustules. This may be caused by an effector that promotes virulence by inhibiting barley ribosomal inactivating proteins which are involved in triggering cell death (Pennington *et al.*, 2016a).

Necrotrophic pathogens gain their nutrition from dead cells. One of the key responses of the plant immune system is the hypersensitive response (HR) which triggers cell death at the site of infection. Necrotrophic fungi such as *Botrytis cinerea* and *Sclerotinium sclerotiorum* are thought to utilise this process to promote pathogenicity (Govrin and Levine, 2000).

Hemibiotrophs combine both biotrophic and necrotrophic lifestyles. Initially, hemibiotrophic pathogens such as *Magnaporthe oryzae* and *Zymoseptoria tritici* exhibit a symptomless phase during which they obtain nutrition in a biotrophic manner before switching to a necrotrophic lifestyle (Luttrell, 1974; Fernandez and Wilson, 2012).

In order to gain nutrition from plant hosts, pathogens need to be able to enter the plant. Some pathogens such as *Cladosporium fulvum,* do not penetrate the cell wall but grow apoplastically (Stotz *et al.*, 2014). Others will penetrate the plant cell wall through a combination of mechanical and enzymatic activity (Pryce-Jones *et al.*, 1999). To assist with this, pathogenic fungi have a range of cell wall degrading enzymes including glucanases, xylanases and pectinases (Kubicek *et al.*, 2014). Many fungi have a specialised complement of cell wall degrading enzymes which is a determinant of the pathogen host range (King *et al.*, 2011). Following penetration of the cell wall, different pathogens have different strategies. *Ustilago maydis*, the corn smut fungus, produces intracellular hyphae which invaginate the cell membrane whereas other pathogens, such as rusts and powdery mildews, form a specialised structure called a haustorium. These differ from intracellular hyphae because they are terminal structures but otherwise appear to have the same function of nutrient uptake (Bushnell, 1972).

1.4 Control of fungal pathogens

Fungal pathogens are controlled either by breeding of resistant varieties or by chemical control.

Resistant varieties are generated by introgression of *R* genes into elite, high yielding lines. In the UK, new varieties are subject to several seasons of trials, during which their resistance to common pathogens is scored. This is then reported in the Recommended List of varieties of the major crops including wheat, barley and oil seed rape (Home Grown Cereal Authority). However, varieties which only contain a single *R* gene against a particular pathogen are vulnerable to the resistance breaking as pathogens evolve to lose recognition. So whilst research continues to identify new *R* genes, they have to be deployed in a responsible manner to maintain resistance for more than a few years. Pyramiding of resistance genes can increase the durability of resistance but pathogens continue to evolve to lose this recognition (Ridout, 2009).

Fungal pathogens can also be controlled by the use of chemical control. Use of chemicals on arable crops has been increasing over the last two decades but in the EU, the majority of chemical usage is on speciality crops such as fruit and vegetables (European Commission,

2007). The chemical with the highest usage as a plant protection product in the EU is inorganic sulphur, mainly used to control powdery mildew on grapevine.

1.5 Powdery mildews (Blumeria and others)

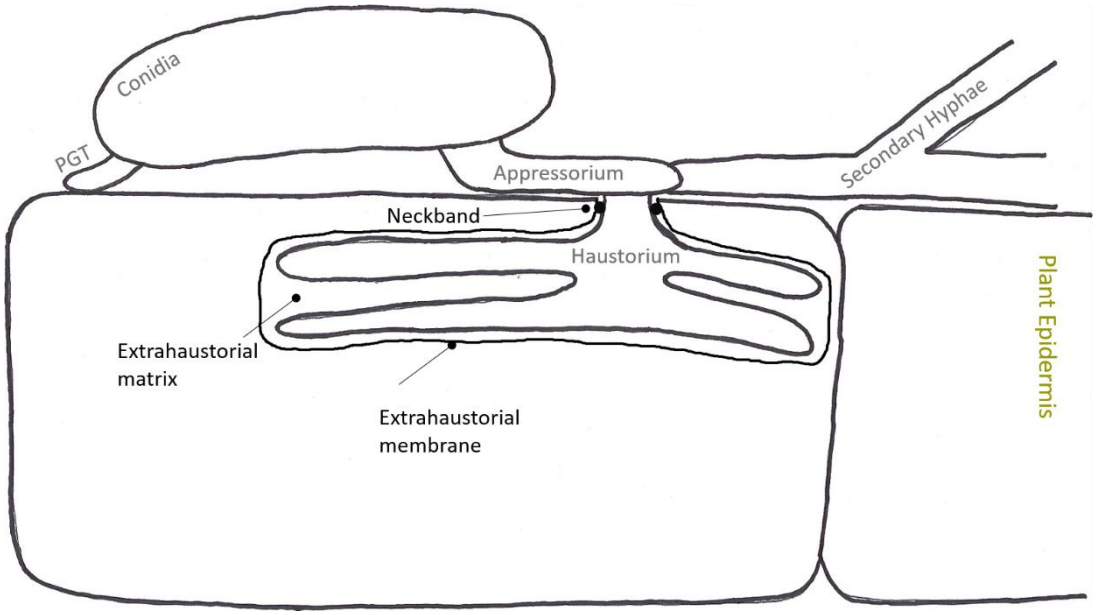
Powdery mildews are ascomycete fungi in the order Erysiphales. Over 400 hundred species in this order, which are all obligate biotrophs, have been found to infect nearly 10000 plant hosts (Takamatsu, 2004). Biotrophs require a living host to acquire nutrients and obligate biotrophs are not able to live outside their host. These differ from hemibiotrophic or necrotrophic organisms which induce necrosis in the host in order to gain nutrition.

The most widely studied species of powdery mildew is *Blumeria graminis* which has *formae specialis* which specifically infect wheat or barley (*B. graminis f.sp tritici* or *B. graminis f.sp hordei; Bgh*). Other well studied powdery mildews include *Erysiphe necator* which infects grapevine, curcubit powdery mildew *Erysiphe cichoracearum* and *Golovinomyces orontii* which infects the model plant *Arabidopsis thaliana.*

The infection process and development of *Bgh* within the plant takes approximately 5-7 days. A primary germ tube (PGT) is formed within hours of the conidia landing on the leaf surface. Following this an appressorial germ tube (AGT) forms within about 8 hours, growing off the conidia. An appressorium forms at the end of the AGT which generates turgor pressure and cell wall degrading enzymes. An infection peg grows from the appressorium to pierce the host cell, allowing the development of the haustoria within the cell (Both *et al.*, 2005). This can occur within 24 hours of infection. The take up of nutrients through the haustorium allows for development of secondary hyphae and within 4-6 days chains of conidia are present on the leaf surface (Ridout, 2009).

Powdery mildews, like many other pathogenic fungi, have specialised feeding structures – haustoria – which allow for efficient uptake of nutrients from the plant (Szabo and Bushnell, 2001). Haustoria are specialised hyphae which form inside the cells of the host plant. Development of the haustorium within the cell results in the invagination of the plasma membrane to form an extrahaustorial membrane (EHM). The EHM is morphologically different from the plant plasma membrane. It has fewer intrinsic membrane proteins and is thickened by associated carbohydrates. Roberts *et al.*, (1993) observed that the EHM was highly convoluted and had extensive branched structures which may increase the surface area for nutrient export. An extrahaustorial matrix is formed between the EHM and the fungal cell wall. This is separated from the apoplast by a haustorial neckband (Koh *et al.*, 2005). This separation allows for a proton gradient to be

generated by haustorial membrane H^+-ATPases, allowing efficient transport of sucrose and other nutrients through the EHM into the haustorium (Godfrey *et al*



., 2009) and secretion of effectors from the haustorium into the plant (Panstruga and Dodds, 2009). Figure 3 shows the structure of the haustoria within a plant cell. Nutrients taken up through the haustoria are transported to the hyphae and allow for growth and production of secondary hyphae (Spanu, 2012).

Figure 1.3 Structure of *Blumeria graminis* f.sp *hordei* 48 hours post inoculation of barley. The conidia lands on the leaf surface and first develops a primary germ tube, then an appressorial germ tube which develops into an appressorium. This produces an infection peg which penetrates the cell wall and a haustorium develops inside the cell. The haustorium is separated from the cytoplasm by the extrahaustorial membrane and the extrahaustorial matrix. Successful formation of an haustorium allows uptake of nutrients and development of secondary hyphae.

Bgh was the first powdery mildew to have its genome fully sequenced. As with other obligate biotrophs such as *Ustilago maydis*, *Bgh* was found to be lacking in several genes encoding enzymes involved with production of secondary metabolites and also plant cell wall degrading enzymes. Despite this gene loss, the overall genome size was larger than the average ascomycete, at around 120 Mb, due to extensive proliferation of transposable elements, making up 64% of the genome (Spanu *et al.*, 2010).

Proteomic studies of *Bgh* have identified around a quarter of the predicted total number of proteins. All of the proteomic studies have identified an abundance of proteins involved in protein biosynthesis, metabolism and modification (Bindschedler *et al.*, 2009, 2011; Godfrey *et al.*, 2009). Analysis of the haustorial proteome showed significantly higher

number of proteins involved with carbohydrate metabolism in this tissue compared to hyphal tissue or conidia (Bindschedler *et al.*, 2009) which would confirm the role of haustoria in nutrient uptake. In particular, the presence of an α-glucoside permease with a sucrose transporter domain indicates that carbohydrates are being transported into the haustorium (Bindschedler *et al.*, 2011). Cell wall degrading enzymes including β-1,3-glucosidases have also been identified in the haustorial proteome (Bindschedler *et al.*, 2011). Haustoria also showed a higher number of proteins involved in immunity and defence and stress responses suggesting that the haustorium may be responding to attempts of the plant to prevent infection (Bindschedler *et al.*, 2009).

1.6 Effector biology

The production of secreted molecules, known as effectors, with various roles in the manipulation of host metabolism and defence response is ubiquitous across plant pathogens. This has made effectors a key area of study in plant-pathogen interactions.

Plant pathogenic bacteria such as *Pseudomonas spp* generally produce between 20 and 30 effector molecules (Chang *et al.*, 2005) and these are secreted into the host cell mostly through a well conserved Type III secretion system, or occasionally a Type IV secretion system (Hueck, 1998; Christie and Vogel, 2000). Many bacterial effectors have been well characterised and their roles in promoting pathogen virulence have been elucidated. These include the inhibition of PTI responses, manipulation of JA responses to prevent SA-mediated responses and some effectors have been shown to have a nuclear localisation signal suggesting that they are directly manipulating host gene expression (Zhu *et al.*, 1998; Kunkel and Brooks, 2002; Hauck *et al.*, 2003). Most bacterial effectors are products of *Avr* genes, as previously described, which are recognised by products of plant resistance (*R*) genes. This recognition has turned their virulence function into an avirulence function.

Filamentous pathogens – fungi and oomycetes – also produce effectors but much less is known about their delivery or function. In oomycetes, a ubiquitous RXLR effector motif was found to be required for translocation of effectors into plant cells and the presence of this motif was used to identify 425 candidate effectors in the potato late blight pathogen *Phytophthora infestans* (Whisson *et al.*, 2007).

The scale of the effector complement in oomycetes is substantially larger than in bacteria and this has also been found in fungal pathogens. Genome analysis identified over 1000

effector candidates in each of two rust fungus species (Duplessis *et al.*, 2011; Hacquard *et al.*, 2012) and over 500 candidate effectors in *Bgh* (Spanu *et al.*, 2010; Pedersen *et al.*, 2012).

Bgh effectors have generally been classified in three ways. The first *Avr* genes identified were *AVRa10* and *AVRk1* and the first classification of putative effectors was the EKA (effectors homologous to *AVRa10* and *AVRk1*) family, which was believed to include over 1350 members (Amselem *et al.*, 2015). This family has proliferated due to their association with transposable elements and the lack of machinery for repeat induced point mutations which would limit genome expansion due to transposons (Sacristán *et al.*, 2009; Spanu *et al.*, 2010; Amselem *et al.*, 2015). Using a proteogenomic approach, a number of *Blumeria* effector candidates (BECs) were identified. BECs were categorised as small proteins with a predicted signal peptide and high haustorial to epiphytic hyphae expression ratio (Bindschedler *et al.*, 2009, 2011). Taking a transcriptomic approach, a third set of putative effectors were identified. These were classed as Candidate Secreted Effector Proteins (CSEPs). The CSEPs are classes as small, secreted proteins, lacking a transmembrane domain and having no homologues outside the powdery mildews (Spanu *et al.*, 2010; Pedersen *et al.*, 2012). There is large overlap between the BECs and the CSEPs with most BECs also being categorised as CSEPs but some were excluded from the CSEP list due to homology in other fungi.

The CSEPs/BECs are predicted to be secreted due to the presence of a signal peptide. Initial evidence of transport of effectors from haustoria into plant cells came from rust fungi (Kemen *et al.*, 2005) and *Bgh* effectors are predicted to be similarly secreted from haustoria (Panstruga and Dodds, 2009). A large proportion of CSEPs/BECs have a conserved YxC motif reminiscent of the oomycete RXLR motif but there has been no direct evidence of the role of this motif in translocation of effectors (Spanu *et al.*, 2010; Pedersen *et al.*, 2012).

Fifty of the *Blumeria* effector candidates were screened using host induced gene silencing (HIGS) to investigate whether they have a virulence function during *Bgh* infection. Using this method, eight BECs were confirmed as virulence factors (Pliego *et al.*, 2013).

Two of these virulence factors *BEC1011* and *BEC1054* (synonym CSEP0264 and CSEP0064) are members of CSEP family 21 with 75% nucleotide similarity and 49% amino acid similarity. HIGS of *BEC1011* produced the most significant effect, with a 70% reduction of haustorial formation while *BEC1054* produced a reduction of 59%. Both these effectors,

along with around 25% of CSEPs, have structural similarity to ribonucleases and are classified as ribonuclease-like protein in haustoria (RALPHs) (Pedersen *et al.*, 2012; Spanu, 2017). No RNase activity has been identified in these effectors although they are predicted to bind RNA. A recent study has identified barley proteins which interact with BEC1054. These include a glutathione-S-transferase, a malate dehydrogenase, pathogenesis related protein 5 and an elongation factor 1γ (Pennington *et al.*, 2016a).

Another BEC validated as a virulence factor was *BEC1019.* This BEC was not identified as a CSEP because homologues of *BEC1019* are found in around 40% of sequenced fungi (Whigham *et al.*, 2015). *BEC1019* is a metalloprotease-like protein with zinc-binding capability but no protease activity has been identified to date. Homologues of *BEC1019* have been identified in human pathogens *Candida albicans* (*Pra1*; Citiulo *et al.*, 2012) and *Aspergillus fumigatus* (*Aps F2;* Amich *et al.*, 2010) as well as plant pathogens including *Zymoseptoria tritici* and *Fusarium graminearum* (Whigham *et al.*, 2015). Homologues of *BEC1019* in other fungi have shown zinc scavenging capabilities and a similar role is predicted in *Bgh*.

AVRa10 was the first *Bgh* avirulence gene identified which is recognised by the barley *Mla10* allele (Ridout *et al.*, 2006). *Bgh* genes recognised by a further two barley *Mla* alleles have recently been identified. *AVRa1,* which is recognised by *Mla1* was identified as *CSEP0008,* and *AVRa13,* recognised by *Mla13,* was identified as *CSEP0372* (Lu *et al.*, 2016). Both *CSEP0008* and *CSEP0372* have high haustorial to hyphal expression ratios (226 and 267, respectively) and both were identified as proteins in haustoria (Bindschedler *et al.*, 2009, 2011; Pliego *et al.*, 2013). CSEP0008 was one of the most abundant proteins in haustoria and was classified as BEC1001 (Bindschedler *et al.*, 2009).

1.7 Host susceptibility genes

The effector complement of a pathogen influences its pathogenicity, but host genes can also be negative regulators of plant defence, also called susceptibility genes.

Several barley genes have been shown to negatively regulate resistance against *Bgh*. The most studied of these is the wild type *Mlo* gene. Mutations in this gene, creating recessive *mlo* alleles, have been shown to provide broad spectrum resistance to all known *Bgh* isolates and has been used durably in agriculture since the 1970s (Jørgensen, 1992). *mlo* promotes cell wall appositions which block fungal penetration in a manner akin to non-host resistance (Humphry *et al.*, 2006).

Another gene associated with *mlo* based resistance is *RACB* and this has also been identified as a susceptibility gene. *RACB* expression interferes with actin remodelling and silencing of *RACB* reduces fungal penetration (Schultheiss *et al.*, 2002; Opalski *et al.*, 2005).

Other genes have been shown to be highly induced upon *Bgh* infection, such as *Blufensin1* and *Bax Inhibitor-1,* and silencing of these genes has been shown to reduce *Bgh* infection, therefore classing them as susceptibility genes (Meng *et al.*, 2009; Eichmann *et al.*, 2010; Xu *et al.*, 2015).

1.8 Gene silencing

RNAi is most commonly mediated through 21-24nt small interfering RNAs (siRNAs) derived from double stranded RNA (dsRNA) or short-hairpin RNAs (Sharp, 2001). The dsRNA or shRNA is processed into siRNA by Dicer or Dicer-like (DCL) enzymes. Dicer was originally identified in *Drosophila* and Dicer or Dicer-like proteins have been shown to have RNaseIII activity and have been identified abundantly in a range eukaryotes (Bernstein *et al.*, 2001). The siRNAs are recruited into the RNA induced silencing complex (RISC). RISC is known to associate with a number of proteins but the fundamental function of RISC has been shown to be conferred by the Argonaut2 protein and the Dicer-derived siRNA (Rand *et al.*, 2004). The RISC binds the complimentary mRNA which is then degraded before it can be translated (Baulcombe, 2004). Argonaut proteins are known to be involved in processing of host microRNA as well as exogenous mRNA.

RNAi silencing initially used viral vectors containing host dsRNA which the virus inserted into the plant and is then processed via Dicer and RISC to induce silencing, known as virus induced gene silencing (VIGS). This has been used in plant pathology to investigate the function of genes involved in defence responses in wheat and barley (Meng *et al.*, 2009; Yin *et al.*, 2011; Lee *et al.*, 2012, 2015; Xu *et al.*, 2015). However, there have been reports that the use of viruses such as barley stripe mosaic virus to induce gene silencing can also trigger a virus-induced immune response, impairing the ability to study the interaction of the silenced plants with other pathogens (Tufan *et al.*, 2011).

After some studies showing that RNA could be taken up by haustoria, Nowara *et al.*, (2010) demonstrated that dsRNA constructs targeting *Bgh* fungal genes, delivered into plant cells could effectively silence the target gene. The HIGS method relied on the construction of RNAi constructs in plasmids which were then delivered to plant cells by microprojectile bombardment along with a GUS reporter construct. This method was used by Pliego *et al.*, (2013) to silence BECs and confirm their role as effectors and by Koch *et al.*, (2013) to

silence fundamental genes in *Fusarium graminearum* (causal agent of fusarium head blight) to reduce growth of the pathogen. Another HIGS method used *Agrobacterium*-mediated transfer of silencing constructs to wheat to study *Puccinia triticina* (Panwar *et al.*, 2013). However, these methods require cloning of silencing constructs before plasmids can be delivered to plants. Given the number of potential effectors identified in fungal pathogens, alternative methods are required for high-throughput screening.

Alternatives which have potential for high-throughput screening involve chemical or enzymatic synthesis (Theis and Buchholz, 2010) and direct delivery of double stranded RNAs for RNAi. These methods do not require cloning of silencing cassettes, transient transformation or biological agents for RNA transfer. In animal research these methods have used liposomal encapsulation of RNAs for transfer into mammalian systems (Castanotto and Rossi, 2009). Transfer of these methods in plant systems were prohibited by the presence of a cell wall preventing delivery of micelles into cells. Early studies used protoplasts to overcome this issue (Vanitharani *et al.*, 2003) but still precluded whole tissue or organism studies and is not viable for high-throughput analysis.

Double stranded RNA has also been used to induce gene silencing in pathogens by transfer from plants. This was initially shown for insect pathogens such as the green peach aphid (*Myzus persicae*) which is able to take up dsRNA while feeding on the plant (Pitino *et al.*, 2011). Later it was shown that dsRNA of nearly 800bp could be taken up by the fungus *Fusarium graminearum* in vitro and resulted in silencing of genes involved in ergosterol biosynthesis (Koch *et al.*, 2013). This was then developed by the application of the same dsRNA by spraying barley leaves which resulted in transport of the dsRNA throughout the leaf and resistance to *Fusarium* infection (Koch *et al.*, 2016). This shows that gene silencing methods are possible without the need for genetic modification of the plant which broadens the applications in agriculture.

Aims

- Validation of a new silencing strategy: to Investigate *Blumeria* Effector Candidates and known barley susceptibility genes
- To use this tool to investigate new potential virulence factors and susceptibility genes in the barley – *Blumeria* interaction

Objectives

1. *Evaluating and comparing the efficacy of non-modified and PTO-modified oligodeoxynucleotides for silencing known fungal effectors (Chapter 3)*

   ODNs or PTOs suitability will be evaluated by setting up a workflow to silence the known and validated fungal effectors *BEC1011*, *BEC1019* and *BEC1054*.

2. *Silencing known effectors to establish and validate the PTO mediated host induced gene silencing (HIGS) workflow (Chapter 3)*
   The silencing impact of PTO silencing of known *Blumeria* virulence factors will be first monitored by using microscopic evaluation of infection, then qRT-PCR to measure relative mRNA levels of targeted genes and estimate the fungal biomass following the treatments. In addition, multiple reaction monitoring mass spectrometry (MRM-MS) will be employed in an attempt to quantify the cognate protein amounts of the targeted effector for silencing.
3. *Transferring the PTO methodology to silence barley susceptibility genes and assessing the impact of silencing on barley susceptibility to Bgh (Chapter 4)*
   The susceptibility genes MLO and Blufensin will be silenced and the methodology assessed for those.
4. *Use the PTO silencing tool to test the virulence role of new gene candidates identified for their virulence function (Chapter 5)*
   AVRa1 and AVRa13 avirulence factors will be scrutinised for their possible virulence function
5. *Investigate the role of PR5, a protein which interacts with the BEC1054 effector (Chapter 6)*

   A barley PR5 isoform, that was found to interact with BEC1054, will be silenced with the PTO silencing workflow to estimate its role in resistance or susceptibility during barley powdery mildew interaction.

6. *Characterise plant defence responses in effector silenced barley leaves (Chapter 7)*
   Since the PTO workflow allows the delivery of PTOs to whole barley excised leaves, the method will be used to investigate the biochemical changes as a consequence of silencing *Blumeria* virulence genes. For this, the production of reactive oxygen species, H_2O_2 will be monitored following infection in *BEC1011* silenced plants.

2 Materials and Methods

All chemicals were obtained from Sigma Aldrich or Fisher Scientific unless otherwise stated. All oligonucleotides were obtained from Sigma Aldrich.

2.1 Plant and fungal material

Barley (*Hordeum vulgare, Hv*) cultivar Golden Promise was selected for this study as it has been shown to be susceptible to all strains of *Bgh*. Seeds were obtained from Prof James Brown (John Innes Centre, Norwich, UK) and stored in air tight containers at 4 °C to maintain seed viability. Approximately 50 seeds were sown in 13 cm diameter pots containing John Innes no.1 compost. Pots were placed in trays filled with water to maintain soil moisture and grown in controlled temperature room with 16 h light, 8 h dark at 22 °C and were watered every two days.

Bgh isolate DH14 (obtained from Prof. Pietro Spanu, Imperial College London, UK) was maintained by weekly transfer of a high spore density inoculum on 7 day old barley seedlings. Although moisture was not controlled, humidity was monitored and locally maintained above 60% to ensure higher infection rate. The isolate DH14 was used as it is sequenced and an assembled, annotated genome draft (version 3) (Spanu *et al.*, 2010) was made publically available initially from the Blugen (www.blugen.org) website and later from EnsemblFungi (http://fungi.ensembl.org). Therefore, ORF, CDS and cDNA sequences could be easily retrieved for oligodeoxynucleotide (ODN) design.

2.2 Design of ODNs

The sequences of barley genes *Mlo, Bln1* and *Pr5* were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/). *Blumeria* cDNA sequences for BEC1011 (CSEP0264), BEC1019, BEC1054 (CSEP0064), AVRa1 (CSEP0008) and AVRa13 (CSEP0372) were retrieved from *EnsemblFungi* (http://fungi.ensembl.org). Accessions of all gene targets are shown in table 1.

Previous plant ODN studies have used software such as mFold for design of ODNs (http://unafold.rna.albany.edu/?q=mfold, (Zuker, 2003; Sun *et al.*, 2005, 2007; Dinc *et al.*, 2011). This software predicts the secondary RNA structure and ODNs can be selected over structures such as free loops where RNA-ODN duplexes could form. However, it has been shown that there is no correlation between this design method and success of an ODN (Matveeva *et al.*, 2003; Shabalina *et al.*, 2006). Therefore, for this study, online software called OligoWalk (http://rna.urmc.rochester.edu/cgi-

bin/server_exe/oligowalk/oligowalk_form.cgi, (Mathews *et al.*, 1999; Lu and Mathews, 2008)) was used to design antisense ODNs from the submitted cDNA sequence. OligoWalk predicts the sequences where binding energy would produce a stable ODN-RNA duplex and where the ODN would be unlikely to form self-structures. This design method has been shown to significantly improve the "hit-rate" of ODNs (Matveeva *et al.*, 2003). Sequences of the designed ODNs are shown in table 2.1.

Table 2.1 accession numbers and antisense ODN sequences for target genes

Gene name	ODN name	CSEP no.	Genomic reference	Uniprot accession	antisense ODN sequence (5' - 3')
BEC1011	11.11	CSEP0264	BGHDH14_bgh06532	N1JJX4	TATCTGGAACTCTATAATC
	11.11 MM1				TATCTGCAACTCTATAATC
	11.11 MM2				TATCTGCAACTCAATAATC
	11.11 shuffle				CATTTAGTTTACGACCAAT
	11.11 25mer				TTTTATCTGGAACTCTATAATCATC
BEC1019	19.11		BGHDH14_bgh03531	N1J6C9	TAAACGATTGTGTAAGAAC
	19.12				TTTGTCTGTGTAGCATTAC
BEC1054	54.11	CSEP0064	BGHDH14_bgh02874	N1JJ94	TTTCAGTACCATCACAATC
BLN1	BLN1_1		Genbank FJ156744	B8X456	TTACATTCTAGATCATAGG
	BLN1_2				TTAATTTACTGCCACAAGG
	BLN1_2 25mer				GATTTAATTTACTGCCACAAGGATG
	BLN1_3				ATATATATAGAAGTAGTGC
MLO	MLO_1		Genbank Z83834.1		TAGTCAACGTACTTGCTGG
	MLO_2				TTGTGGAAGTCGAACTTGC
AVRa1	AVRa1_1	CSEP0008	BGHDH14_bgh00029		TATTTGAAGTAAAGACAGC
	AVRa1_2				TTTCGGTTATTTCGATAGC
AVRa13	AVRa13_1	CSEP0372	BGHDH14_bghG0028610 00001001		TAAAAATCTCCACTACGCC
	AVRa13_2				TATCTCGCACCATTGTAAC

PR5	PR5.1		GenBank AJ001268.1	O23997	TTGAAGAACATTGAGTAGT
Barley seed protein Z	Z		Genbank X97636.1	P06293	AAGCGGTTGAGCACTGAA
M13 primer sequence	M13				CGCCAGGGTTTTCCCAGTC

Potential ODN sequences were checked against barley and *Blumeria* databases for potential off targets using BLASTn which adjusts parameters for short input sequences. ODNs with matches to barley or *Blumeria* genes longer than 14 nucleotides in length were discarded. This length was used because prediction of efficiency using OligoWalk falls sharply from around 0.95 to between 0.1 and 0.2 when predicting sequences shorter than 14 nt.

The secondary mRNA structure of *BEC1011, BEC1019* and *BEC1054* were predicted using mFold software (http://unafold.rna.albany.edu/?q=mfold, Zuker, 2003). The mRNA sequence was inputted into the web server with the default parameters and the probable secondary structure was predicted based on free energy estimations. The ODNs designed using OligoWalk were mapped onto the predicted secondary structure.

2.3 ODN/PTO treatment workflow to silence *Blumeria* and barley genes in planta

To silence barley or *Blumeria* genes in planta, ODNs were delivered to excised barley leaves through loading into the vascular tissue. The workflow was modified from Sun *et al.*, (2005, 2007) and Dinc *et al.*, (2011). This system allows for delivery of ODNs throughout the leaves, targeting whole tissues.

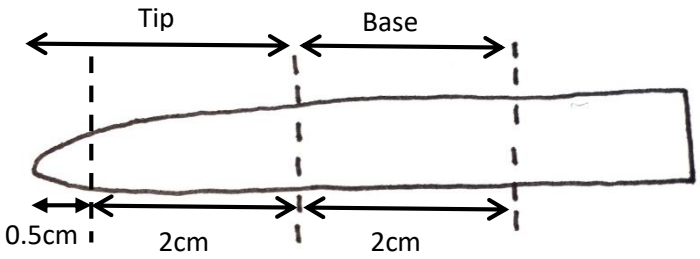
Primary leaves of seven-day old barley (*Hordeum vulgaris* cv. Golden Promise) were cut into 8 cm segments in a petri dish containing water to prevent air embolism in the xylem. Up to three leaves were placed in 2 ml tubes with 1 ml 10 µM ODN or phosphorothioate (PTO) modified ODN, three leaves per tube. Leaves were incubated for 24 h under continuous light.

After the treatment period, leaves were cut to 6 cm – removing the submerged portion – and laid adaxial face up on 20 mg/l benzimidazole (from 20 mg/ml stock in ethanol) 0.6% agar plates (12 x 12 cm square Petri dishes). Glass Pasteur pipettes were taped across the top and bottom of the leaves in order to keep them flat and ensure even spore distribution.

Plates were inoculated with *Bgh* spores by placing the open dishes in an inoculation chamber with pots of barley with sporulating *Bgh* and using a hair dryer to blow spores onto the Petri dishes. Inoculation density was recorded using a haemocytometer. Inoculated plates were incubated for 48 hours in a 16 h/8 h light/dark cycle at 22 °C before leaf sections were sampled for microscopy or frozen in liquid nitrogen for RNA or protein extraction. 2 cm leaf sections were cut, retaining 0.5 cm at the tip to distinguish the upper and lower sections, as shown in figure 2.1.

Figure 2.1 indicating the cutting of leaves to be stained for microscopy

Leaves were stained in lactophenol cotton blue (50% glycerol, 25% lactic acid, 25% phenol, 500 mg/L aniline blue) (Pro-Lab Diagnostics, Wirral, UK) and then destained in 3:1 ethanol:



acetic acid. Leaf sections were mounted on glass slides with 20% glycerol in water and infection scoring was performed by bright field transmission microscopy at 250x magnification.

The number of non-germinated conidia (NG), conidia with appressoria (App) and conidia with appressoria and secondary hyphae (SH) were counted in five passes across the leaf along the length of each of the tip or base leaf section. The proportion of secondary hyphae (%SH) are used to estimate the rate of successful infection as an alternative to observing the proportion of haustoria, as haustoria do not stain well and are more difficult to observe. However, as secondary hyphae only form after successful formation of a haustorium, this is a useful alternative.

A general linear mixed model was used to assess the significance of the difference between the silenced and negative control treatments. This was calculated in R Studio (https://www.r-project.org/) using the glmer function from the lme4 package. An example of the preparation of the data for analysis is shown in supplementary table 1. The number of conidia with secondary hyphae out of number of germinated conidia was defined as the response variable. This variable was treated as a binary variable as the data is germinated conidia with or without secondary hyphae, therefore a binary error structure was used. A GLMM using raw count data, rather than proportions which are transformed with arcsine

transformation, is recommended with data such as this (Warton and Hui, 2011) and other fixed and random factors can be incorporated into the model. The type of treatment and density of the *Bgh* spore inoculum were considered as fixed factors, while the date of the experiment was included as a random factor. This model was coded as follows:

glmer (proportion SH~Treatment*Density+(1|Date), data=mydata, family=binomial)

The full code of the GLMM, using the example data set from supplementary figure 1, is shown in supplementary figure 1.

2.4 RNA extraction from barley leaves

Leaves were sampled for RNA extraction generally two days post inoculation (dpi), or at indicated times, and immediately flash frozen in liquid nitrogen, and kept at -80 °C until further use. A maximum of 100 mg leaf tissue was ground in liquid nitrogen with a small amount of quartz sand and RNA was extracted using the RNeasy Plant mini kit (QIAGEN, Crawley,UK) according to the manufacturer's protocol. Buffer RLT supplemented with 10 µl/ml 14.3 M β-mercaptoethanol was used to resuspend the ground powder before loading onto QIAshredder spin column. This was centrifuged for two minutes at 9000 g and the flow through collected. The flow through was mixed with 0.5 volume 100% ethanol and loaded onto RNeasy spin column and centrifuged again. Following this step, the RNA is bound to the column membrane and was washed with the RW1 buffer and then RPE buffer provided in the kit. RNA was eluted in 40 µL RNase-free water. The RNA yield was quantified by measuring the absorbance at 260 nm in 2 µl of extract using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The 260/280 nm absorbance ratio gave an indication of purity of the RNA sample, with a ratio above 1.8 being optimal.

2.5 cDNA synthesis by reverse transcription

Reverse transcription of 1 µg extracted RNA into cDNA was performed using Quantitect Reverse Transcription kit (QIAGEN) according to the manufacturer's protocol. RNase free water was added to 1 µg RNA to a volume of 12 µl and 2 µl of the gDNA wipeout buffer was added prior to incubation at 42 °C for five minutes. Following this incubation, the sample was placed on ice and 1 µl Quantiscript reverse transcriptase, 1 µl dNTPs and 4 µl Quantiscript reverse transcription (RT) buffer were added. The sample was incubated at 42°C for 20 minutes before the reaction was stopped by heating at 90 °C for three minutes.

2.6 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was performed on prepared cDNA. Reference genes used were *Bgh* GAPDH and *Hv* GAPDH as the expression level of these have been shown to be most stable in these conditions (Pennington *et al.*, 2016b). Primers for gene targets BEC1011, BEC1019 and BEC1054 were taken from Pliego *et al.*, (2013) and Pennington, Li, *et al.*, (2016). Primers for PR5 were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Sequences for all primers are in Table 2.2.

Table 2.2 Forward and reverse primers used for quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Gene	Forward primer	Reverse primer	Reference
Bgh GAPDH	GGAGCCGAGTACATAGTAGAGT	GGAGGGTGCCGAAATGATAAC	(Pennington *et al.*, 2016b)
Hv GAPDH	CTGATTGAGAAGGCTGATGGAT	AGAGCAGGAGCGTCATTGA	(Pennington *et al.*, 2016b)
BEC1011	TCATGGAGCATCTGCATTGTC	CATGCTCTCCTTGCCAGTTT	(Pliego *et al.*, 2013)
BEC1019	TCCTACGACTGGACAACACCT	CATGCTGAGCAAGGGTTACA	(Pliego *et al.*, 2013)

The qRT-PCR reaction was prepared by adding: 20 ng of the prepared cDNA in 5 µl (assuming equal efficiency of reverse transcription in all samples), 0.6 µl 10 µM forward and reverse primers and 10 µl 2x PrecisionPlus SY master mix (Primerdesign, Southampton, UK). The qRT-PCR was performed on a RotorGene Q qPCR machine (QIAGEN). The procedure included a 2 minute hold at 95°C and 35 cycles of 14 seconds 95°C then 60 seconds 60°C for annealing and extension. To check for the PCR primer specificity based on the detection of a single amplicon of expected size, a melt curve was performed by ramping the temperature from 65-95°C. At the melting point of the PCR amplicon the fluorescence drops because the DNA is no longer double stranded and the SYBR green fluorescent stain only binds to double stranded nucleic acids. For each sample a single peak is expected when rate of change in fluorescence is plotted as a function of temperature. A single peak indicates that a single product has been amplified and the melting temperature is dependent on the length of the amplicon and is used to indicate whether the correct product has been amplified. For example, it is possible to distinguish primer dimers as this results in a peak of lower temperature in the melt curve as the primers are shorter than the amplified product.

The QIAGEN Rotorgene Q software reports the CT value ("Take off point"), the number of cycles required for the SYBR green fluorescence to reach a threshold above background fluorescence, and the efficiency (E) of the primers in each sample. Samples with an E value below 1.7 were removed from analyses.

The relative levels of barley and *Bgh* cDNA were calculated using the following formula from Pfaffl (2001).

$$ratio = \frac{(E_{target})^{\Delta CT_{target}(control-sample)}}{(E_{ref})^{\Delta CT_{ref}(control-sample)}}$$

Figure 2.2 shows the equation used to calculate the ratio of target *Bgh* transcript to barley reference. E refers to the efficiency of the PCR for each set of primers (target *Bgh* and reference barley). ΔCT is the difference in number of PCR cycles required for fluorescence to reach a 'take-off point (CT)' – defined as being significantly above the background fluorescence – between the PTO treated samples and the PTO Z control samples.

2.7 Protein Extraction from barley leaves

Proteins were extracted from 100 mg of frozen leaf samples (kept at -80 °C) ground in liquid nitrogen by addition of 1ml 10% w/v trichloroacetic acid (TCA), 0.07% v/v β-mercaptoethanol in cold acetone. After mixing, the resuspended powder was incubated at -20 °C overnight before centrifuging at 9000 g for 30 minutes at 4 °C. The supernatant was removed and the pellet washed twice with 1 ml ice cold acetone with 0.07% v/v β-mercaptoethanol. The pellet was air dried to remove remaining acetone and the pellet was resuspended in 50 µl urea extraction buffer (5 M urea, 2 M thiourea, 20 mM Tris, 20 mM dithiothreitol (DTT)), centrifuged for 20 minutes at 9000 g and the protein-containing supernatant collected and stored at -20 °C.

Protein amount was estimated using a Bradford assay. Concentrated Bradford reagent (5x; Bio-Rad, Watford, UK) was diluted with water and 1 ml aliquots used for estimation. Five microlitres of protein sample was added to the reagent, gently mixed, and incubated for ten minutes to allow the reaction to develop. A spectrophotometer was used to measure absorbance at 595 nm, blanked to Bradford reagent with no protein added. A standard curve was performed with varying concentrations of bovine serum albumin (BSA, Sigma Aldrich) between 1 µg/µl and 10 µg/µl and used to estimate protein amount in the sample extracts.

2.8 Tricine SDS PAGE

Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) gels were prepared according to Schagger (2006) using the Mini-PROTEANIII gel casting system (Bio-

Rad). The separation gel composition was 1 M Tris-HCl pH 8.45, 10% glycerol, 0.1% SDS, 12% acrylamide protogel (19:1 acyrylamide:bis-acrylamide; Sigma Aldrich), 0.05% TEMED and 0.05% ammonium persulphate. The stacking gel contained 1 M Tris-HCl pH 8.45, 0.1% SDS, 12% acrylamide protogel, 0.05% TEMED and 0.05% ammonium persulphate.

Samples were prepared with 20 µg protein per well. Loading buffer (5x Tricine sample buffer, Bio-Rad) with 100 mM DTT was added in a total volume of 20 µl and samples were heated at 42°C for 10 minutes. A broad range protein marker (0.5 µl) was used for colloidal coomassie blue stained gels or Precision plus protein dual extra standard marker (both Bio-Rad) for western blot. The cathode buffer (1 M Tris, 1 M Tricine, 1% SDS, pH 8.25; Bio-Rad) and the anode buffer (1M Tris-Hcl pH 8.9, 1% SDS) were diluted 10x with water. Samples were loaded onto the gel and a voltage of 50 V was applied until the migration front was beyond the stacking gel and into the separation gel. The voltage was then increased to 100 V for 45 minutes, or until the migration front (visible as the blue dye from the loading buffer) had reached the lower end of the gel. A colloidal coomassie blue stain (Instant Blue, Expedeon, San Diego, CA, USA) was used if gels required staining.

2.9 Western Blot for detection of BEC1011 or BEC1019 effectors

SDS PAGE gels were transferred to the blotting system for Mini-Protean gels (Bio-Rad) between sponge pads, filter paper and PVDF membrane (Roche, Mannheim, Germany, activated in methanol), all soaked in transfer buffer (25 mM Tris, 192 mM glycine (Flowgen Biosciences, Nottingham, UK), 20% v/v methanol). The tank was filled with cold transfer buffer and an ice pack and the transfer was performed for 45 min at 100 V with magnetic stirring. Following transfer, the membrane was placed into blocking buffer consisting of 5% skimmed milk powder (Marvel, London, UK) in TBST (25 mM Tris, 150 mM NaCl, 0.05% Tween20) on an orbital shaker at 65 rpm at room temperature for 1 h. The blocking buffer was drained and all residual liquid removed with a pipette. The membrane was incubated with a primary polyclonal antibody directed against BEC1011 or BEC1019 at 1:2000 dilution (rabbit serum, Covalab, Cambridge, UK) in 0.1% BSA TBST on an orbital shaker at 65 rpm at 4 °C overnight. The primary antibody was removed and the membrane washed four times in TBST, removing residual liquid each time. The membrane was incubated with the secondary antibody (Swine anti-rabbit IgG conjugated to horseradish peroxidase; 1:2000 dilution, Dako, Ely, UK) in 0.1% BSA TBST for 2 h at room temperature. After a further 4 washes in TBST, the excess liquid was removed by blotting the PVDF membrane briefly on blotting paper prior to placing it between transparent plastic sheets. A chemiluminescence based kit was used for the detection of peroxidase activity by adding 1.5 ml developing

solution (1:1 SuperSignal West Pico stable peroxidase:luminol enhancer, ThermoScientific, Waltham, MA, USA) to the membrane and air bubbles carefully removed by gently rolling with a Pasteur pipette. The membranes were incubated in the dark for 5-10 min. In the dark room, an autoradiogram film (ThermoScientific) was placed on top of the membrane in the hypercassette, exposed for 3 minutes and developed using an automatic developer (Photon Imaging Systems, Swindon, UK). A new film was placed immediately for longer exposure time if necessary.

2.10 In solution tryptic digest

Plant protein extracted using the TCA/acetone precipitation protocol described above (2.7) was digested with trypsin to generate tryptic peptides for targeted protein quantification by multiple reaction monitoring mass spectrometry, as an alternative to semi-quantitative western blot analysis.

Protein extracts containing 20 µg protein (as estimated by Bradford assay) was incubated with 100 mM ammonium bicarbonate and 10 mM DTT for 15 minutes at 50 °C. Iodoacetamide was then added to reach a final concentration of 20 mM and incubated at room temperature in darkness for 15 minutes.

Trypsin, supplied as lyophilised powder (Sigma Aldrich), was reconstituted in 1 mM HCl to a concentration of 200 ng/µL. Trypsin was added in 1:50 ratio to the protein sample and digests were incubated over night at 37 °C. Following this, a further 1:50 volume of trypsin was added and incubated for 2 hours at 37 °C. The reaction was stopped with 10% formic acid to get a final concentration of 0.6% to obtain a final pH around 2.

2.11 Protein expression in *E. coli*

Recombinant BEC1011 and BEC1019 with an N-terminal His-tag and C-terminal Strep tag was expressed in *E. coli.* BEC1011 or BEC1019 in pET53 plasmid (Invitrogen, Carlsbad, CA, USA) was grown in *E. coli* strain Bl21 on agar plates containing 100 µg/ml ampicillin. Plasmids containing the effector insert was provided by Pietro Spanu, Imperial College London, UK. Colonies from these plates were used to inoculate 10 ml liquid LB and grown overnight at 37 °C. An equal volume of 60% glycerol was then added and 1 ml aliquots were stored at -80 °C.

For protein induction, 1 ml glycerol stocks were used to inoculate 40 ml LB medium and grown at 37°C to an OD of 0.4. 1 mM IPTG was then added and the culture was incubated on a shaker at 37 °C for a further 2 to 3 hours or at 25 °C overnight.

The culture was then divided into 10 ml aliquots and centrifuged at 4 °C, 4000 rpm for 15 minutes. The supernatant was discarded and the pellets were stored at -20 °C.

2.12 Extraction of recombinant proteins from *E. coli*

Recombinant BEC1011 or BEC1019 protein was extracted from *E. coli* using two extraction buffers – a native buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mg/ml lysozyme (Sigma Aldrich) and DNAseI (Sigma Aldrich, added after freeze-thaw cycle) and a denaturing buffer containing 7 M urea, 20 mM Tris pH 7.5, 100 mM NaCl and 10mM DTT.

The bacteria were initially extracted in the native buffer on ice with a freeze thaw step to lyse the cells. This was centrifuged at 9000 g for 25 minutes at 4 °C. The supernatant contains the soluble extracted proteins. The pellet was then resuspended in the denaturing buffer and centrifuged again to separate the insoluble protein fraction.

Protein content was estimated against a Bradford assay (Biorad) standard curve measured at 595 nm as previously described and then all extracts run on 12% tricine SDS PAGE gel.

2.13 In gel digestion of recombinant proteins

Protein bands corresponding to BEC1011 or BEC1019 were excised from tricine SDS PAGE gels and cut into 1 mm^2 for in gel tryptic digestion. Gel pieces were sequentially washed in 100 µl 50 mM ammonium bicarbonate (ABC), 100 µl 25 mM ABC, twice in 50 µl 33% acetonitrile (ACN) in 25 mM ABC and twice in 50% ACN in 25 mM ABC before vacuum drying for 20 minutes.

Gel pieces were incubated and rehydrated at 50°C in 30 µl 20 mM DTT in 25 mM ABC for 45 minutes to reduce cysteines. Excess liquid was removed and cysteines were then alkylated by incubating the gel pieces in 30 µl 10 mg/ml iodoacetamide in 25 mM ABC in darkness for 1 hour. Gel pieces were then washed three times in 50% ACN in 25 mM ABC and vacuum dried for 20 minutes.

The dried gel pieces were rehydrated in 25 µl 25 mM ABC containing 10 ng/µl sequencing grade trypsin (Sigma Aldrich) for 15 minutes before a further 15 µL 25 mM ABC was added and incubated at 37 °C overnight.

The digestion reaction was quenched by adding one volume 50% ACN in 5% trifluoroacetic acid (TFA) and incubated for 10 minutes. The supernatant was collected. Three further

washes in 50% ACN/5% TFA were performed and the supernatants were pooled. The pooled supernatants were vacuum dried for 1-2 hours and then stored at -80 °C.

2.14 Multiple Reaction Monitoring Mass Spectrometry

In silico digestion of target proteins was performed using Skyline software (https://skyline.ms/project/home/software/Skyline; MacLean *et al.*, 2010). Barley and *Blumeria* databases were uploaded and used to check that potential peptides were unique.

MRM-MS was performed by Mark Bennett (Imperial College London, UK). Methods are as describe in Young *et al.*, (2014). Digested protein samples (20 µl) were injected onto a 3 µm C_{18} Luna 100 × 2 mm column (Phenomenex, Macclesfield, UK) on an Agilent (Stockport, UK) 1100 Binary LC system, linked to an Applied Biosystems (Foster City, CA, USA) Q-Trap hybrid mass spectrometer fitted with a TurboIonspray (electrospray; Applied Biosystems) source in positive ion MRM mode.

2.15 DAB staining for detection of H_2O_2

3,3-diaminobenzidine (DAB; Sigma Aldrich) was used as a stain to detect H_2O_2 produced following infection of barley leaves with *Bgh* spores.

Excised barley primary leaves that were first treated with PTOs were sampled 18 hours post inoculation, 2.5 cm tip sections were cut and stained with DAB in a method adapted from Thordal-Christensen *et al.*, (1997), Bindschedler *et al.*, (2006) and Daudi and O'Brien, (2012).

The DAB solution was prepared fresh for each use and kept wrapped in foil as DAB is light sensitive. A 1 mg/ml solution was prepared in H_2O with 1:10 volume of 0.1 M HCl to acidify the solution to about pH 3.8 to allow for the solubilisation of DAB. The solution was shaken for 1-2 hours on a rotating platform before use. Following the solubilisation of the DAB, a sodium phosphate buffer (pH 7) to a final concentration of 10 mM was added to increase the pH of the DAB solution. Four 2.5 cm leaf tips were submerged in 4 ml 1 mg/ml DAB supplemented with 0.05% Tween-20 in a 15 ml falcon tube. A hole was made in the lid to allow for vacuum infiltration. The leaves were vacuum infiltrated by placing the tube in a conical flask with a side arm. The top was closed with a rubber bung and the side arm connected to a vacuum water pump attached to the main water supply. Formation of air bubbles on the leaf surface indicated that sufficient vacuum had been reached. The vacuum was broken by removing the rubber tube from the side arm. To check that leaves were fully infiltrated they were examined, and where liquid was infiltrated leaves looked

darker and more translucent. Following the DAB infiltration leaves were laid on damp filter paper under light for four hours for precipitation of DAB. Leaves were then stained with lactophenol cotton blue for *Blumeria* detection and cleared with 3:1 ethanol:acetic acid as previously described.

H_2O_2 production, visible as light yellow to dark brown stain following precipitation of DAB, was observed using a light microscope at 400x magnification. The proportion of full or partial cell staining was recorded and staining associated with papillae at site of potential penetration was recorded for at least 100 spores per leaf, in at least four leaves per treatment.

3 Optimisation of gene silencing technique

3.1 Introduction

Following the publication of the genome of *Blumeria graminis* f.sp *hordei (Bgh)* (Spanu *et al.*, 2010), over 500 potential effectors (Candidate Secreted Effector Proteins – CSEPs (Pedersen *et al.*, 2012) – and *Blumeria* Effector Candidates – BECs (Bindschedler *et al.*, 2009, 2011)) have been identified.

Gene function studies generally use gene knockouts; however powdery mildews have no stable transformation protocol. Attempts to generate transformants (Chaure *et al.*, 2000; Vela-Corcía *et al.*, 2015; Martínez-Cruz *et al.*, 2017) have been unstable or had poor efficiency and were therefore not applicable for large-scale screening studies. A transient Host-Induced Gene Silencing (HIGS) system using biolistic transformation of excised barley leaves was shown to be successful in transient expression of short hairpin silencing constructs targeting *Bgh* effector genes AVR_{k1} and AVR_{a10} (Nowara *et al.*, 2010). This system was then applied to screen a number of the putative *Bgh* candidate effectors (Pliego *et al.*, 2013; Ahmed *et al.*, 2015, 2016; Aguilar *et al.*, 2016), confirming the function of some of these effectors in pathogenicity by showing reduction in haustorial formation following silencing. However, such an approach could not be applied to wheat powdery mildew studies, as the biolistic transformation causes stress and affects the wheat responses to the pathogen (Patrick Schweizer, IPK Gatersleben, personal communication).

Whilst the development of the HIGS system was a useful development in functional genomics in powdery mildew pathogens, it has a number of flaws which limit its applicability for large scale analysis such as this. Firstly, construction and cloning of silencing constructs is time consuming when there are no automation platforms available. Secondly, biolistic transformation only results in transformation of a small proportion of cells. This limits any downstream analysis. In order to effectively and efficiently analyse the potential pathogenicity function of such a large number of candidate effectors an alternative method is required for high-throughput silencing which allows for global analysis of whole tissues.

One alternative system of transient gene silencing that has been widely used in mammalian systems uses short antisense oligonucleotides (ODNs). ODNs have been used widely – particularly in mammalian studies – both as a genomic tool to understand gene function and increasingly as therapeutic agents (Hu *et al.*, 2002; Carroll *et al.*, 2011). ODNs have also been used in plants (Sun *et al.*, 2005, 2007; Dinc *et al.*, 2011; Liao *et al.*, 2013) but to a

much lesser extent and have never been used to silence fungal genes or to target pests or pathogens.

ODNs are typically 18-25 nucleotide synthetic single-stranded DNA molecules which bind to complementary RNA, thereby silencing that gene. There are two different mechanisms by which this is thought to occur. The RNA-DNA heteroduplex can be destroyed by RNase H (Wu *et al.*, 2004) or the binding of the ODN can prevent translation through steric hindrance (Baker *et al.*, 1997). RNase H is known to degrade only the RNA in an RNA:DNA heteroduplex (Hausen and Stein, 1970).

Efficacy of ODNs for silencing can be limited because they are vulnerable to degradation by nucleases within the cell meaning that they are destroyed before they can bind the target RNA. Modifications to the backbone of the molecule can prevent this degradation, and therefore increase the efficacy of the ODN. One early first generation modification is a phosophorothioate (PTO) modification which replaces one of the oxygen molecules in the phosophodiester bond with a sulphur atom (Chan *et al.*, 2006).

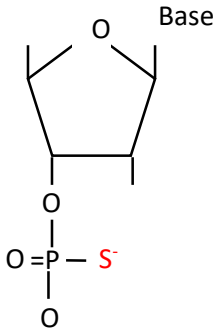


Figure 3.1 structure of the phosphorothioate modified phosphodiester bond. The non-bridging oxygen ion is replaced with a sulphur ion with no overall change in charge.

This modification has the benefit of prolonging the half-life of the ODN in cell culture (Campbell *et al.*, 1990) whilst maintaining the base pairing on which RNase H recognition and activity is based (Kurreck, 2003). However, PTO modified ODNs have also been shown to have off target effects and interact with some proteins. For example, Rockwell *et al.,* (1997) showed PTO modified ODNs interacted with epidermal growth factor receptors and stimulated their phosphorylation.

Given these problems, a number of second and third generation modifications have been developed. Second generation modifications are generally based on 2'-alkyl modifications of the ribose (Chan *et al.*, 2006). The two most widely used second generation

modifications are a 2'-O-methyl (2'-OMe) RNA or 2'-O-methoxy-ethyl (2'-MOE) RNA. These increase the binding efficiency of the ODN for the mRNA but have been shown to be unable to mediate cleavage via RNase H (Hogrefe *et al.*, 1990). However, the use of 2'-OMe or 2'-MOE modified bases in regions flanking a PTO modified ODN has been shown to increase the longevity of the ODN and maintain RNase H activity as it is able to bind between the flanking regions (McKay *et al.*, 1999). These modifications also gave rise to alternative uses of ODNs in which cleavage by RNase H is not useful but by blocking an intron-exon junction aberrant splicing can be repaired. This technique of exon skipping is being utilised as a potential treatment of disorders such as Duchenne Muscular Dystrophy (DMD) (Cirak *et al.*, 2011).

Third generation ODN modifications are mostly based on modifications to the furanose ring and have further improved the stability of ODNs (Chan *et al.*, 2006). Examples include locked nucleic acids and phosphoroamidate morpholino oligomers. As with the second generation modified ODNs, these are not substrates for RNase H but they have been used in studies utilising steric hindrance and to flank PTO modified ODNs which are RNase H substrates (Kurreck *et al.*, 2002).

There are fewer examples of ODN application in plants – possibly due to the lack of applicability of delivery methods from animal studies which generally deliver ODNs into cells via liposomes. The presence of a cell wall in plants prevents entry of liposomes and thus alternative delivery methods had to be devised. At the start of the project a number of studies have shown that naked ODNs can be taken up by plants (Sun *et al.*, 2005, 2007; Dinc *et al.*, 2011; Liao *et al.*, 2013). These have largely been gene function studies and all have targeted plant genes but there is potential for therapeutic applications in plants too.

Although this system has never been used to silence fungal genes, the success of the HIGS system and the development of ODN silencing systems in plants indicate that this could be a viable alternative for gene silencing in *Bgh.*

In this study, ODNs were designed to target a number of *Blumeria* effector candidates (BECs) whose virulence function had already been confirmed using HIGS (Pliego *et al.*, 2013) in order to verify the ODN methodology being developed. The BEC targets are BEC1011, BEC1019 and BE1054. BEC1011 and BEC1054 were identified as proteins specifically expressed in haustoria (Bindschedler *et al.*, 2011) and were suggested to be Candidate Secreted Effector Proteins (CSEP0264 and CSEP0064, respectively) as they are predicted to be secreted, small proteins which lack transmembrane domains and do not

have homologues outside the powdery mildews (Pedersen *et al.*, 2012). BEC1011 and BEC1054 are both RNase-like proteins in haustoria (RALPHs) and are members of the same CSEP superfamily, family 21, with high sequence homology at both the DNA and protein level. A number of barley proteins have been identified which interact with BEC1054, including a malate dehydrogenase, a glutathione S transferase, and a pathogenesis related 5 protein isoform which are all known to be involved with plant immune responses (Pennington *et al.*, 2016a).

Unlike BEC1011 and BEC1054, BEC1019 was not defined as a CSEP because homologues of BEC1019 exist in 40% of sequenced fungi (Whigham *et al.*, 2015). BEC1019 is a metalloprotease-like protein with three zinc binding residues, although no protease or zinc-binding activity has been identified in the *Bgh* homologue.

A control oligonucleotide – ODN Z – was used. This is an ODN designed to target barley seed protein Z and has been used as a control in other ODN studies in plants (Sun *et al.*, 2005, 2007). This protein is only expressed in seeds so silencing should have no effect.

Multiple reaction monitoring is a targeted mass spectrometry technique which allows for quantification of specific proteins within a sample. This is done by predicting and identifying unique peptides of a target protein. Using a triple quadrupole mass spectrometer, the unique peptide is detected in the first quadrupole and allowed to pass into the second quadrupole. Here it is fragmented and the fragment ions enter the third quadrupole where the target ions are specifically detected (Lange *et al.*, 2008). Due to the targeted identification of specific peptides, this technique is better at quantification of lower abundance proteins than shotgun proteomics approaches where lower abundance proteins are masked by high abundance proteins such as RUBISCO (Bindschedler and Cramer, 2011; Boja and Rodriguez, 2012).

3.2 Aims and Objectives

Aim – To investigate whether ODNs are successful in silencing targeted effectors *in planta*

Objective – To design and deliver ODNs targeting effectors BEC1011, BEC1019 and BEC1054 to *Blumeria graminis* f.sp *hordei* and assess the effect of silencing these effectors on fungal virulence and target transcript and protein abundance.

Aim – To investigate the optimal conditions to produce an effective ODN silencing methodology

Objective – to test different lengths of ODN and to compare non-modified ODNs with phosphorothioate (PTO) modified ODNs to optimise the silencing effect.

3.3 Materials and Methods

3.3.1 Development of ODN silencing workflow

Oligonucleotides were designed using OligoWalk software (http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi, (Mathews *et al.*, 1999)), as described in Chapter 2.2. A cut leaf delivery system was used as established by Sun *et al.*, (2005, 2007) and Dinc *et al.*, (2011) where excised barley leaves are placed in a solution of ODNs which are taken up by transpiration. The use of fluorescent tagged ODNs showed this system to be effective for uniform delivery of ODNs throughout the leaf (Sun *et al.*, 2005; Dinc *et al.*, 2011) and also reduces the stress to the leaves which can occur with infiltration. Following ODN or PTO modified ODN uptake under continuous light for 24 h, leaves were placed back in the normal light regime (16 h light, 8 h dark) on 0.6% agar plates with 20 µg/ml benzimidazole (bdz) and immediately inoculated with *Bgh* conidia. Bdz is an anti-fungal agent which has been shown to delay senescence of excised leaves on agar without affecting *Blumeria* development (Kar and Mishra, 1976; Limpert *et al.*, 1988) and was used to reduce growth of mould on the plates post inoculation. Leaves were laid flat with the adaxial surface up and inoculated with *Bgh* spores by shaking spores from heavily infected plants. Plants used for inoculation were infected at 7 days after sowing and used seven days post inoculation. Pots on infected plants were shaken 24 hours before use to ensure that fresh conidia are used for inoculation. Inoculation was performed between 4pm and 6pm, towards the end of the illumination phase of the photoperiod, to ensure no difference in infection efficiency due to circadian rhythms. The specific effect of circadian rhythm or photoperiod on pathogen infection has not been clearly defined but there are examples of pathogen related genes which are rhythmically expressed and that are also induced following infection with pathogens including *Bgh* (Molina *et al.*, 1997; Roden and Ingle, 2009). Due to this, infection time was controlled to remove outside variation. Spore density was measured with a haemocytometer with a target density between 30 and 60 spores per mm^2. Inoculated leaves were kept at 22°C in a 16 h/8 h light/ dark cycle for 48 hours before sampling for microscopy and RNA and protein analysis.

Leaves sampled for microscopy were cut into upper and lower sections and stained for 30 minutes in lactophenol cotton blue at 90°C, destained in 3:1 ethanol: acetic acid and mounted in 20% glycerol. Leaves were viewed at 250x magnification. Number of non-

germinated conidia, conidia with appressoria and conidia with secondary hyphae were scored on at least five passes across the upper and lower leaf sections, recording at between 300 and 500 conidia per leaf.

Microscopy was used to evaluate the impact of gene silencing on *Bgh* pathogenicity. This was also used as an initial screen to evaluate the efficiency of this new silencing method. The mode of action of ODN/PTO silencing can be through two different mechanisms. Either through RNase H degradation of the target mRNA, or through steric hindrance, by blocking translation of the targeted effector transcript. If silencing is mediated though RNase H degradation of the mRNA of the targeted gene, then this would lead to a reduction in target mRNA which should be detectable using qRT-PCR. However, if ODNs/PTOs work by blocking translation of the target mRNA into protein, then the target mRNA would not be reduced and the effect would be seen in a reduction in cognate protein amount. Whichever mode of action, successful silencing should result in a reduction in the proportion of conidia with secondary hyphae out of germinated conidia. Regardless of the mode of action, silencing of effectors or other virulence factors would result in a reduction in the proportion of secondary hyphae. This phenotype can be easily scored using microscopy.

The significance of difference in proportion of secondary hyphae between silenced and control leaves was estimated using a General Linear Mixed Model (GLMM). This was calculated in R Studio using the glmer function from the lme4 package. The ratio of conidia with secondary hyphae out of germinated conidia was used as the response variable, treatment and *Bgh* spore density as fixed factors and date of experiment as a random factor.

3.3.2 RNA extraction and qRT-PCR

RNA was extracted from PTO treated leaves using RNeasy Plant mini kit (QIAGEN) as described in chapter 2.4. Reverse transcription was performed on extracted RNA using Quantitect Reverse Transcription kit (QIAGEN) to synthesise cDNA.

qRT-PCR was used to assess transcript abundance of the targeted genes normalised to expression of housekeeping genes, as well as to make a proxy estimation of fungal biomass following silencing treatment. qRT-PCR was performed on a Rotorgene Q real time PCR cycler (QIAGEN) as described in chapter 2.6. Transcript abundance was calculated using the Pfaffl method relative to control PTO Z treatment, using *BghGAPDH* as a reference gene (Pfaffl, 2001; Pennington *et al.*, 2016b).

As *HvGAPDH* and *BghGAPDH* have been shown to be stably expressed during the susceptible barley-*Bgh* interaction (Pennington *et al.*, 2016b), the ratio of these genes, in leaf samples which contain a mixture of barley and *Blumeria* RNA, can therefore be used as a proxy for the fungal biomass in a given leaf sample.

3.3.3 Protein extraction and western blotting

Proteins were extracted from infected leaf material as described in chapter 2.9 using precipitation in TCA/acetone and resuspension of proteins in a denaturing buffer containing 5 M urea, 2 M thiourea, 20 mM Tris and 20 mM DTT. SDS-PAGE was performed using 12% tricine acrylamide gels and proteins blotted onto PVDF membrane. The membrane was blocked in 5% skimmed milk powder in TBST before incubation with primary antibodies (1:2000) specific to BEC1011 or BEC1019 at 4°C overnight. The membrane was washed with TBST before incubation of the secondary antibody at room temperature for two hours. SuperSignal West Pico stable peroxidase:luminol enhancer (thermoScientific) was used to visualise bands on the autoradiography film. The film was exposed for 5 min – 1 h and developed using an automatic developer.

3.3.4 Tryptic digestion and Multiple Reaction Monitoring Mass Spectrometry

Extracted proteins were subjected to digestion with trypsin as described in chapter 2.10 to obtain peptides for multiple reaction monitoring mass spectrometry (MRM-MS). MRM-MS was performed by Mark Bennett at Imperial College London, as described in chapter 2.14.

The process of assay development for protein quantification by MRM-MS is shown in figure 3.2.

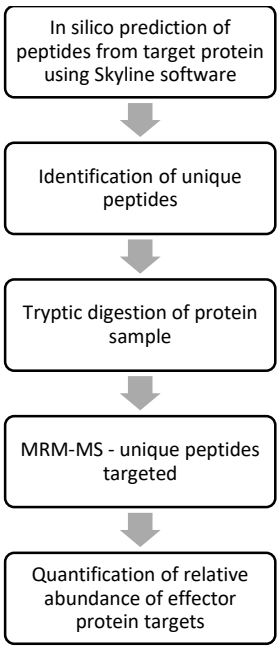


Figure 3.2 Workflow for development of a multiple reaction monitoring mass spectrometry assay for quantification of effector proteins following PTO silencing

The process of identification and quantification of target peptides in MRM-MS is summarised in figure 3.3.

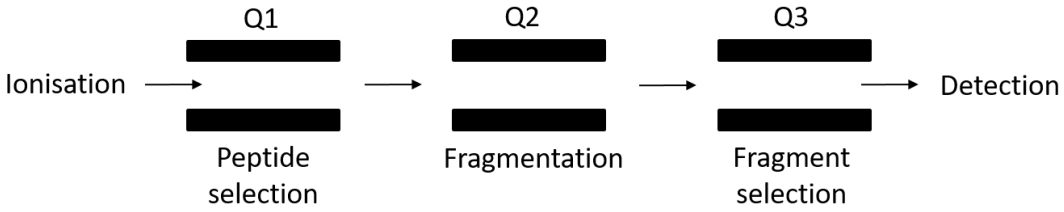


Figure 3.3 The multiple reaction monitoring mass spectrometry process using a triple quadrupole mass spectrometer

The digested protein sample is injected into the LC column, ionised by electrospray ionisation and the ions enter the mass spectrometer. The targeted peptides are selected in the first quadrupole (Q1) and are then fragmented in the second quadrupole (Q2). The fragment ions are selected in the third quadrupole (Q3) and the signal abundance produced is used to quantify the relative abundance of the target protein in the sample.

3.4 Results

3.4.1 Design of oligonucleotides

In order to validate the silencing workflow described in 3.3.1, 19mer ODNs were designed using OligoWalk software, as described above, to target two different types of *Blumeria* effectors first confirmed as virulence factors by HIGS (Pliego *et al.*, 2013). *BEC1011* and

BEC1054 are *Blumeria* specific CSEPs belonging to CSEP family 21 with an RNase-like fold, and *BEC1019* a metalloprotease-like effector which is common to 40% of sequenced fungi (Pedersen *et al.*, 2012; Whigham *et al.*, 2015). *BEC1019* has been further validated using virus induced gene silencing (VIGS) (Whigham *et al.*, 2015).

The antisense ODNs designed to target *BEC1011, BEC1019* and *BEC1054* were mapped onto the predicted mRNA structure, predicted using mFold software (http://unafold.rna.albany.edu/?q=mfold, (Zuker, 2003) (Fig. 3.4, 3.5 and 3.6).

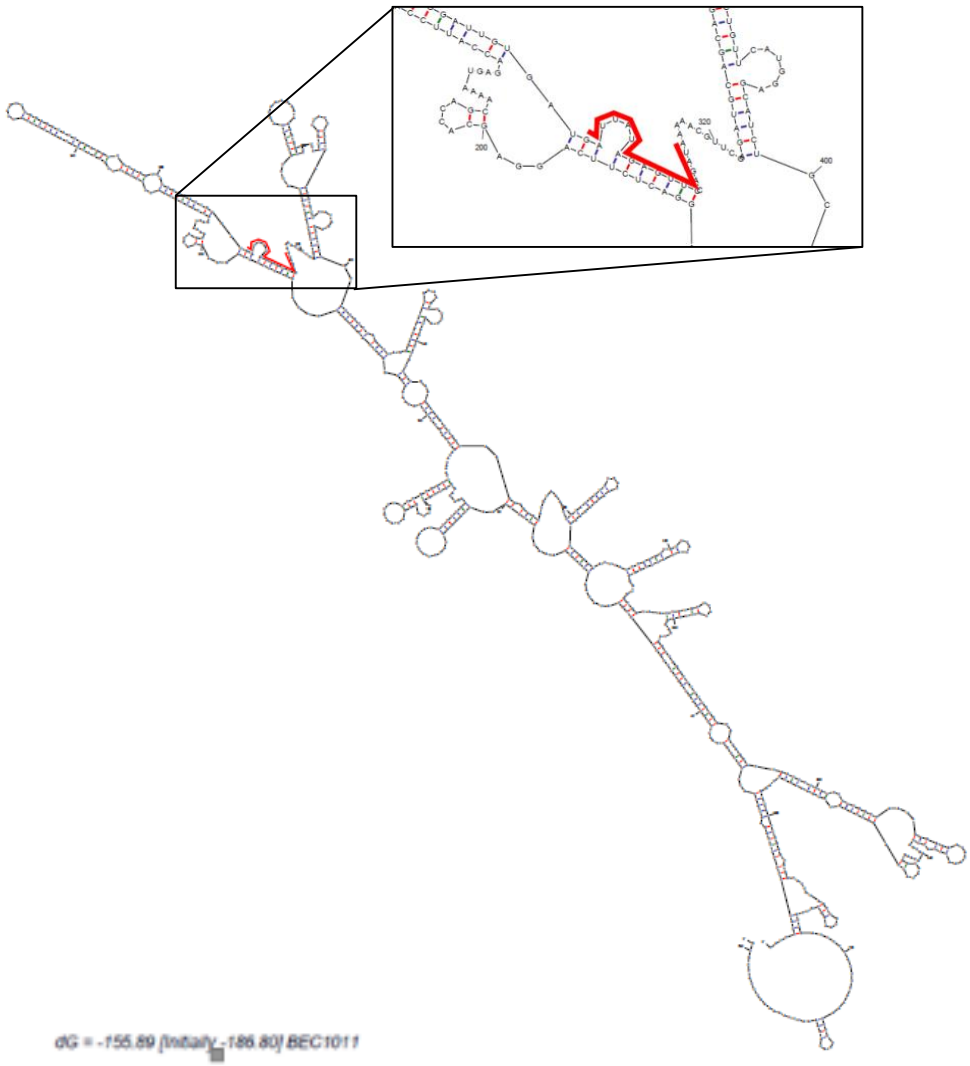


Figure 3.4 Modelled structure of the *BEC1011* effector mRNA, predicted using mFold software. The position of the ODN 11.11 oligonucleotide is indicated in red.

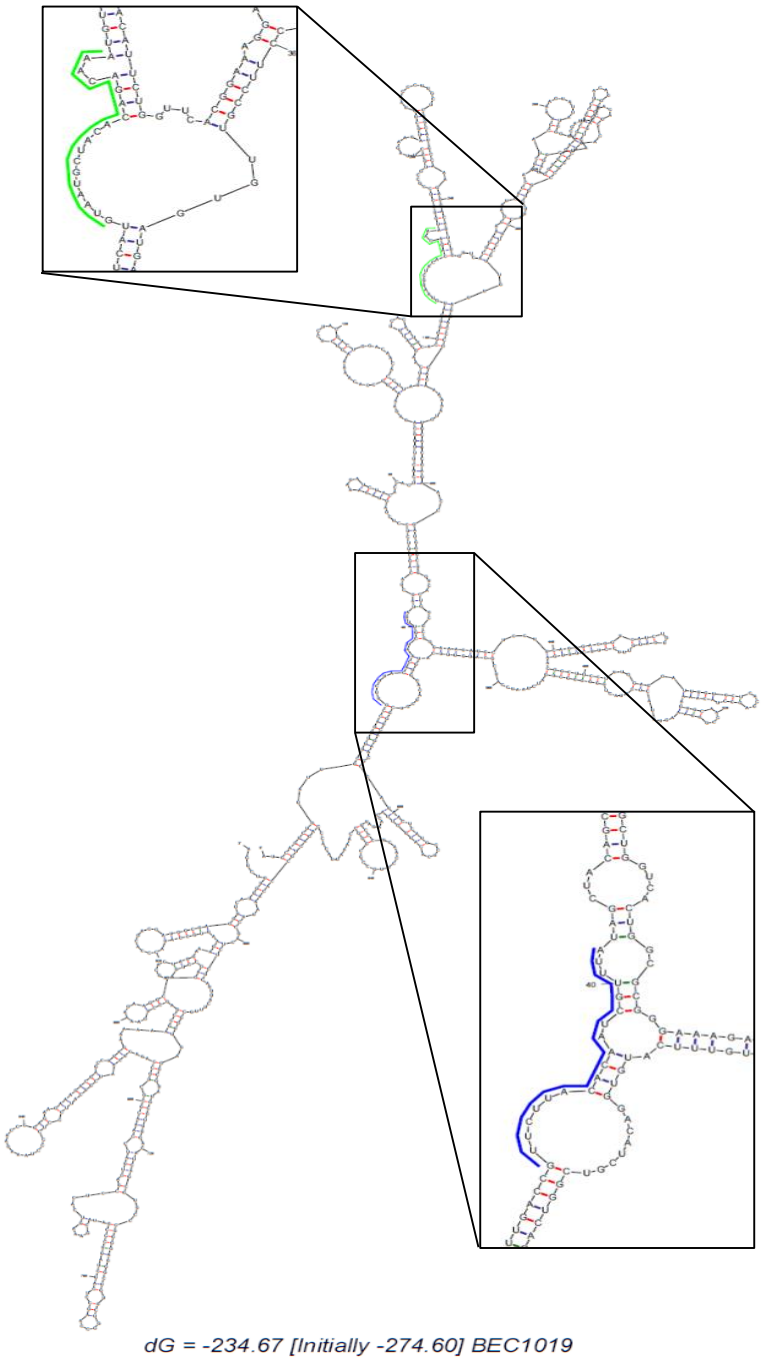
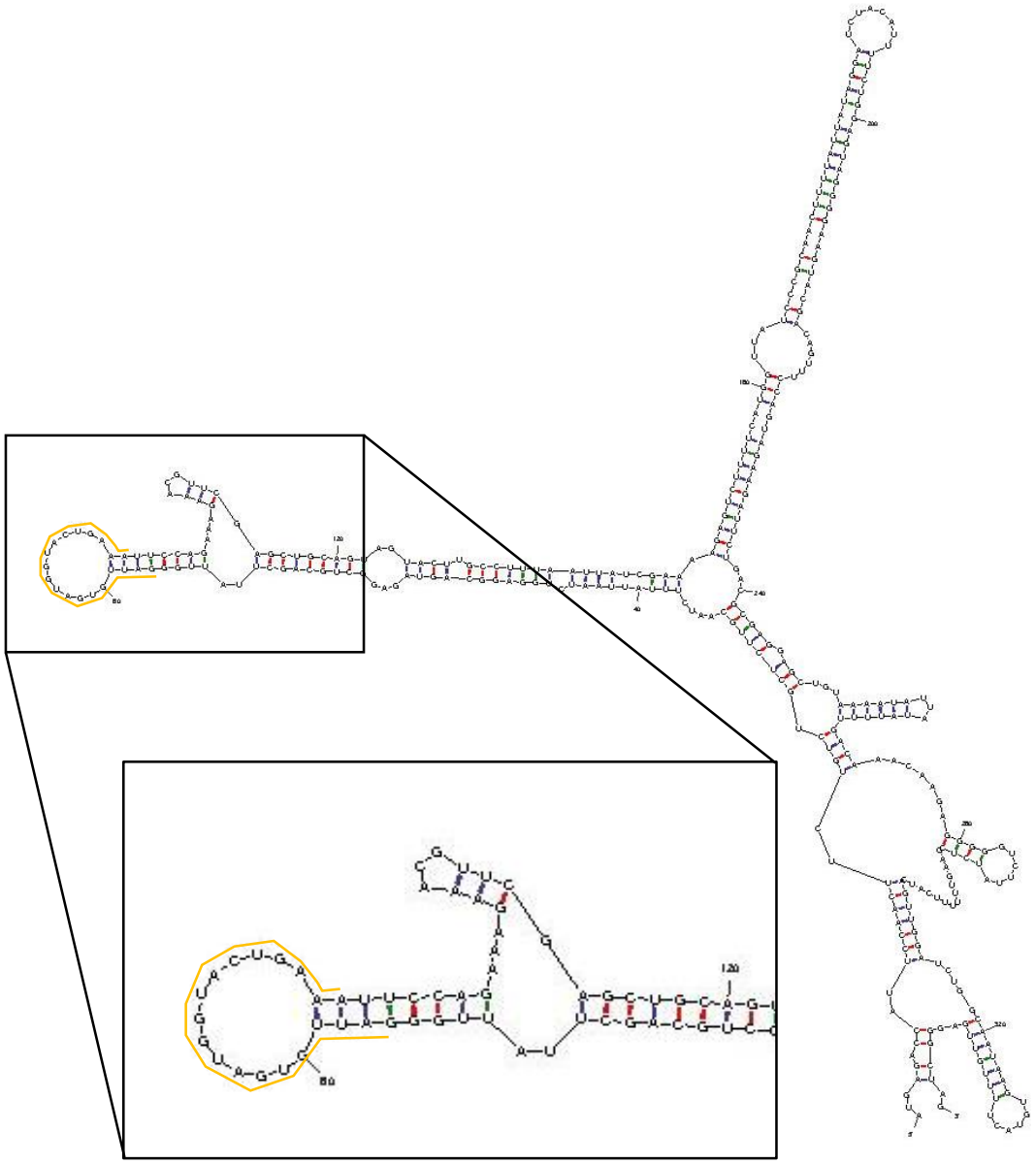


Figure 3.5 Modelled structure of the *BEC1019* effector mRNA, predicted using mFold software. The position of ODN 19.11 is shown in blue and ODN 19.12 is shown in green.



dG = -77.25 [Initially -89.30] BEC1054

Figure 3.6 Modelled structure of the *BEC1054* effector mRNA, predicted using mFold software. The position of PTO 54.11 is shown in yellow

The position of the ODNs designed using OligoWalk software are predicted to be over open loops in the mRNA structure. Although ODNs can be designed using just the secondary structure prediction from software such as mFold, the success of an ODN has shown to be higher when there is stable binding of the ODN-RNA duplex and minimal self-structure of the ODN (Matveeva *et al.*, 2003; Shabalina *et al.*, 2006). OligoWalk uses these variables to predict the optimal ODN sequence.

Unlike previous attempts using ODNs designed with mFold, the non-modified ODNs designed with OligoWalk software to target *BEC1011, BEC1019* and *BEC1054* were shown

to significantly reduce the growth of secondary hyphae in preliminary experiments, suggesting that the target effectors had been successfully silenced (Moritz Bomer, University of Greenwich, pers comm.).

3.4.2 Comparison of non-modified and phosphorothioate modified oligonucleotides

To investigate whether the silencing effect could be improved by using ODNs with a phosphorothioate modified backbone, a non-modified ODN targeting BEC1011 – ODN 11.11 was compared with a PTO modified version of the same ODN (PTO 11.11), described in table 1. Excised primary leaves from 7-day old barley were treated with 10 µM of non-modified ODN or PTO modified ODN targeting *BEC1011* or respective non-modified or PTO modified control ODN/PTO Z. Following the ODN/PTO treatments, leaves were infected with *Bgh* as described above. Between two and eight leaves were used per treatment in each experiment. The treatment workflow was the same for non-modified and PTO modified ODNs but it was noted that leaves treated with PTO modified ODNs had lower uptake of ODN solution than leaves treated with non-modified ODNs. This was noted by measuring the volume of liquid remaining in tubes following 24 h treatment. When non-modified ODNs were used, the volume of solution remaining was generally 100 – 200 µL, whereas when using PTO modified ODNs, the volume remaining was around 400 – 500 µL.

Infection was scored microscopically by recording the number of non-germinated conidia, conidia with an appressorium and conidia with secondary hyphae – indicating formation of a successful haustoria. There were no marked differences in proportion of non-germinated conidia or proportion of conidia with an appressoria out of total conidia between different treatments. This may indicate that the ODNs/PTOs are not able to reach the fungal structure before formation of the haustorium or it may be because the effectors targeted are important only for haustorial formation. Results shown are the percentage of conidia which had formed secondary hyphae out of germinated conidia (%SH) (Fig. 3.7).

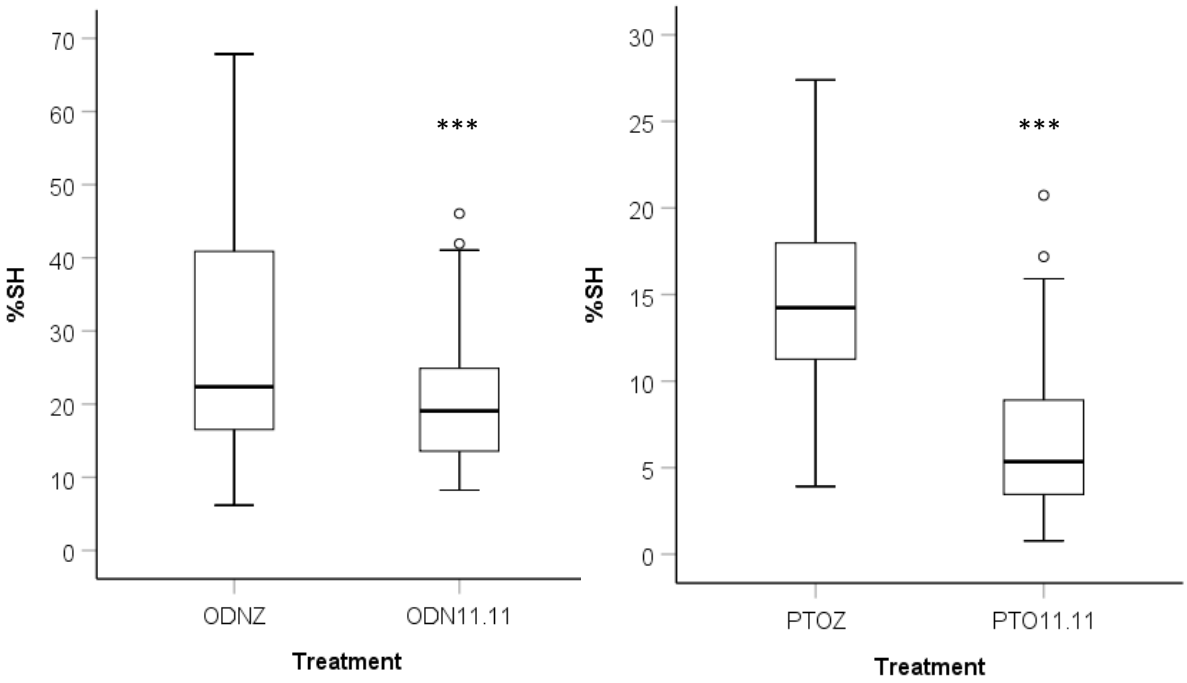


Figure 3.7 Effects of non-modified and PTO modified oligodeoxynucleotides of the same sequence targeting the BEC1011 effector on percentage of conidia forming secondary hyphae, out of germinated conidia, measured microscopically. (N=20 leaf tip segments from seven independent experiments for ODN 11.11, N=65 leaf tip segments from 18 independent experiments for PTO 11.11. A minimum of 300 conidia were scored per leaf segment). Results are presented as Boxplot showing median, interquartile range (IQR) and minimum/maximum values, circles represent outliers of more than 1.5x IQR above the box. Significance was estimated with GLMM and * indicates P<0.001**

Occurrence of secondary hyphae in leaves treated with the non-modified ODN 11.11 was reduced by an average of 24% compared to leaves treated with control ODN Z, whereas the reduction of secondary hyphae between leaves treated with PTO-modified ODN 11.11 and the PTO modified control, Z averaged 54%. These results suggest that PTOs are potentially more efficient than non-modified ODNs. Therefore, the use of PTOs was favoured over non-modified ODNs and used in further experiments.

3.4.3 Effect of length on ODN efficiency

In RNAi silencing systems, the silencing molecules are usually 21-25 nucleotides in length, whereas most ODN studies have used 19mer ODNs. Therefore, an experiment was carried out to assess the effect of ODN length on silencing efficiency. For this, 25mer versions of the ODN targeting BEC1011 was designed by extending the 19mer sequence by 3 nucleotides at both the 5' and 3' ends, complementary to the respective gene sequence. A 25mer ODN targeting the barley susceptibility gene *BLUFENSIN1* (*Bln1*; see chapter 4) was

also designed. The effect of these ODNs were compared to a 25mer control ODN M13 which was based on the M13 bacteriophage forward (-47) sequencing primer (Fig. 3.8).

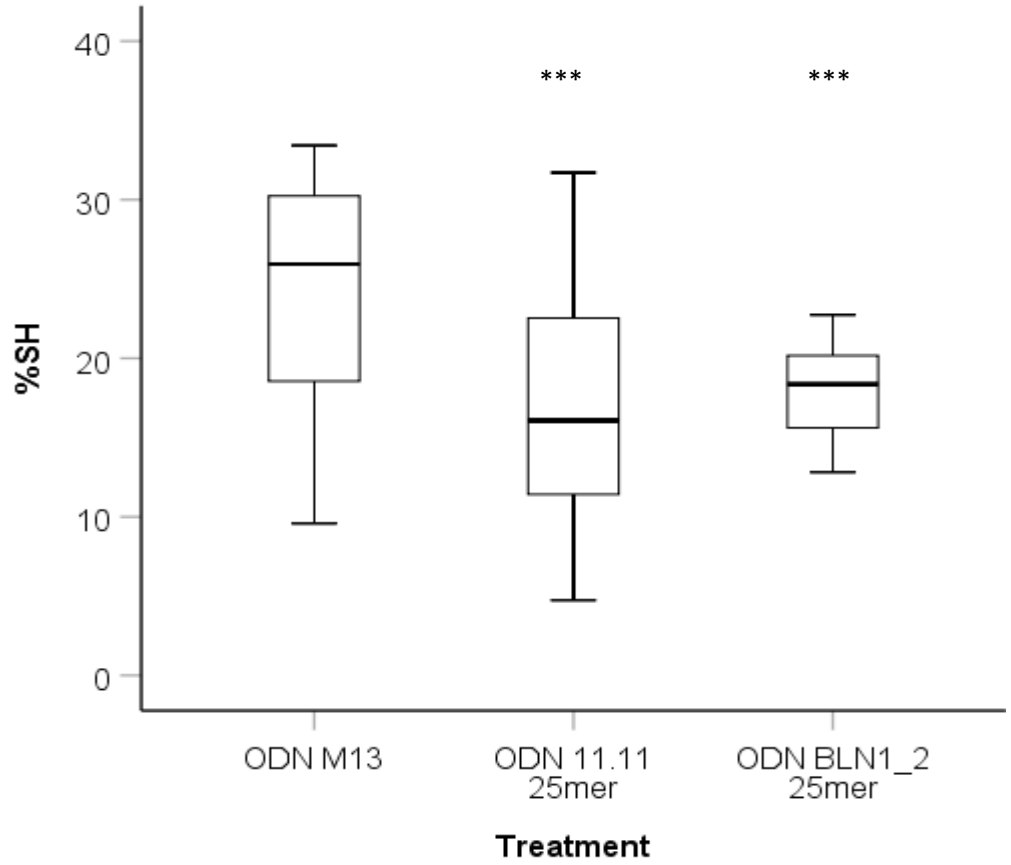


Figure 3.8 Percentage of conidia producing secondary hyphae (%SH) following treatment with 25mer ODNs targeting BEC1011 (ODN 11.11 25mer) and BLN1 (ODN BLN1_2 25mer) compared to the 25mer control ODN M13. N=14 leaf tip segments from three independent experiments. A minimum of 300 conidia were scored per leaf section. Results are presented as Boxplot showing median, IQR and minimum/maximum values, circles represent outliers of more than 1.5x IQR below the box. Significance was estimated with GLMM and * indicates P<0.001**

The proportion of conidia with secondary hyphae in leaves treated with 25mer ODNs targeting either BEC1011 or BLN1 was significantly reduced compared to leaves treated with control 25mer ODN M13 with 33% and 26% reductions in %SH respectively. Table 3.1 shows a comparison of the reduction in production of secondary hyphae between leaves treated with the 19mer or 25mer versions of ODNs targeting *BEC1011* or *Bln1.* Although there is a significant difference between the 19mer and 25mer versions of non-modified ODN11.11 and ODNBLN1_2 (P=0.027 and 0.002, respectively), using a PTO modified version of the 19mer ODN is more efficient in reducing the %SH, following gene silencing of either *BEC1011* or *Bln1*, than increasing the length of the ODN. Given the more potent effects on the infection phenotype when using 19mer PTOs, those were favoured over longer ODNs.

However, due to the substantial increase in price of PTOs compared to ODNs, 25mer PTOs were not tested.

Table 3.1 Comparison of reduction in percentage of conidia with secondary hyphae (%SH) relative to control ODN treatment in leaves treated with 19mer or 25mer ODNs targeting *BEC1011* or *Bln1*. 19mer ODN targeting *Bln1* is detailed in chapter 4.

Gene target	Relative reduction in %SH using 19mer ODN	Relative reduction in %SH using 25mer ODN
BEC1011	24%	33%
Bln1	22%	26%

3.4.4 Validation of PTO controls

To evaluate the validity of the controls being used, in particular to assess the direct effect of PTOs on the virulence of *Bgh,* the chosen controls, PTO Z and PTO M13 described in table 1, were compared to a mock treatment with water.

PTO Z targets barley seed protein Z (GenBank X97636), a serine protease inhibitor protein which is only expressed in seeds (Østergaard *et al.*, 2004; Druka *et al.*, 2006) and therefore should have no effect in leaves. PTO M13 (a PTO modified, 19mer version of the ODN used for ODN length testing) targets the M13 bacteriophage and has no target in barley or *Bgh*. Comparison of the PTOs with a mock control should indicate any effect of the PTO chemistry as both PTOs were screened for off targets and should therefore not have any sequence-specific effects.

Seven-day old barley primary leaves were treated as for standard silencing experiments, with 10 µM PTO Z, PTO M13 or mock control (water) for 24 hours before inoculation with *Bgh* conidia. Proportion of conidia with secondary hyphae was scored after 48h (Fig. 3.9).

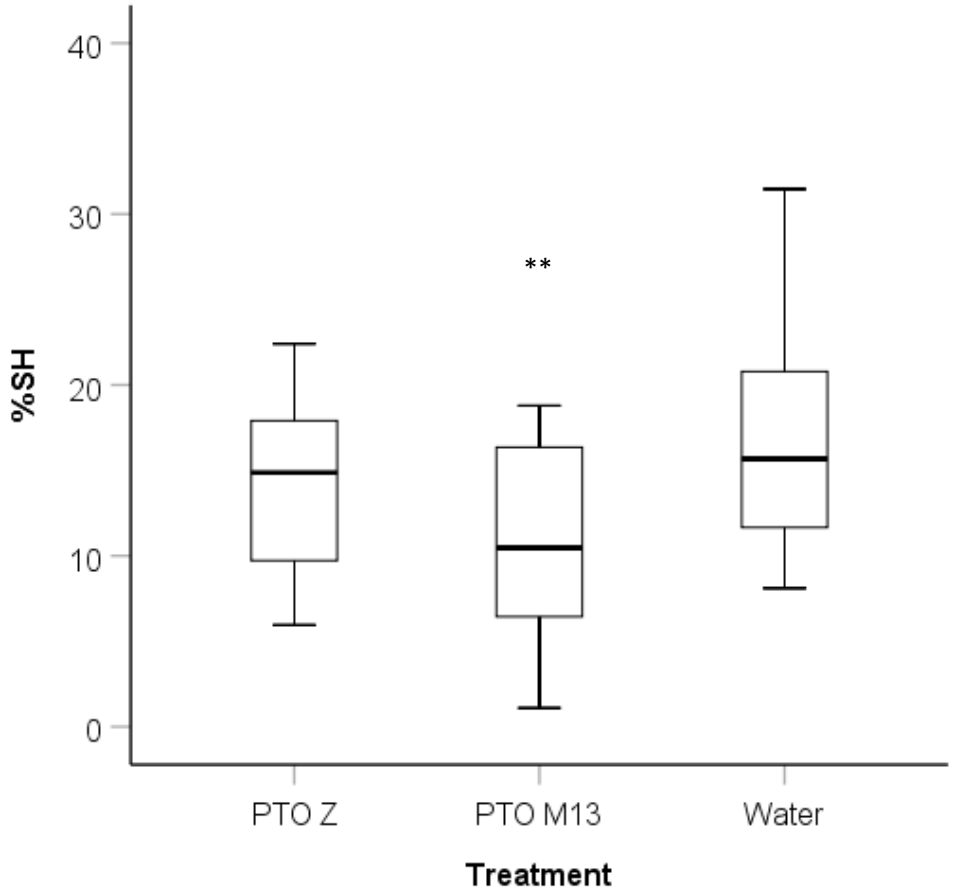


Figure 3.9 Percentage of conidia producing secondary hyphae (%SH) in leaves treated with control PTOs Z and M13 or with water. For PTO Z and PTO M13, N=22 leaves from six independent experiments. For Water, N = 18 leaves from six independent experiments. Results are presented as Boxplot showing median, interquartile range and minimum/maximum values. Significance was estimated with GLMM and ** indicates P<0.01

The percentage of conidia producing secondary hyphae (%SH) in leaves treated with PTO Z control was not significantly different to the %SH in the mock treatment, i.e. leaves treated with water, suggesting that PTO Z is an appropriate control. In leaves treated with PTO M13 the %SH was significantly different to %SH of leaves treated with either PTO Z or water, indicating that this was a less suitable control and therefore was not used in further experiments. Despite these differences, leaves treated with PTO 11.11, PTO 19.11, PTO 19.12 or PTO BLN1_2 (targeting *BEC1011, BEC1019* or *Bln1*) in the same experiments as PTO M13 still showed a significant reduction relative to PTO M13 control (P<0.001, N=10, data not shown).

3.4.5 Assessing specificity of PTO modified ODNs

In order to assess the specificity of the PTOs that were designed, one or two mismatched bases were introduced to the PTO 11.11 sequence to produce PTO 11.11 MM1, with one mismatched base, and PTO 11.11 MM2, with two mismatched bases, as described in table 1. A further PTO was designed by shuffling the PTO 11.11 sequence (PTO 11.11 shuffle). Leaves were treated with 10 µM of these new PTOs as well as PTO Z and the original PTO 11.11. The effect of the different PTO treatments were compared by monitoring microscopically the proportion of conidia with secondary hyphae (Fig. 3.10).

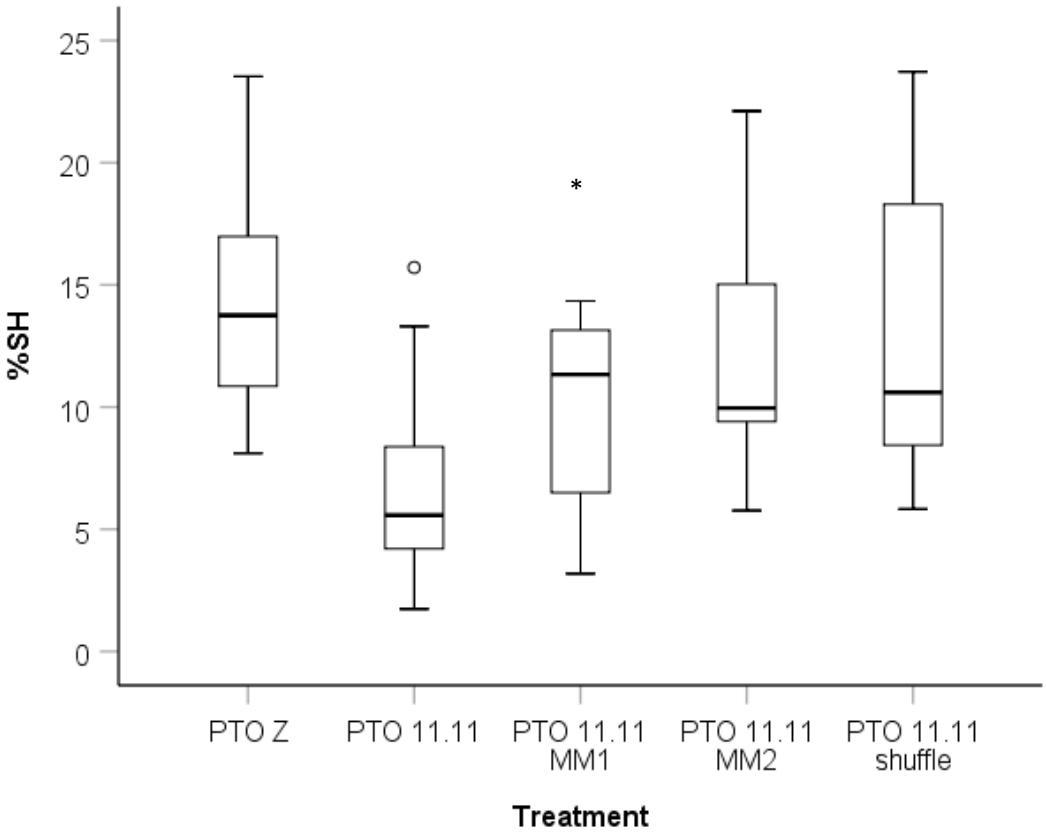


Figure 3.10 Assessing the specificity of PTOs. (N=19 leaf tip segments from three independent experiments. A minimum of 300 conidia were scored er leaf segment). Results are presented as Boxplot showing median, IQR and minimum/maximum values, circles represent outliers of more than 1.5x IQR above the box. Significance was estimated with GLMM and * indicates P<0.05, * indicates P<0.001**

The reduction in percentage of conidia with secondary hyphae in PTO treated leaves relative to PTO Z control is shown in table 3.2.

Table 3.2 Relative reduction in percentage of conidia with secondary hyphae between leaves treated with PTO Z control and versions of the PTO 11.11 ODN with one or two mismatches or a shuffled sequence

PTO	Relative reduction in percentage of conidia with secondary hyphae	Significance
PTO 11.11	53%	P<0.001
PTO 11.11 MM1	32%	P<0.05
PTO 11.11 MM2	14%	ns
PTO 11.11 shuffle	10%	ns

PTO 11.11 with one mismatch (PTO 11.11 MM1) showed a reduced effect compared to PTO 11.11. PTO 11.11 with two mismatches (PTO 11.11 MM2) or a shuffled sequence showed no significant difference to PTO Z control. This indicates that the sequences designed to target the desired gene are highly specific and that PTOZ is a suitable control.

3.4.6 Assessing the robustness of the PTO silencing assay

In order to assess the robustness of the PTO silencing assay, the variation in the data was examined. One factor that is expected to have an effect on secondary hyphae formation is the density of *Bgh* inoculum. As first line of defence, different plant cultivars have various degrees of basal resistance to pathogens, which pathogens have evolved to overcome (Jones and Dangl, 2006), including barley to its powdery mildew (Aghnoum *et al.*, 2010). But even where pathogens have no effective counter to basal resistance, high disease pressure can overcome basal resistance allowing pathogen colonisation (Newton *et al.*, 2002; Ahmad *et al.*, 2010). To examine the effect of spore density on formation of secondary hyphae, the percentage of conidia with secondary hyphae was plotted against inoculation density for PTO Z (control) and PTO 11.11 treated leaves (Fig. 3.11).

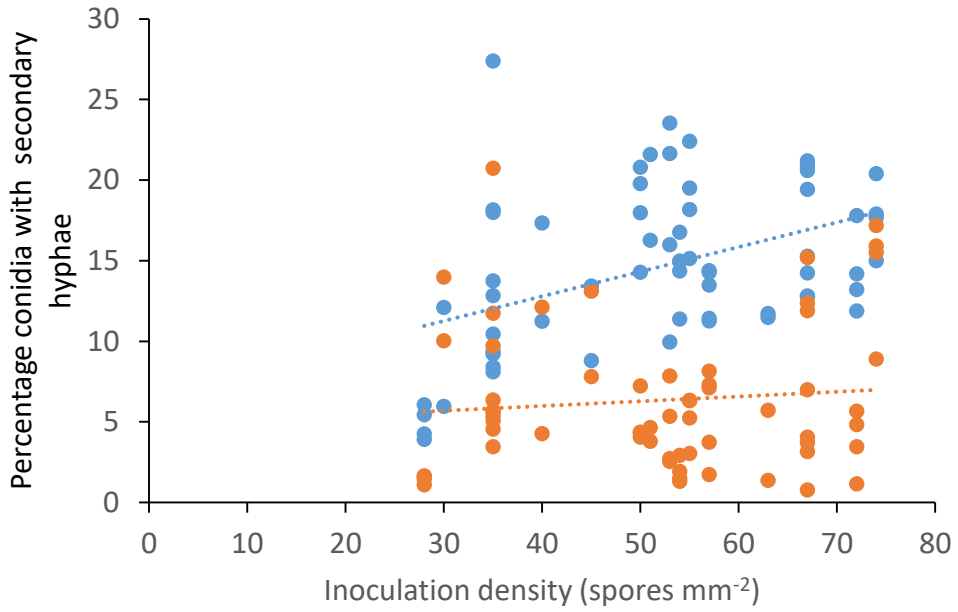


Figure 3.11 Percentage of conidia with secondary hyphae plotted against density of Bgh spore inoculum. Each point represents an individual leaf section. Blue points are leaves treated with control PTO Z. Orange points are leaves treated with PTO 11.11 targeting the BEC1011 effector. *R^2 value for PTO Z = 0.189, R^2 value for PTO 11.11 = 0.008.*

A GLMM statistical analysis was performed to assess whether spore density affects the proportion of conidia with secondary hyphae. This showed a significant effect of density for leaves treated with PTO Z (P=0.0016) but not for leaves treated with PTO 11.11 (P=0.303). The trends here show that for the negative control treatment (PTO Z) there is higher percentage of conidia with secondary hyphae when inoculation density is higher, suggesting that at higher disease pressure, plants are likely to be more susceptible than at low disease pressure, as previously described (Newton *et al.*, 2002; Aghnoum *et al.*, 2010; Ahmad *et al.*, 2010). However, that trend is absent for leaves treated with PTO 11.11 This strongly suggests that the silencing of the *BEC1011* effector overcomes the effect of increasing disease pressure, but it does not lead to the complete abolishment of the pathogen virulence.

As this analysis showed that density of the inoculum has a direct effect on the response variable (i.e. the percentage of conidia forming secondary hyphae) in some cases, the parameter “spore density” was included in the GLMM, when density data is available, and the interaction between treatment and density was also analysed. Density only had a significant effect on conidia with secondary hyphae when comparing leaves treated with PTO Z and PTO 11 and in leaves treated with PTO Z and PTOs targeting AVRa1 and AVRa13 (described in Chapter 5). Density did not have a significant effect in any other treatments tested. Where there was a significant effect of density, there was no significant interaction

between individual treatments and spore density. This is supported by the examination of the effect of spore density on reduction number of conidia with secondary hyphae in leaves treated with PTO 11.11 compared with leaves treated with PTO Z control. The average reduction in conidia with secondary hyphae as a result of treatment with PTO 11.11 (to silence *BEC1011*) relative to the PTO Z control, was compared in 17 independent experiments to investigate whether density of the inoculum influenced the decreased infection phenotype. The %SH reduction was plotted against density of inoculum to examine whether density affects the efficacy of the treatment (Fig 3.12).

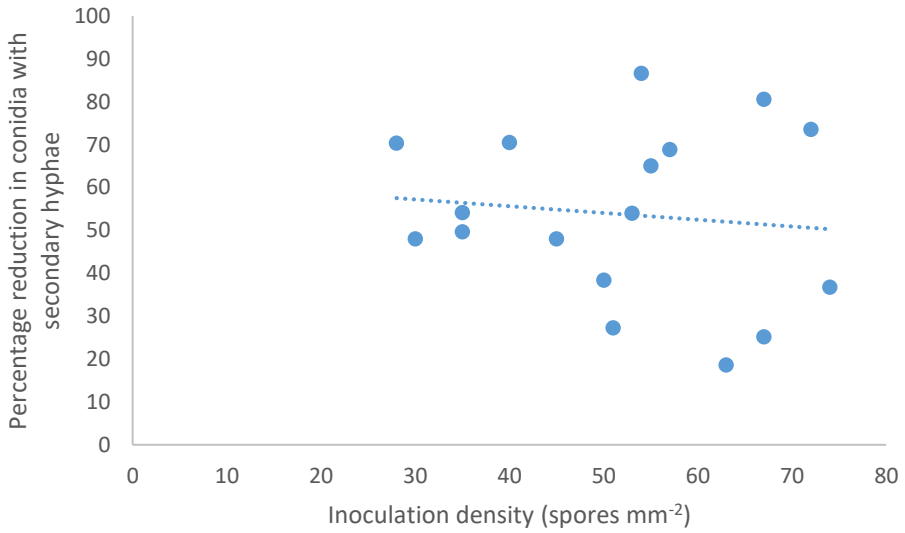


Figure 3.12. Average percentage reduction of conidia with secondary hyphae between leaves treated with PTO 11.11 compared to leaves treated with PTO Z control plotted against density of Bgh spore inoculum. Each point represents one independent experiment with between two and six leaf segments.

This suggests that the efficacy of the treatment is not influenced by the density of the inoculum and that the effect of PTO silencing the *BEC1011* effector is not affected by density of the inoculum. It may also indicate that *BEC1011* has a role in suppression of basal immunity because silencing leads to reduction in infection even when disease pressure is low. Overall, this indicates that although there can sometimes be an effect of inoculation density, the effect of PTO treatment is independent of density of the inoculum.

3.4.7 Targeting known effectors with PTO modified ODNs

Following validation of the method, PTO modified ODNs were used to target *BEC1011*, *BEC1019* and *BEC1054*. *BEC1011* and *BEC1054* were each targeted with one PTO (PTO 11.11 and PTO 54.11, respectively) and two different PTOs were tested for *BEC1019* (PTO 19.11 and PTO 19.12). Leaves treated with PTOs targeting each of these three effectors

showed a reduction in percentage of conidia with secondary hyphae relative to PTO Z treatment (Fig. 3.13)

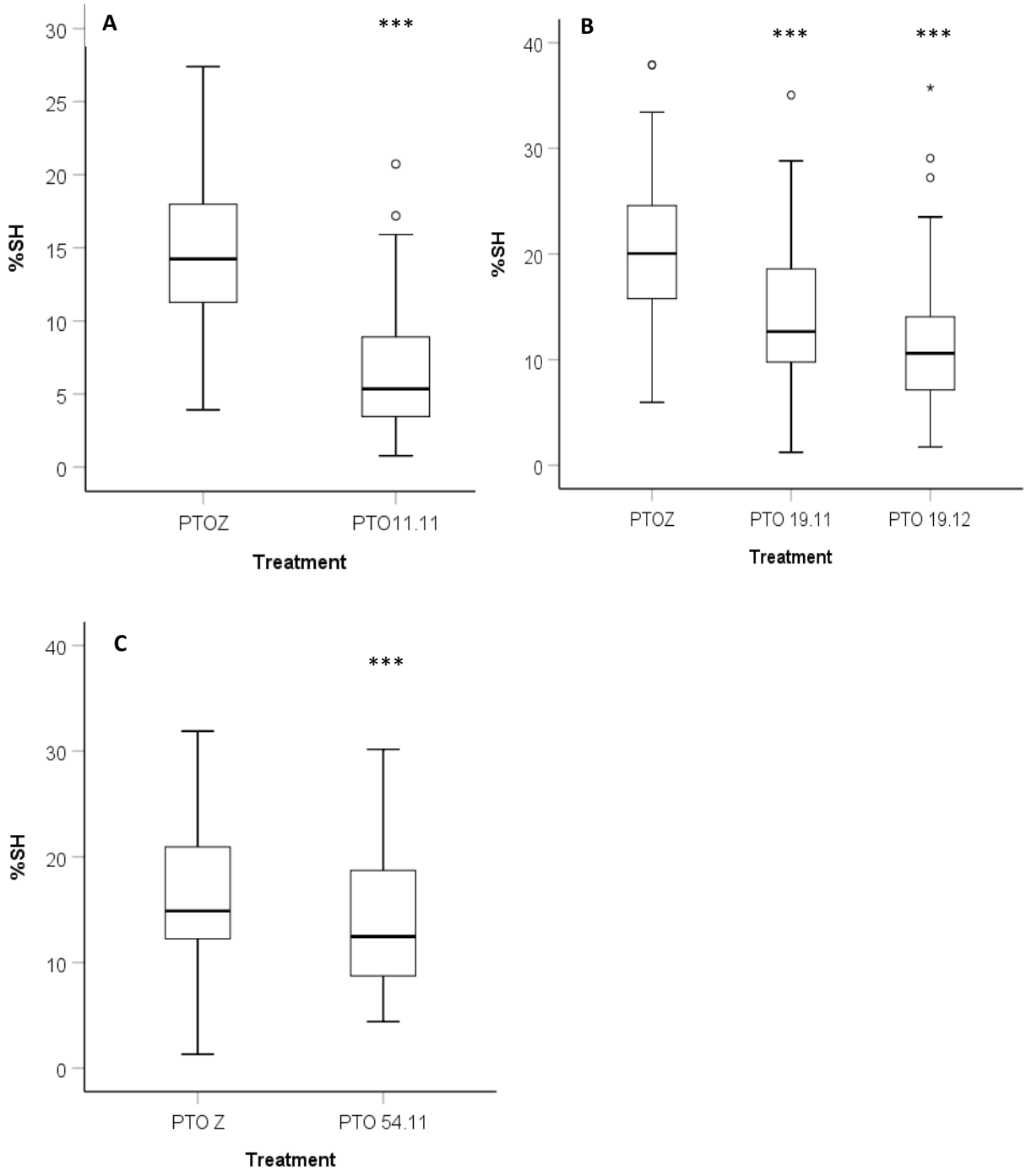


Figure 3.13 Effects of PTO treatment on *Bgh* infection, as measured microscopically by measuring the proportion of conidia either secondary hyphae (%SH) for plants treated with the (A) PTO 11.11 to target *BEC1011*, N=65 leaf tip segments, (B) PTO 19.11 and PTO 19.12 to target *BEC1019,* N=36 leaf tip segments for PTO 19.11 and 40 leaf tip segments for PTO 19.12, or (C) PTO 54.11 to target *BEC1054*, N=35 leaf tip segments. A minimum of 300 conidia were scored per leaf segment Results are presented as Boxplot showing median, interquartile range and minimum/maximum values, circles represent outliers of more than 1.5x IQR above the box. Significance was estimated with GLMM and * indicates P<0.001**

Targeting *BEC1011* with PTO 11.11 resulted in a 53% reduction of conidia with secondary hyphae relative to PTO Z treated leaves. Using PTO 19.11 to target *BEC1019* resulted in 32% reduction of conidia with secondary hyphae, whereas PTO 19.12, also targeting *BEC1019,* resulted in a slightly stronger reduction of 41% on average, relative to PTO Z treated leaves. Targeting *BEC1054* with PTO 54.11 resulted in 21% reduction of conidia with secondary hyphae.

3.4.8 Assessing fungal biomass using qRT-PCR

Targeting *BEC1011* and *BEC1019* led to decreased infection, quantified microscopically by the reduction in formation of secondary hyphae. This phenotype is also expected to be detectable as a decrease in fungal biomass in infected barley leaves following PTO treatment targeting *BEC1011* or *BEC1019.*

qRT-PCR was used to estimate fungal biomass in leaves treated with the two PTOs which showed the greatest reduction in secondary hyphae – PTO 11.11 and PTO 19.12 (Fig. 3.14).

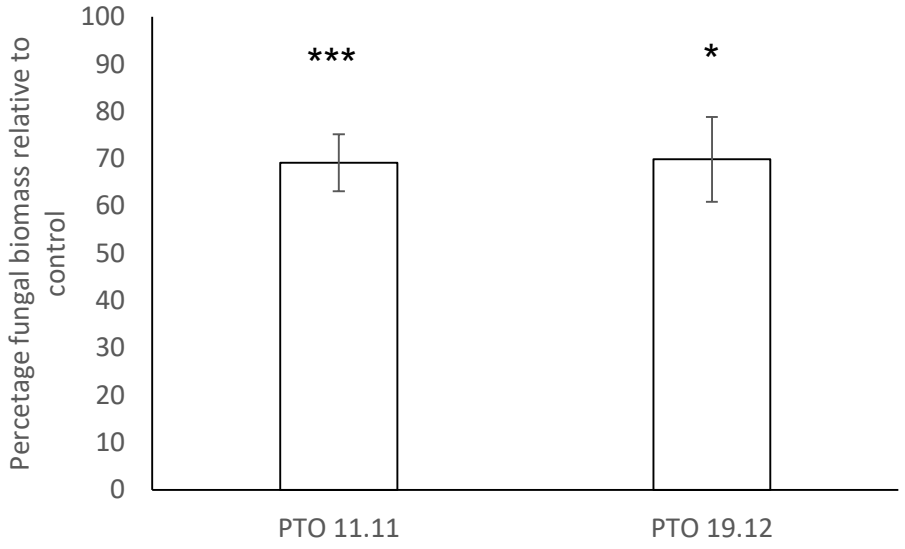


Figure 3.14 Relative percentage of fungal biomass in leaves treated with PTO 11.11 (N=11 biological replicates of four pooled leaves, each including three technical replicates) or PTO 19.12 (N=4 biological replicates, each including three technical replicates) compared to leaves treated with PTO Z, calculated as the ratio between BghGAPDH and HvGAPDH housekeeping genes. Error bars show standard error of the mean. Significance was calculated using a T-Test, * indicates P<0.05, * indicates P<0.001.**

The ratio of *BghGAPDH* and *HvGAPDH* housekeeping gene transcript abundance was used as a proxy measurement for fungal biomass. These housekeeping genes were selected as they have been shown to be the most stable reference genes (Pennington *et al.*, 2016b).

The sequence of all primers used are shown in table 2.2 The ratio of *BghGAPDH* to *HvGAPDH* was reduced by an average of 31% in leaves treated with PTO 11.11 relative to leaves treated with PTO Z and in leaves treated with PTO 19.12 the reduction was 30%. For leaves treated with PTO 11.11 there was a significant correlation between the relative percentage of secondary hyphae measured microscopically and the relative percentage fungal biomass measured by qRT-PCR, as shown in figure 3.15.

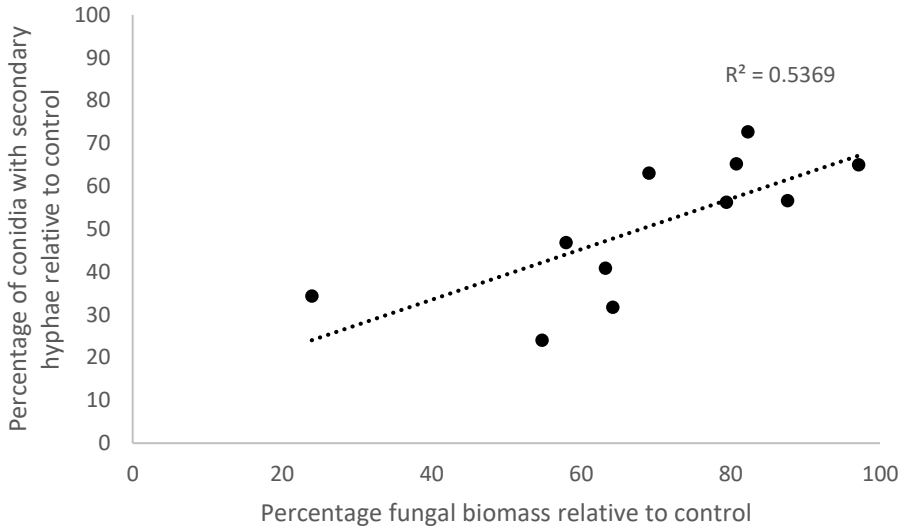


Figure 3.15 Correlation between percentage conidia producing secondary hyphae and fungal biomass in PTO 11.11 treated leaves relative to PTO Z treated leaves. R=0.7328, N=11, P=0.016

For PTO 19.12 treated leaves, there were only three data points and therefore it was not possible to accurately analyse possible correlation.

3.4.9 Assessing target transcript abundance using qRT-PCR

If PTO silencing is mediated through recruitment of RNase H to degrade mRNA then it should be possible to detect this reduction in abundance of the targeted mRNA. qRT-PCR was used to assess transcript abundance of targeted genes following treatment. *BEC1011* transcript abundance was measured relative to *BghGAPDH* in leaves treated with PTO Z and PTO 11.11 at 48 hpi (Fig. 3.16). Using qRT-PCR to assess reduction in *Bgh* transcript was challenging because there is an inherently low abundance of fungal RNA in leaf samples compared to barley RNA and the result of successful silencing is a reduction in fungal biomass. This means that the comparison of *Bgh* target genes to barley housekeeping genes will be skewed with the cumulative effect of any transcript reduction and the reduction in overall *Bgh* RNA within the plant samples. Therefore, relative reduction is measured against a *Bgh* housekeeping gene which is stably expressed during infection (Pennington *et al.*, 2016b) and the abundance of which should change by the same amount due to reducing *Bgh* biomass.

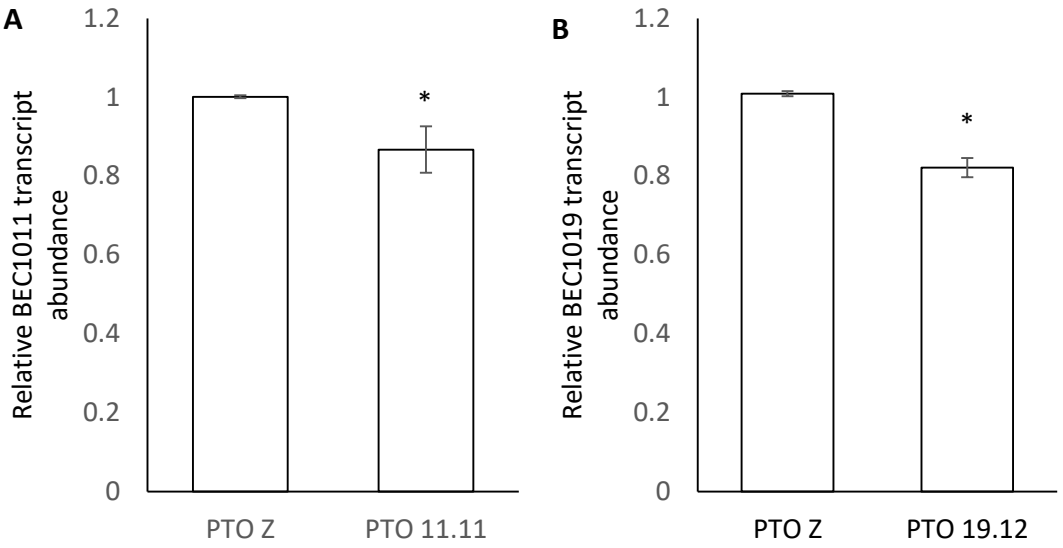


Figure 3.16 Relative (A) *BEC1011* or (B) *BEC1019* transcript abundance in leaves treated with (A) PTO Z or PTO 11.11, N=10 biological replicates, each including three technical replicates or (B) PTO Z or PTO 19.12, N=3 biological replicates, each including three technical replicates. Significance was calculated using one sample T-test. * represents P<0.05. Error bars show standard error in means between biological replicates.

Leaves treated with PTO 11.11 showed a 13% reduction in *BEC1011* transcript compared to leaves treated with PTO Z. Leaves treated with PTO 19.12 showed an average reduction of 19% compared to PTO Z treated leaves.

3.4.10 BEC1011 and BEC1019 proteins can be detected in infected leaves

Proteins were extracted in both native and denaturing conditions from barley leaves 4 and 9 dpi with *Bgh* spores or leaves which had not been infected. Western blot was performed on these protein extracts using BEC1011 and BEC1019 primary antibodies. After one hour exposure of the autoradiograph film, bands can be seen for both BEC1011 and BEC1019 (Fig. 3.17).

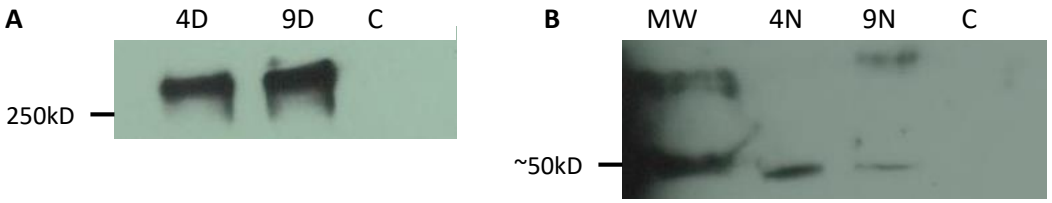


Figure 3.17 showing Western Blots with BEC1011 (A) and BEC1019 (B) antibodies. 4 and 9 indicate days post inoculation and N and D denote native or denaturing extraction buffers. C= control (non-infected), MW= molecular weight standard

BEC1011 was only seen in the protein extraction performed in denaturing conditions which may indicate it is not soluble in native conditions, whereas BEC1019 was seen in native extraction. BEC1011 bands are stronger than BEC1019 bands which could be due to a lower protein concentration in the samples or due to lower efficiency of antibodies. BEC1011

bands are larger than expected (~250 kD band seen, actual size 14 kD) which may be due to polymerisation of the protein or formation of complexes with other proteins. This production of a “big BEC” band in BEC1011 western blots have also been identified in other groups (Laurence Bindschedler, RHUL; Pietro Spanu, Imperial College London, personal communication). However, it cannot be excluded that this band seen is not BEC1011 but results from aspecific recognition of another protein. The difficulty of extracting BEC1011 as a monomer, even in denaturing conditions, meant this was not pursued further.

Multiple Reaction Monitoring (MRM) mass spectrometry could be used on protein extracted from these high molecular weight bands to identify whether this is BEC1011.

Proteins were also extracted in denaturing conditions from a time course of leaves 1, 2, 4 and 8 days post inoculation and non-inoculated leaves and a western blot was performed using BEC1011 antibodies.

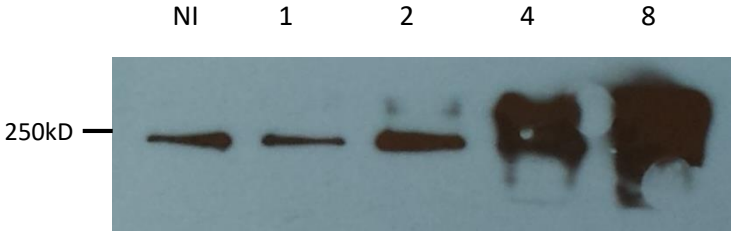


Figure3.18. Western blot of BEC1011 protein extracted from non-infected barley leaves (NI) and leaves 1, 2, 4 and 8 days post inoculation in denaturing conditions.

Bands seen are of a similar size to previous western blots using the BEC1011 antibody (Fig. 3.18). Samples taken 4 and 8 days post inoculation appear to show two bands. A band can be seen in the non-inoculated sample which is not seen in fig. 19. This may be due to contamination with inoculated samples. It appears that protein can be detected 1 day post inoculation but it cannot be ruled out that this is also due to contamination. Due to these issues, this was not continued further.

3.4.11 Assessing target protein amount following PTO silencing using Multiple Reaction Monitoring Mass Spectrometry

Although qRT-PCR measurements did show a significant reduction in the abundance of the targeted transcript following silencing treatment of the cognate gene, these reductions were relatively moderate when compared to the phenotype analysis showing decreased infection level by microscopically monitoring proportion of hyphae formed. In order to further assess the outcomes of PTO silencing, Multiple Reaction Monitoring Mass Spectrometry (MRM-MS) was used to assess protein abundance following silencing.

MRM-MS allows quantification of a protein through detection of peptides known to be unique to that protein. MRM-MS can more accurately quantify protein amount than techniques such as western blot, which is at best only semi-quantitative, or ELISA. The specificity of these techniques is reliant on antibody detection of the target protein and often cannot rule out aspecific identification of another protein.

A protein standard of purified recombinant protein was used to optimise the MRM assay. Recombinant BEC1011 and BEC1019 proteins were produced by generating pET53 plasmids containing *BEC1011* and *BEC1019* genes with an N-terminal His-tag and C-terminal Strep tag. Plasmids were transformed into *E. coli* and protein production was induced using IPTG. Proteins were extracted in denaturing conditions, tricine SDS-PAGE was performed and bands excised for in gel tryptic digestion. The digested peptides were analysed by LC-MS to optimise MRM protocols.

In silico digestion of *BEC1011* and *BEC1019* was performed using Skyline software to predict unique peptides to use in MRM-MS (Mark Bennett, personal communication). These were checked against other proteins in barley and *Bgh* to ensure they are unique. Unique peptides were identified for both *BEC1011* and *BEC1019* but the *BEC1019* predicted peptide did not produce clear peaks from mass spectrometry analysis. Suitable peptides need to produce a clear peak, good ionisation and good fractionation in MS/MS.

The tryptic peptide identified for BEC1011 (DAAVFAFSK) was easily detectable as it was found to produce a clear peak and did not co-elute with any other peptides, as can be seen in Figure 3.19.

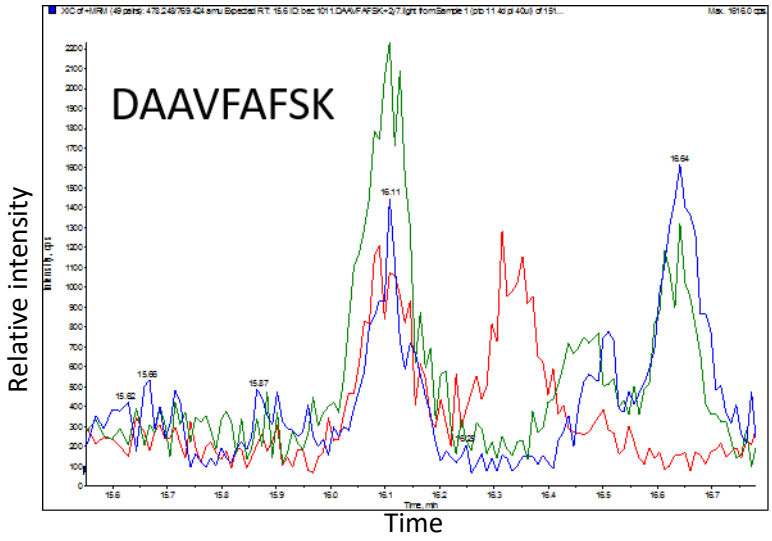


Figure 3.19 MRM-MS chromatogram showing intensity of the targeted peptide (DAAVFAFSK) from purified recombinant BEC1011. Green, blue and red lines indicate different fragment ions of the peptide (y5, y6 and y7) and the intensity indicates the abundance of the peptide within the sample.

Having identified a suitable peptide from a sample of purified recombinant BEC1011 protein, a time course of infected leaf material was sampled, proteins extracted, digested with trypsin followed by MRM-MS/MS analysis to evaluate whether the BEC1011 peptide could be identified in whole leaf samples. Results showed that the BEC1011 peptide could be identified in infected whole leaf samples from 3 dpi onwards but with a relative intensity three-fold lower than from purified recombinant protein. Intensity of the DAAVFAFSK peak was too low at earlier time points to distinguish from background signal. Unlike systems to analyse DNA or RNA, such as RT-PCR, protein analysis does not involve replication of the proteins so methods are limited by the ability of the system to detect the protein/ peptide of interest.

Leaves were treated with PTO Z and PTO 11.11 as previously described and leaves were sampled 2, 3 and 4 dpi. Proteins were extracted using TCA/acetone precipitation and suspended in a urea extraction buffer as described in chapter 2.7. Tryptic digestion was performed to produce peptides for MRM-MS. The same amount of protein was digested for each sample to allow for relative quantification of protein amount between samples but absolute quantification was not possible as no labelled peptide was available for use as an internal standard.

Samples from five PTO treatment experiments were analysed by MRM-MS. The BEC1011 peptide was detected in 3 dpi and 4dpi samples in three experiments. The analyte peak area indicates the abundance of the protein in the samples and this is shown for PTO 11.11 treated samples relative to the PTO Z control at the same time point after inoculation (Fig. 3.20).

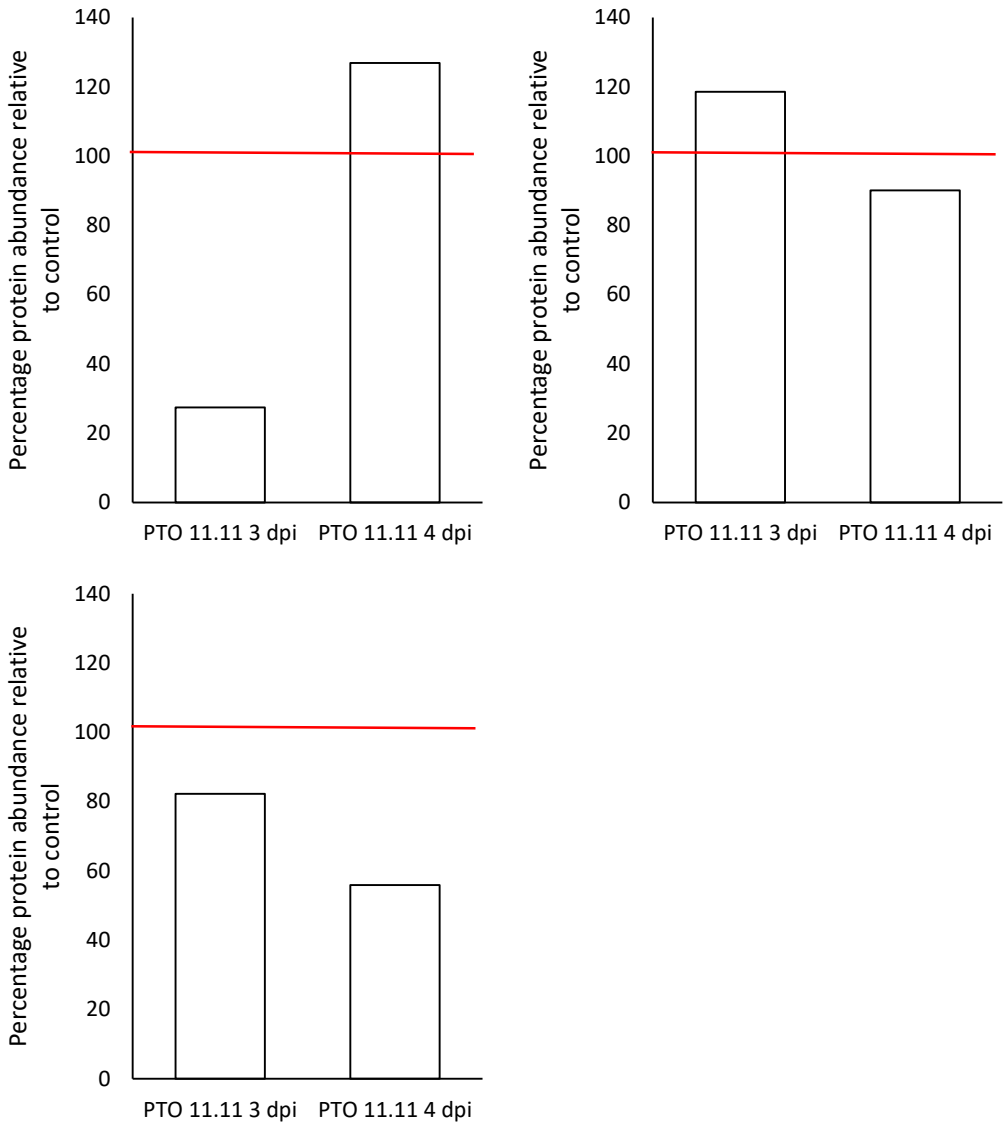


Figure 3.20 BEC1011 protein abundance in leaves treated with PTO 11.11 relative to PTO Z control treatment at 3 and 4 days post inoculation in three replicate experiments. Red bar indicates BEC1011 protein in PTO Z treated leaves, adjusted to 100%.

In two other experiments overall protein concentration level was too low to detect BEC1011 peptides in all samples so comparisons between treatments could not be made.

In each of the three experiments, BEC1011 abundance was reduced in PTO 11.11 treated leaves relative to PTO Z treated leaves in at least one time point. The reduction relative to control varied from 10% to over 70% in different experiments and in some samples BEC1011 abundance was increased following PTO 11.11 treatment. This gives no observable trend to suggest when protein level is affected following PTO treatment. It does indicate that PTO silencing does last up to four days after treatment as differences could still be seen at this time point.

3.5 Discussion

This study aimed to establish and validate a system for silencing *Bgh* genes using antisense oligonucleotides (ODNs). Three candidate effectors which had previously been shown to be virulence factors during *Bgh* infection – BEC1011, BEC1019 and BEC1054 (Pliego *et al.*, 2013) - were selected to test whether it was possible to use ODNs to silence *Bgh* genes in planta. ODNs had been designed to target these effectors and preliminary results showed a reduction in growth of secondary hyphae. The use of ODNs with a PTO modified backbone was investigated to improve the efficacy of silencing treatment. A PTO modified version of ODN 11.11 increased the reduction in growth of secondary hyphae by a further 30%.

The effect of length on the efficacy of silencing was also assessed. Using a 25mer version of ODN 11.11 or ODN BLN1_2 showed reductions in proportion of conidia with secondary hyphae of 33% and 26% respectively. The 19mer versions of each of these ODNs resulted in 24% and 22% reduction, respectively. There was a significant difference in the reduction seen using the 25mer ODNs versus the 19mer ODNs, but this difference was much smaller than the difference between non-modified ODNs and PTO-modified ODNs. Given that the difference in price of 19mer and 25mer ODNs can be up to £19 per tube with PTO modifications (Sigma Aldrich, £3.20 per base for PTO modified ODN at 1µM synthesis scale) , using 25mer ODNs was not a justifiable choice.

Two different PTO controls have been used in these experiments, as well as a mock water control. When comparing the three controls, there was no significant difference in percentage of secondary hyphae in leaves treated with PTO Z or with water. This indicates that differences observed in percentage secondary hyphae in leaves treated with PTOs targeting effectors are solely due to the sequence specificity of the PTO and not an undesirable toxic effect of the PTO chemistry itself. Leaves treated with PTO M13 did show a significant difference in growth of secondary hyphae compared to either PTO Z or water. BLAST analysis showed 13 bp matches between the M13 PTO sequence and *Bgh* genes. Whilst this was below the criteria of 14 bp matches set out for exclusion of PTO sequences, aspecific effects of this PTO cannot be ruled out. This indicates that this is not a suitable control and will not be used further. More evidence that PTO Z is a suitable negative control was obtained when using a shuffled version of PTO 11.11 as negative control, as using such PTO did not show any significant difference when compared to PTO Z. Using a BLAST similarity search against the barley and *Blumeria* genomes, neither PTO Z nor PTO 11.11 shuffle sequence predicted any off targeted. Therefore, both negative controls were considered valid, as discussed below.

The specificity of the PTOs designed was tested by designing PTOs with the same sequence as PTO 11.11 but with one mismatched base, two mismatched bases or a shuffled sequence. Results indicated that one mismatch in the sequence still produced a significant reduction in growth of secondary hyphae relative to PTO Z control, albeit markedly less than the reduction in leaves treated with the original PTO 11.11. Leaves treated with PTOs with two mismatched bases or a shuffled sequence showed no significant difference in growth of secondary hyphae compared to PTO Z control. This indicates that using PTOs as silencing molecules is highly specific and off target effects can be ruled out as long as sequences have been checked for the possibility of fewer than two mismatches.

As it has been previously shown that PTOs have the ability to interact in a non-sequence specific manner (Rockwell *et al.*, 1997), it was particularly important to test the effect of the PTO controls compared to mock water treatment as well as the specificity of the PTOs designed to specifically silence their targets. The results of both these experiments indicate that the PTOs are not having any non-sequence specific effects.

A screen of fifty candidate effectors using HIGS validated eight effectors as pathogenicity factors during *Bgh* infection (Pliego *et al.*, 2013). Three of these confirmed virulence factors were selected to test this new silencing method – *BEC1011, BE1019* and *BEC1054*. The effect of the silencing was measured by recording the proportion of conidia which produced secondary hyphae – an indicator that a successful haustoria has formed. This is a proxy measurement similar to the haustorial index used by Pliego *et al.*, (2013) but as secondary hyphae stain better than haustoria, secondary hyphae are easier to measure. Hyphae may also be a better measure as they show that the haustoria is successfully established and providing nutrients for the fungus to grow whereas the mere presence of a haustorium does not mean that it is going to be compatible.

HIGS was used to confirm the pathogenicity of *BEC1011, BEC1019* and *BEC1054*. Table 3.4 compares the reduction in haustoria formation following HIGS (haustorial index) and the reduction in proportion of conidia producing secondary hyphae following PTO silencing.

Table 3.4 comparison of silencing effects using HIGS and PTO silencing

Effector target	Reduction in haustoria formation by HIGS (Pliego *et al.*, 2013)	Reduction in proportion of conidia producing secondary hyphae following PTO silencing	Difference between HIGS and PTO silencing
BEC1011	70%	53%	-17%
BEC1019	47%	41%	-6%
BEC1054	59%	21%	-38%

The results of silencing *BEC1011,* or *BEC1019* using PTO modified ODNs were similar to the results achieved using HIGS with a significant reduction in proportion of conidia producing secondary hyphae when these effectors were targeted. Differences seen in the effect of silencing the effector targets may also be due to the different *Bgh* isolates used in this study (isolate DH14) and the HIGS study (isolate CH4.8) where the virulence effects of *BEC1011, BEC1019* and *BEC1054* were confirmed. Isolate specific roles or differences in expression of effectors between different isolates has been shown in other studies (Pliego *et al.*, 2013; Aguilar *et al.*, 2016).

Using PTOs also has the advantage that the design process is simpler and faster for PTO design than for design of HIGS constructs. The process of screening the effectors targeted using PTOs or using HIGS are very similar and using PTOs has the secondary advantage that whole tissues are treated and downstream analyses such as qRT-PCR can be used. Targeting *BEC1011* or *BEC1019* resulted in significant reduction in target mRNA and in fungal biomass – results that could not be quantified if the effectors were targeting using HIGS.

A proxy measurement of fungal biomass was made by recording the ratio of barley and *Bgh* housekeeping gene transcript abundance following silencing using PTO 11.11 to target *BEC1011* or PTO 19.12 to target *BEC1019.* The housekeeping genes selected were *HvGAPDH* and *BghGAPDH* which were found to be the most stably expressed during the infection process (Pennington *et al.*, 2016b). Using this method, fungal biomass was found to be reduced by 30% following PTO 11.11 or PTO 19.12 treatment.

BEC1011 and *BEC1019* transcript abundance was also measured using qRT-PCR following treatment with PTO 11.11 or PTO 19.12. Effector transcript abundance was measured relative to the *BghGAPDH* housekeeping gene to determine that the reduction was due to specific reduction in the target mRNA, not an artefact of the reduction in biomass caused by the PTO treatments.

Although the reductions in transcript abundance were significant, they were still relatively modest. This may indicate that the PTO silencing is not occurring through RNase H degradation of mRNA but by causing translational arrest because the mRNA is blocked from processing through the ribosome because of the presence of the PTO bound to the mRNA. Previous studies using ODNs or PTOs in plants have generally indicated that silencing was mediated by RNase H recruitment. Sun *et al.*, (2005) showed reduction in mRNA of up to 70%, with the highest reduction being at the 5’ end of the mRNA. This would be indicative

of the 5'-3' action of RNase H. Dinc *et al.*, (2011) showed a reduction in both mRNA and protein in the genes they targeted but the mRNA reduction was greater than the reduction in protein. This would indicate that the mRNA was being degraded by RNase H and this resulted in the reduction in protein. Other studies also found a reduction in the expression of the targeted genes (Liao *et al.*, 2013; Mizuta and Higashiyama, 2014). Another possibility as to why the mRNA reduction seen was only minimal could be because of the time of measurement. Addition of the PTO modification has been shown to extend the longevity of oligonucleotides but the longest that they have been reported is 19 hours following treatment (Campbell *et al.*, 1990). Dinc *et al.*, (2011) measured the effect of silencing at 8 and 24 hours after treatment. They showed a greater reduction in both phenotype and target mRNA expression at 8h than 24h after treatment. After 24h the reduction in mRNA was only around 20% which is similar to the reductions in *BEC1011* and *BEC1019* transcript following PTO treatment. Mizuta and Higashiyama, (2014) measured target expression up to 9 hours after treatment and saw a reduction in the effect at the later time points. In this study, the transcript abundance and microscopic phenotype were recorded 48 hours post inoculation with *Bgh* spores. This time point is the earliest that the phenotype can be easily examined and that the *Bgh* biomass is great enough to be able to accurately detect *Bgh* genes by qRT-PCR from samples which are a mixture of barley and *Bgh* RNA.

MRM-MS was used to assess BEC1011 protein abundance following PTO 11.11 treatment. This was measured 3 and 4 days after treatment and relative reductions in BEC1011 protein abundance were observable. This indicates that the effect of PTO silencing last at least four days at the protein level. This effect may be dependent on the turnover of individual proteins.

The results of BEC1011 protein quantification by MRM-MS were very variable and in some samples peptides could not be detected. The relative abundance of *Bgh* effectors in samples of whole infected leaves is very low and it would appear that this is below the limit of detection by MRM in some cases. The data collected have given a good indication PTO silencing leads to a reduction in target protein but further optimisation of the workflow is needed to give true quantification of this effect.

One way of improving accuracy of protein quantification is by the use of internal standards. Artificial proteins can be designed with concatenated tryptic peptides of target proteins and of reference proteins. These are labelled with ^{15}N and used to spike samples which allows for absolute, rather than relative, quantification of the protein of interest (Beynon *et*

al., 2005). A protein with concatenated peptides from *Bgh* effectors BEC1011, BEC1019 and BEC1054 and barley and *Bgh* reference proteins, including GAPDH, is being designed to allow for absolute quantification of *Bgh* effectors in future experiments.

There are also ways in which detection of BEC1011 peptides can be improved. *Bgh* infection is restricted to the epidermal tissue and therefore this is where *Bgh* will be found. However, in this study proteins were extracted from whole leaves meaning that *Bgh* proteins were diluted with higher abundance plant proteins such as RUBISCO, which can make up 40% of total protein content (McCabe *et al.*, 2001; Bindschedler and Cramer, 2011). By using epidermal strips instead of whole leaves or methods such as chloroform/methanol precipitation to concentrate proteins, *Bgh* proteins should be easier to detect.

This MRM-MS technique may be easier to apply to barley susceptibility proteins than to effector proteins because they will have higher abundance in samples.

4 Using PTO silencing to investigate known susceptibility genes

4.1 Introduction

Resistance or susceptibility to a pathogen is not only determined by pathogenicity of the pathogen, but also by specific genes within the plant which contribute to susceptibility. These susceptibility genes are often upregulated following infection. Susceptibility genes have been found across a wide range of plant species, leading to susceptibility to a range of pathogens including bacteria, fungi and nematodes (Yang *et al.*, 2006; Hewezi *et al.*, 2008; Antony *et al.*, 2010; Raiola *et al.*, 2011).

The same susceptibility gene can confer susceptibility to several different pathogens – even from different kingdoms. *Arabidopsis thaliana* gene *PECTIN METHYLESTERASE 3 (PME3)* can be recruited by fungi (*Botrytis cinerea*), bacteria (*Pectobacterium carotvorum*) (Raiola *et al.*, 2011) and nematodes such as *Heterodera schactii* to help degrade the plant cell wall to facilitate infection (Hewezi *et al.*, 2008).

As well as genes which assist in physical penetration of the host, pathogens often recruit genes which help the pathogen gain nutrition from the host. The *SWEET* family of sugar transporters are found across numerous plant species and also have homologues in humans and *Caenorhabditis elegans.* Two *SWEET* genes have been shown to be induced by infection with the bacterial pathogen *Xanthomonas oryzae oryzae (Xoo)* in rice. This was found to be due to bacterial TAL effectors interacting with effector response elements in the promoter region to manipulate *SWEET* gene expression during infection (Streubel *et al.*, 2013). In *Arabidopsis* different *SWEET* genes have been shown to be induced following infection with bacterial and fungal pathogens such as *Pseudomonas syringae, Botrytis cinerea* and *Erysiphe cichoracearum* (Chen *et al.*, 2010). The *SWEET* sugar transporters are involved with sugar efflux from the phloem and therefore pathogens may induce these genes to increase the accessible sugars for their nutrition (Chen *et al.*, 2012).

One of the best known and most exploited susceptibility genes in barley would be wild type *Mildew Locus O (Mlo)*. As early as 1942, mutations in this gene were found to confer resistance to barley powdery mildew (Freisleben and Lein, 1942) and have been used for broad, durable powdery mildew resistance in barley breeding since the 1970s (Jørgensen, 1976). The recessive resistance phenotype conferred is non race specific and works by the formation of cell wall appositions at the site of penetration (Jørgensen, 1992). The MLO protein has seven transmembrane domains and homologues have subsequently been identified in many other plant species including wheat, rice, soybean and *Arabidopsis*.

These homologues have also been linked with susceptibility to powdery mildews in their wild type state (Devoto *et al.*, 1999). In *Arabidopsis* a partial resistance phenotype exhibited by mutation of *AtMLO2.* This is supplemented in double mutants *Atmlo2/mlo6* or *Atmlo2/mlo12* and a full resistance phenotype is seen in the *Atmlo2/mlo6/mlo12* triple mutant (Consonni *et al.*, 2006). Whilst *mlo* mutants have only been deployed in barley, this discovery showed that there is potential for durable powdery mildew across monocots and dicots through mutations at the *MLO* locus.

Two other barley genes have been identified which are required for *mlo* resistance in barley – *Ror1* and *Ror2* (Freialdenhoven *et al.*, 1996). In addition to MLO, a barley RAC/ROP small GTP-binding protein, RACB, has been shown to be involved, along with wild type MLO, in the prevention of actin remodelling associated with resistance to fungal penetration (Opalski *et al.*, 2005). Resistance conferred by recessive *mlo* alleles are not only associated with penetration resistance, but there are also pleiotropic effects on cell death and H_2O_2 accumulation (Peterhansel *et al.*, 1997; Piffanelli *et al.*, 2002; Consonni *et al.*, 2010). Overexpression of the negative regulator of cell death, *BAX INHIBITOR-1* (*BI-1*) is able to restore the susceptibility phenotype in *mlo* barley (Hückelhoven *et al.*, 2003). This suggests that modulation of cell death is another important factor of *mlo* based resistance.

Whilst *mlo* provides effective resistance to the biotrophic powdery mildews, there have been reports that the *mlo* mutation actually confers susceptibility to necrotrophic or hemi-biotrophic pathogens, including *Magnaporthe oryzae* (Jarosch *et al.*, 1999), *Fusarium spp.* (Jansen *et al.*, 2005) and *Ramularia collo-cygni* (McGrann *et al.*, 2014). However, there have also been studies which refute this assertion, stating that there is no effect of *mlo* alleles on virulence of other pathogens (Hofer *et al.*, 2015).

Mlo is not the only gene conferring susceptibility in barley during powdery mildew infection. The *BLUFENSIN1* and *BLUFENSIN2* (*Bln1* and *Bln2*) genes were identified as being amongst the most highly induced barley genes following infection with *Bgh* as well as *Fusarium graminearum* – the causal agent of Fusarium head blight (Boddu *et al.*, 2006; Meng *et al.*, 2009) but their role in the plant is unknown. Blufensins are also present in wheat (*Tritcum aestivum*), which has six blufensin genes, and in rice (*Oryza sativa*) which has three. Blufensins are knottin-like, small (~55 amino acids), cysteine-rich proteins with three disulphide bonds forming the characteristic knot structure that gave the protein its name (Xu *et al.*, 2015). All the identified blufensin peptides have high sequence similarity in the signal peptide, specific conserved residues within the mature peptide, including two

cysteine residues, between which is a conserved intron (Meng *et al.*, 2009). Both *Bln1* and *Bln2* are secreted to the apoplast and have been shown to interact with each other but also with calmodulin. The initial study showed that silencing of *Bln1*, but not *Bln2*, using virus induced gene silencing (VIGS) increased resistance to *Bgh* indicating that *Bln1* acts as a susceptibility factor for *Bgh* (Meng *et al.*, 2009). This made *Bln1* the focus of our interest. However, the later study into these blufensins showed that barley stripe mosaic virus (BSMV) mediated overexpression of *Bln2* resulted in increased susceptibility to *Bgh,* but not overexpression of *Bln1* (Xu *et al.*, 2015), suggesting that *Bln2* also plays a role in conferring susceptibility to barley during *Bgh* infection. To further investigate *Bln1,* it was silenced in two different barley cultivars (Xu *et al.*, 2015). Six genes were identified as differentially expressed in both cultivars following *Bln1* silencing. Two of these six genes – *Importin α-1b* and *Sec61 γ* – are involved in protein transport (Xu *et al.*, 2015). This indicates two possible functions of *Bln1.* It may interact with calmodulin to alter signalling pathways, or it may be involved with protein trafficking – possibly for the import of effectors.

Alcohol dehydrogenase 1 (*ADH1*) has also been shown to be induced following *Bgh* infection and knocking down *ADH1* expression was reported to reduce fungal penetration (Pathuri *et al.*, 2011). Therefore, *ADH1* may also play a role as a susceptibility factor. *ADH1* is involved with maintenance of ATP levels during aerobic respiration. Therefore, this suggest that upregulation of this enzyme would be beneficial for nutrient acquisition and growth of the pathogen.

4.2 Aims and Objectives

Aim: Validating the use of ODN mediated gene silencing as a valid tool to investigate susceptibility genes *Mlo* and *Bln1* during the infection of barley with powdery mildew.

Objective: To design and deliver ODNs to target *Mlo* and *Bln1* and quantify the effect on *Bgh* virulence.

4.3 Materials and Methods

4.3.1 ODN design

ODNs were designed to target *Bln1* and *Mlo* as previously described in chapter 2.2.

The ODNs designed to target the *Mlo* and *Bln1* susceptibility genes are shown in table 1. ODNs targeting *Bln1* were manually checked to ensure they did not target the related gene *Bln2.*

4.3.2 ODN/PTO treatment and phenotype scoring

ODN/PTO treatments were performed as previously described in chapter 2.3. Seven-day old barley primary leaves were treated with 10 µM ODN/PTOs targeting *Bln1* or *Mlo* for 24 h before being laid on 0.6% water agar plates with 20 mg/ml benzimidazole and inoculated with *Bgh* conidiospores. Leaves were sampled 48 hpi and stained with lactophenol cotton blue before destaining with 3:1 ethanol:acetic acid. The number of conidia, conidia with appressoria and conidia with secondary hyphae were recorded using light microscopy at 250x magnification. Proportion of conidia with secondary hyphae is used to estimate the infection success rate as secondary hyphae are only produced following formation of a successful haustorium.

4.4 Results

4.4.1 Different ODNs targeting *Bln1* have different efficiencies

Three different ODNs were designed to target *Bln1* using OligoWalk software, ODN BLN1_1, ODN BLN1_2 and ODN BLN1_3. These were used to treat seven-day old barley primary leaves as previously described. Small, non-significant reductions of 14% and 18% were seen when comparing proportion of conidia producing hyphae between leaves treated with ODN BLN1_1 or ODN BLN1_3 and ODN Z, respectively. Two of the three ODNs tested – ODN BLN1_2 and ODN BLN1_3 showed a significant reduction (21% and 22%, respectively) in proportion of conidia with secondary hyphae compared to leaves treated with ODN Z control (Fig. 4.1).

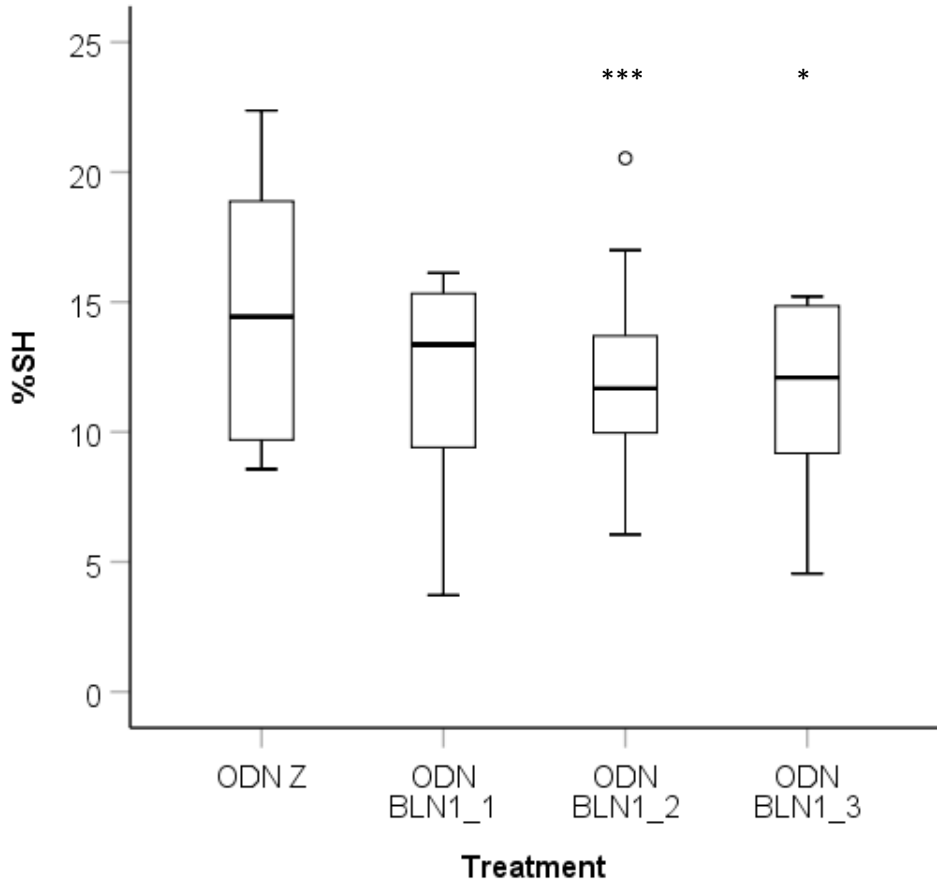


Figure 4.1 Effect of three ODNs targeting *Bln1* on proportion of conidia producing secondary hyphae (%SH) compared to ODN Z control. N=8 leaf tip segments from two independent experiments for ODN BLN1_1 and BLN1_3. N=14 leaf tip segments from three independent experiments for ODN BLN1_2. A minimum of 300 conidia were scored per leaf segment. Results are presented as Boxplot showing median, IQR and minimum/maximum values, circles represent outliers of more than 1.5x IQR above the box. Significance was calculated using GLMM. * indicates P values <0.001. * indicates P value <0.05.**

Interestingly, the BLN1_2 ODN was the only ODN present in the intron rather than the coding sequence. Despite this, it still had the most significant effect on development of secondary hyphae of the ODNs tested. Having screened the potential ODNs, a PTO modified version of ODN BLN1_2 was used.

4.4.2 Using PTOs to target *Bln1* decreases susceptibility to *Bgh*

Seven-day old barley primary leaves were treated with 10 µM PTO BLN1_2 or PTO Z and inoculated with *Bgh* spores as previously described. The proportion of conidia with secondary hyphae was monitored microscopically (Fig. 4.2).

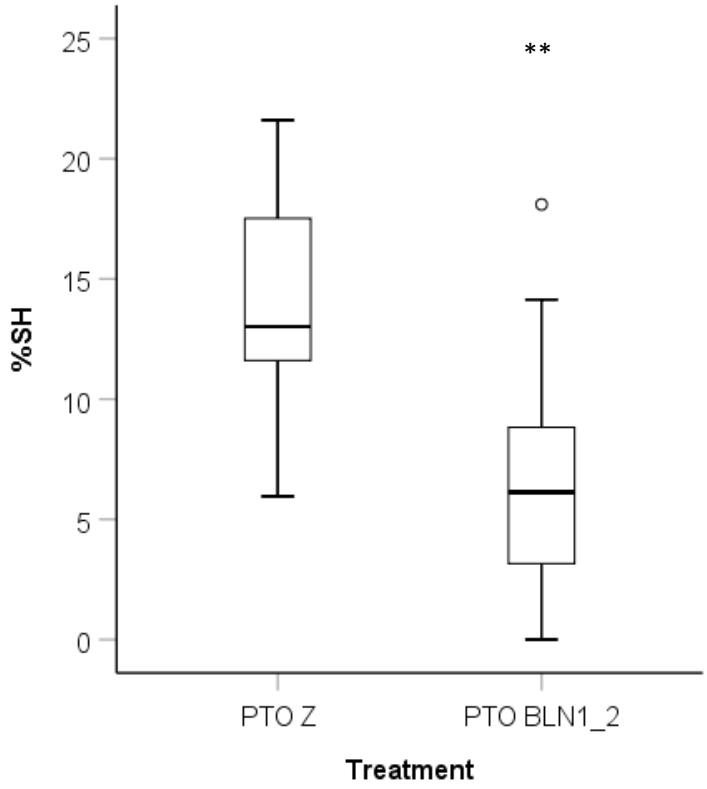


Figure 4.2 Effect of targeting *Bln1* with PTO BLN1_2 on proportion of conidia with secondary hyphae (%SH) compared with leaves treated witih PTO Z control. N=24 leaf tip segments from eight independent experiments. A minimum of 300 conida were scored per elaf segment. Results are presented as Boxplot showing median, IQR and minimum/maximum values, circles represent outliers of more than 1.5x IQR above the box. Significance was calculated using GLMM. ** indicates P values <0.01.

Treating leaves with PTO BLN1_2 resulted in a 54% reduction in proportion of conidia with secondary hyphae compared to leaves treated with PTO Z control.

4.4.3 Targeting *Mlo* using PTOs reduces barley susceptibility to *Bgh*

Two PTOs were designed to target the *Mlo* susceptibility gene – PTO MLO_1 and PTO MLO_2. The PTOs were designed to target the loops rather than the transmembrane domains of the MLO protein. PTO MLO_1 is targeted to a part of the gene encoding an extracellular loop between the second and third transmembrane domains and PTO MLO_2 is targeted to a region of the gene encoding an intracellular loop between transmembrane domains 3 and 4. Seven-day old barley primary leaves were treated with either 10 µM PTO MLO_1, PTO MLO_2 or the PTO Z control. The proportion of conidia with secondary hyphae was assessed microscopically (Fig. 4.3).

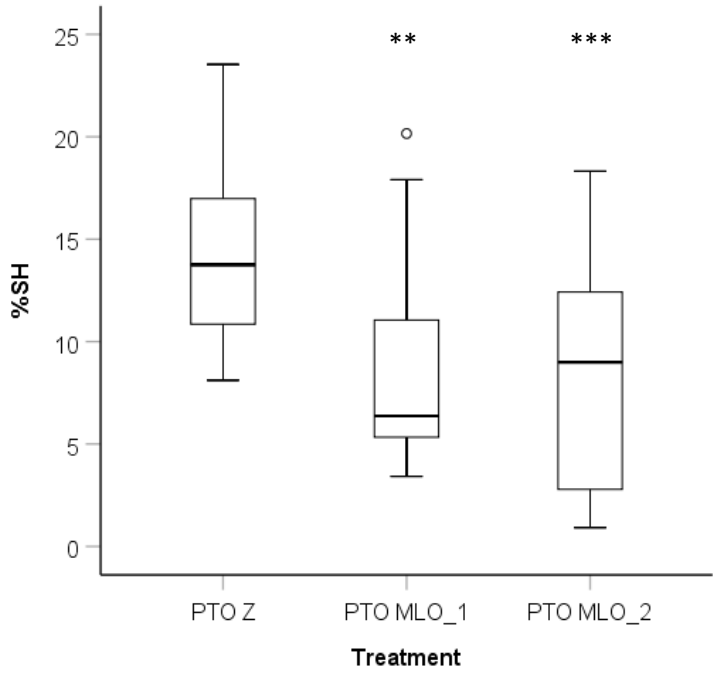


Figure 4.3 Effect of targeting *Mlo* with PTO MLO_1 or PTO MLO_2 on secondary hyphae production, compared to leaves treated with PTO Z control. N=19 leaf tip segments from four independent experiments. A minimum of 300 conidia were scored per leaf segment. Results are presented as Boxplot showing median, IQR and minimum/maximum values, circles represent outliers of more than 1.5x IQR above the box. Significance was calculated using GLMM. **indicates P<0.01, * indicates P <0.001.**

Targeting *Mlo* with PTO MLO_1 or PTO MLO_2 showed a 38% and 43% reduction of conidia with secondary hyphae when compared to PTO Z control treatment, suggesting that gene silencing with PTO modified oligodeoxynucleotides is suitable to study or discover barley genes involved in susceptibility during barley powdery mildew infection.

4.5 Discussion

Mlo and *Bln1* are two genes which have previously been identified as susceptibility factors in the interaction between barley and barley powdery mildew. Mutant *mlo* varieties are used widely in agriculture as they are resistant to powdery mildew (Jørgensen, 1992) and more recently, silencing *Bln1* has been shown to reduce susceptibility to *Bgh* (Meng *et al.*, 2009). These two susceptibility genes were therefore good targets to validate the use of PTO silencing to investigate genes involved in the barley – *Bgh* interaction.

Three non-modified ODNs were first evaluated for their efficiency to target *Bln1* in order to select the most effective ODN sequence. One of the three ODNs – ODN BLN1_2 – was successful in producing a significant reduction in secondary hyphae production. Of the three ODNs tested, this was the only ODN whose sequence was within the intron, whereas all other ODNs used so far were from within the coding sequence of their respective gene. This result may give an indication that the mode of action of the ODN does not necessarily

rely on interfering with the mature mRNA sequence. Oligonucleotide-mediated silencing is proposed to work mainly through two possible modes of action. Either the binding of the ODN to the mRNA prevents binding or processing through the ribosome, blocking translation, or the ODN/RNA duplex is targeted by RNase H and enzymatically degraded (Baker *et al.*, 1997; Wu *et al.*, 2004). As the intron is spliced from the mature mRNA before translation, an ODN in the intron would not be able to induce silencing through translational arrest. This would indicate that the silencing effect observed by targeting *Bln1* is caused by RNase H degradation of the immature mRNA.

Using PTO BLN1_2 to target *Bln1* resulted in 54% reduction in secondary hyphae production. This is comparable to the original study which used VIGS to confirm *Bln1* as a susceptibility factor which showed a 50% reduction in penetration efficiency (Meng *et al.*, 2009).

Two PTOs were designed to target *Mlo* and both resulted in a significant reduction in secondary hyphae production. PTO MLO_1 treatment resulted in 38% reduction and PTO MLO_2 resulted in 43% reduction. *Mlo* is a transmembrane protein with intra- and extracellular loops. The PTOs were targeted to one extracellular (PTO MLO_1) and one intracellular (PTO MLO_2) loop rather than to sequences coding for the transmembrane domains as the loops are less conserved than the transmembrane domains (Devoto *et al.*, 1999) and therefore off target gene silencing and cross silencing are less likely to occur.

Naturally occurring and induced *mlo* barley mutants are variable in their level of resistance to powdery mildew, with some conferring total resistance and some only partial resistance (Lyngkjaer *et al.*, 2000; Piffanelli *et al.*, 2002). It has been reported that the second and third intracellular loops are particularly sensitive to mutation and therefore critical to *Mlo* susceptibility (Reinstädler *et al.*, 2010). As PTO MLO_2 is targeted towards the second intracellular loop, this may account for the slightly higher response seen using PTO MLO_2 rather than PTO MLO_1 but neither PTO resulted in the level of resistance commonly seen in commercial *mlo* mutant varieties. This may be because there is a reduced amount of MLO protein but the MLO protein that is there is still functional. These results are, however, comparable to previous studies which silenced *Mlo* using barley stripe mosaic virus mediated gene silencing and resulted in approximately 50% reduction in haustorial formation rate (Ahmed *et al.*, 2016). Another study induced transient silencing of *Mlo* by bombardment of RNAi constructs. This resulted in 90% reduction in haustorial formation but only when leaves were inoculated 4 days post bombardment (Douchkov *et al.*, 2005).

This may indicate that MLO protein abundance needs to be depleted prior to *Bgh* inoculation. The longevity of mature MLO protein in cells in unknown so this partial phenotype may be due to the presence of functional MLO protein in the cells allowing for some resistance even if new MLO production is impaired through PTO silencing.

This methodology has shown significant reductions in secondary hyphae production by targeting the *Mlo* and *Bln1* susceptibility genes and it is therefore a useful tool for barley-*Bgh* functional genomics but further development of the PTO delivery system may improve the efficacy for longer term studies.

Targeting *Mlo* and *Bln1* has shown comparable results to silencing known *Bgh* effectors. This shows that the PTO silencing method is useful for investigating virulence or susceptibility factors on both sides of the barley-*Bgh* interaction.

5. Using PTO silencing to validate new *Blumeria* effectors

5.1 Introduction

Plant resistance (*R*) genes are known to interact with pathogen avirulence (*AVR*) genes in a gene-for-gene manner in order to prevent infection (Flor, 1971). As pathogens evolve to avoid recognition of their *AVR* genes, so plants are under pressure to maintain their resistance. This can lead to large numbers of *R* gene alleles within a species which recognise different *AVR* genes. One such example of this in barley are the alleles of the *Mla* locus, of which at least 23 have been characterised (Lu *et al.*, 2016). These alleles have over 90% sequence identity and encode nucleotide-binding leucine rich repeat (NB-LRR) type receptors with an N terminal coiled-coil domain (Lu *et al.*, 2016). These barley *R* genes recognise *Bgh AVRa* alleles.

AVRa10 was the first *Bgh* avirulence gene to be identified – along with the *AVRk1* gene which is recognised by barley *Mlk1* (Ridout *et al.*, 2006). These two homologous pathogen genes were found to be part of a larger family of *Bgh* genes. The EKA (effectors homologous to *AVRk1* and *AVRa10*) family has over 1350 members in the *Bgh* genome and are predicted to arise from degenerate copies of class I – LINE retrotransposons (Sacristán *et al.*, 2009; Amselem *et al.*, 2015).

Two further *AVR* alleles were identified through a genome wide association study of 17 *Bgh* lines – *AVRa1* and *AVRa13*. The avirulence function of both these genes was confirmed by expression in barley lines containing the corresponding *Mla* alleles (*Mla1* and *Mla13*) which resulted in HR, whereas expression in barley without the corresponding *Mla* allele showed no cell death (Lu *et al.*, 2016). Both *AVRa1* and *AVRa13* were found to be genes previously identified as CSEPs – CSEP0008 and CSEP0372, respectively. CSEP0008 is one of 84 CSEPs which do not belong to a family, whereas CSEP0372 was placed in family 34 (Pedersen *et al.*, 2012). Both CSEP0008 and CSEO0372 have been classified as ribonuclease-like effectors due to structural similarity to fungal ribonucleases (Pedersen *et al.*, 2012; Praz *et al.*, 2017).

Both genes have high haustoria to epiphytic expression ratio and CSEP0008 was previously identified as a protein in haustoria (synonym BEC1001) (Bindschedler *et al.*, 2009; Pedersen *et al.*, 2012). CSEP0008 was included in the panel of candidate effectors that were silenced using HIGS but CSEP0372 has never been silenced (Pliego *et al.*, 2013).

5.2 Aims and Objectives

Aim: To investigate the virulence function of newly identified *Bgh AVR* effector genes

Objective: To use PTOs to target *AVRa1* and *AVRa13* and quantify the effect on *Bgh* virulence

5.3 Materials and Methods

5.3.1 PTO design

Two PTOs were designed to target each *AVR* – PTO AVRa1_1, PTO AVRa1_2, PTO AVRa13_1 and PTO AVRa13_2. The sequences of the ODNs designed are shown in table 2.1.

5.3.2 PTO treatment and phenotypic observation

PTO treatment of excised barley leaves was performed as previously described using 10 µM PTO. Barley primary leaves were treated for 24 h, inoculated with *Bgh* spores and sampled for microscopic observation 48 hpi. Leaf sections were stained in lactophenol cotton blue, detained in ethanol/acetic acid and mounted in 20% glycerol. Number of non-germinated conidia, conidia with appressoria and conidia with secondary hyphae were recorded with a light microscope at 250x magnification.

5.4 Results

Seven-day old barley primary eaves were treated with 10 µM PTO AVRa1_1, PTO AVRa1_2, PTO AVRa13_1, PTO AVRa13_2 or PTO Z control for 24 h before inoculation with *Bgh* spores. Leaves were sampled 48 hpi and percentage of conidia with secondary hyphae was scored microscopically (Fig. 5.1).

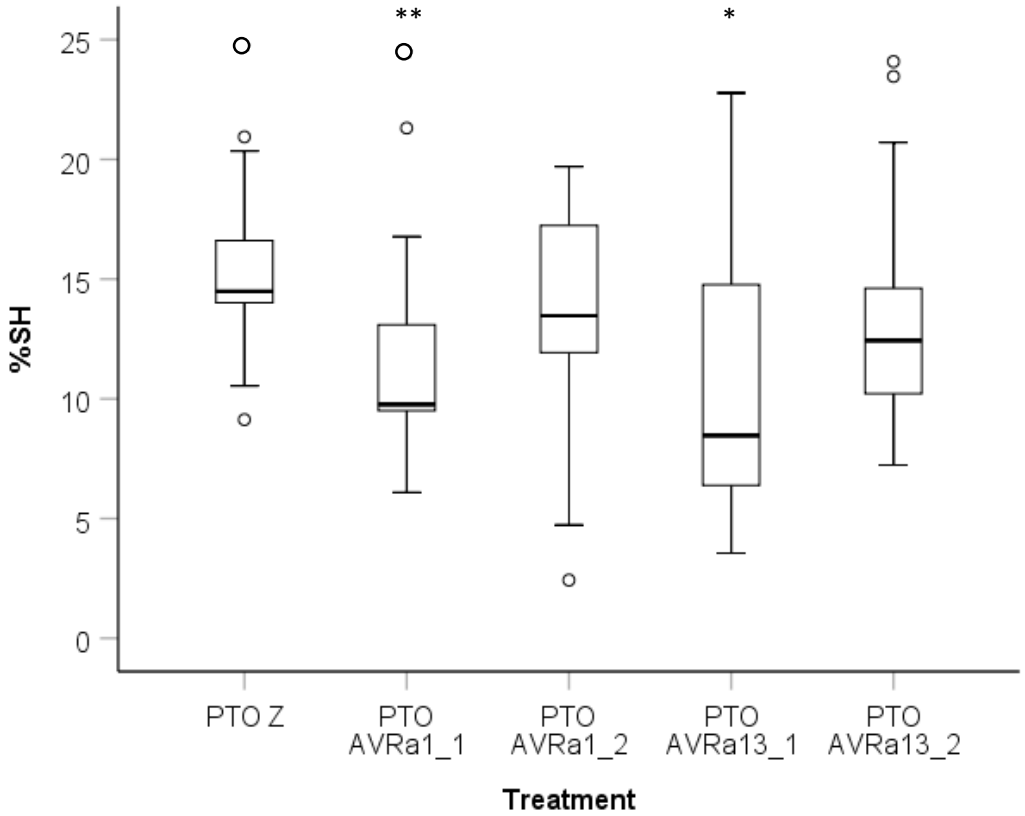


Figure 5.1 Effect of PTOs targeting *AVRa1* and *AVRa13* avirulence genes on secondary hyphae production. N=13 leaf tip segments from three independent experiments. A minimum of 300 conidia were scored per leaf segment. Results are presented as Boxplot showing median, IQR and minimum/maximum values, circles represent outliers of more than 1.5x IQR above the box. Significance was estimated with GLMM and * indicates P<0.05, ** indicates P<0.01.

When compared with PTO Z control, PTO AVRa1_1 and PTO AVRa1_2 reduced secondary hyphae production by 22% and 16%, respectively. PTOs targeting *AVRa13* (PTO AVRa13_1 and PTO AVRa13_2) reduced secondary hyphae production by 31% and 12%, respectively. Two out of the four PTOs tested produced significant reductions, however, only PTO AVRa13_1 showed significant reduction in each of the three independent experiments. Neither of the PTOs targeting *AVRa1* showed reproducibly significant reductions although the cumulative results did show significance.

5.5 Discussion

5.5.1 Virulence of the *AVRa1* and *AVRa13* avirulence genes

Although the *Mla*/*AVRa* resistance gene/avirulence gene interaction has been known about for many years, *AVRa1* and *AVRa13* have only recently been identified (Lu *et al.*, 2016). Recognition of these avirulence genes in cultivars with the cognate R gene leads to the hypersensitive response, preventing fungal proliferation (Skamnioti and Ridout, 2005). In cultivars which do not have the cognate R gene, the pathogen is not detected and infection is able to proceed. The presence of *Mla* alleles across may barley lines would put *Bgh* under

evolutionary pressure to mutate or lose these *AVR* genes which are being detected. The fact that these *AVR* alleles are still present in diverse *Bgh* isolates (Lu *et al.*, 2016) would suggest that these genes have another function in infection.

The *Bgh* isolate used in this study – DH14 – has *AVR* genes which are recognised by *Mla1, Mla6, Mla7, Mla13* and *Mla15* (Ridout *et al.*, 2006; Lu *et al.*, 2016). The barley cultivar, Golden Promise, is fully susceptible to this isolate as it only contains the *Mla8* allele (Seeholzer *et al.*, 2010). This pathosystem is therefore well suited to study the function of these newly isolated *AVR* genes.

Two PTOs were tested to target *AVRa1* and these resulted in moderate reductions in secondary hyphae production. PTO AVRa1_1 was slightly more effective than PTO AVRa1_2 with 22% and 16% reductions compared to PTO Z. However, neither of these PTOs were reproducibly significant in each of the individual experiments completed. *AVRa1* had previously been silenced using HIGS. That study showed a haustorial index of 96% relative to control which indicated there was no virulence function for *AVRa1* (Pliego *et al.*, 2013). Further investigation is needed to determine whether *AVRa1* has maintained any virulence function in *Bgh* or whether its recognition by *Mla1* has driven mutation or loss-of-function to prevent detection.

Two PTOs were also tested to target *AVRa13.* One of these PTOs – PTO AVRa13_1 – showed a significant reduction in secondary hyphae production relative to control treatment. PTO AVRa13_2 resulted in only a 12% reduction compared to PTO Z control indicating that this PTO sequence is not effective at targeting *AVRa13*. The significant reduction in secondary hyphae production seen following PTO AVRa13_1 treatment shows that *AVRa13* has a virulence function which has not been determined. This virulence function must outweigh the detrimental effect that the presence of *AVRa13* has in the presence of the *Mla13* resistance gene allele.

The AVRa13_1 PTO sequence is located across the intron/exon junction which shows that unlike the *Bln1* PTO, this must be acting on the mature mRNA once the intron has been spliced. This may indicate that different modes of action may be used in different cases but whether this is on a gene by gene basis or whether different mechanisms are in use in plants and fungi cannot be determined from this.

These *AVR* genes have structural similarity to ribonucleases, as do the *BEC1011* and *BEC1054* effectors. Sequence homology did not initially suggest that these effectors/*AVR*

genes were related (Lu *et al.*, 2016) but the presence of a conserved intron in these four genes, as well as homologous *AVR* genes from *Blumeria graminis* f.sp. *tritici,* suggests that they may have had a common ancestor which was a ribonuclease (Spanu, 2017). Many of these ribonuclease-like effectors were identified as proteins in infected barley epidermis (Bindschedler *et al.*, 2009) and this abundance may account for why these proteins were targeted by *R* genes. In the process of diversification of these RNase-like proteins, potentially driven by evolutionary pressure of *R* gene recognition, these may have found new functions or have lost their function altogether.

5.5.2 Future work

Further investigation, including the use of qRT-PCR may help to elucidate any virulence function of *AVRa1* or whether the avirulence effect in the presence of *Mla1* has led to loss of function of this gene.

6 Using PTOs to investigate other potential factors in the barley – powdery mildew interaction

6.1 Introduction

Pathogenesis-related (PR) proteins are proteins produced by plants in response to pathogen attack (Antoniw *et al.*, 1980). Seventeen families of PR protein have been described based on their biological and biochemical activity (van Loon *et al.*, 2006) with each family based on an archetypal member, generally the first identified member. Many of the identified PR families have members in barley which are implicated in the interaction with *Bgh* (Table 6.1).

PR family	**Type member**	**Activity**	***Hv-Bgh* example?**	**Ref**
PR1	Tobacco PR-1a	Unknown	Yes	(Antoniw *et al.*, 1980; Gregersen *et al.*, 1997; Schultheiss *et al.*, 2003)
PR2	Tobacco PR2	B-1,3-glucanase	Yes	(Antoniw *et al.*, 1980; Gregersen *et al.*, 1997)
PR3	Tobacco P, Q	Chitinase type I,II,IV,V,VI,VII	Yes	(Gregersen *et al.*, 1997)
PR4	Tobacco "R"	Chitinase type I,II	Yes	(Gregersen *et al.*, 1997)
PR5	Tobacco S	Thaumatin-like	Yes	(Hejgaard *et al.*, 1991; Gregersen *et al.*, 1997)
PR6	Tomato Inhibitor I	Proteinase inhibitor		
PR7	Tomato P_{69}	Endoproteinase		
PR8	Cucumber chitinase	Chitinase type III		
PR9	Tobacco "lignin-forming peroxidase"	Peroxidase		
PR10	Parsley "PR1"	Ribonuclease-like		
PR11	Tobacco "class V" chitinase	Chitinase type I		
PR12	Radish Rs-AFP3	Defensin		
PR13	Arabidopsis TH12.1	Thionin		
PR14	Barley LTP4	Lipid transfer protein		
PR15	Barley OxOa	Oxalate oxidase		
PR16	Barley OxOLP	Oxalate oxidase-like		
PR17	Tobacco PRp27	Unknown	Yes	(Christensen *et al.*, 2002; Zhang *et al.*, 2012)

Table 2.1 Type members and activity of the seventeen known families of PR proteins (modified from van Loon *et al.*, 2006).

Some of the PR proteins which have been found to be upregulated during *Bgh* infection have also been shown to specifically interact with certain *Bgh* effectors. Barley PR1 and PR17c proteins were found to specifically interact with CSEP0055 (Zhang *et al.*, 2012). Having been confirmed as a virulence factor, interactors of *CSEP0055* were investigated using a yeast-two-hybrid screen. PR17c was identified as a potential interactor and confirmed using biomolecular fluorescence complementation (BiFC). PR17c accumulates apoplastically around the site of papilla formation where it interacts with CSEP0055 following infection. Silencing of PR17c resulted in increased susceptibility to *Bgh* whereas overexpression reduced *Bgh* infection, indicating PR17c has a role in barley defence against powdery mildew (Zhang *et al.*, 2012).

Another PR protein – PR5 – was shown to interact with the BEC1054 effector. A protein pull down identified a number of potential interactors and PR5 was confirmed to interact with BEC1054 by a yeast-two-hybrid screen (Pennington *et al.*, 2016a). The PR5 family of PR proteins were identified as having high sequence similarity with thaumatin, a sweet tasting protein from the plant *Thaumatococcus daniellii,* and are therefore called thaumatin-like proteins (TLPs) (Velazhahan *et al.*, 1999). TLPs have been identified in both monocot and dicot plants and also in fungi, insects and nematodes (Liu *et al.*, 2010). They are not only induced following pathogen infection but also in response to abiotic stresses such as drought, cold or wounding (Velazhahan *et al.*, 1999; van Loon *et al.*, 2006). This would indicate a broad role in plant protection.

A number of PR5 proteins have been structurally analysed and contain a high number of cysteine residues which are predicted to form eight disulphide bonds (Breiteneder, 2004). The protein structure has three domains (as shown in figure 6.1) and a characteristic cleft between the first and second domain which is predicted to be the site of ligand binding (Liu *et al.*, 2010). This cleft is acidic in PR5 proteins which have shown antifungal activity and it is predicted that basic clefts in other PR5 proteins may facilitate binding of different molecules and have different function (Batalia *et al.*, 1996; Koiwa *et al.*, 1999; Liu *et al.*, 2010).

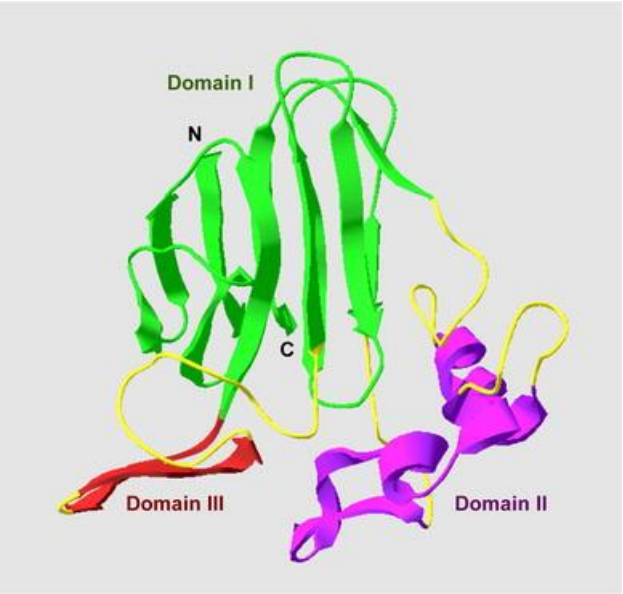


Figure 6.1 Structure of PR5 proteins with three domains. Diagram taken from (Liu *et al.*, 2010)

PR5 isoforms in numerous plant species have antifungal activity against a range of pathogens including *Candida albicans, Trichoderma viridae* and *Botrytis cinerea* (Hejgaard *et al.*, 1991; El-kereamy *et al.*, 2011; Rout *et al.*, 2016). Increased expression of *PR5* proteins have also been recorded in response to other pathogens including *Fusarium oxysporum, Puccinia triticina* and *Blumeria graminis* (Hejgaard *et al.*, 1991; Li *et al.*, 2015; Rout *et al.*, 2016). Although *PR5* has increased expression following *Bgh* infection, no antifungal activity against *Bgh* has been identified (Bryngelsson and Green, 1989; Collinge *et al.*, 1997) and the function of PR5 proteins has yet to be elucidated.

6.2 Aims and Objectives

Aim: To investigate the role of barley *Pr5* during *Bgh* infection

Objective: To design phosphorothioate modified ODNs to target *Pr5* and quantify the effect on development of *Bgh* secondary hyphae and *Pr5* transcript abundance.

6.3 Materials and methods

6.3.1 PTO design and treatment

OligoWalk software was used to design a 19mer PTO to target a barley *PR5* isoform (Genbank KP293850) which has been shown to interact with BEC1054. PTO PR5.1 was used to treat seven-day old barley primary leaves. Following 24 h treatment, leaf sections were laid on 0.6% agar plates with 20 mg/ml benzimidazole and inoculated with *Bgh* spores. Spore density was measured with a haemocytometer. Leaf sections were sampled 48 hpi and either flash frozen in liquid nitrogen for RNA extraction or stained with lactophenol cotton blue and destained in ethanol:acetic acid. Stained leaf sections were mounted on

glass slides in 20% glycerol and examined under a light microscope at 250x magnification. Number of conidia, conidia with appressoria and conidia with secondary hyphae were recorded. Successful infection was quantified as proportion of conidia with secondary hyphae as secondary hyphae are only produced following successful haustorial formation.

6.3.2 RNA extraction, cDNA synthesis and qRT-PCR

Flash frozen leaves were ground in liquid nitrogen with quartz sand and RNA extracted using RNeasy plant mini kit (QIAGEN) as previously described in chapter 2.4. RNA was eluted in 40 µl RNase free water and yield quantified using a Nanodrop-1000 spectrophotometer (ThermoScientific).

cDNA was synthesised by reverse transcription of 1 µg RNA using Quantitect Reverse Transcription kit (QIAGEN) as previously described in chapter 2.5

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on 20 ng cDNA using Rotorgene Q quantitative PCR cycler (QIAGEN) as described in chapter 2.6.

6.4 Results

6.4.1 Silencing of *PR5* reduces barley susceptibility to *Bgh*

Seven-day old barley primary leaves were treated with 10 µM PTO targeted to *PR5* or 10 µM control PTO Z for 24 h before inoculation with *Bgh* spores. Leaf sections were sampled 48 hpi and stained with lactophenol cotton blue to visualise fungal structures on the leaf surface. Success of *Bgh* infection was quantified as proportion of conidia which produced secondary hyphae as this indicates there has been successful haustorial formation (Fig. 6.2).

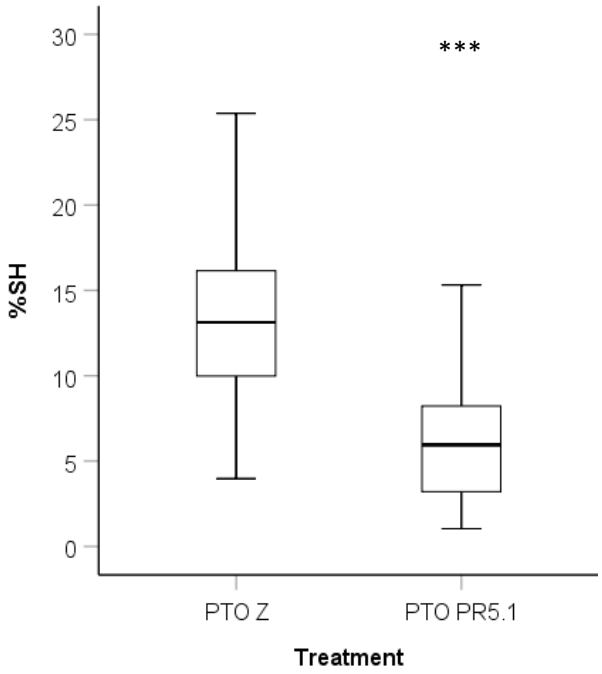


Figure 6.2 Effect of targeting *PR5* with PTO silencing molecules on secondary hyphae percentage. N=43 leaf tip segments from seven independent experiments. A minimum of 300 conidia were scored per leaf segment. Results are presented as Boxplot showing median, IQR and minimum/maximum values. Significance was estimated with GLMM and * indicates P<0.001.**

Targeting *PR5* with PTO PR5.1 resulted in 54% reduction in secondary hyphae production relative to PTO Z control treatment. This indicates that *PR5* has a negative effect on barley resistance.

6.4.2 *Pr5* transcript abundance is reduced following PTO PR5.1 treatment

Leaf samples treated with PTO PR5.1 or PTO Z control and inoculated with *Bgh* spores were sampled 48 hpi and RNA extracted. qRT-PCR was performed to assess abundance of *PR5* transcript in PTO Z and PTO PR5.1 treated leaves. Transcript abundance was calculated using the Pfaffl method relative to the barley housekeeping gene *GAPDH* (Pfaffl, 2001) (Fig. 6.3).

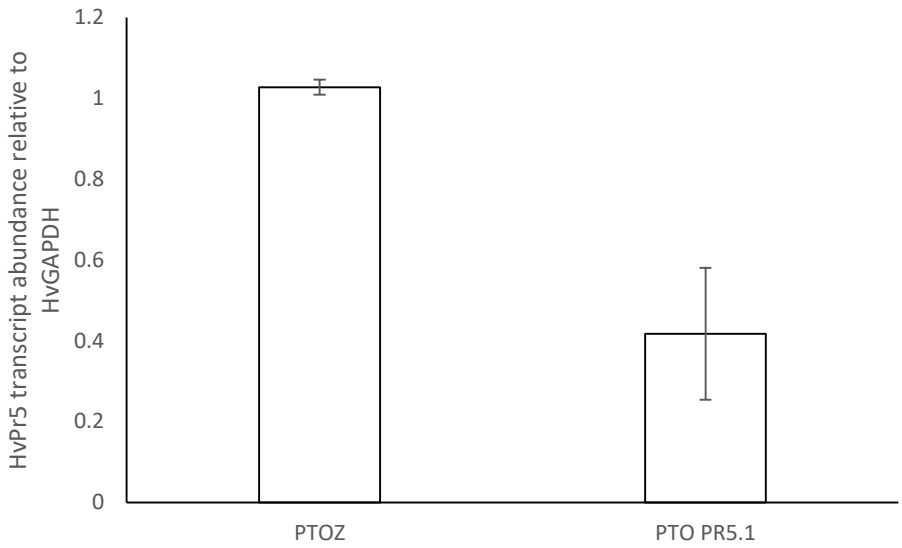


Figure 6.3 Relative *PR5* transcript abundance in leaves treated with PTO Z or PTO PR5.1, N=4 independent experiments, each with 3 technical replicates. Error bars show standard error of the mean.

Leaves treated with PTO PR5.1 showed a 60% reduction in *PR5* transcript abundance relative to leaves treated with PTO Z. This is comparable to the reduction in secondary hyphae production seen microscopically. This shows that PTO PR5.1 is successful in silencing *PR5.*

6.4.3 *PR5* transcript abundance is reduced following *BEC1011* or *BEC1054* silencing

PR5 transcript abundance was also measured in leaves treated with PTO 11.11 or PTO 54.11 targeting *BEC1011* or *BEC1054,* respectively (Fig. 6.4).

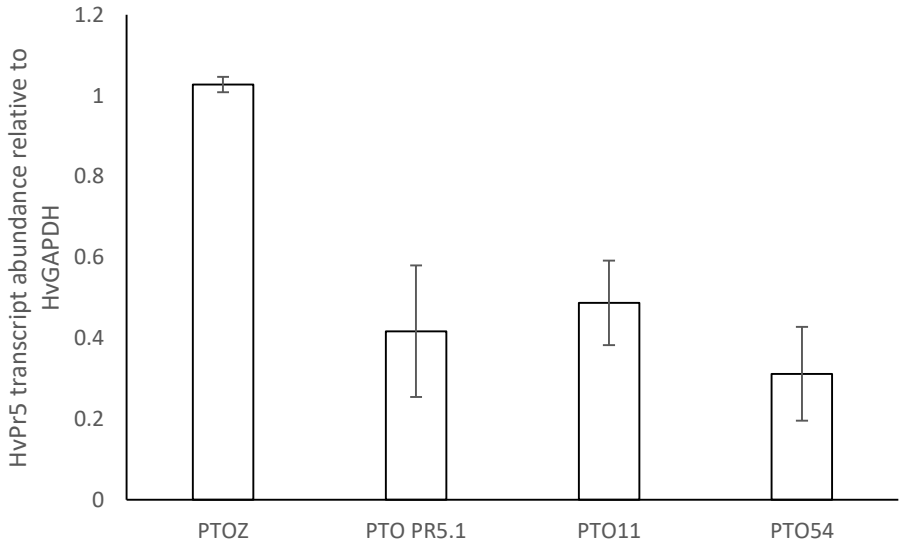


Figure 6.4 Relative *Pr5* transcript abundance in leaves treated with PTO Z, PTO PR5.1 (N=4 independent experiments with three technical replicates), PTO 11.11 (N=3 independent experiments with three technical replicates) or PTO 54.11 (N=2 independent experiments with three technical replicates). Error bars show standard error of the mean.

qRT-PCR showed 53% reduction in *PR5* transcript abundance following silencing of *BEC1011* and 70% reduction in *PR5* transcript abundance following silencing of *BEC1054.* The effect of *BEC1054* silencing on *PR5* transcript abundance may confirm that BEC1054 interacts with PR5 but the similar effect seen with *BEC1011* silencing may suggest that BEC1011 and PR5 also interact.

6.4.4 PR5 protein abundance is increased in infected leaf material

Specific peptides to identify PR5 proteins using MRM-MS have been developed and were included in two MRM-MS experiments. These showed between two and ten-fold increase in PR5 protein in samples infected with *Bgh* compared to non-infected samples, as shown in figure 6.5.

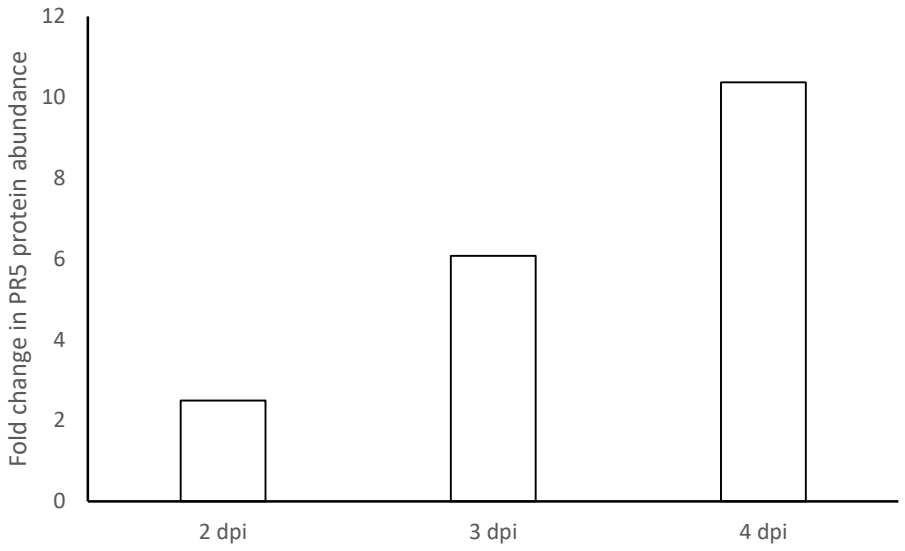


Figure 6.5 Fold change in PR5 protein abundance in leaves 2, 3 and 4 days post inoculation with *Bgh* spores, relative to PR5 protein abundance in non-infected leaves, measured using MRM-MS.

The PR5 protein continued to accumulate up to the latest time point tested which was four days post inoculation. This might suggest that PR5 is involved in later stages of infection.

6.5 Discussion

6.5.1 *PR5* acts as a negative regulator of resistance in barley

Silencing of *PR5* using PTO PR5.1 resulted in a significant reduction in production of secondary hyphae relative to control treatment and reduction in *PR5* transcript abundance. Previous research has shown that PR5 proteins and thaumatin-like proteins have anti-fungal properties (Hejgaard *et al.*, 1991). This led to the assumption that the interaction between PR5 and BEC1054 was as a function of the defence response against *Bgh*.

However, the reduction in secondary hyphae production following *PR5* silencing suggests that *PR5* is actually acting as a susceptibility factor. Secondary to this, the reduction in *PR5* transcript following *BEC1011* or *BEC1054* silencing suggests that *PR5* is not upregulated in response to *Bgh* infection but that these effectors recruit *PR5* to promote virulence.

Barley *PR5* was identified in barley challenged with *Bgh* (Bryngelsson and Green, 1989) and PR5 proteins have been shown to be antifungal against multiple pathogens (Hejgaard *et al.*, 1991; Vigers *et al.*, 1992; El-kereamy *et al.*, 2011; Rout *et al.*, 2016) but has not been shown to be anti-fungal against *Bgh* specifically (Collinge *et al.*, 1997). PR5 proteins that have shown antifungal activity have an acidic cleft between the first and second domains of the protein, however, this barley PR5 is basic and this is predicted to allow for other, but as yet unspecified functions (Breiteneder, 2004; Liu *et al.*, 2010).

Given this evidence, it is possible that this *PR5* isoform is acting as a susceptibility genes during the interaction with *Bgh*. Like *Bln1*, *PR5* is induced following infection and silencing results in a reduction in infection. However, much further investigation is required to elucidate the specific role of PR5 in this interaction.

A more detailed analysis of PR5 protein accumulation during infection using MRM-MS should give an indication of which stage of infection is affected. Other proteomics techniques such as protein pull down or yeast-two-hybrid screens could be used to identify both barley and *Blumeria* proteins that are interacting with PR5.

Understanding this possible appropriation of what has been described as a ubiquitous defence mechanism by this pathogen could help to give a much broader understanding of plant pathogen interactions.

7 Analysis of hydrogen peroxide production in infected leaves following PTO treatment

7.1 Introduction

The production of reactive oxygen species (ROS), such as hydrogen peroxide, has long been linked with plant defence against pathogen attack. An oxidative burst is produced as a characteristic response of PTI following detection of PAMPs such as flagellin, chitin or elongation factor Tu by pathogen recognition receptors (Boller and Felix, 2009). This first phase of ROS production occurs in both compatible and incompatible interactions and a second phase of ROS production occurs in incompatible reactions following *R* gene recognition (Bolwell and Daudi, 2009). The oxidative burst is produced by respiratory burst oxidase homologues (RBOHs). A barley *RBOHF2* gene is induced in response to biotrophic and necrotrophic pathogens (Trujillo *et al.*, 2006; Lightfoot *et al.*, 2008). Knockdown of this *HvRBOHF2* increases susceptibility to *Bgh* but this has also been shown to be dependent on the physiological age of the leaves, with older leaves being less susceptible than younger leaves (Proels *et al.*, 2010; Torres *et al.*, 2017).

Specific roles of ROS have been shown in the interaction of barley and *Bgh.* H_2O_2 production has been shown in two specific locations during *Bgh* infection of barley. Firstly, it is found in papillae at the site of attempted penetration where it is involved in cross-linking of glycoproteins to prevent penetration (Levine *et al.*, 1994; Thordal-Christensen *et al.*, 1997; Daudi *et al.*, 2012). Secondly, it is present in epidermal cells undergoing HR following attempted penetration (Thordal-Christensen *et al.*, 1997).

Cellular production of H_2O_2 can be visualised using 3,3-diaminbenzidine stain which produces a brown stain in the presence of H_2O_2. The staining of plant cell wall appositions, called papillae, that successfully prevent penetration have been shown to produce a stronger colour of stain than papilla at the site of successful penetration (Hückelhoven and Kogel, 2003).

7.2 Aims and Objectives

Aim: To investigate the effect of silencing *BEC1011* or *PR5* on production of hydrogen peroxide.

Objective: To use DAB to stain infected barley leaves treated with PTO 11.11 or PTO PR5.1 and microscopically assess production of hydrogen peroxide.

7.3 Materials and Methods

7.3.1 PTO treatment of barley primary leaves

In order to assess the effect of PTO silencing on the production of hydrogen peroxide following infection of barley with *Bgh*, seven-day old barley primary leaves were treated with PTOs targeting the *Bgh* effector *BEC1011,* barley pathogenesis-related gene *PR5* or control PTO Z as previously described. Leaf sections were sampled 18 hpi for staining with DAB.

7.3.2 Staining leaves with 3,3-diaminobenzidine

A 1 mg/ml solution of 3,3-diaminobenzidine (DAB) was prepared as described in chapter 2.15. DAB was solubilised in water with the addition of 0.1 M HCl to achieve a pH around 3.8. After solubilisation sodium acetate buffer (pH 7) was added to increase pH for more favourable conditions for the leaves. The tips of the leaf sections were submerged in DAB solution and vacuum infiltrated before placing on damp filter paper for 4 h to allow DAB stain to develop. Leaf tips were then stained with lactophenol cotton blue, as previously described, for visualisation of *Bgh* and destained in ethanol: acetic acid.

Leaf sections were mounted on glass slides in 20% glycerol and viewed with a light microscope at 400x magnification. Number of non-germinated conidia and conidia with appressoria were counted (secondary hyphae have not formed by 18 hpi), as well as DAB stained papillae associated with germinated conidia and whole or partial cells stained with DAB associated with germinated conidia.

7.4 Results

7.4.1 Leaves treated with PTO 11.11 or PTO PR5.1 to silence *BEC1011* and *PR5* show increased H_2O_2 production

Following treatment with PTO 11.11 or PTO PR5.1, or water or PTO Z controls, the percentage of germinated conidia associated with cells showing whole cell or partial cell DAB staining was assessed microscopically. Figure 7.1 shows a barley epidermal cell stained with DAB indicating the HR response following attempted penetration by *Bgh.*

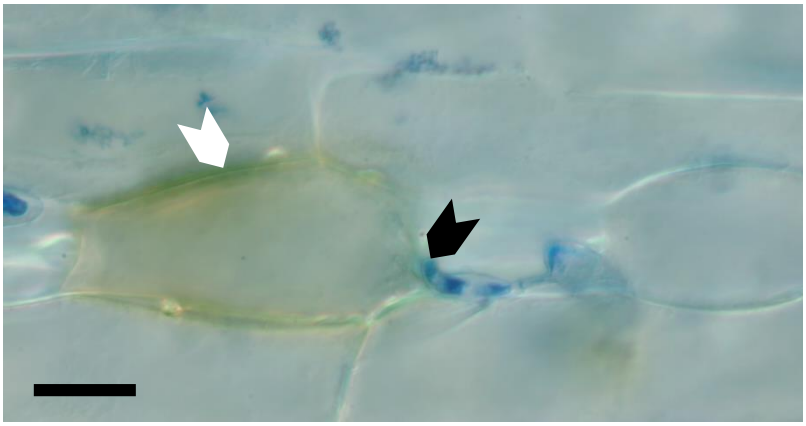


Figure 7.1 Light microscopy image of infected barley leaf stained with 3,3-diaminobenzidine to stain hydrogen peroxide and lactophenol cotton blue to stain fungal structures. An epidermal cell is stained pale yellow (white arrow) with DAB indicating whole cell hydrogen peroxide production in response to attempted penetration by *Bgh* appressorium (black arrow). Bar =20 µm. Photo courtesy of Alessio Bertalone, MSc student, RHUL.

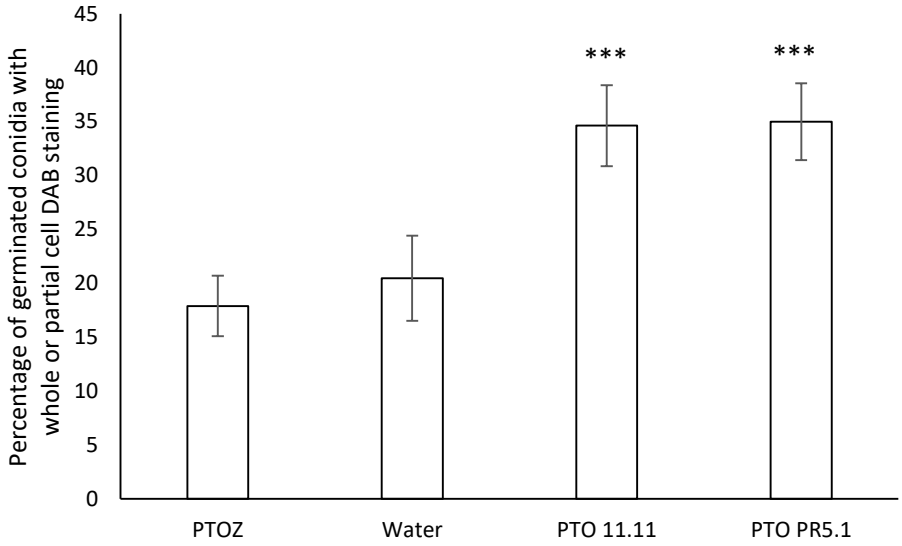


Figure 7.2 Percentage of germinated conidia associated with whole or partially DAB stained cells in leaves treated with PTO Z or water controls or PTO 11.11 or PTO PR5.1. N=11 leaves from two independent experiments. Error bars show standard error of the mean. Significance calculated with GLMM. * indicates P<0.001.**

In leaves treated with PTO 11.11 or PTO PR5.1, there was whole or partial cell DAB staining associated with germinated conidia almost twice as often as in control (PTO Z or water) treated leaves. This would indicate that the plant is reacting to pathogen attack at a higher rate (Fig. 7.2).

DAB staining is also present around the papilla. The intensity of this staining was recorded in a semi-quantitative way using a colour scale from light yellow to dark brown with 1 = light yellow, 2 = dark yellow, 3 = light brown and 4 = dark brown. Figure 7.3 shows examples of papillae with staining at the four different recorded colour intensities.

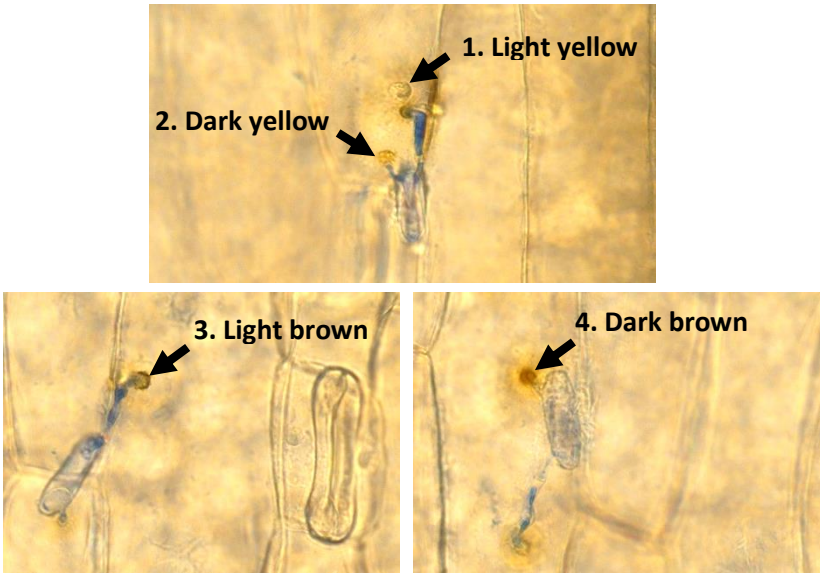


Figure 7.3 Light microscopy of infected barley leaves stained with DAB and lactophenol cotton blue. Arrows indicate examples of papillae with staining of the four different colour intensities recorded.

The number of papilla at each colour intensity was summed and divided by the total number of papillae to give a colour intensity index. The colour intensity index was calculated for leaves treated with PTO Z, water, PTO 11.11 or PTO PR5.1 (Fig. 7.4).

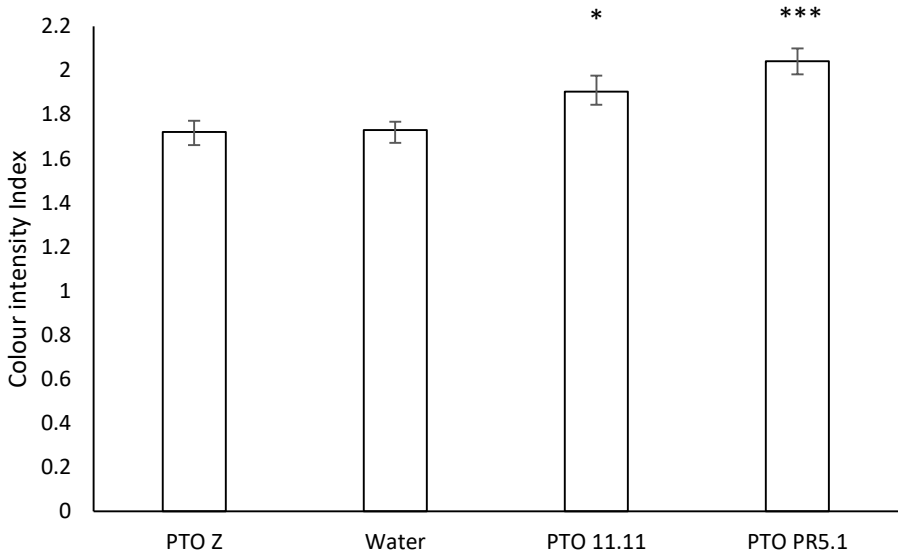


Figure 7.4 Colour intensity index of DAB stained papillae in leaves treated with PTO Z, water, PTO 11.11 or PTO PR5.1. Error bars show standard error of the mean. Significance calculated with X^2 test. * indicates P<0.05, * indicates P<0.001.**

Leaves treated with PTO 11.11 or PTO PR5.1 have a significantly higher colour intensity index than control (PTO Z or water) treated leaves (Fig. 7.4). This indicates that the plants are showing a stronger response to infection when *BEC1011* or *PR5* are silenced.

7.5 Discussion

Hydrogen peroxide is known to accumulate around papillae and cells undergoing HR following infection of barley with *Bgh*. DAB staining was used to visualise H_2O_2 in leaves treated with PTO Z, water, PTO 11.11 or PTO PR5.1. Silencing of *BEC1011* or *PR5* resulted in a significant increase in whole or partial cells stained with DAB, indicating that they are undergoing HR. A colour intensity index was calculated to assess the intensity of DAB staining around papillae in PTO treated leaves because stronger DAB staining is associated with successful prevention of penetration (Hückelhoven and Kogel, 2003). Silencing of *BEC1011* or *PR5* also resulted in a stronger H_2O_2 response around papillae. This would indicate an improved physiological response of the plant to infection. H_2O_2 is associated with protein cross-linking to form physical barriers to fungal penetration and silencing of this pathogen effector or this barley susceptibility factor have resulted in increased production of H_2O_2. Whether or not BEC1011 and PR5 directly contribute to H_2O_2 suppression during successful infection, for example by blocking production of H_2O_2, or indirectly, by manipulating the processes which ordinarily initiate the ROS burst, cannot be determined from this work. Given that one is a pathogen effector and one a plant pathogenesis-related protein which may be a susceptibility factor, it would seem unlikely that they are both having a direct effect on ROS production but that whatever their role in virulence or susceptibility leads to this phenotype.

8 General Discussion and conclusion

The aim of this project was to develop a methodology to investigate virulence and susceptibility factors in the barley-powdery mildew pathosystem. Antisense oligonucleotides were chosen to target genes involved in this interaction and to see if this technology was applicable to plant pathogen interactions.

8.1 PTO silencing of factors involved in *Blumeria* infection of barley

A cut leaf assay was optimised based on previous studies (Sun *et al.*, 2005, 2007; Dinc *et al.*, 2011) for delivery of antisense oligonucleotides to barley leaves to target *Bgh* effectors and barley susceptibility genes. The use of phosphorothioate modified ODNs were found to be more effective than non-modified ODNs. The PTO modification has been used widely in animal and plant studies (Campbell *et al.*, 1990; McKay *et al.*, 1999; Carroll *et al.*, 2011; Dinc *et al.*, 2011; Liao *et al.*, 2013) because it prolongs the longevity of the ODN in the cell without affecting recruitment of RNase H, one of the ways ODNs are predicted to induce gene silencing. Clinical applications of ODNs often use second or third generation modifications such as phosphorodiamidate morpholinos, 2'-O-methyoxyethyl modification or locked nucleic acids to increase the efficiency of ODNs and substantial research has been done to improve the delivery of ODNs into cells by nanoparticle delivery or cholesterol conjugation, for example (McClorey and Wood, 2015). The use of the PTO modification in the barley-*Bgh* system has led to significant phenotypes and would suggest that more expensive modifications are not necessary in a functional genomics context.

The PTO silencing method was initially tested using three *Bgh* effectors which had already been confirmed as virulence factors – *BEC1011, BEC1019* and *BEC1054* (Pliego *et al.*, 2013). Following confirmation of the efficacy of the PTO silencing effect by microscopically monitoring formation of secondary hyphae, two further candidate effectors were targeted with PTOs. These had been identified as avirulence factors – *AVRa1* and *AVRa13* which are recognised by barley resistance genes from the *Mla* locus but any potential virulence function has not been investigated (Lu *et al.*, 2016). PTO silencing of *AVRa13* showed a significant reduction in formation of secondary hyphae suggesting that *AVRa13* maintains a virulence function in *Bgh* despite the recognition by barley *Mla13* alleles leading to resistance. Targeting of *AVRa1* with PTOs did result in some reduction in formation of secondary hyphae which was significant overall but had lower reproducibility.

Although Golden Promise does not contain *Mla* alleles which would recognise either *AVRa1* or *AVRa13,* genes such as these that are detected by the host are under diversifying

selection. Logic would suggest that as these are proteins which are secreted by *Bgh*, they would have a function in infection and the fact that *AVRa13*, and possibly *AVRa1*, have maintained an effect on infection suggests that to lose these genes would be more costly to the pathogen than recognition by some barley lines (Brown, 2003; Ridout *et al.*, 2006). Diversifying selection may also lead to redundancy amongst avirulence genes which could also account for limits in the effect of silencing.

Due to lack of recognition by plant resistance genes, it might be expected that BECs or CSEPs which are not *AVR* genes would be under less pressure to diversify but it has been shown that CSEPs are under varying degrees of diversifying selection with haustorially expressed CSEPs under the highest pressure and family 21, of which *BEC1011* and *BEC1054* are members, shows the highest tendency towards diversification (Pedersen *et al.*, 2012; Hacquard *et al.*, 2013).

The size of the *Bgh* candidate effector complement (over 500) would suggest a high level of redundancy or that many have lost their virulence function. This raises a significant question. Why does silencing one effector out of 500 have such a great effect on pathogen virulence? Silencing single effectors using HIGS, VIGS or PTO based silencing has resulted in up to 70% reduction in *Bgh* infection rate and over 20 have a virulence phenotype (Zhang *et al.*, 2012; Pliego *et al.*, 2013; Ahmed *et al.*, 2015, 2016; Whigham *et al.*, 2015; Aguilar *et al.*, 2016). There are several proposed ideas which may contribute to this.

The original CSEP candidate list contained 491 genes and was identified using the genome of the DH14 isolate of *Bgh* (Spanu *et al.*, 2010; Pedersen *et al.*, 2012). Subsequently a further two isolates of *Bgh* have been sequenced and their respective effector complements compared with that of DH14 (Hacquard *et al.*, 2013). This study found that of the 491 CSEPs identified in DH14, only 5 or 6 were not found in the genomes of isolates A6 or K1. This shows that despite wide geographic distances between where these isolates are found, the effector complement has remained stable and may indicate that most if not all have an important role in fungal virulence or fitness.

Another suggested answer to the question of the effect on silencing single effectors is that may work together, by forming complexes, in signalling cascades or in infection processes (Ahmed *et al.*, 2016). In these situations, loss of individual effectors may disrupt the outcome even where multiple factors are involved. One observation that may support this idea is the “big BEC” theory – that the high molecular weight bands seen in western blots

for BEC1011 are due to complex formation of BEC1011 with other proteins, although there is no direct evidence of this as yet.

Whilst HIGS has been able to confirm the virulence function of 21 CSEPs/BECs, a further 61 CSEPs have been silenced using HIGS and showed no reduction in haustorial formation (Pliego *et al.*, 2013; Aguilar *et al.*, 2016; Ahmed *et al.*, 2016). This may be because these CSEPs really do not have a role in virulence but it may be due to limitations of the HIGS system that PTO silencing may improve. Single cell bombardment used in HIGS limits the observable phenotype to whether individual cells are penetrated and haustoria successfully form. In this situation, effectors involved in nutrient uptake or fungal fitness after haustorial formation would not produce a phenotype. Using a PTO silencing approach, it is possible to visually assess growth of *Bgh* past the stage of haustorial formation where effectors which have a role in nutrient uptake would show a phenotype of reduced secondary hyphal growth. Whole leaf treatment using PTOs also allows for assessment of target mRNA or protein, giving further confirmation of visual phenotypes recorded and that silencing has been successful. Although PTO silencing of elements of the barley-*Bgh* pathosystem is still limited to the early stages of infection, recent studies showing spray application of dsRNA can induce silencing (Koch *et al.*, 2016), it seems possible that the PTO silencing workflow can be modified to investigate effectors involved at later stages of infection by using spray treatment rather than cut leaf assays.

The decrease virulence phenotype observed following silencing of the known effectors *BEC1011* and *BEC1019* was also confirmed using qRT-PCR to assess target transcript abundance and fungal biomass. PTO silencing of either of these effectors resulted in significant decrease in fungal biomass and a moderate reduction in effector transcript abundance.

Silencing these *Bgh* genes resulted in significant effects and therefore it can be deduced that the PTOs are transported across the extrahaustorial membrane, through the extrahaustorial matrix and the fungal cell wall into the haustorium. However, there is no indication of the mechanism of uptake. Some studies using ODNs in plants reported that sucrose was required for uptake into cell, suggesting that they are taken up through sucrose transporters (Sun *et al.*, 2005, 2007). This observation was not corroborated by other studies (Dinc *et al.*, 2011) and sucrose was not found to be required for uptake in this system (Laurence Bindschedler, RHUL, personal communication) but it might be that PTOs are taken up from plant cells into the haustoria through the same transporters that the

fungus uses for nutrient uptake from the plant. All of the *Bgh* effectors targeted are expressed highly in the haustoria so from this data alone it cannot be confirmed that PTOs are transported further than the haustoria to other fungal tissues. Previous reports using VIGS to silence *Puccinia* genes in wheat showed that significant results were only seen for genes expressed in haustoria and not those constitutively expressed or expressed highly in other fungal tissues (Yin *et al.*, 2011). However, initial results have shown that silencing of two constitutively expressed, fundamental structural or enzymatic genes, *GAPDH* or *actin* in *Bgh* using PTOs results in similar effects on *Bgh* virulence to those seen when effectors have been targeted (Shaoli Das Gupta, RHUL, personal communication). This may be because the two silencing systems use different mechanisms and therefore it would be predicted that PTOs are transported differently to dsRNA but would also indicate another advantage of PTO silencing over VIGS based systems.

The PTO silencing methodology was also used to silence barley genes which have been shown to confer susceptibility to *Bgh* – *Mlo* and *Bln1* (Jørgensen, 1992; Meng *et al.*, 2009). These barley proteins are recruited by *Bgh* in order to promote virulence. Wild type MLO protein is redistributed within the plasma membrane towards the site of penetration where Ca^{2+} dependent binding of calmodulin appears to supress defence responses (Kim *et al.*, 2002; Bhat *et al.*, 2005). Recruitment of *Bln1* by *Bgh* leads to increased *Bln1* expression in response to infection. It is thought that *Bln1* may have a role in protein trafficking and possibly the transport of effectors (Meng *et al.*, 2009; Xu *et al.*, 2015). Silencing of both these genes using PTO modified ODNs resulted in a reduction in fungal virulence.

Another class of protein that is induced upon *Bgh* infection is the pathogenesis-related proteins. Many of the families of PR proteins have been shown to have anti-fungal properties. This includes the thaumatin-like PR5 family. A barley PR5 isoform interacts with the BEC1054 effector and given the current thinking on the role of PR proteins, it was thought that silencing this PR5 isoform may inhibit the barley defence response and increase susceptibility. However, the virulence phenotype seen following PTO silencing indicates that *PR5* is acting as a susceptibility gene in this interaction.

PR5 induction is reliant on salicylic acid signalling which is linked to the response to biotrophic pathogens (Thomma *et al.*, 1998). Members of the PR5 family have been shown to have an antifungal effect against numerous fungal and oomycete pathogens but these are largely necrotrophic pathogens such as *Alternaria brassicicola, Botrytis cinerea, Rhizoctonia solani* and *Macrophomina phaseolina* (Hejgaard *et al.*, 1991; El-kereamy *et al.*,

2011; Li Liu, 2011; Acharya *et al.*, 2013; Rout *et al.*, 2016). PR5 mediated resistance has also been linked to an increase in phytoalexins such as camalexin in *Arabidopsis* (El-kereamy *et al.*, 2011). Phytoalexins are well-known plant defence compounds but are linked more to response to necrotrophic pathogens than biotrophs (Glazebrook, 2005). This would seem to present contrasting mechanisms surrounding PR5. On one hand, the salicylic pathway is necessary for *PR5* induction but the characterised responses to PR5 are all related to necrotrophs. Increased *PR5* expression has been reported in response to biotrophic or hemibiotrophic pathogens including *Puccinia triticina, Fusarium oxysporum* and *Blumeria graminis* but no direct antifungal activity has been reported against these pathogens (Li *et al.*, 2015; Rout *et al.*, 2016).

Our results have shown up to a ten-fold increase in PR5 protein accumulation following *Bgh* infection but *PR5* gene expression is reduced following silencing of *BEC1011* or *BEC1054* suggesting that these effectors may be involved in recruitment of PR5. The fact that *PR5* silencing also results in an increase in production of reactive oxygen species suggests that PR5 recruitment by *Bgh* allows *Bgh* to avoid detection by barley defence systems.

Combined, this evidence suggests that *PR5* is a negative regulator of plant defence and that the PR5 family may play different roles during infection by biotrophic or necrotrophic pathogens. This all contributes to our understanding of the interaction between barley and *Blumeria*. Effectors such as BEC1011, BEC1019, BEC1054 and AVRa13 are secreted by *Bgh*, probably from the haustoria, and promotes *Bgh* virulecne through interaction with barley. This may include inducing expression of PR5 through direct interaction with BEC1054 and possibly BEC1011. The role of PR5 in this interaction is unclear but the evidence suggests that, similar to *Mlo* and *Bln1,* it negatively regulates resistance of barley to *Bgh.* Figure 8.1 represents the current understanding of the role of PTO silencing and effectors and suseptibility genes in the barley – *Blumeria* interaction.

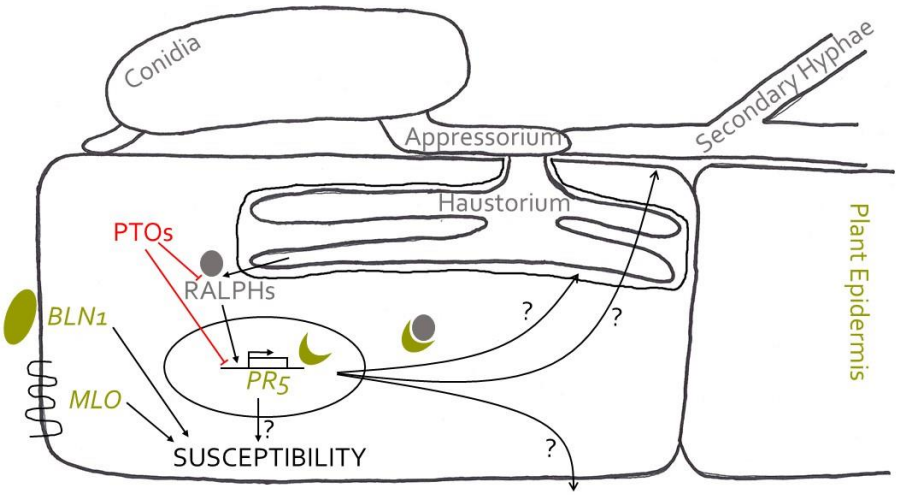


Figure 8.1 Diagrammatic representation of the interaction of effectors and susceptibility genes in the barley-*Bgh* pathosystem

8.2 Mechanism of PTO silencing

In general, there are two ways in which ODNs/ PTOs are thought to induce gene silencing. Either by directing degradation of the DNA/RNA duplex by RNase H, or by binding to the mRNA and preventing ribosome binding and blocking translation (Kurreck, 2003).

Although these experiments were not designed to directly investigate the mechanism of action of PTO silencing in barley or *Bgh,* there are two pieces of evidence that contribute to our understanding of this. Firstly, the PTO targeted to *Bln1* was complementary to a sequence in the intron of the *Bln1* gene and resulted in significant reduction in *Bgh* virulence. As the intron is spliced prior to maturation of the mRNA, it would indicate that the silencing effect occurs before translation, most likely by recruitment of RNase H targeting the immature mRNA for degradation.

It was not known whether silencing would be taking place by the same mechanism in both barley and *Bgh*. Genes encoding RNase H are in the *Bgh* genome so either mode of action is possible in *Blumeria* as well as in barley. One PTO designed to target the *Bgh* gene *AVRa13*, and the PTO that had the greatest effect for this gene, is targeted to the intron/ exon junction. There are two possibilities of how this affects *AVRa13*. Similar to the suggestion for the barley gene *Bln1*, the positioning of this PTO may allow for RNase H targeting of the immature mRNA so it is degraded before translation. However, there is another alternative possibility. The positioning of the PTO across the intron/ exon junction could prevent splicing of the intron leading to a truncated or non-functional protein. This is similar to the

exon skipping techniques that are being developed to treat human diseases that are due to aberrant protein synthesis, such as Duchenne muscular dystrophy (Cirak *et al.*, 2011).

8.3 Application of PTO silencing in research and in agriculture

During this project, I have developed methodology for using PTO silencing as a functional genomic tool for investigating the role of effectors and susceptibility factors in the barley – powdery mildew interaction. This has proved to be a useful tool for investigating plant pathogen interactions, particularly where stable transformation methods are lacking. Investigation of the barley – *Blumeria* interaction had been advanced with the discovery of host induced gene silencing (HIGS) which allowed for transient silencing of *Blumeria* genes through bombardment of barley leaves with silencing constructs targeted to *Bgh* effector genes (Nowara *et al.*, 2010). However, this technique has several limitations. It requires construction of silencing cassettes to target the genes of interest and silencing constructs are delivered to the plant by co-bombardment with GUS-containing plasmids. This means that only single cells are transformed and only provides a very restricted analysis of gene function. It is not possible to analyse the function of genes beyond the stage of haustorial formation and global analysis of target gene downregulation and other downstream effects are very limited. The other means of transfer of silencing constructs to *Bgh* is by virus mediated silencing. However, the effects of using a virus to deliver silencing constructs can affect plant defences and mean that analysis of plant pathogen interactions can be flawed (Tufan *et al.*, 2011).

Using a PTO mediated technique to silence *Bgh* and barley genes allows for whole tissues to be targeted and although the workflow we have developed does not allow for analysis of long term effects, it does allow for determination of the establishment of secondary hyphae and early colonies beyond the formation of haustoria that can be measured in HIGS studies. Treatment of whole tissues rather than single cells also allows for further downstream analysis of the silencing effect. In this study, qRT-PCR was used to assess fungal biomass and target transcript abundance, a workflow for protein quantification by MRM-MS was developed and the effect of silencing on the barley immune response was measured using DAB to stain reactive oxygen species.

As a tool with potential agricultural applications there are also benefits to PTO mediated silencing over silencing methods which require genetic transformation. Regulations pertaining to GM crops, particularly in the EU, are very restrictive and this would limit the application of gene silencing technology through a HIGS method. The use of techniques

that do not contain foreign DNA or genome edited crops are in a less clear situation in terms of EU regulation. Sweden was recently the first country to give an interpretation of the EU legislation to mean that crops which do not contain foreign DNA would not be subject to GM regulations. It is unclear whether short oligodeoxynucleotides targeted towards pathogen genes without any effect on the DNA of the crop plant would be subjected to any degree of regulation.

There are examples of direct applications of dsRNA to crops in what has been termed spray-induced gene silencing. These have generally been applied against insect pests but there have been uses to target fungal pathogens both in academia and in industry (Palli, 2014; Koch *et al.*, 2016; San Miguel and Scott, 2016). There is no reason to think that the cut leaf assay we have used for PTO treatment could not be developed into a spray based treatment. The use of PTOs as a spray based treatment would also have benefits over using dsRNAs as ODNs and PTOs are easier and cheaper to prepare and are more stable than RNA based molecules. A spray treatment would also allow for longer term studies because there is less problem of senescence than with cut leaf assays.

This all indicates that the use of PTO silencing as a functional genomic tool is well established but has scope for further development to broaden the applicability and the development of systems for direct application of dsRNA shows that there is both an industrial interest and the means to develop PTO silencing as an agricultural tool.

8.4 Conclusions

This study has been successful in the development of a workflow to design and deliver phosphorothioate modified antisense oligodeoxynucleotides to silence effectors and susceptibility genes involved in the interaction between barley and the fungal pathogen *Blumeria graminis* f.sp. *hordei*. PTOs have shown a significant effect when used to target genes on both the plant and pathogen sides of the interaction, leading to reduced pathogen virulence, reduced fungal biomass and an increase in the plant immune response of reactive oxygen species production. A specific silencing effect was confirmed by assessing target transcript abundance of two of the effector genes silenced. A mass spectrometry technique was used to assess protein abundance of one of the effectors silenced and although the outputs were limited, further optimisation should result in a useful tool for proteomic analysis of plant pathogen interactions.

Supplementary figures and tables

Supplementary Table 1 Example data set showing the formatting of data for GLMM analysis in R Studio. Each row represents one leaf section. Exp is the date of the experiment, Density is the density of *Bgh* spore inoculum, Conidia is the number of non-germinated conidia counted in a leaf section, App is the number of conidia with an appressorium counted in a leaf section, SH is the number of conidia with secondary hyphae counted in a leaf section, Total is the total number of conidia of all classes in each leaf section, Germ is the number of geminated conidia (conidia with appressorium and conidia with secondary hyphae) in a leaf section.

Treatment	Exp	Density	Conidia	App	SH	Total	Germ
PTOAZ	19/11/2013	35	44	221	49	314	270
PTOAZ	19/11/2013	35	70	251	26	347	277
PTOAZ	19/11/2013	35	33	106	40	179	146
PTOAZ	19/11/2013	35	48	82	18	148	100
PTOAZ	27/01/2014	54	111	335	59	505	394
PTOAZ	27/01/2014	54	93	286	48	427	334
PTOAZ	27/01/2014	54	67	298	60	425	358
PTOAZ	27/01/2014	54	58	296	38	392	334
PTOAZ	24/02/2014	28	103	260	15	378	275
PTOAZ	24/02/2014	28	163	225	10	398	235
PTOAZ	24/02/2014	28	117	196	8	321	204
PTOAZ	24/02/2014	28	105	186	12	303	198
PTOAZ	11/03/2014	67	173	266	69	508	335
PTOAZ	11/03/2014	67	147	279	41	467	320
PTOAZ	11/03/2014	67	80	253	42	375	295
PTOAZ	11/03/2014	67	168	273	40	481	313
PTOAZ	07/04/2014	72	118	266	44	428	310
PTOAZ	07/04/2014	72	120	267	36	423	303
PTOAZ	07/04/2014	72	138	256	39	433	295
PTOAZ	07/04/2014	72	127	254	55	436	309
PTOAZ	20/05/2014	4	150	248	22	420	270
PTOAZ	20/05/2014	4	174	277	27	478	304
PTOAZ	20/05/2014	4	156	278	39	473	317
PTOAZ	20/05/2014	4	108	288	31	427	319
PTOAZ	04/06/2014	40	135	267	56	458	323
PTOAZ	04/06/2014	40	126	284	36	446	320
PTOAZ	10/06/2014	63	111	286	38	435	324
PTOAZ	10/06/2014	63	97	285	37	419	322
PTOAZ	11/11/2014	51	171	247	48	466	295
PTOAZ	11/11/2014	51	247	225	62	534	287
PTOAZ	17/11/2014	67	120	266	48	434	314
PTOAZ	17/11/2014	67	128	246	65	439	311
PTOAZ	17/11/2014	67	119	249	67	435	316
PTOAZ	17/11/2014	67	89	257	62	408	319
PTOAZ	25/11/2014	74	214	277	71	562	348

PTOAZ	25/11/2014	74	162	266	58	486	324
PTOAZ	25/11/2014	74	153	265	57	475	322
PTOAZ	25/11/2014	74	182	238	42	462	280
PTOAZ	12/01/2015	30	199	218	30	447	248
PTOAZ	12/01/2015	30	144	237	15	396	252
PTOAZ	09/03/2015	45	65	219	34	318	253
PTOAZ	09/03/2015	45	87	238	23	348	261
PTOAZ	23/03/2015	55	152	261	58	471	319
PTOAZ	23/03/2015	55	143	258	46	447	304
PTOAZ	23/03/2015	55	130	239	69	438	308
PTOAZ	23/03/2015	55	119	231	56	406	287
PTOAZ	29/03/2016	57	209	263	41	513	304
PTOAZ	29/03/2016	57	257	252	32	541	284
PTOAZ	29/03/2016	57	247	244	41	532	285
PTOAZ	29/03/2016	57	116	234	39	389	273
PTOAZ	29/03/2016	57	234	225	29	488	254
PTOAZ	11/04/2016	53	98	226	43	367	269
PTOAZ	11/04/2016	53	99	208	23	330	231
PTOAZ	11/04/2016	53	110	217	60	387	277
PTOAZ	11/04/2016	53	61	208	64	333	272
PTOAZ	14/06/2016	35	77	224	33	334	257
PTOAZ	14/06/2016	35	61	229	21	311	250
PTOAZ	14/06/2016	35	49	232	37	318	269
PTOAZ	14/06/2016	35	97	256	26	379	282
PTOAZ	14/06/2016	35	88	240	28	356	268
PTOAZ	14/06/2016	35	73	238	21	332	259
PTOAZ	06/09/2016	50	36	216	36	288	252
PTOAZ	06/09/2016	50	108	235	58	401	293
PTOAZ	06/09/2016	50	65	219	48	332	267
PTOAZ	06/09/2016	50	103	217	57	377	274
PTOB11	19/11/2013	35	54	231	11	296	242
PTOB11	19/11/2013	35	67	243	13	323	256
PTOB11	19/11/2013	35	63	130	34	227	164
PTOB11	19/11/2013	35	95	265	18	378	283
PTOB11	27/01/2014	54	81	300	4	385	304
PTOB11	27/01/2014	54	52	263	4	319	267
PTOB11	27/01/2014	54	69	265	8	342	273
PTOB11	27/01/2014	54	97	253	5	355	258
PTOB11	24/02/2014	28	140	181	2	323	183
PTOB11	24/02/2014	28	188	244	4	436	248
PTOB11	24/02/2014	28	73	237	4	314	241
PTOB11	24/02/2014	28	120	201	3	324	204
PTOB11	11/03/2014	67	128	245	8	381	253
PTOB11	11/03/2014	67	160	258	2	420	260

PTOB11	11/03/2014	67	143	259	10	412	269
PTOB11	11/03/2014	67	115	260	11	386	271
PTOB11	07/04/2014	72	149	258	3	410	261
PTOB11	07/04/2014	72	114	276	14	404	290
PTOB11	07/04/2014	72	133	251	9	393	260
PTOB11	07/04/2014	72	137	250	15	402	265
PTOB11	20/05/2014	4	130	283	39	452	322
PTOB11	20/05/2014	4	168	291	13	472	304
PTOB11	20/05/2014	4	149	286	4	439	290
PTOB11	20/05/2014	4	177	297	18	492	315
PTOB11	04/06/2014	40	159	279	11	449	290
PTOB11	04/06/2014	40	176	267	13	456	280
PTOB11	10/06/2014	63	78	274	37	389	311
PTOB11	10/06/2014	63	90	279	21	390	300
PTOB11	11/11/2014	51	188	240	43	471	283
PTOB11	11/11/2014	51	221	248	35	504	283
PTOB11	17/11/2014	67	148	245	45	438	290
PTOB11	17/11/2014	67	112	259	49	420	308
PTOB11	17/11/2014	67	89	266	26	381	292
PTOB11	17/11/2014	67	127	265	55	447	320
PTOB11	25/11/2014	74	184	240	39	463	279
PTOB11	25/11/2014	74	204	242	27	473	269
PTOB11	25/11/2014	74	92	252	38	382	290
PTOB11	25/11/2014	74	167	236	20	423	256
PTOB11	12/01/2015	30	171	223	7	401	230
PTOB11	12/01/2015	30	136	251	17	404	268
PTOB11	09/03/2015	45	97	237	16	350	253
PTOB11	09/03/2015	45	89	253	14	356	267
PTOB11	23/03/2015	55	123	261	20	404	281
PTOB11	23/03/2015	55	235	248	22	505	270
PTOB11	23/03/2015	55	132	241	19	392	260
PTOB11	23/03/2015	55	133	232	9	374	241
PTOB11	29/03/2016	57	171	227	4	402	231
PTOB11	29/03/2016	57	239	270	23	532	293
PTOB11	29/03/2016	57	263	230	13	506	243
PTOB11	29/03/2016	57	212	230	6	448	236
PTOB11	29/03/2016	57	231	215	6	452	221
PTOB11	11/04/2016	53	150	248	15	413	263
PTOB11	11/04/2016	53	72	220	13	305	233
PTOB11	11/04/2016	53	154	203	27	384	230
PTOB11	11/04/2016	53	99	223	24	346	247
PTOB11	14/06/2016	35	147	223	8	378	231
PTOB11	14/06/2016	35	77	248	14	339	262
PTOB11	14/06/2016	35	89	218	17	324	235

PTOB11	14/06/2016	35	101	237	10	348	247
PTOB11	14/06/2016	35	74	220	10	304	230
PTOB11	14/06/2016	35	67	220	10	297	230
PTOB11	06/09/2016	50	61	220	41	322	261
PTOB11	06/09/2016	50	127	200	15	342	215
PTOB11	06/09/2016	50	139	215	33	387	248
PTOB11	06/09/2016	50	92	215	21	328	236

```
> propSH<-cbind(PTO11$SH,PTO11$Germ)
> glmer(propSHPTO11~PTO11$Treatment*PTO11$Density+(1|PTO11$Exp),data=PTO11,f
amily=binomial)
Generalized linear mixed model fit by maximum likelihood (Laplace Approximat
ion) ['glmerMod']
 Family: binomial  ( logit )
Formula: propSHPTO11 ~ PTO11$Treatment * PTO11$Density + (1 | PTO11$Exp)
   Data: PTO11
      AIC       BIC    logLik  deviance  df.resid
1073.1932 1087.5309 -531.5966 1063.1932       125
Random effects:
 Groups    Name        Std.Dev.
 PTO11$Exp (Intercept) 0.3118
Number of obs: 130, groups:  PTO11$Exp, 18
Fixed Effects:
                       (Intercept)                PTO11$TreatmentPTOB11
PTO11$Density  PTO11$TreatmentPTOB11:PTO11$Density
                         -2.544539                           -0.712472
0.011242                          -0.001052
> model2<-glmer(propSHPTO11~PTO11$Treatment*PTO11$Density+(1|PTO11$Exp),data
=PTO11,family=binomial)
> summary(model2)
Generalized linear mixed model fit by maximum likelihood (Laplace Approximat
ion) ['glmerMod']
 Family: binomial  ( logit )
Formula: propSHPTO11 ~ PTO11$Treatment * PTO11$Density + (1 | PTO11$Exp)
   Data: PTO11

     AIC      BIC   logLik deviance df.resid
  1073.2   1087.5   -531.6   1063.2      125

Scaled residuals:
    Min      1Q  Median      3Q     Max
-3.3816 -1.1153 -0.3905  0.8002  5.3849

Random effects:
 Groups    Name        Variance Std.Dev.
 PTO11$Exp (Intercept) 0.09719  0.3118
Number of obs: 130, groups:  PTO11$Exp, 18

Fixed effects:
                                   Estimate Std. Error z value Pr(>|z|)
(Intercept)                       -2.544539   0.230976 -11.016  < 2e-16 **
*
PTO11$TreatmentPTOB11             -0.712472   0.118449  -6.015  1.8e-09 **
*
PTO11$Density                      0.011242   0.004418   2.545   0.0109 *
PTO11$TreatmentPTOB11:PTO11$Density -0.001052   0.002152  -0.489   0.6250
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
             (Intr) PTO11$TrPTOB11 PTO11$D
PTO11$TrPTOB11 -0.171
PTO11$Dnsty    -0.943  0.154
PTO11$TPTOB11:  0.162 -0.950         -0.160
```

Supplementary Box 1 Coding of the GLMM in R Studio. Code sections are sown in blue. Output is shown in black.

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