Phyto-gene therapy using antisense oligonucleotides to control cereal fungal disease by silencing virulence factors and their regulators

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Declaration of Authorship

I, Shaoli Das Gupta, 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: 4th September 2020.

Abstract

With increasing concerns regarding food security, alternative solutions are required for disease control in crops, including those caused by fungal pathogens. Antisense single stranded short oligodeoxynucleotides (ASO) based gene therapy is widely used in medicine but is still emerging in plant sciences. The ASO gene silencing approach using phosphorothioate modified oligodeoxynucleotides (asPTOs) delivered to excised barley leaves was first devised as a tool for *in planta* transient host induced gene silencing (HIGS) to query the virulence role of genes from the biotrophic fungal pathogen, *Blumeria graminis* f.sp. *hordei* (*Bgh*), the causal agent of barley powdery mildew.

Following this, our project aimed at exploiting the HIGS approach for discovering new key players for virulence of *Bgh* and some of the major wheat pathogens, *B. graminis f.sp. tritici (Bgt)* and *Fusarium graminearum,* the causal agent of Fusarium head blight. The ASO gene silencing approach was also evaluated for its suitability to protect wheat against fungi by targeting host susceptibility genes.

AsPTOs to silence vital *Bgh* genes (*actin*, *GAPDH*, *2-Glycosyl transferase*) successfully reduced powdery mildew infection in several barley cultivars. Similarly, silencing the metallo-protease-like effector *BEC1019* impacted on *Bgh* and *Bgt* virulence in barley and wheat respectively. Following promoter sequence analysis of *Bgh* effectors expressed in haustoria, the HIGS approach allowed to confirm the implication of ZAP1 and PacC transcription factors in regulating *BEC1019* and *BEC1011* effector expression, while affecting *Bgh* virulence.

To adapt ASO based gene silencing for disease control, *in planta* gene silencing of *F. graminearum* known virulence genes was attempted but with no convincing impact. However, asPTOs to silence *BEC1011* were delivered into whole barley seedlings by root uptake resulting in reduced powdery mildew infection. This suggests that asPTOs based HIGS could be further investigated as a strategy to control fungal diseases in crops.

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Table of Contents

List of figures

List of tables

List of Equations

Chapter 1 Gene silencing strategies for the control of plant fungal diseases

1.1 General overview on plant diseases

With the world population projected to reach 8 billion in 2050, food security is a major issue for all countries alike. The World Bank predicted that cereal production must increase by 50% by 2030 to meet global food requirements (FAO).

Historically, fungal pathogens have had tragic impacts on agriculture and hence on national economies and food security. Fungal pathogens are amongst the main factors responsible for food and goods shortages, famines as well as production of unsafe food containing potent neurotoxins. Crop diseases such as ergot *(Claviceps purpurea)* in Middle-Age Europe and brown spot in rice (*Cochliobolus miyabeanus*) in present Bangladesh, amongst others, have resulted in millions dying of starvation and mass scale immigration

For crops that are used as commodities, fungal diseases have caused huge economic impacts and habit changes in entire population. For instance, Ceylon was famous for coffee production since the 1658 when the coffee plantations were started by the Dutch. These plantations were later taken over by the British and by 1870, Ceylon was exporting 100 million pounds of coffee, especially to England. However, in 1876, coffee rust caused by *Hemileia vastatrix* hit the Ceylon coffee plantations, causing complete loss of crop and economical ruination of the farmers. Few farsighted farmers, including the Scotsman Thomas Lipton, saw the potential of converting the coffee plantations into tea gardens and introduced tea production in Ceylon in 1890. Ever since the raging of coffee rust, Ceylon is known as the exporter of the world's finest teas and the coffee rust also led to the English switching from being coffee-drinkers to tea-drinkers (Arneson, 2000).

Similar outbreaks of devastating fungal diseases have also been observed in the more recent years. For instance, wheat stem rust caused by *P. graminis* f. sp. *tritici* can be a devastating disease which destroy entire fields of wheat cultivars. However, use of resistance lines had kept it under check over decades, until in 1999, when a highly virulent Ug99 strain of wheat rust were identified in the wheat fields of Uganda. Ug99 overcame multiple wheat resistance genes including Sr31 and by 2007 spread to all East Africa and also into Yemen in the middle East (FAO). Similarly, the outbreak of wheat blast caused by *Magnaporthe oryzae Triticum* (MoT pathotype) was first reported in Asia in February 2016, particularly in Bangladesh where 42% of the world's wheat is produced (Callaway, 2016). Before its outbreak in Asia, wheat rust was limited to the South American countries. In 2016, wheat

rust was observed in 8 districts of Bangladesh, which hen spread to another 7-8 neighbouring districts 2017-18 and acreage used for wheat production reduced by 21% within a year of occurrence of this devastating disease. No wheat blast was observed in neighbouring India in 2016, which is the world's second largest producer of wheat. However, in February 2017, the emergence of wheat blast was observed on about 1,000 hectares of the wheat fields in Murshidabad and Nadia districts of West Bengal (https://bit.ly/2HtIVcs and https://bit.ly/2AqhcdA; Last accessed: Sep. 23, 2019). Presently, wheat rust is big threat to the world's wheat production and the government of these countries are working with the experts to control the spread of this devastating disease (Islam et.al.,2019).

Other fungal crop pathogens such as *Magnaporthae oryzae* causing rice blast, *Botrytis cinerea* causing grey rot, *Zymoseptoria tritici* causing wheat leaf blotch, *Fusarium graminearum* causing Fusarium head blight , *Blumeria graminis* causing powdery mildew, *Phakospora pachyrhizi* causing soyabean rust, *P. infestans* causing potato late blight, amongst others, are also of great economic importance, resulting in food security issues due to high yield loss and deterioration of grain quality of major cereal crops (Dean et al., 2012; Fisher et al., 2012).

The study of the molecular interaction between plant hosts and invading pathogens is an important aspect of phytopathology. Plants have developed robust and complex signalling networks to detect invaders and for the triggering their innate immune system to defend themselves against different kinds of pathogens such as bacteria, oomycete, fungi and viruses, parasitic plants, whether they have a biotrophic or a necrotrophic life style (Jones & Dangl, 2006; Zhang et.al., 2013). Similarly, pathogens have adapted various strategies for evading plant immune surveillance. This includes the expression of virulence genes, facilitating successful host invasion, colonisation and disease development while compromising the plant immune system (Doehlemann & Hemetsberger, 2013; Toruño et.al., 2016). The increasing understanding of plant-pathogen interactions has resulted in recognition of various fungal virulence genes as well as plant susceptibility genes that play an important part in pathogenesis of these phytopathogens. Using this knowledge, this project aims at exploiting and adapting novel gene silencing strategies that will reduced the mRNA transcript or compromise the translation of a selection of fungal virulence and plant susceptibility genes in order to control some of the main wheat and barley fungal diseases like *Blumeria graminis* f. sp. *hordei, Blumeria graminis* f. sp. *tritici, Fusarium graminearum* and *Zymoseptoria tritici*. In addition, the gene silencing tool and gene deletion will be utilised to investigate the virulence role of *B. graminis* virulence factor *BEC1019* during development of these wheat diseases. Gene silencing will be further utilised to investigate the regulation of *Bgh BEC1019* by zinc sensitive transcription factor ZAP1 and pH sensitive transcription factor PacC in *B. graminis*.

1.2 Traditional approaches to control fungal diseases

1.2.1 Breeding for *R*-gene resistance

Traditionally, disease resistance breeding has been used for decades for protecting the crop against fungal diseases. The resistance varieties are obtained by introducing resistance genes (*R-genes*) into elite high yielding varieties of crops. *R*- genes code for plant immune receptors which play a role in pathogen recognition and neutralisation, resulting in resistance to that pathogen. These elite varieties are then tested for susceptibility against various pathogens for multiple generations, before being released into the market.

Resistance can be either qualitative or quantitative, depending on the phenotypic expression and the nature of the resistance. Qualitative resistance is conferred by single *R*-genes responsible for pathogen recognition resulting in complete or near complete resistance. *R*-genes are therefore, also called major genes. These *R*-genes can be either dominant or recessive (reviewed by Nelson et al., 2018). For instance, in rice, presence of the dominant major *R* genes *Xa1, Xa4, Xa21* and the recessive major *R* genes *xa5, xa13* confer race-specific resistance to *Xanthomonas oryzae* p.v. *oryzae* (Zhang & Wang, 2013). On the other hand, quantitative disease resistance (QDR) confers incomplete resistance and is controlled by multiple genes, each with a partial effect (Nelson et al., 2018). Genes conditioning QDR are known as minor genes and map to quantitative trait loci (QTLs) or sometimes called quantitative resistance loci (QRL) (Poland et al., 2009). The genetic dissection of QDR is challenging and the relationship between the associated genes and the resistance phenotype is not very well understood (Nelson et al., 2018). However, mounting evidence shows an involvement of QRLs in pathogen recognition and therefore, the boundaries between the mechanisms underlying qualitative and quantitative resistance are not very clear (Poland et al., 2009).

The initial research used major *R*-genes for resistance against a specific race of pathogens. However, the use of single dominant *R*-genes led to pathogen evolution resulting in loss of these resistance traits. For instance, a recent outbreak of a new strain of wheat stem rust (caused by *Puccinia graminis* race Ug99) on wheat cultivars carrying widely deployed *R*-genes, highlighted the need for durable resistance against wheat rust, that is not conferred by dominant *R*-genes (Ayliffe et al., 2008). Similarly, potato late blight pathogen *Phytophthora infestans*, has rapidly overcome the resistance conferred majorly by *R*-genes (Sliwka & Zimnoch-guzowska, 2013). Not only does this subject the pathogen populations to high levels of selection pressure, but the presence of *R*-genes also shifts the trait distribution in such a way that the underlying QRLs cannot be detected. This phenomenon has led to the intentional elimination of R-genes in some breeding programs so that QDR can be more effectively assessed and advanced (Sliwka & Zimnoch-guzowska, 2013). Therefore,

resistance breeding now utilises race nonspecific minor gene resistance to ensure long-term durability of disease resistance. *R*-gene pyramiding is also being used to ensure durable disease resistance (reviewed by Burdon et.al., 2014). This involves stacking multiple genes leading to the simultaneous expression of more than one gene in a variety to develop durable resistance expression (Joshi and Nayak, 2010).

1.2.2 Fungicides

The first reported use of fungicides was in the 17th century, when brining of grain with saltwater followed by liming was used to control bunts. This occurred before Tillet discovered that root fungus *Tilletia tritici* caused blunt in wheat (Morton and Staub, 2008). The traditional fungicides also include a combination of copper sulphate and lime used for spraying grapevines to control downy mildew in grapes (Morton & Staub, 2008) The traditional fungicides which were used up till 1940s, were discontinued as they were mostly inorganic mixes prepared crudely by the user and therefore, posed toxicity issues for the user. The introduction of new chemistries like thiocarbamates and phthalimides between 1940-1970 marked the beginning of a safer modern fungicide (Morton & Staub, 2008). Therefore, fungicides have been used for a long time as the main method of crop protection as either a preventive or curative measure, improving the productivity and marketability of the plant products (reviewed by Yang et.al., 2011). According to the European Environmental agency, in a single year of 2016, 1.8 billion tons of fungicide active ingredients was sold in the EU (European Environmental Agency).

These fungicides have different modes of actions based on the protein or structure they target. For instance, methyl benzimidazole carbamate (MBC) group of fungicides (within benzimidazoles), such as carbendazim affect the fungal cytoskeleton by targeting β-tubulin protein of fungi (Qiu et al., 2011). Similarly, the succinate dehydrogenase inhibitor (SDHI) group of fungicides affect the fungus respiration and energy acquisition by targeting succinate dehydrogenase, an enzyme from the TCA cycle (Xiong et al., 2015). Fungal respiration is also affected by the quinone outside inhibitor (QoI) group of fungicides that target the mitochondrial cytochrome complex (Miles et.al., 2012). Fungicides groups such as the de-methylation inhibitors (DMI) group have also been developed to target the fungal sterol biosynthesis pathway and include the triazole fungicide triadimefon that can target major phytopathogens (Y. Chen et al., 2015).

A summary of groups of fungicides, based on their mode action is included in Table 1-1.

Table 1-1: Fungicides and their mode of action.

Data reproduced from Yang et al., 2011**.**

Mode of Action	Group name	Target site and code	Fungicide chemical group	Common name
Lipid, sterol, and other membrane components	AH-fungicides	Cell peroxidation (proposed)	Aromatic hydrocarbons	Dicloran
	Heteroaromatics		1,2,4-thiadiazoles	Etridiazole
	DMI-fungicides (De-methylation Inhibitors) (SBI: Class I)	Sterol C14-demethylase in sterol biosynthesis (*erg11/cyp51*)	Triazoles	Triadimefon
				Triticonazole
			Cinnamic acid amide	Dimethomorph
			Triazole	Hexaconazole
			Morpholine	Fenpropimorph
			Triazole	Propiconazole
				Tebuconazole
Amino acid and protein synthesis	Glucopyranosyl antibiotic	Protein synthesis (ribosome, initiation step)	Glucopyranosyl antibiotic	Streptomycin
	Tetracycline antibiotic	Protein synthesis (ribosome, elongation step)	Tetracycline antibiotic	Oxytetracycline

Signal transduction	PP-fungicides (PhenylPyrroles)	MAP/Histidine- Kinase in osmotic signal transduction (os-2, HOG1)	Phenylpyrroles	Fludioxonil
	Dicarboximides	MAP/Histidine- Kinase in osmotic signal transduction (*os-1, Daf1*)	Dicarboximides	Iprodione
				Vinclozolin
Respiration	Pyrimidinamines	NADH oxido-reductase (Complex I) inhibitors	Pyrimidinamines	Diflumetorim
	SDHI (Succinate-dehydrogenase inhibitors)	Succinate-dehydrogenase (Complex II) inhibitors	Pyridine carboxamides	Boscalid
			Benzamides	Flutolanil
			Gxathiin carboxamides	Carboxin
		Oxidative phosphorylation uncouplers	2,6-dinitroanilines	Fluazinam
			Dinitrophenyl crotonate	Dinocap
Mitosis and cell division	MBC - fungicides (Methyl Benzimidazole Carbamates)	ß-tubulin assembly in mitosis	Methyl benzimidazole carbamate	Benomyl
				Carbendazim
	Phenylureas	Cell division (target unknown)	Phenylurea	Pencycuron
	PA fungicides	RNA polymerase I inhibitors	Acylalanines	Metalaxyl

Nucleic acids synthesis	(Phenyl Amides)		Oxazolidinones	Oxadixyl
	Hydroxy-2-amino-pyrimidines	Adenosine deaminase	Hydroxypyrimidines	Ethirimol
Multisite activity			Dithiocarbamate	Mancozeb
			Phthalimide	Captan
			Dithiocarbamate	Thiram
			Anthraquinone	Dithianon
			Copper	Copper sulphate

1.2.3 Fungicide resistance build-up

Fungicide resistance is the one most significant risk faced by modern day agriculture. Natural selection of pathogenic fungi as well as excessive fungicide use are believed to be the main causes of rapid development of fungicide resistance (Lucas et.al., 2015). Traditional fungicides, including copper sulphate and other inorganic mixtures, have a single fungal target as implied by the definition. These single-site fungicides are highly potent *i.e.* they confer resistance at very low doses. Therefore, at low doses, these fungicides kill most of the fungal strains which are originally susceptible, but then result in a strong selection for resistance build-up, creating increasingly resistant strains (Lucas et al., 2015). Multi-site fungicides, as the name suggests, targets multiple metabolic pathway and are therefore more difficult to develop resistance against. However, they can target multiple fungi, resulting in regulatory issues. Examples include chlorothalonil and fludioxynil (Lucas et al., 2015).

Resistance to single-site fungicides is mostly due to alteration in the target protein or gene. For instance, in the fungal pathogens like *Zymoseptoria tritici*, point mutations have been identified in all four subunits of the fungal succinate dehydrogenase enzyme, rendering them resistant to the DMI group of fungicides (Scalliet et al., 2012) (Figure 1-1, 1). *Z. tritici* has also gained resistance to azole fungicides by overexpressing the target sterol 14α-demethylase gene (Cools et.al., 2012) (Figure 1-1, 2). In some instances, fungicide resistance is mediated by increased efflux of the fungicide, as seen in *C. albicans,* where resistance to azole fungicides is conferred by over-expression of ABC transporters resulting in increased efflux of the fungicide (Hiller et.al., 2006; Sanglard et al., 1995) (Figure 1-1, 3). In certain cases, fungicide resistance is also induced by degradation of fungicides by fungal metabolic enzymes, as seen in QoI fungicide degradation by the fungal esterases in *Venturia inequalis* (Jabs et.al., 2001) (Figure 1-1, 4).

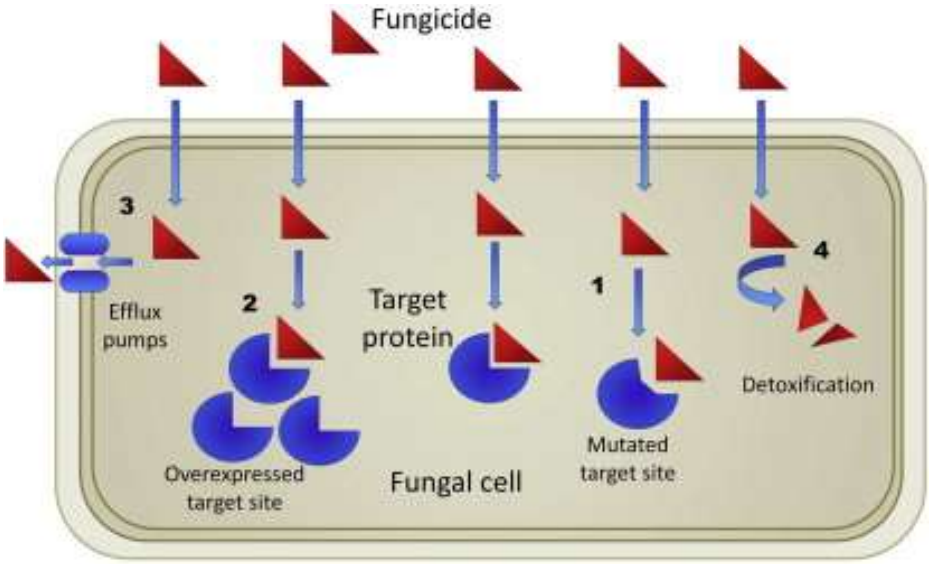

Figure 1-1: Mechanisms of resistance for single-site fungicides.

1. Alteration of target protein preventing the binding of fungicide. 2. Overexpression of target gene necessitating a higher dose of fungicide for fungal control. 3. Efflux of the fungicide from fungal cell due to overexpression of transporters. 4. Detoxification of fungicide by fungal metabolic enzymes. Diagram obtained from (Lucas et al., 2015)**.**

Fungicide resistance is prevalent amongst crop threatening phytopathogens. In most cases, this is occurring as the result of excessive fungicide required for crop protection. For instance, the European strains of *Z. tritici* have developed resistance to both benzimidazole and QoI group of fungicides (Fraaije et al., 2005). The European strains of *F. graminearum* have intrinsic resistance against the QoI fungicide trifloxystrobin (Dubos et.al., 2011) as well as the SDHI fungicide isopyrazam (Dubos et al., 2013). Moreover, they have now developed resistance against few DMI (Ammar et al., 2013) and MBC fungicides (Chen et al., 2009), then restricting the availability of effective fungicides to control Fusarium head blight. Similarly, *B. graminis* strains with resistance to azole fungicides have been discovered in the lab and the fields (Wyand & Brown, 2005). The pre and post-harvest pathogen *B. cinerea*, on the other hand, have evolved strains that are resistant to multiple fungicides (reviewed by Hahn, 2014). The case of grape downy mildew is quite interesting as disease control involves the use of a disease prediction model along with traditional and single site fungicides used either pre or post infection. The pre-infection fungicides include traditional fungicides like copper sulphate and no resistance has been developed for these fungicides, However, Downy mildew oomycete pathogen *Vitis vinifera* has developed resistance to post-infection single-site fungicides like QoI and CAA (Ash, 2000)

Therefore, the current fungicide research is focussed on understanding of the basis of fungicide resistance development, to develop molecular strategies and implement agricultural practises to prevent further fungicide resistance development in different fungal phytopathogens. Multi-site targeting fungicides are also being recommended to British farmers, as they are less prone to

resistance development by single point mutation (AHDB). Moreover, resistance gene-based breeding, genetically modified crops and gene silencing are also being explored as alternatives for crop protection in different countries.

1.3 Biology of Fungal pathogens

1.3.1 Biotrophic, Necrotrophic and Hemibiotrophic fungal pathogens

The fungal phytopathogens can be classified into three main groups based on their mode of nutrition. Most common and problematic biotrophic fungal pathogens comprise of the rusts, which include wheat rust caused by *Puccinia sp., Asian soybean rust caused by Phakopsora pachyrhizi* and powdery mildews, including cereal powdery mildews caused by *Blumeria graminis spp*, amongst others. Such pathogens are obligate biotrophs that can only derive nutrients and proliferate only on live plant tissue. This means that, instead of killing the host, the pathogenesis of such fungi involves keeping the plant cells alive for derivation of nutrients for fungal proliferation. These fungi produce specialised feeding structures called haustoria, within cells of the invaded host tissue. The host extra-haustorial and the fungal haustorial membranes, separated by the extrahaustorial matrix, act as a medium for nutrient uptake required for fungal growth as well as the secretion of virulence factors, in particular effectors, facilitating or mediating host colonisation (reviewed by Chisholm et.al., 2006; Pavan et.al., 2010; Hou et.al., 2011).

Necrotrophic fungal pathogens include *Botrytis cinerea, Ralstonia* species, for example. Necrotrophs derive their nutrition from dead host tissue. To succeed in the infection process, necrotrophs, therefore, produce toxins or cell wall degrading enzymes resulting in host tissue degradation, which in turn, acts as a direct source of nutrients for fungal proliferation (reviewed by Chisholm et.al., 2006;Pavan et.al., 2010; Hou et.al., 2011).

Hemi-biotrophs, on the other hand, are characterised by “an initial biotrophic phase followed by a necrotrophic stage”, as it is the case for *Zymoseptoria tritici* or *Fusarium graminearum*. Unlike other hemi-biotrophs, no specialised feeding structures have been reported in the biotrophic phase of these two fungi (Perfect & Green, 2001)

The mode of nutrition of these phytopathogens have a significant influence on their pathogenesis, plant-pathogen interactions as well as the plant immune response. For instance, localised programmed cell death (PCD) at the infection site is used as a plant immune response to control biotrophic pathogens. However, PCD generated debris acts a source of nutrients for the necrotrophs, facilitating their growth and infection development. Therefore, fungal lifecycle is a

major consideration for developing crop protection strategies for these fungal diseases (Pavan et al., 2010; Vleeshouwers & Oliver, 2014).

1.3.2 *Blumeria graminis* life cycle

Blumeria graminis is an important group of biotrophic fungal pathogens causing powdery mildew in cereal crops. The different formae specialis have very high host specificity i.e. f.sp. *hordei* will only infect barley, while they cannot infect wheat or other cereals. Powdery mildew symptomscan be mainly identified as white powdery pustules on infected leaf surfaces (Both et al., 2005).

Figure 1-2: Barley Powdery mildew symptoms.
Barley leaf showing powdery postules due to Blumeria graminis f. sp. hordei infection.
(https://www.mpg.de/622228/pressRelease201012081)

As mentioned above*, Blumeria graminis* is an obliage biotroph. ascomycete, Erysiphales fungus. It usually overwinters on crop debris by forming cleistothecia, one of the typical sexual fruiting body of Ascomycetes. However most of the life cycle and further spreading of the disease occurs mostly asexually with asexual conidia or mycelia developing on the areal part of the plant, epiphytically, while only haustoria are located within live host cells as depicted in Figure 1-3 and

Figure 1-4 (Both et.al., 2005). Both wind-borne ascospores (sexual) and conidiospores (asexual) can act as an inoculum for powdery mildew disease propagation. Immediately after encountering

wheat/barley leaves, the conidia produce extracellular matrix that helps adhesion to the leaf and perception of the hard/ hydrophobic leaf surface (Carver et.al., 1999; Wright et.al., 2002) . The cuticular wax of the barley leaf surface influence the penetration as well as germination of *Blumeria* spores, as evidenced by the 20% reduction in spore germination and differentiation on removal of the epicuticle (Zabka et al., 2007). Particularly, very-long-chain-aldehydes present in the barley cuticle was shown to be important for *B. graminis* spore germination and differentiation (Hansjakob et.al., 2010). Within half an hour post inoculation (hpi), the spore germinates to form a primary germ tube with the role of recognising the surface of spore germination (Jankovics et al., 2015; Edwards, 2002; Nielsen et.al., 2000). By 4 hpi, a secondary germ tube forms a hook and bulges and transforms into an appressorium at 8 hpi. A penetration peg emerges underneath the appressorium at 15 hpi, which penetrates the host epidermis cell wall by enzyme secretion as well as turgor pressure (Pryce-Jones et.al., 1999; Jankovics et al., 2015; Both et.al., 2005; Edwards, 2002). If the penetration is successful, by 24hpi, the fungus forms the first haustorium by expansion of the penetration peg in the plant periplasmic space. The haustorium acts as a extensively branched feeding structure absorbing nutrient from the host for fungal growth, resulting in host colonisation and generation of hyphae on the leaf surface (epiphytic mycelium) (Godfrey et. al., 2009; Szabo & Bushnell, 2001). At the same time, the haustorium is also believed to inject effectors into host cells, to favour virulence by avoiding perception and set up of successful resistance by the host (Panstruga & Dodds, 2009). A haustorium is surrounded by a plant derived extrahaustorial membrane. A gel-like carbohydrate-rich extrahaustorial matrix (EHM) is present between the extrahaustorial membrane and the fungal cell wall forming the haustorium (Szabo & Bushnell, 2001). The EHM is separated from the apoplast and the plant plasma membrane by a haustorial neckband (Kunoh & Akai, 1969). The neckband facilitated the haustorial membrane H+-ATPase mediated proton gradient generation that promotes nutrient uptake (Godfrey et al., 2009) and effector secretion (Panstruga & Dodds, 2009). Secondary haustoria are formed for sustained fungal growth. At 4-5 days post inoculation (dpi), powdery mildew symptoms are visible as powdery pustules containing mycelia and conidia bearing conidiophores on the leaf surface. These wind-dispersed conidia facilitate the spread of the disease over large areas (Both et. al., 2005; Hückelhoven & Panstruga, 2011).

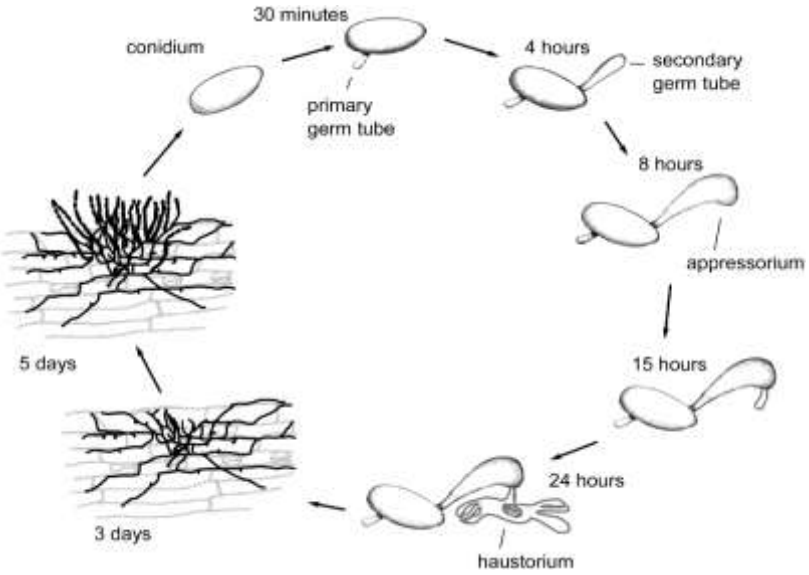

Figure 1-3:* Asexual life cycle of *Blumeria graminis
(Both et. al. 2005).

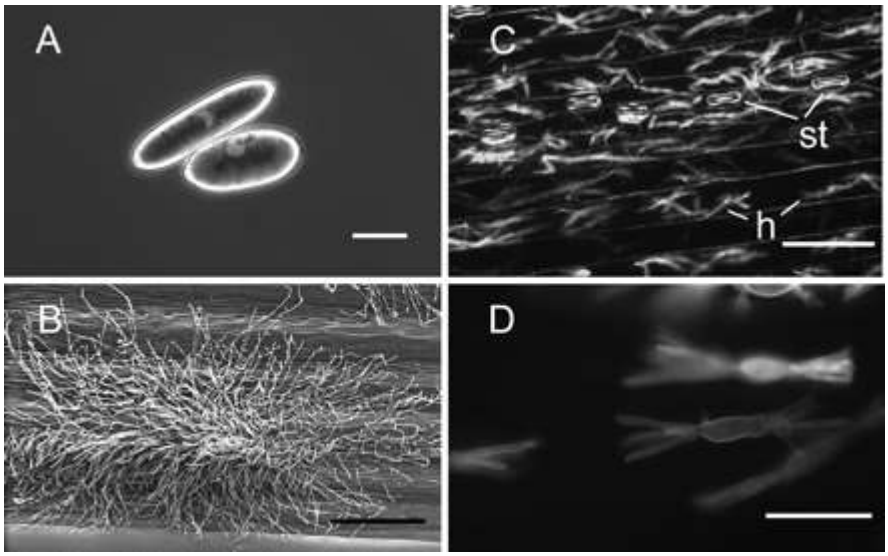

Figure 1-4: Different stages of B. graminis development during infection of barley leaves.
A. Conidia (bar, 10 µm) B. Epiphytic growth of Bgh hyphae on barley leaf surface (bar, 100 µm) C. Haustorial structures within epithelial cells (bar, 20 µm) D. Detail picture of Bgh haustoria (bar, 10 µm). (Bindschedler, 2009).

1.3.3 *Fusarium graminearum* life cycle

Fusarium graminearum (syn *Giberella zeae*) is the causal agent of Fusarium Head Blight (FHB), a devastating cereal disease in wheat, durum wheat, barley and oat in Europe, Asia and America

(reviewed by Schmale III et.al., 2003). *F. graminearum* belongs to the ascomycota phylum, ascomycetes class and telomorphs *of* Fusarium are classified under the genus *Gibberella* and some under the genera *Hemanitricia* and *Amentricia* (Moretti, 2009). FHB symptoms include premature discolouration of cereal spikelet, followed by appearance of black dots on spikelets and formation of shrunken and wrinkled grains lack of gluten and albumin proteins (Figure 1-5) (Schmale III et.al., 2003). This results in yield loss as well as production of low-quality grains. Moreover, *F. graminearum* produces mycotoxins in the cereals that renders them unsafe for human and cattle intake as well as malting purposes (Kimura et.al., 2007).

Figure 1-5: Fusarium head blight (FHB) disease symptoms.
A and B: Discoloured spikelets due to FHB infection. C: Pathogen sporodochia (pink) on glumes. D: Shrunken diseased grains (right) as compared to healthy ones (Agriculture and Agri Food, Canada http://www.agrgc.ca).

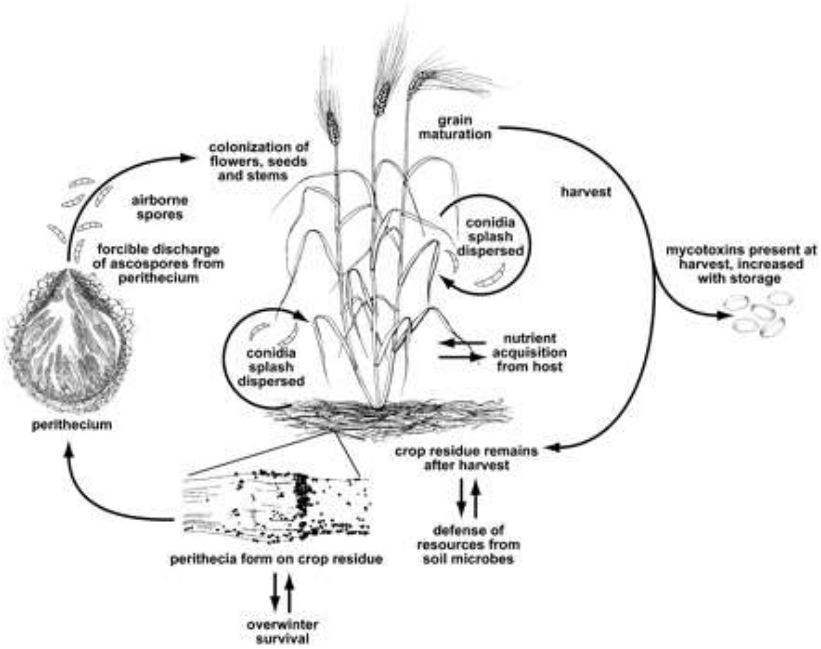

Figure 1-6: Disease cycle of Fusarium graminearum (Trail, 2009).

F. graminarum are ascomycete fungi, producing both sexual (ascospores) and asexual spores (macroconidia). It is a hemibiotrophic fungal pathogen characterised by an initial asymptomatic

biotrophic phase after host penetration followed by a necrotrophic phase (N. A. Brown et al., 2011). The fungus overwinters on infected crop debris as a mycelium, producing macroconidia and perithecia containing sexual ascospores. Spore dispersal occurs through wind and rain splash onto floral tissues of the host plants, initiating a new disease cycle (Figure 1-6) (Sutton, 1982). Therefore, coincidence of warm moist weather with flowering time of wheat crops facilitates disease epidemics (Pritsch et.al., 2000). Once deposited as spores on spikelets, the fungus initially hyphenates on the spikelet surface, forming small hyphae that directly penetrate through epidermal penetration (Boenisch et.al., 2011). Once fungi reach the spikelet rachilla, *Fusarium* undergoes a biotrophic, asymptomatic and intercellular growth phase with colonisation of the host in between the cortical cells surrounding the vascular tissue, which is characteristic of most of the infected tissues (N. A. Brown et al., 2011). During this stage, homogenous hyphal networks are formed on caryopses until 1-2 dpi, on paleas until 4-5 dpi and until 6-7 dpi on lemmas and glumes. After these time points, the running hyphae containing foot structures, lobate appressoria and infection cushions are formed which invade the cortical cells and the vasculature. This host cell colonisation coincides with plant cell death adjoining the infection cushions visualised as tissue necrosis and host tissue discolouration. Lesions appear at 3 dpi on caryopses, at 5 dpi on paleas and at 7 dpi on glumes and lemmas (Boenisch et.al., 2011). By 8 dpi, the hyphae colonise beyond regions with visible disease symptoms and colonise the entire rachis by 12 dpi. During this period of 8- 16 dpi there is a reduction in active fungal biomass and redistribution of resources to the growing hyphal tip (N. A. Brown et al., 2011). The different parts of the wheat spikelet and fungal disease structures are shown in

Figure 1-7 and

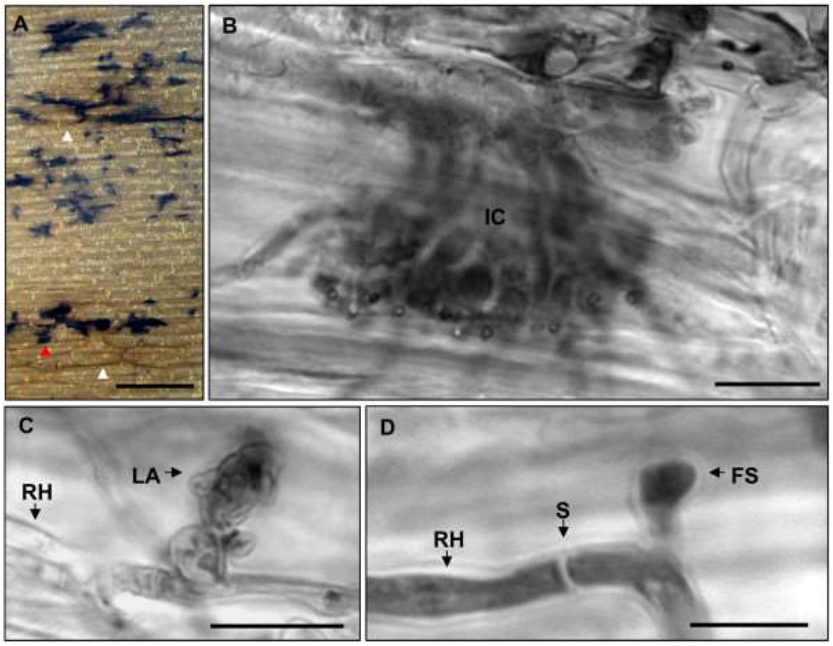

Figure 1-8. *F.graminearum* also produces trichothecene mycotoxins such as deoxynivalenol (DON) which acts as a virulence factor by promoting necrosis (M. Kimura et al., 1998). Expression of DON synthesising TRI gene expressions have been shown to localise at the growing hyphal tip during host colonisation by *F. graminearum* (N. A. Brown et al., 2011). DON accumulation in seeds is also correlated with shrivelled and undersized grain production (Proctor et.al., 1995).

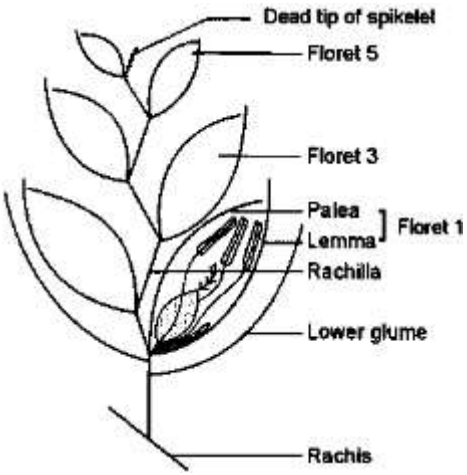

Figure 1-7: Different parts of a wheat spikelet.

Source: Kirby and Appleyar, 1987

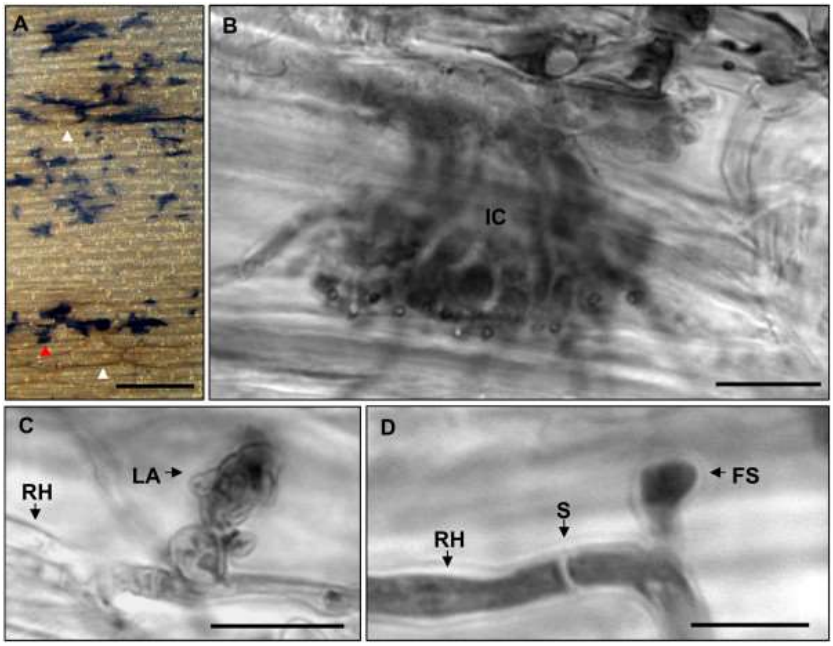

Figure 1-8: Stages of F. graminearum infection development.

A-D Light microscopy of infected palea after trypan blue staining of mycelium at 10 dpi. A. Abundance of infection cushions on the surface of palea. Different sizes of infection cushions and necroses of plant cells. B Magnification of blue stained fungal structure (red arrowhead in A) showing a typical infection cushion. C Typical cellular structure of a lobate appressorium and D foot structure arising from runner hyphae. Abbreviations: FS Foot structures, IC infection cushions, LA lobate appressorium, RH runner hypha, S septum.(Diagram from Boenisch et al., 2011).

1.3.4 *Zymoseptoria tritici* life cycle

Zymoseptoria tritici (also known as *Mycosphaerella graminicola*) is an ascomycete fungus causing Septoria tritici blotch (STB), a major wheat foliar disease. The diseased leaves initially develop chlorotic patches, which then enlarge and produce necrotic lesions called pycnidia. There are sometimes green patches in the leaf called “green islands” which are regions without leaf necrosis (Figure 1-9). About 70% of fungicide input in European cereal production is directed towards STB control (Fones and Gurr, 2015) and it costs US agriculture around $276 million annually (Driscoll et.al., 2014).

Figure 1-9: STB symptoms on wheat plants.

Wheat leaves showing necrosis with pycnidia lesions. Also visible, green islands with no necrosis. Picture obtained from http://www.apsnet.org/edcenter/intropp/lessons/fungi/

As an ascomycete, *Zymoseptoria* lifecycle is characterised by the presence of both sexual (ascospores) and asexual spores (pycnidiospores/conidia). Ascospores are produced in fruiting bodies called pseudothecia, whereas pycnidiospores are present in pycnidia producing necrotic lesions, the main symptom of STB (

Figure **1-9**). The vegetative form of *Z. tritici* include macroconidiospores and microconidiospores. Macro-conidiospores are the most common form grown in lab, sometimes called "yeast-like cells", even though they are multi-septate with 4-8 elongated cells (Steinberg, 2015). However, *Z. tritici* also form unicellular, small microconidiospores which fit the "yeast-like cell" description (Steinberg, 2015). Wind and rain splash carried spores act as primary inoculum for STB infection and germinate 12 hours post leaf contact under highly humid conditions.

Unlike other hemibiotrophic phytopathogens, *Z. tritici* does not form any specialised infection structures during host penetration (Yang et.al., 2013). Hyphae formation and high relative humidity for 20 hours are mandatory for successful host penetration, as the fungus solely enters the host through stomatal opening (Kema, 1996). However, there is some evidence of *Z. tritici* penetrating the leaves at the junction of epidermal cells (Cohen and Eyal, 1993; Rohel et.al., 2001). The transition from unicellular or multi-septate conidiospores to hyphae can also be induced in liquid culture by nutrient deprivation and elevated temperatures (Mehrabi et.al., 2006; Motteram et al., 2011).

As an hemibiotroph, *Z. tritici* has a long intracellular asymptomatic biotrophic growth phase, followed by a sudden switch to necrotrophy. No evidence of haustoria (feeding structure) has been

found during its biotrophic growth (Kettles and Kanyuka, 2016). During the first 12-24 h the hyphae are restricted to sub-stomatal cavity which is followed by penetration into the leaf mesophyll tissue where they grow in the intercellular spaces in the tissue (Kema, 1996; Keon et al., 2007). MAP kinases *Zt*Slt2 (syn. MgSlt2) and *Zt*Ste12 (syn. MgSte12) have been shown to play an important role in hyphal colonisation. Deletion mutants of both *ZtSlt2* and *ZtSte12* were limited to the sub-stomatal cavity, following failure to branch and colonise the mesophyll cells (Kramer et.al., 2009; Mehrabi et al., 2006). The asymptomatic phase is also called the latent phase as the fungal biomass hardly increases during the initial stages of the infection (Keon et al., 2007). Some studies suggest that there is no changes in the apoplastic nutrient composition during this phase (Keon et al., 2007). Others like Rohel et.al. have suggested an uptake of apoplastic soluble carbohydrates by *Z. tritici* during this phase. GFP gene expressed under a carbon source repressed promoter in *Z. tritici* was repressed between host penetration and sporulation during wheat infection (Rohel et al., 2001). Recent transcriptomic and metabolomic studies of wheat cultivars infected with *Zymoseptoria* suggest that during this biotrophic phase *Z. tritici* may derive its nutrition from stored lipids and fatty acids, as indicated by upregulation of lipid metabolism enzymes during this phase (Palma-Guerrero et al., 2016; Rudd et al., 2015). The asymptomatic phase also has upregulation of secreted effector proteins including the chitin-motif containing *Zt*3Lysm effector, which plays an important role in establishment of this latent phase by protecting the fungus from recognition by the plant defence mechanisms (Marshall et al., 2011; Palma-Guerrero et al., 2016). The putative effectors *MgECP2, MgTrp18* and *MgTrp21* are also upregulated during this phase (Palma-Guerrero et al., 2016).

This latent phase lasts up to 9 dpi in the laboratory and between 6-36 days in the fields, at which point it switches to necrotrophy due to unknown triggers (Duba et.al., 2018; Steinberg, 2015). The necrotrophic phase is characterised by pycnidia formation and host alterations that are more apoptotic than necrotic. However, *Z. tritici* necrotic proteins (NEP1, ZtNIP1) are expressed during this phase, indicating at an interaction between pathogen necrotic factors and host immune response at this point (Kettles and Kanyuka, 2016). During this phase, the cell nutrients leak into the apoplastic phase accelerating fungal growth (Palma-Guerrero et al., 2016).

Pycnidia formation starts in the sub-stomatal space with excessive fusion of hyphae. Pre-pycnidia formation occurs between 5-9 dpi. At 9 dpi, the fungus switches to an aggressive necrotrophic phase resulting in spore bearing pycnidia lesion formation on the leaf surface by 10-12 dpi (Kema, 1996). The lesions are regions of high programmed-cell death in host tissue that acts as a source of nutrients for pathogen growth (Keon et al., 2007). MAP kinase *Zt*Fus3 play an important role in pycnidia formation, as deletion mutants are unable to form pycnidia *in vitro* (Cousin et al., 2006). Similarly, catalytic and regulatory subunit of protein kinase A - *ZtTpk2* and *ZtBcy1*, respectively (syn.

MgTpk2 and MgBcy1) also contribute to *in vivo* pycnidia formation (Mehrabi and Kema, 2006). Over time, the mature asexual fruiting bodies – pycnidia, produce macro-conidiospores that disperse into the canopy by rain splash, supporting the spread of STB in temperate and humid regions. *Z. tritici* overwinters on crop residues as pycnidia and crop-free as pseudothecia structures containing ascospores. These ascospores are then wind-dispersed and support the spread of STB to wider areas (Driscoll et al. 2014; Steinberg 2015). The lifecycle of *Z. tritici* is described in Figure 1-10.

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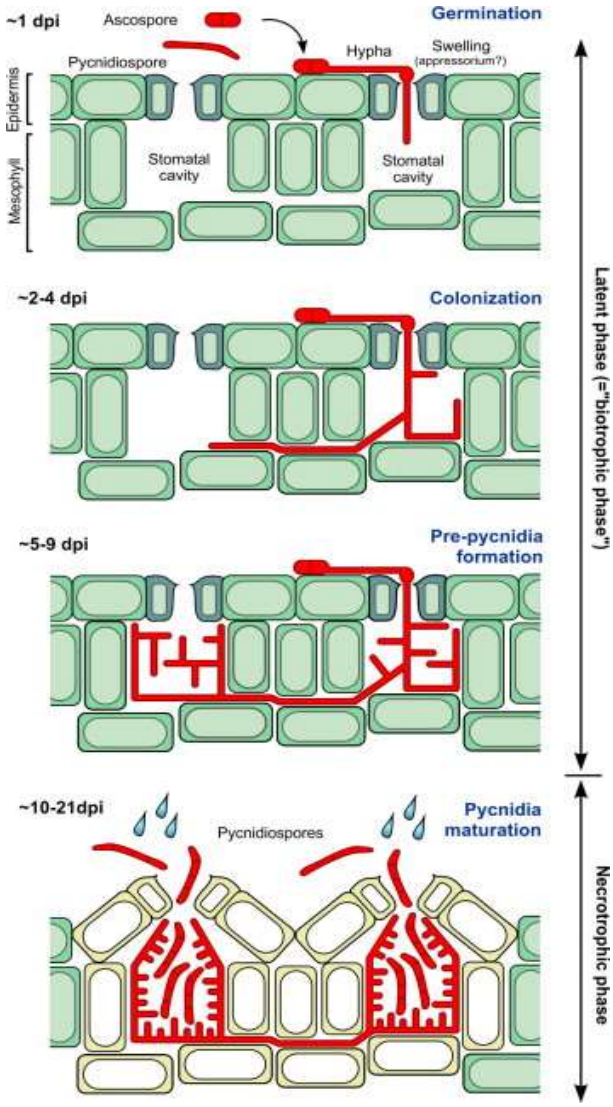


Figure 1-10: Zymoseptoria tritici lifecycle in planta showing latent biotrophic and necrotrophic phases. *(Steinberg, 2015)*

1.4 Fungal effectors and virulence genes favouring infection

Pathogenic fungi possess virulence genes, whose products enable a microorganism to establish itself within a host and cause disease (Van der Does and Rep 2007). Virulence genes are key players of the infection process to secure the successful establishment of the pathogen inside the host (Van der Does

and Rep 2007). Their presence also reflects the evolutionary battle between plants and pathogens. Disease pressure has led to evolution of host resistance genes which in turn created a resistance pressure, which then has resulted in the duplication and divergent evolution of multiple pathogen virulence genes (Van der Does and Rep 2007). Many of these virulence factors target host susceptibility proteins. Host susceptibility proteins are plant proteins that are targeted by pathogen's virulence factors to potentially alter the physiology of the host plants, facilitating either pathogen entry, host colonisation or disease development (Van der Does and Rep 2007). Depending on the mode of nutrition of the pathogen, these virulence factors may either encode secretory proteins that interact directly with host proteins or enzymes producing species-specific necrotic toxins compromising the plant immune response, as described later. Virulence factors can be either species-specific or non-specific. The species specific or sometimes even isolate specific virulence factors are many a times implicated in determining the host specificity at species or at cultivar level. These species-specific subgroup of virulence factors are called effectors and have been reported in bacterial, oomycete and fungal phytopathogens (Jones & Dangl, 2006). Although virulence factors might also contribute to the pathogen fitness, effectors, on the contrary, are defined as being solely involved in virulence (reviewed by Rovenich et.al., 2014). However, for biotrophic pathogens, the role in virulence cannot be easily distinguished from a vital role for fungal survival due to the dependency of the biotrophs on their host for survival. *B. graminis* effector BEC1011 is one such species-specific effector (Pliego et al., 2013). However, there are certain virulence factors are found in multiple species. This includes the necrosis and ethylene-inducing protein (NEP1)-like proteins (NLPs) which are conserved in many pathogenic bacteria, fungi, and oomycetes (Gijzen & Nürnberger, 2006) (explained in more detail later in this section).

Depending on their host target site, effectors can be broadly classified as apoplastic and cytosolic effectors (Kamoun, 2006). The apoplastic effectors function in the apoplast/plant extracellular space where they translocate and target plant extracellular targets and receptors (Kamoun, 2006). Some apoplastic effectors contain LysM domains that sequester chitin to prevent fungal recognition by plant immune system (Marshall et al., 2011). The cytosolic effectors, on the other hand, are predicted to have a signal peptide for secretion and are translocated into the plant cells via specialised infection vesicles or haustoria. Once within the invaded cells, they target host components in various sub-cellular compartments (Figure 1-11) (Kamoun, 2006). The apoplastic effectors have mostly been characterised as small secretory cysteine-rich proteins (SSCP). Their high content in cysteine residues allow them to forming many disulphide bridges which make them resistant against apoplastic proteases (Lu and Edwards, 2016).

In biotrophic and hemi-biotrophic pathogens, many effector proteins belong to a functional group of avirulence genes (*Avr*-genes). Virulence genes act as *Avr* genes when they are perceived by an incompatible host which has evolved to possess a specific corresponding resistance gene (*R*-gene) able to specifically recognise that *Avr*-gene, first described as the gene for gene theory (Flor, 1971). Here *R*-genes are resistance genes whose presence confer resistance to different microbial diseases in plants. Plant immunity involves PAMP-triggered immunity (PTI), which recognises certain pathogen associated molecular patterns (PAMPs) like the fungal chitin or bacterial flagellin to mount the first line of defence (Jones & Dangl, 2006). Fungal effectors can mask the fungal pathogen against PTI recognition, resulting in the need for a second line of defence in the form of effector triggered immunity (ETI). In an incompatible interaction, the *R*-gene driven recognition of the *Avr* gene initiates effector-triggered-immunity (ETI) resulting in the hypersensitive response (HR) characterised by localised programmed cell death (PCD) at the site of infection (Jones & Dangl, 2006). However, the plant defence response, which is accompanied by the host production of reactive oxygen species leading to necrotic cell death is an inefficient strategy against necrotrophs such as *Botrytis cinerea* or *Sclerotinia sclerotiorum,* as they exploit localised cell death for nutrient derivation and host colonisation. Similarly, a compatible interaction results when the host is unable to recognise these effectors, due to the absence of the cognate *R* gene. In this case, effectors act as virulence factors compromising or supressing the host immunity. This phenomenon is called effector-triggered susceptibility (ETS) (Jones & Dangl, 2006). These species-specific effectors are mostly expressed during host penetration and seem to play an important role during the biotrophic stage. For instance, Avr-Piz-T effector in the hemibiotrophic *Magnaporthae oryzae* targets the RING E3 ubiquitin ligase APIP6 to inhibit PTI in rice (C.-H. Park et al., 2012). Moreover, Avr-Piz-T can suppress Bax-Inhibitor mediated PCD in tomato plants, facilitating biotrophic growth (Li et al., 2009). Another example is the species specific and cysteine-rich Avr2 effector of the biotrophic fungus *Cladosporium fulvum*, which inhibits basal defences in tomato plant by interacting and inhibiting a group of plant extracellular cysteine proteases (Koeck et.al., 2011).

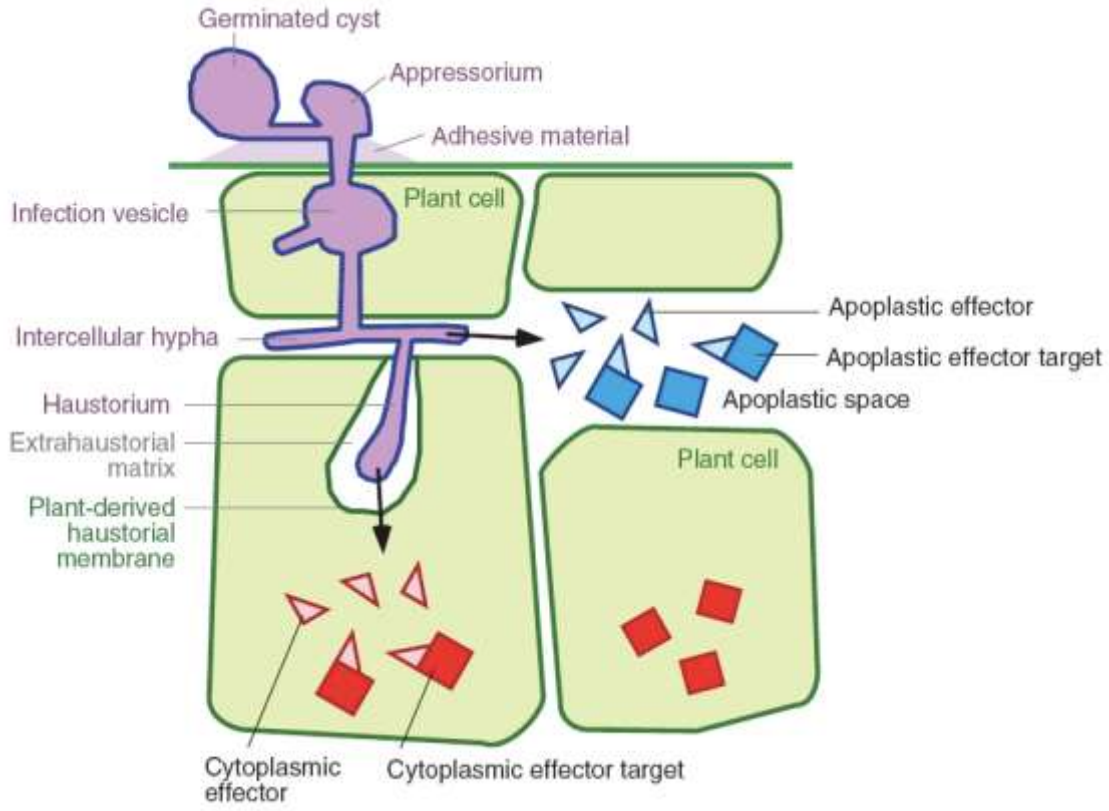


Figure 1-11: Apoplastic and cytoplasmic effectors involved in promoting pathogen virulence.

Diagram obtained from http://kamounlab.dreamhosters.com/images/effectors.jpg

The presence of a protein motif that triggers delivery of Avr effectors into host plant cells was first unearthed in oomycete effectors, including Avr3a from *Phytophthora infestans*. Avr3a entry is mediated by the N-terminal RxLR host targeting signal (HTS), conserved in many oomycete effectors (Morgan & Kamoun, 2007). The HTS was later shown to bind to phosphatidyl inositol phosphates (PIPs) on the outer layer of plant cell membrane, resulting in endocytotic uptake of the effector by the plant cell (Kale et al., 2010). Similarly, a N-terminal HTS mediated pathogen-independent uptake of fungal effector was reported in the biotrophic flax rust fungus, *Melampsora lini* (Rafiqi et al., 2010). These N-terminal HTS are not conserved among fungi and not much is known about their delivery role of Avr effectors in other pathogens (Morgan & Kamoun, 2007).

In necrotrophic pathogens, one of the main class of effectors characterised so far are host-specific toxins that induce host cell death and necrosis to facilitate the acquisition of nutrients for fungal proliferation (Wang et.al., 2014). In contrast, biotrophic effectors are involved in the avoidance of the pathogen perception by the host, which can lead to ETI, usually ending up with local cell death or HR (Wang et.al., 2014). In addition, effectors from both biotrophs and necrotrophs, effectors also promote pathogen proliferation and host colonisation by interacting with specific host susceptibility genes. For instance, the necrotrophic effector toxin victorin produced by *Cochliobolus victoriae*,

induces necrosis in oat hosts by interacting with the host glycine decarboxylase complex in the presence of host susceptibility gene Lov1 (Lorang et.al., 2007). Interestingly, *Lov1* encodes for an NBS-LRR protein related to *R* genes, thus illustrating co-evolution of biotrophic *Avr* genes and necrotrophic host-specific toxins.

Although most effectors identified are species specific virulence factors, there are plenty of virulence factors conserved in an array of phytopathogens. Such exceptions include the necrosis and ethylene-inducing protein (NEP1)-like proteins (NLPs), as mentioned above (Gijzen & Nürnberger, 2006). Deletion of an NLP protein in the fungal pathogen *Verticillium dahliae* decreased its virulence towards *Arabidopsis,* tomato and *Nicotiana benthamiana* (Santhanam et al., 2013). NLP1 from the oomycete *Hyaloperonospora arabidopsidis* causing downy mildew in *Arabidopsis*, triggers tissue necrosis in *Arabidopsis* leaves. This pathogen, however, expresses 10 other non-cytotoxic NLPs, all of which can trigger host immune responses. For instance, the ectopic expression of *Hyaloperonospora* specific non-toxic HaNLP in *Arabidopsis*, resulted in increased resistance to the biotrophic oomycete pathogen. The immune response is thought to be mediated by a central region of the NLP protein that acts as a PAMPS. This region is conserved in both cytotoxic and non-cytotoxic NLP proteins (Oome et al., 2014). NLPs induce cell lysis in dicotyledonous plants by plasma membrane destabilisation (Ottmann et al., 2009). Interestingly, expression of NLP protein homologues from the wheat pathogen *Zymoseptoria tritici* (MgNLP) into wheat leaves did not trigger necrosis. Moreover, NLP knock-out did not affect the virulence of *Z. tritici*, suggesting that NLP proteins of all monocotyledonous plant pathogens do not influence their pathogenesis (Motteram et al., 2009).

LysM (Lysine containing domain M) effectors or virulence factors are another conserved group of effectors, characterised by a common chitin binding LysM domain. Chitin, a component of the fungal cell wall, acts as a pathogen associated molecular pattern (PAMP), triggering host PTI during fungal attack. The LysM effector Ecp6 of *Cladosporum fulvum* sequesters chitin derived from fungal cell wall fragments, preventing their detection by host immune receptors such as chitin elicitor receptor kinases (CERKs), hence avoiding the activation of chitin-derived immunity (de Jonge et al., 2010). Similarly, chitin based immunity is also perturbed by *Magnaporthae oryzae* effector Slp1 and Mg3Lysm effector of *Zymoseptoria tritici* (described in section 1.4.3), suggesting a common role for all LysM effectors described so far (Mentlak et al. 2012; Marshall et. al. 2011).

1.4.1 *Blumeria graminis* effectors

The publication of the *Blumeria graminis* f. sp. *hordei* genome (Spanu et al 2010) and later the *B. graminis* f.sp. *tritici* genome (Wicker et al., 2013) paved the way for effector discovery. In the initial study by Spanu et. al., 248 candidate effector proteins were identified, distinguished by the presence

of a signal peptide and absence of a transmembrane domain in the *Blumeria graminis* f. sp. *hordei* genome. No clustering of candidate effectors on any chromosomes was observed in this study (Spanu et al 2010). Further bioinformatics analysis resulted in the identification of 491 Candidate Secretory Effector Proteins (CSEPs) in *B. graminis* f.sp. *hordei,* which amounted to 7% of the annotated genome (Pedersen et al., 2012). Another improved bioinformatic study identified 734 and 732 candidate effectors in *B. graminis* f.sp *tritici* and f.sp. *hordei* respectively amounting to 10% of the fungal genomes (Menardo et.al., 2017). These CSEPs were primarily small haustorial proteins and were characterised by the presence of signal peptides, absence of trans-membrane domain and being exclusive to the powdery mildews (Spanu et.al., 2010; Pedersen et.al., 2012). Mass spectrometry based proteomic analysis of the *B. graminis* f.sp. *hordei* haustorium led to the discovery of 71 haustoria-specific proteins, 63 of which were *Blumeria* Effector Candidates (BECs) (Bindschedler et al. 2009; 2011). Most of these BECs were characterised as encoding small secretory proteins, expressed exclusively in haustoria. The relevance of this is that haustoria are thought to play an exclusive role in effector delivery in biotrophs. BECs have a big overlap with CSEPs, but also contain virulence factors such as BEC1019 and BEC1005 that are present in fungi other than the powdery mildews (reviewed by Bindschedler et.al., 2016; Bindschedler et al., 2011).

Some of the *B. graminis* f.sp. *hordei* CSEPs were characterised as RNase-like proteins in haustoria (RALPHs) for possessing homology to RNase-like 3D structure fold, despite a very low sequence similarity to microbial RNAses, except the RNA binding domain. They also have a intron whose position is highly conserved (Pedersen et al., 2012). These RALPH effectors and the conserved introns are also present in grapevine powdery mildew which diverged from the cereal powdery mildews about 70 mya ago, suggesting that the RALPHs have evolved from a single ancestor similar to a canonical fungal RNase T1 (Bourras et.al., 2018). The study of the *B. graminis* f.sp. *tritici* CSEPs led to the discovery of a new class of effectors with structural homology with the MD2-related lipid-recognition (ML) domain, predicted to bind specific lipids (IPR003172). The ML-like CSEPs are conserved across different lineages of cereal powdery mildews, leading to another group of CSEPs with a common ancestry (Bourras et al., 2018; Menardo et al., 2017). Evolutionarily, the effector families of cereal powdery mildews are more diversified than the non-effectors. Within a family, the sequences are highly variant although the structures are conserved (Bourras et al., 2018; C. Pedersen et al., 2012). Protein sequence identity is restricted to the signal peptide and an N-terminal YxC motif, reminiscent of the oomycete RxLR domain important for secretion of oomycete effectors (Godfrey et.al., 2010; Pedersen et al., 2012; Spanu et.al., 2010). However, YxC does not contribute to *Blumeria* effector secretion by haustoria.

In *B. graminis* f.sp. *hordei,* an initial functional analysis of 50 *Blumeria* effector candidates (BECs) was carried out by HIGS mediated by hairpin RNA introduced using biolistic bombardment. The leaves

were then inoculated with *Blumeria* spores and disease development was monitored. Out of this initial screen, the silencing of eight BECs, namely BEC1005, BEC1011, BEC1016, BEC1018, BEC1019, BEC1038, BEC1040 and BEC1054, reduced formation of functional haustoria (haustorial index) confirming their roles as effectors (Pliego et al., 2013). In particular, BEC1011 (CSEP0264) and BEC1054 (CSEP0064) encoded structurally related ribonuclease-like proteins in haustoria (RALPHs) that have 75% nucleotide homology and belong to CSEP family 21 (Pedersen et al., 2012). BEC1019 is a metalloprotease-like effector, whereas BEC1005 codes for a protein similar to β-1, 3-endoglycosidases (Pliego et al., 2013). Like BEC1011 and BEC1054, the remaining BECs did not show any sequence similarity to known proteins outside the Erysiphales clade (Pedersen et al., 2012). BEC1011 silencing led to maximum reduction of the haustorial index followed by BEC1019. BEC1011 has been shown to prevent host cell death (Pliego et al., 2013) whereas BEC1019 has been hypothesised to be an universal virulence factor with role in inhibition of ROS and PCD (Whigham et al., 2015). To understand the mode of action of BEC1054, pull-down assays and yeast-2-hybrid were carried out showing its interaction with a pathogenesis related protein (PR5), glutathione-S-transferase (GST), a malate dehydrogenase (MDH) and an elongation factor 1 gamma protein (eEF1G). Therefore, BEC1054 seems to function by targeting various host proteins, but the actual mechanism is yet to be deciphered (Pennington et al., 2016). Another set of unrelated *B. graminis* f.sp. *hordei* effectors, CSEP0105 and CSEP0162 were shown to be important for *Blumeria* virulence using HIGS. Yeast two-hybrid assay and microscopic colocalization studies confirmed their interaction stress related small heat shock protein chaperones HSP16.9 and HSP17.5 in barley. Interestingly, CSEP105 interfered with the accumulation of the heat shock proteins HSP16.9 important for heat tolerance, confirming its role in compromising plant immunity (Ahmed et al., 2015). Similarly, BEC1004 was shown to interferes with host vesicle trafficking (Schmidt et al., 2014). Therefore, *Blumeria* effector candidates promote fungal virulence by targeting different host physiological processes.

Detailed classical genetic studies consisting of eleven crosses between *B. graminis* f. sp *hordei* strains indicated the presence of 30 *Avr* genes in *B. graminis* f. sp. *hordei* (Skamnioti et al., 2008). Most of these *avr* genes map as single characteristic of the standard gene-for-gene pattern. This is characterised by an F1 progeny with 1:1 segregation ratio. However, deviations of *Avr* segregation from the classical 1:1 single gene model were frequently observed, indicating the involvement in avirulence of at least two or three (Brown et al., 1996). For instance, in a classical genetics study, a cross between *B. graminis* resistant isolate CC151 and wildtype isolate DH14, MLA6 segregated in the Avirulence: Virulence: Intermediate avirulence ratio of 3:0:1 (Brown et al., 1996). Two regions of the *B. graminis* f. sp. *hordei* genome are known to contain clusters of linked *avr* genes. These include the *Avr* genes associated to EKA effector class which are clustered in the Avr_{K1} and Avr_{A10} locii (Ridout et

al., 2006; Ridout, 2009). Avr_{A10} and Avr_{K1}, encode proteins that act as both avirulence genes and effectors. Their recognition by host *R*-genes *MLA10* and *MLK1* results in a hypersensitive reaction typical of gene for gene interactions (Ridout et al., 2006). Their effector role is proved by increased powdery mildew susceptibility in barley plants transiently expressing Avr_{A10} and Avr_{K1} in the absence of the corresponding *R*-genes (Ridout et al., 2006). Moreover, silencing of Avr_{A10} and Avr_{K1} led to decreased percentage of haustorium formation (Nowara et al., 2010). In *B. graminis* f.sp. *tritici*, Avr effectors include *AvrPm3a2/f2*, and *AvrPm2* which are recognized by the wheat *Pm3a/f* and *Pm2 R* genes (Bourras et al., 2015; Parlange et al., 2015). *AvrPm3a2/f2* is highly conserved in worldwide *B. graminis* f.sp. *tritici* populations, supporting its role as putative effector candidate during wheat powdery mildew infection development (McNally et al., 2018). *B. graminis* f.sp. *tritici* also possesses RNase-like effector SvrPm3a1/f, which supresses PM3a/f-mediated race-specific resistance in wheat against powdery mildew. SvrPm3a1/f prevents the recognition of AvrPm3a/f recognition by the host *R*-gene PM3a/f, suppressing ETI and promoting ETS (Bourras et al., 2015).

Recently, a study also demonstrated the effect of *Ustilago maydis* secreted Pep1 effector in *B. graminis* infection of maize (Hemetsberger et al., 2015). Pep1 is thought to inhibit plant peroxidases and oxidative burst, facilitating pathogen entry and establishment of biotrophic growth in different smut fungi (Doehlemann et al. 2009, Hemetsberger et al. 2015). Another study also identified small RNAs in both barley and wheat powdery mildew fungi which may have targets in their respective hosts, suggesting a possibility of presence of sRNA effectors in *B. graminis* (Kusch et.al., 2018).

Blumeria effectors characterised so far are listed in Table 1-2.

1.4.2 *Fusarium graminearum* effectors and virulence factors

The manual annotation of genes in the latest version of the *F. graminearum* genome sequence assembly led to the discovery of 13,718 genes (Wong et al., 2011) of which 1,853 proteins are predicted to be secreted (King et.al., 2015). A refined analysis of this predicted secretome led to the identification of 574 secreted proteins of which 278 belonged to a group of putative secreted enzymes. 102 of these proteins are thought to be involved in degradation of components of plant cell wall, plasma membrane, fat bodies, starch bodies and protein bodies, which might be important for pathogenicity and virulence of the fungal pathogen. Moreover, a recent study has identified 190 small secreted cysteine-rich proteins (SSCPs) as potential effector candidates in *F. graminearum* infection, two of which are homologous to the *C. fulvum* LysM chitin binding effector Ecp6. Thirty-four SSCPs were expressed in infected wheat heads and half of them were upregulated during FHB disease development (Lu & Edwards, 2016).

Amongst the SSCPs, a secreted effector lipase *FGL1* was shown to act as a virulence factor for *Fusarium* infection in wheat. *FGL1* was shown to release free fatty acids for inhibiting plant innate immunity mediated callose deposition against FHB infection. Despite high DON secretion, the mutant strain colonisation was restricted to the inoculated spikelet as compared to the whole spike colonisation by wild type *F. graminearum* (Blümke et al., 2014).

The secreted trichothecene mycotoxin, deoxynivalenol (DON), is an importance virulence factor for *F. graminearum* infection in wheat ears, as it inhibits protein synthesis in eukaryotes (M. Kimura et al., 1998). The genes of the trichothecene mycotoxin loci also play an important role in *Fusarium* head blight virulence. Deletion of trichodiene synthase (*Tri5*) gene restricted *F. graminearum* colonisation to the inoculated spikelet, preventing further spread of the disease and host colonisation (Maier et al., 2006).

MAP kinases are important signalling molecules shown to have a role in fungal growth and pathogenicity (Román et.al., 2007). In *F. graminearum*, two MAP kinases *Mgv1* and *Map1* (the latter also called *gpmk1)* contribute to fungal virulence. Deletion of *Mgv1* (homologue of *Slt1* in *S. cerevisiae)* resulted in *F. graminearum* strains with weakened cell walls, sterility, reduced vegetative growth on solid media, self-incompatibility, incapability to form perithecia *in vitro*, reduced mycotoxin formation and virulence (Hou et al., 2002). Similarly, in two different studies, *Map1* (also called *gpmk1)* disruption/deletion resulted in apathogenic/non-virulent *F. graminearum* strains with reduced conidia formation and no sexual reproduction (Jenczmionka et.al., 2003; Urban et.al., 2003). This was not surprising, as *Map1* is homologue of *PMK1,* a MAP kinase previously shown to be required for virulence in *M. grisea,* affecting virulence by compromising appressorium formation (J. R. Xu & Hamer, 1996). Therefore, MAP kinase signalling contributes towards cell wall integrity, sexual reproduction and virulence in *F. graminearum*.

Another virulence factor affecting *F. graminearum* growth and morphogenesis in the enzyme 2-glycosyl transferase (GT2). *GT2* deletion mutants failed to penetrate the host due to lack of hyphae formation rendering *F. graminearum* avirulent (King et al., 2017). GT2 was postulated to be involved in fungal cell wall biosynthesis, as the mutants showed cell wall deformities (King et al., 2017).

Similarly, topoisomerase 1 (*Top1*) is another important virulence factor in *Fusarium graminearum*, as *top1* deletion mutants showed restricted fungal colonisation to inoculated spikelet, in spite of DON production (Baldwin et.al., 2010). Therefore, FHB infection requires a combination of the DON toxin and other virulence factors for full virulence of the pathogen.

1.4.3 *Zymoseptoria tritici* effectors and virulence factors

The initial asymptomatic phase of *Z. tritici* infection suggests the pathogen ability to evade host recognition in the early stages of host colonisation, very likely using effector-triggered- susceptibility strategies (Morais do Amaral et.al. 2012). Therefore, initial effector studies were based on the understanding of the asymptomatic phase. The publication of the *Zymoseptoria tritici* reference genome led to the discovery of 491 proteins expressed during the initial asymptomatic phase (Morais do Amaral et.al. 2012). Protein homology studies led to the discovery of the first effector protein - Mg3LysM – a homolog of Ecp6 effector of the biotrophic fungus *Cladosporium fulvum* (described above). Mg3LysM contains a chitin binding lysine-rich domain and plays a role in pathogen evasion of chitin- elicited PTI. Its role in *Z. trtici* virulence was evidenced by gene deletion studies producing *Z. tritici* strains severely impaired in host colonisation, incapable of inducing lesion formation and forming sexual spores (Marshall et al., 2011). Studies are being carried out to identify other *Z. tritici* virulence factors responsible for host ETI evasion and the switch to necrotrophic phase, but so far little progress has been made (Driscoll et al., 2014).

Recently, the first cytotoxic effector *Zt6* was described as cytotoxic against both plants and bacteria, indicating at a role in both virulence and niche protection (Kettles et al., 2018).

MAP kinases have been shown to play a significant role in STB disease development and spread by regulating fungal hyphal development and branching. Components of the Fus3 MAP kinase pathway like *ZtFus3, ZtSt50, ZtSt11* amongst others, when deleted, blocked hyphal branching on leaf surface preventing host penetration. Deletion of *ZtSt12,* on the other hand did not affect host penetration but restricted host colonisation due to absence of hyphal branching. Deletion of these MAP kinases also prevented pycnidia formation (Cousin et al., 2006; Kramer et al., 2009). A similar inability to colonise mesophyll cells was also observed post deletion of another MAP kinase *ZtSlt2* (Mehrabi et.al., 2006). Other MAP kinases like *ZtHOG1* affects the dimorphic switch essential for *Z. tritici* virulence. Deletion of this gene blocked hyphae formation on leaf surface preventing host penetration (Mehrabi et al., 2006).

Z. tritici hyphenation on leaf surface was also shown to be dependent on a 2-glycosyl transferase enzyme (GT2). *ZtGT2* deletion mutants (Δ*ZtGT2)* were unable to hyphenate on leaf surface, preventing host penetration and rendering them non-pathogenic. Δ*ZtGT2* mutants also had cell wall abnormalities and induced untimely expression of the effector Mg3Lysm on the leaf surface instead of during the latent phase, indicating at a probable role of GT2 in cell wall synthesis and *Z. tritici* transcriptional regulation post leaf sensing (King et al., 2017).

Few transcription factors have been shown to affect *Z. tritici* pathogenicity. *ZtWOR1*, which regulates small secreted proteins (SSPs), when deleted rendered the fungus avirulent due to inability to penetrate the host due to lack of hyphenation (Mirzadi Gohari et al., 2014). *Zt107320,* a homologue of the *M. oryzae* MoCOD1 transcription factor, was shown to regulate dimorphic switch i.e. transformation from spore stage to hyphae, and reduced *Z. trtici* virulence when deleted (Habig et.al., 2019).

Therefore, fungal virulence is dependent on a combination of effectors, vital genes, enzymes, signalling molecules and transcription factors. The virulence factors of the different fungal pathogens being studied are described in Table 1-2.

Table 1-2: Effectors and virulence factors characterised for the fungal pathogens B. graminis f.sp. *hordei (Bgh), B. graminis* f.sp. *tritici (Bgt), F. graminearum (Fg) and Z. tritici (Zt).*

Gene	Protein	Pathogen	Pathogen life cycle	Plant	Phenotype	Publication
BEC1005	β-1,3-Endoglycosidase-like-protein	*Bgh*	Biotrophic	Barley	HIGS - Reduced haustorial index	Pliego et al., 2013
BEC1011 (CSEP0264)	Ribonuclease-like-protein	*Bgh*	Biotrophic	Barley	Reduced haustorial index due to silencing of BEC1011. Supresses host PCD.	Pliego et al., 2013
BEC1016		*Bgh*	Biotrophic	Barley	HIGs -Reduced haustorial index	Pliego et al., 2013
BEC1018		*Bgh*	Biotrophic	Barley	Reduced haustorial index	Pliego et al., 2013
BEC1019	Metallo-protease-like-protein	*Bgh*	Biotrophic	Barley	Reduced haustorial index due to silencing. Conserved in a third of sequenced fungal species. Putative universal virulence factor in fungi.	Pliego et al., 2013 Whigham et al., 2015
BEC1038		*Bgh*	Biotrophic	Barley	HIGS - Reduced haustorial index	Pliego et al., 2013
BEC1040		*Bgh*	Biotrophic	Barley	HIGS - Reduced haustorial index	Pliego et al., 2013
BEC1054 (CSEP0064)	Ribonuclease-like-protein	*Bgh*	Biotrophic	Barley	HIGS - Reduced haustorial index	Pliego et al., 2013
CSEP0065	Ribonuclease-like-protein	*Bgh*	Biotrophic	Barley	asPTO based HIGS – reduced fungal secondary hyphae	Snehi Gazal, Master thesis, RHUL.

					formation; increased host ROS production.	Orman, Das Gupta et. al., manuscript in prep
CSEP0066	Ribonuclease-like-protein	*Bgh*	Biotrophic	Barley	asPTO based HIGS – reduced fungal secondary hyphae formation; increased host ROS production.	Snehi Gazal, Master thesis, RHUL. Orman, Das Gupta et. al., manuscript in prep
CSEP0081		*Bgh*	Biotrophic	Barley	Cytoplasmic effector – important for haustoria formation and infection development	Ahmed et.al., 2016
CSEP0254		*Bgh*	Biotrophic	Barley	Cytoplasmic effector – important for haustoria formation and infection development	Ahmed et al., 2016
CSEP0007		*Bgh*	Biotrophic	Barley	Knock down- Reduced odds ratio of haustorium formation	Aguilar et.al., 2015
CSEP0025		*Bgh*	Biotrophic	Barley	Knock down- Reduced odds ratio of haustorium formation	Aguilar et.al., 2015
CSEP0128		*Bgh*	Biotrophic	Barley	Knock down- Reduced odds ratio of haustorium formation	Aguilar et.al., 2015
CSEP0247		*Bgh*	Biotrophic	Barley	Knock down- Reduced odds ratio of haustorium formation	Aguilar et.al., 2015

Gene	Protein	Pathogen	Pathogen life cycle	Plant	Phenotype	Publication
CSEP0345		*Bgh*	Biotrophic	Barley	Knock down- Reduced odds ratio of haustorium formation	Aguilar et.al., 2015
CSEP0105		*Bgh*	Biotrophic	Barley	Chaperon activity of a heat shock protein	Ahmed et al., 2015
CSEP055		*Bgh*	Biotrophic	Barley	Interacts with the barley pathogenesis-related protein PR1 and PR17c Promotes fungal aggressiveness by suppressing defense.	Zhang et al., 2012
BEC1		*Bgh*	Biotrophic	Barley		Schmidt et al., 2014
BEC2		*Bgh*	Biotrophic	Barley		Schmidt et al., 2014
BEC3		*Bgh*	Biotrophic	Barley		Schmidt et al., 2014
BEC4		*Bgh*	Biotrophic	Barley	Interferes with host associated vesicle trafficking	Schmidt et al., 2014
$\boldsymbol{Avr_{K1}}$	Avirulence gene	*Bgh*	Biotrophic	Barley	Supresses PCD in the absence of *Hv* R gene MLK1	Ridout et al., 2006
$\boldsymbol{Avr_{A10}}$	Avirulence gene	*Bgh*	Biotrophic	Barley	Supresses PCD in the absence of *Hv* R gene MLA10	Ridout et al., 2006
ep1	Penetration enhancing protein 1	*Bgh*	Biotrophic	Barley	Suppress peroxidase activity and ROS generation	Hemetsberger et al., 2015

					Overexpression - Increased powdery mildew susceptibility	
Bcg1	Part of the AvrPm3 locus	*Bgt*	Biotrophic	Wheat	*Bcg1* mutation – disrupts AvrPm3-Pm3 mediated powdery mildew resistance	Parlange et al., 2015
AvrPm3$^{a2/f2}$	Avirulence effector	*Bgt*	Biotrophic	Wheat	Supresses ROS in the absence of wheat R gene Pm3.	Bourras et al., 2015
SvrPm3a1/f	Suppressor of AvrPm3a2/f2-Pm3 interaction	*Bgt*	Biotrophic	Wheat	SvrPm3a1/f associated with virulent AvrPm3a2 recognised *Blumeria* isolates	McNally et al., 2018
AvrPm2	Avirulence effector	*Bgt*	Biotrophic	Wheat	Suppressed PCD in the absence of wheat R-gene Pm2.	Praz et al., 2017
FGL1	Secreted lipase	*Fg*	Hemibiotrophic	Wheat	Reduced callose deposition (PTI). Infection restricted to infected spikelet.	Blümke et al., 2014
FgTom1	Tomatinase enzyme	*Fg*	Hemibiotrophic	Wheat	Deletion – reduced virulence	Carere et al., 2017
FTL1	Transducin beta-like gene Homologue of *S. cerevisiae* SIV2 involved in ascospore formation	*Fg*	Hemibiotrophic	Wheat	Deletion – reduced conidiation and virulence	Ding et al., 2009

FgSSP6	Chitin binding small secretory protein	*Fg*	Hemibiotrophic	Wheat	Accumulation of wheat fructans.	Hammond-Kosack2016- MPMI 2016 talk
FgSSP7					Prevents chitin elicited plant immune response	Hammond-Kosack2016- MPMI 2016 talk
					Silencing of fructan biosynthesis genes reduced FHB disease	
gpmk1/MAP1	MAP kinase	*Fg*	Hemibiotrophic		Deletion mutant avirulent	Jenczmionka et al., 2003; Urban et al., 2003
Tri genes (Tri5)	DON biosynthesis and regulation	*Fg*	Hemibiotrophic	Wheat	Tri 5 disruption reduced virulence	Proctor et al., 1995
FgGT2	2-Glycosyl transferase Importance in cell wall synthesis	*Fg*	Hemibiotrophic	Wheat	Deletion -Reduced hyphal growth on slid surface, Avirulent strain on wheat heads	King et al., 2017
FgMkk1	MAP kinase kinase	*Fg*	Hemibiotrophic	Wheat	Deletion – reduced virulence, hyphal growth, pigmentation, conidiation and DON production; increased sensitivity to cell wall damaging agents, osmotic and	Yun et al., 2014

					oxidative stress, *Trichoderma sp.*	
Mg3Lysm	Chitin binding effector	*Zt*	Hemibiotrophic	Wheat	Deletion - impaired colonisation, sexual sporulation and pycnidia formation. Subverts recognition through CERK1 and CeBP1	Marshall et.al. 2011 Lee et.al., 2014
Zt6	Secreted Ribonuclease	*Zt*	Hemibiotrophic	Wheat	Upregulated during *Zt* infection of wheat. Cytotoxic on wheat leaves; Cytotoxic to *E.coli*, yeast, monocots and dicots using transient expression systems (phytotoxic and anti-microbial)	Kettles et al., 2018
Zt GT2	2-Glycosyl transferase Importance in cell wall synthesis	*Zt*	Hemibiotrophic	Wheat	Deletion – non-pathogenic; no hyphal development on leaf surface; cell wall abnormalities; constitutive overexpression of Mg3Lysm effector and alpha-1,3-glucan on leaf surface.	King et al., 2017
ZtWOR1	Transcription factor	*Zt*	Hemibiotrophic	Wheat	Deletion – non-pathogenic; reduced sporulation *in vitro*;	Gohari et al., 2014

					regulates expression of specific SSPs.	
MgHOG1	MAP-kinase	*Zt*	Hemibiotrophic	Wheat	Deletion - unable to switch to filamentous growth on water agar; no germ-tube initiation on leaf surface reducing fungal virulence.	Mehrabi et al., 2006
MgSlt2	MAP-kinase	*Zt*	Hemibiotrophic	Wheat	Deletion – reduced virulence; no branching post host penetration, reducing invasiveness; no pycnidia formation; defective polarised growth *in vitro*; increased sensitivity to several fungicides.	Mehrabi et.al., 2006
ZtAde5	*De novo* purine synthesis	*Zt*	Hemibiotrophic	Wheat	Deletion – reduced virulence; reduced growth *in vitro*	Yemelin et al., 2017
MgTpk2 ***MgBcy1***	Catalytic and regulatory regions of protein kinase A	*Zt*	Hemibiotrophic	Wheat	Deletion – reduced virulence; no pycnidia formation	Mehrabi and Kema, 2006
MgSte11 ***MgSte7*** ***MgSte50***	Components of Pmk1/Fus3 MAP kinase pathway	*Zt*	Hemibiotrophic	Wheat	Deletion of *MgSte11, MgSte7 MgSte50* – reduced virulence; reduced filamentous growth on	Kramer et al., 2009

MgSte12					leaf surface preventing host penetration. *MgSte12* deletion – reduced infection spread due to lack of branching post host penetration.	
MgFus3	MAP kinase	*Zt*	Hemibiotrophic	Wheat	Deletion – non-pathogenic; absense of hyphal branching preventing host penetration; no pycnidia formation; lack of melanisation and aerial hyphae formation *in vitro*.	Cousin et al., 2006
ZtSSK1	Part of HOG pathway	*Zt*	Hemibiotrophic	Wheat	Deletion – reduced virulence; increased susceptibility to osmotic and oxidative stress; reduced germ-tube formation; increased sensitivity to phenylpyrrole fungicide; role in cell membrane biogenesis and dimorphic switch.	Yemelin et al., 2017

Zt107320	Homologue of *MoCOD1* transcription factor (Zn(II)2Cys6 family) from *M. oryzae.*	*Zt*	Hemibiotrophic	Wheat	Lower expression in incompatible interaction as compared to compatible interaction. Deletion – reduced fungal virulence in compatible interactions (reduced pycnidia); reduced fungal growth *in vitro* and under osmotic stress; fungal hyphal growth even in yeast-like growth inducing media, confirm a role in switch between yeast and hyphal stages.	Habig et.al., 2019

1.5 Plant immunity

Plants and pathogens are involved in a continuous co-evolutionary struggle amongst each other. Mostly, the plants can keep these pathogens at bay by recognising and responding to some conserved or variable pathogen elicitors. But sometimes, the pathogens can break or dampen the plant defences by secretion of a battery of virulence factors that manipulate the host immune responses and cause diseases, resulting in a compatible interaction. However, these pathogens are not able to infect all plants. Plants are mostly resistant to a plethora of microorganisms because of the incapability of these microbes to infect certain plants due to host incompatibility. Therefore, disease development is dependent on both host susceptibility and resistance as well as pathogenicity of the pathogen (Jones & Dangl, 2006; Rovenich et al., 2014)

Host compatibility determines a pathogen's host range whereas host incompatibility is conferred by non-host resistance. The host range of a pathogen can vary from a single plant species (small host range) to multiple plant species (broad host range). Usually, a pathogen can infect multiple host species which are phylogenetically closer to each other (Gilbert & Webb, 2007). However, the molecular mechanisms that decide the host range of a pathogen is not very well understood.

Non-host resistance (NHR) is a very durable form of resistance mediated by both constitutive barriers as well as inducible immune responses (reviewed by Nurnberger and Lipka, 2005). The heterologous pathogens i.e. the pathogens that have an incompatible interaction with a host plant (any cultivar of that species in the case of NHR), are first stopped by physical barriers like cuticle wax, cell wall and production of antimicrobials. In case if the heterologous pathogen is able to bypass the physical barriers it encounters the cell membrane associated receptors that recognise the pathogen-associated molecular patterns (PAMPs) like bacterial flagellin or chitin from the fungal cell wall and mount a pathogen triggered immune response (PTI) that neutralises the pathogen (reviewed by Nurnberger and Lipka, 2005). This PTI mediated immune response conferring a durable non-host resistance is an innate form of plant immunity.

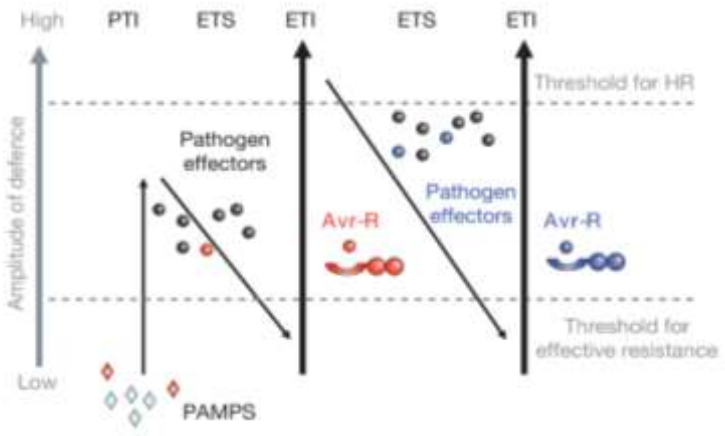


Figure 1-12: The zig zag model of plant immunity.

The pathogen associated molecular patterns (PAMPs) are recognised by PAMPS recognising receptors (PRRs) to induce PAMPS triggered immunity (PTI). The pathogens bypass the PTI by secreting effectors giving rise to Effector triggered susceptibility (ETS). The plants counter ETS by triggering Effector triggered immunity (ETI) by recognising the effectors or avirulence genes through nucleotide–binding leucine-rich receptors (NLRs) or R genes resulting in hypersensitive reaction (HR). (Jones & Dangl, 2006)

During compatible interactions, according to the simple zig-zag model described by Jones and Dangl (Jones & Dangl, 2006) (Figure 1-12), the innate immunity works at different levels through two different perception systems – Pathogen Associated Molecular Pattern (PAMPS) triggered immunity (PTI) and Effector triggered immunity (ETI). PTI is activated by perception of highly conserved PAMPS such as bacterial flagellin or chitin in fungal cell wall by plant membrane PAMPS-recognising receptors (PRR) and acts as first line of defence. Some PRRs are leucine rich repeat receptor kinases (LRR-RK) with an intercellular kinase domain and a LRR domain on the cell surface (Zipfel & Robatzek, 2010). Bacterial flagellin (fls22) is recognised by detection of highly conserved N-terminus region by FLS2 receptors in *Arabidopsis* (Gómez-Gómez & Boller, 2002) (Figure 1-13A). The fungi recognising chitin receptors Chitin Elicitor Binding Protein (CEBiP) and Chitin Elicitor Receptor Kinase 1 (CERK1), on the other hand, have chitin binding lysine domains (Miya et al., 2007). The chitin receptor CEBiP differs from other PRRs for the absence of intracellular kinase domains but it works in combination with CERK1 to induce MAP kinase signalling cascade. CERK1 can also function independently (Shimizu et al., 2010) (Figure 1-13B). PTI, among other responses, leads to stomatal closure, activation of MAP kinase signalling cascade, transcription of pathogen-responsive (PR) genes, callose deposition and ROS generation (Hou et al. 2011, Chisholm et al. 2006). PTI results in pathogen recognition and neutralisation before host invasion.

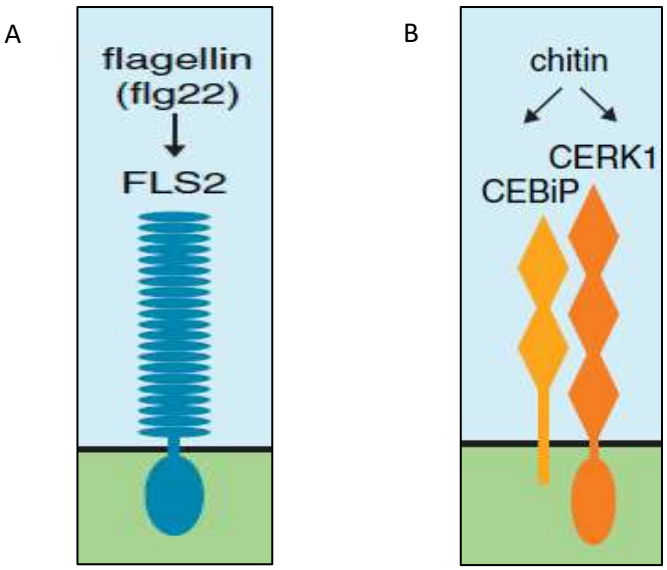


Figure 1-13 Examples of a bacterial and a fungal PAMPs recognising receptors (PRRs).

A. FLS2 is a bacterial PRR that recognises the 22 amino acids long flagellin peptide (flg22) present in bacterial flagella.

B. The fungal PRRs include CEBiP/CERK1. Chitin the main component of the fungal cell wall is detected by LysM domains of the CEBiP/CERK1 PRRs that induce kinase signalling. Diagram reproduced from (Monaghan & Zipfel, 2012).

Diseases usually occur when pathogens evade PTI by secretion of effector proteins either in the host cytosol or apoplast before or during the invasion, in order to mask PAMPS to prevent or delay their recognition by the host immune receptors. This phenomenon is called Effector Triggered Susceptibility (ETS). To counter this, plants have evolved Effector triggered immunity (ETI), which usually recognises species specific pathogen effectors in a gene-for gene specific manner (Panstruga & Dodds, 2009). This perception is mediated by intracellular nucleotide–binding leucine-rich receptors (NLR) encoded by host species or even cultivar specific resistance genes (*R*-genes) (Jones & Dangl, 2006).

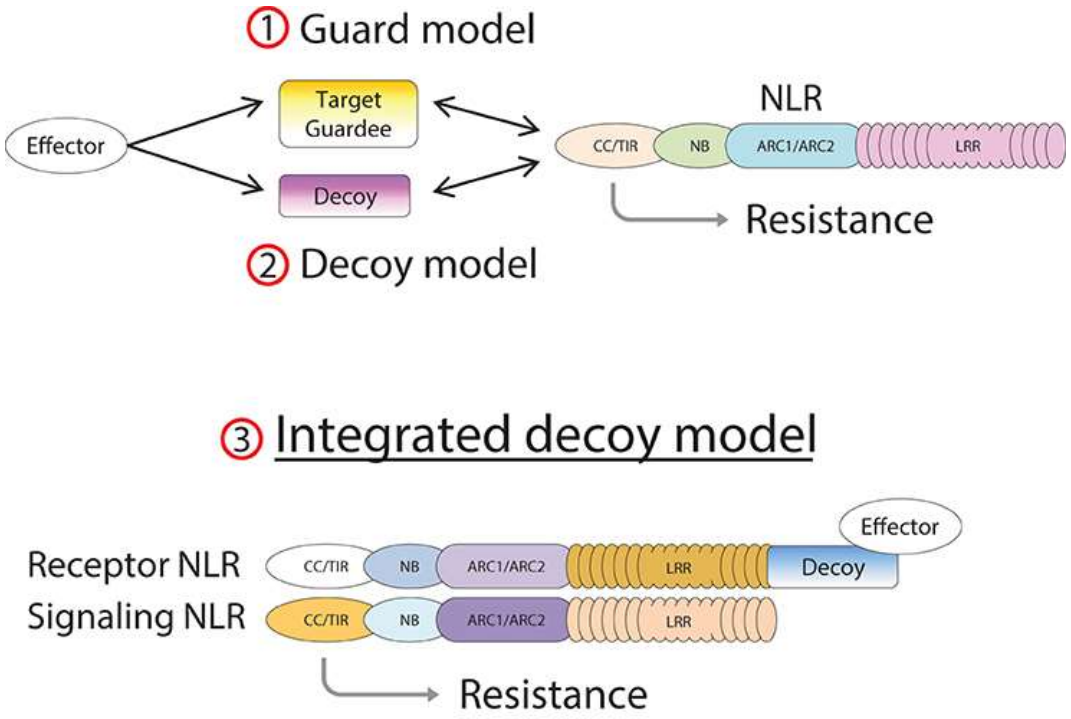


Figure 1-14: Models for describing the indirect recognition of effectors by the NLRs.

According to the Guard model, the NLRs monitor perceive effectors by monitoring the effector mediated alteration of target proteins that are guarded by the NLRs or when NLRs detect the change in a decoy protein caused by effector interaction. Alternatively, the Integrated decoy model suggests that these decoys may be structurally integrated into receptor component of the NLR allowing effector/Avr protein recognition by direct binding. Diagram reproduced from Cesari et.al., 2014.

NLRs are believed to perceive pathogen effectors using different mechanisms. Some NLRs directly perceive an effector protein as a non-self and induce an immune response. Tobacco *N*, a TIR-NB-LRR *R* gene that confers resistance to tobacco mosaic virus by recognising the p50 helicase, is one such NLR whose effector specificity is dependent on its TIR domain (Burch-Smith et al., 2007). Some NLRs perceive pathogens indirectly by recognising "modified self" i.e. alterations in host proteins induced by the pathogen effector. This is a much more efficient method of disease perception, as effectors of multiple pathogens target similar host proteins (Baggs et.al., 2017). This is called the 'Guard model' of NLR activity (Figure 1-14). Activation of this group of NLRs is regulated though an associated interacting host protein, which is either called a "guardee" if it retains a role in the host immunity/cellular processes or a "decoy" if it is only involved in NLR activation by acting as an effector bait (Baggs et al., 2017; Cesari et al., 2014). For instance, during *P. syringae* infection of *Arabidopsis,* the NLR RPS2, perceives the effector AvrRpt2 mediated degradation of the NLR associated RIN4 protein as a signal for mounting an ETI response against the bacterial pathogen (Axtell & Staskawicz, 2003). The activity of other altered self-recognising NLRs can be explained by the Integrated decoy model (Figure 1-14). In these NLRs, the bait is fused to the receptor in the form

of an additional non-canonical domain (Baggs et al., 2017; Cesari et al., 2014). For instance, the rice NLR Pik1 perceives the *P. syringae* effector Avr-PikD through its heavy metal-associated binding (HMA) domain, which results in a conformational change in the NLR (Maqbool et al., 2015). This conformational change in turn activates another physically linked NLR Pik2, resulting in a signalling active NLR complex (Baggs et al., 2017).

Some of these NLRs, like the *Arabidopsis* NLR RPS2, act alone to perceive the effector and trigger an immune response (Axtell & Staskawicz, 2003). Others act in pairs as the Pik1/Pik2 rice NLRs described above. Like Pik1/Pik2, one of the partners of the paired NLRs is involved in pathogen perception and trigger, whereas the other recognises the change in the confirmation of the first to remove the switch that induces the downstream immune response (Maqbool et al., 2015). The receptors of the paired NLRs have been shown to form heterogenous proteins during the initial effector perception stage (Williams et al., 2014).

These NLRs have a downstream helper NLR, to regulate the downstream immune signalling pathways. The helper NLRs are different from paired NLRs, as they are not physically linked to any sensory NLR receptors. The helper NLRs are receptors that safeguard immune signalling pathways downstream to different signalling receptors including the NLRs (Bonardi et al., 2011). Even the NLRs that regulate their function at the ligand perception level, need these helper NLRs to regulate downstream signalling (Gabriëls et al., 2007). So far three different helper NLRs have been characterised. These include the ACTIVATED DISEASE RESISTANCE 1 (ADR1), N REQUIREMENT GENE (NRG1) and NB-LRR REQUIRED FOR HYPERSENSITIVE RESPONSE-ASSOCIATED CELL DEATH 1 (NRC1). Both ADR1 and NRG1 belong to the CC_R-NLRs superfamily of helper NLRs which are characterised by the presence of an N-terminal regions similar to resistance to powdery mildew 8 (RPW8) domain which contains coiled coil (CC) motifs (reviewed by Baggs et al., 2017). The CC_R-NLRs are conserved across plant species indicating at a universal role in immune response signalling (Collier et al., 2011). NRC1 on the other hand belongs to the CC-NLR NRC helper clade that in specific to the Solanaceae family (Wu et al., 2016). The molecular mechanism triggering the activation of these helper NLRs is still not understood. But it is speculated that they may either perceive conformational changes in the receptor NLRs either directly or through changes in a shared signalling cascade (reviewed by Baggs et al., 2017). Interestingly, some NLRs can perceive disruptions in downstream MAP kinase cascade (Zhang et al., 2017). Therefore, helper NLRs which respond to these sensor NLRs can act as hubs which regulate immune signalling as a whole and not just for ETI response.

The ETI response is often more robust than PTI culminating into a hypersensitive reaction characterised by activation of MAP kinase cascade, localised expression of PR proteins, ROS,

resulting in programmed cell death at the infection site and callose deposition limiting the spread of infection (Eckardt 2002, Chisholm et al. 2006, Hou et al. 2011, Jones & Dangl 2006).

The downstream signalling to both PTI and ETI responses is regulated by plant hormones like jasmonic acid (JA) and salicylic acid (SA). The JA signalling pathway mediates resistance to necrotrophs and insect pests by suppressing host mediated ROS, whereas SA signalling pathway induces ROS to mediate resistance to biotrophs. These signalling pathways negatively regulate each other (reviewed by Glazebrook, 2005). Therefore, resistance to biotrophs can render a plant susceptible to necrotrophs. For instance, the resistance to biotrophic pathogen *Blumeria graminis* gained by silencing of the barley susceptibility gene MLO1 which negatively regulated host PCD, renders the barley susceptible to the necrotrophic pathogen *Ramularia collocygni* (McGrann et al., 2014).

Finally, the plant immune system induces systemic acquired resistance (SAR). SAR is also induced by SA signalling pathway and ensures broad-spectrum resistance to pathogens by transcription of PR proteins at sites away from the initial infection site (Eckardt 2002, Chisholm et al. 2006, Hou et al. 2011, Jones & Dangl 2006).

1.6 Susceptibility genes

Disease development occurs as a direct effect of host-pathogen interaction. It is equally dependent on pathogen virulence factors as well as the presence and expression of functional host susceptibility genes. Many plant susceptibility genes have first been identified by their over-expression during successful infection in compatible interactions between the pathogen and the plant host. In some cases, these susceptibility genes encode proteins which act as negative regulator of the host immune response. For instance, the barley mildew locus 1 (MLO1), a barley susceptibility gene for *B. graminis* f. sp. *hordei*, is a negative regulator of HR and programmed cell death (Büschges et al., 1997). MLO1 mutations inhibits host cell penetration by *B. graminis* f. sp. *hordei* (Piffanelli et al., 2002) associated with the formation of oversized papilla beneath penetration pegs (produced by *Blumeria* appressoria) at the site of attempted penetration. Although, powdery mildew resistance conferred by MLO1 mutation additionally requires the expression of ROR-1 and ROR-2 genes (Freialdenhoven et.al., 1996)., express receptor tyrosine kinases which contribute to SNARE-dependent non-host penetration resistance against powdery mildew (Collins et al., 2003).

Interestingly, MLO1 mutation renders the barley plants susceptible to hemibiotrophic and necrotrophic pathogens such as *Magnaporthae oryzae* and *Ramularia collocygni* respectively (McGrann et al., 2014). The second cost of using mlo mutations in spring barley is an enhanced susceptibility to several facultative pathogens, including *Magnaporthe oryzae* (blast; Jarosch et.al.,

1999), *Cochliobolus sativus* (spot blotch; Kumar et.al., 2001), and *Fusarium graminearum* (head blight; Jansen et al. 2005; Makepeace, 2006).

Products of susceptibility genes are likely targeted by effectors for evading host surveillance or alter host physiology in order to promote pathogenesis. For instance, the wheat susceptibility gene *Snn1* is targeted by the necrotrophic effector Sn-Tox1 from *Stagnospora nodorum,* a causative agent of *Stagnospora nodorum* blotch (SNB), resulting in oxidative burst. *Snn1* mutation prevents host penetration by *S. nodorum* and upregulation of wheat PR genes (Z. Liu et al., 2012). There is increasing studies evidencing many more novel plant susceptibility factors favouring fungal, oomycete or bacterial invasion. Therefore, silencing of host susceptibility genes is emerging as a strategy with high potential for the generation of disease resistant crop lines (Pavan et al. 2010; Vleeshouwers & Oliver 2014).

The fate of a gene to be considered as a susceptible factor, depends largely on the pathogen nutritional mode. Thus, the strategy of controlling crop diseases by silencing susceptibility genes, will be challenging, as it very likely differs between biotrophs, hemibiotrophs and necrotrophs. Biotrophic lifestyles requires a long-term symbiotic-like relationship with the life host for the acquisition of nutrients, whereas necrotrophs induce host cell necrosis for nutrient derivation. Hemibiotrophs need a combination of both for proliferation and disease development. For instance, the identified susceptibility genes favouring infection by the biotrophic *B. graminis,* such as Blufensin 1 and Bax Inhibitor 1, suppress ROS and PCD at the infection site to ensure living tissues for nutrient supply for fungal proliferation. (Meng et al. 2009; Ishikawa et al. 2011). On the other hand, this increase in antioxidant potential prevents tissue necrosis caused by PCD and confers resistance to necrotrophic and hemi-biotrophic pathogens, as shown by increased resistance to hemibiotrophic *Fusarium sp.* in barley varieties overexpressing *B. graminis* susceptibility gene bax inhibitor 1 (Ishikawa et al., 2011). This hypothesis is further supported by increased resistance to biotrophs and increased susceptibility to necrotrophs in *Arabidopsis* lines harbouring mutation in autophagy genes (ATG) involved in PCD (Lenz et al., 2011). Similarly, a recent study showed that overexpression of wheat resistance genes Lr34 and Lr46 for resistance against the biotrophic rust fungi *Puccinia sp.* enhanced susceptibility to the hemi-biotrophic *Zymoseptoria tritici* (Bansal, 2014).

Susceptibility genes that favour both biotrophic and necrotrophic growth would represent ideal targets for silencing or suppression in order to develop universal strategies for control of phytopathogens harbouring different lifestyles. One such example might be the *Arabidopsis* homoserine kinase, encoded by the downy mildew resistance gene, *DMR1*. Deletion of DMR1 results in foliar resistance to the biotrophic pathogens *Hyaloperenospora arabidopsis* and *Oidium neolycopersici* (Huibers et al., 2013). Similarly, mutation of different alleles of DMR1 result in silique

resistance and decreased leaf colonisation by hemibiotrophic *Fusarium graminearum and Fusarium culmorum* (Brewer et.al., 2014). These mutants were characterised by accumulation of homoserine in the disease resistant silique tissue. Interestingly, resistance could also be induced by external application of L-serine. However, the mutants showed smaller leaves and delayed senescence, which might mediate the resistance to *Fusarium sp* (Brewer et al., 2014).

An alternative strategy could be to target susceptibility genes affecting pathogens with one particular lifestyle, for instance, by targeting plant proteins associated with development of biotroph specific structures such as haustoria. One such example is the extra-haustorial protein REMORIN 1.3, which has recently been associated with a role in integrity of extra-haustorial membrane and susceptibility to the biotrophic oomycete *Phytophthora infestans.* Silencing of REMORIN 1.3 resulted in increased resistance to the oomycete in both tomato and *Nicotiana benthamiana* plants (Bozkurt et.al., 2014). However, Remorin 1.3 belongs to a superfamily of proteins with high sequence similarity and therefore, silencing might result in off targets.

A summary of plant susceptibility genes in included in Table 1-3.

Table 1-3: Summary of candidate susceptibility genes against different plant pathogens obtained by literature review.

Gene	Protein	Function	Pathogen	Pathogen life cycle	Plant	Phenotype	Publication
RAC4	OsRAC4 and OsRAC5	ROP GTPase activating protein	*Magnaporthae oryzae*	Hemibiotrophic	Rice	Silencing - increase rice blast resistance	Chen et. al. 2010
MLO1	Mildew Locus 1	Negative regulator of programmed cell death	*B. graminis* f.sp. *hordei*	Biotrophic	Barley	Silencing - Increased ROS, PCD, increased powdery mildew resistance and increased susceptibility to *Ramularia* spot disease	Büschges et al. 1997
BLN 1	Blufensin – 1		*B. graminis* f.sp. *hordei*	Biotrophic	Barley	Overexpression increased powdery mildew susceptibility	Meng et al., 2009
BI1	Bax Inhibitor-1	Broad spectrum cell death suppressor in plants	*B. graminis* f.sp. *hordei*	Biotrophic	Barley	Overexpression – Increased host penetration by *B. graminis* and abolished mlo resistance.	Babaeizad et al. 2009 Hückelhoven et.al., 2003
RACB	ROP GTPase activating protein	Role in fungal entry through plant cell membrane	*B. graminis* f.sp. *hordei*	Biotrophic		Knock out - Decreased haustoria formation	Schultheiss et al. 2002
Mds1	Mayetiola destructor susceptibility 1	Heat shock protein	*B. graminis* f.sp. *tritici*	Biotrophic	Wheat	Silencing - reduced lesion formation during infection	Liu et al. 2013
PMR5	Powdery Mildew Resistant gene 5		*Erisiphe sp.*	Biotrophic	*Arabidopsis*	Deletion – Lesser pectin modification in cell wall as compared to wild-type - reduced pathogen penetration of cell wall, Reduced cell size due to compromised cell expansion.	Vogel et al. 2004

Gene	Protein	Function	Pathogen	Pathogen life cycle	Plant	Phenotype	Publication
PMR6	Powdery Mildew Resistant gene 6		*Erisiphe cichoracearum*	Biotrophic		Mutation - More pectin than modified pectin in cell wall - reduced pathogen penetration of cell wall	Vogel et al. 2002
DMR1	Downy mildew resistance gene 1	Homoserine kinase	*Fusarium graminearum*	Hemibiotrophic	*Arabidopsis*	Knock-out increases resistance to biotrophs and Hemibiotrophs Reduced leaf size	Brewer et al. 2014
			Fusarium culmorum	Hemibiotrophic			
			Hyaloperonospora arabidopsis	Biotrophic			
			Oidium neolycopersici	Biotrophic			
			Oidium neolycopersici	Biotrophic	Tomato	Silencing – Increased tomato PM resistance, Severely reduced plant growth	Huibers et al., 2013
Lr34, Lr46	ABC transporter	Resistance gene to wheat rust fungus	*Zymoseptoria tritici*	Hemibiotrophic	Wheat	Silencing - Resistance to biotrophic powdery mildew and wheat rust. Susceptibility to STB	Bansal, 2014
			Magnaporthae oryzae				
REM1.3	Remorin protein	Membrane binding domain imp for Phytophthora susceptibility by influencing perihautorial membrane integrity	*Phytophthora infestants*	Biotrophic		Overexpression - susceptibility to biotrophs	Bozkurt et al. 2014
			Pseudomonas infestans				

Gene	Protein	Function	Pathogen	Pathogen life cycle	Plant	Phenotype	Publication
ZmGF14-6	14-3-3 protein -	Signalling pathway regulators	*Fusarium verticilliodes*	Hemibiotrophic	Maize	Overexpression -Increased pathogen susceptibility and drought tolerance	Campo et al. 2012
			Magnaporthae oryzae		Rice		
PROVIR 1-13	Pro-virulence factor	Unknown function	*Plectosperilla cucumerina*	Necrotrophic	*Arabidopsis*	Overexpressed during disease	Dobón et al. 2015
PSY 1	Plant peptide containing sulphated tyrosine	Hormone peptide	*Plectosperilla cucumerina*	Necrotrophic	*Arabidopsis*	Overexpressed during disease	Dobón et al. 2015
AGP24	Arabino galactan protein	Control plant growth and development by AGP anchorage to lipid in plant plasma membrane	*Plectosperilla cucumerina*	Necrotrophic	*Arabidopsis*	Overexpressed during disease	Dobón et al. 2015
GRP3	Extracellular matrix associated Glycine-rich proteins	Potential role in Pathogenesis signal transduction by interaction with WRK1 protein kinase receptors involved in *Arabidopsis* disease resistance	*Plectosperilla cucumerina*	Necrotrophic	*Arabidopsis*	Overexpressed during disease	Dobón et al. 2015
RALF23	Rapid Alkalization Factor	Apoplastic alkalisation; plant cell growth	*Plectosperilla cucumerina*	Necrotrophic	*Arabidopsis*	Overexpressed during disease	Dobón et al. 2015
DVL3	DEVIL3	Regulation of polar cell proliferation and	*Plectosperilla cucumerina*	Necrotrophic	*Arabidopsis*	Overexpressed during disease	Dobón et al. 2015

		longitudinal growth of organs					
Gene	**Protein**	**Function**	**Pathogen**	**Pathogen life cycle**	**Plant**	**Phenotype**	**Publication**
EDR1	Enhanced disease resistance 1	MAPKK kinase Negatively regulates the ATL1 E3 Ubiquitin ligase to suppress cell death Negatively regulates SA-inducible defence responses.	*Erisiphe cichoracearum*	Biotrophic	*Arabidopsis*	Loss of function mutation – Increased reistance to powdery mildew, enhanced PCD during biotic and abiotic stress	Frye & Innes, 1998 Frye et al. 2001 Serrano et. al. 2014
Tsn1		Effector/toxin Tsn-ToxA recognition	*Stagnospora nodorum*	Necrotrophic	Wheat	SNB susceptibility based on host-toxin interaction	Friesen et al. 2009
Snn1		Confers susceptibility to toxin SnTox1	*Stagnospora nodorum*	Necrotrophic	Wheat	SNB susceptibility based on host-toxin interaction	Friesen et al. 2009
Snn2		Confers susceptibility to toxin SnTox2	*Stagnospora nodorum*	Necrotrophic	Wheat	SNB susceptibility based on host-toxin interaction	Friesen et al. 2009
Snn3		Confers susceptibility to toxin SnTox3	*Stagnospora nodorum*	Necrotrophic	Wheat	SNB susceptibility based on host-toxin interaction	Liu et al. 2009
Snn4		Confers susceptibility to toxin SnTox4	*Stagnospora nodorum*	Necrotrophic	Wheat	SNB susceptibility based on host-toxin interaction	Abeysekara et. al. 2009
Snn5		Confers light-dependent susceptibility to toxin SnTox5	*Stagnospora nodorum*	Necrotrophic	Wheat	SNB susceptibility based on host-toxin interaction	Friesen et al. 2012

Gene	Protein	Function	Pathogen	Pathogen life cycle	Plant	Phenotype	Publication
Snn6		Confers light-dependent susceptibility to toxin SnTox6	*Stagnospora nodorum*	Necrotrophic	Wheat	SNB susceptibility based on host-toxin interaction	Gao et al. 2015
PMR4	Powdery mildew resistance gene 4	Callose synthase	*Powdery mildew fungi*	Biotrophic	*Arabidopsis*	Overexpression – early callose deposition, complete penetration resistance	Ellinger et. al. 2013
			Oidium neolycopersici	Biotrophic	Tomato	Silencing -Normal plant growth and reduced susceptibility to tomato PM	Huibers et al. 2013
			Psuedomonas syringae	Biotrophic	*Arabidopsis*	Deletion – Increased basal resistance	Flors et. al. 2007
			Alternaria brassicicola	Necrotrophic	*Arabidopsis*	Deletion – Increased susceptibility	
LOV1		Resistance gene involved in disease resistance	*Cochliobolus victorae*	Necrotrophic	*Arabidopsis*		Lorang et al. 2007
MYB46		Regulates transcription of pathogen induced ep5c gene coding for a membrane bound peroxidase	*Botrytis cinerea*	Necrotrophic	*Arabidopsis*		Ramírez et al. 2011
DS1	Phosphatidic Acid Phosphatase	Regulation of lipid biosynthesis and lipid-based signalling molecules	*Ralstonia solanacearum*	Necrotrophic	*Nicotiana benthamiana*	Knock-out increases resistance to *Ralstonia*	Nakano et al. 2013

Gene	Protein	Function	Pathogen	Pathogen life cycle	Plant	Phenotype	Publication
GhWRKY40		Stress inducible transcription factor	*Rhizoctonia solani*	Necrotrophic	*Nicotiana benthamiana*	Overpression – increased susceptibility to *Rhizoctonia solani;* reduced tolerance to drought stress	Wang et al. 2014
TaNac1	Transcription factor	Supresses stress signalling through Ja and SA signalling pathways	*Psuedomonas syringae*	Biotrophic	*Arabidopsis*	Knock down - Resistance biotroph *Puccinia,* Overexpression- Increased susceptibility and reduced SAR for biotrophic *Psudomonas syringae*	Wang et al. 2015
			Puccinia striiformis f. sp. *tritici*	Biotrophic			
HvPP2C	Protein phosphatase 2C gene	Role in plant signal transduction	*Puccinia graminis* f. sp. *tritici*	Biotrophic	Barley	Expression – reduced RPG5 protein kinase mediated resistance against wheat rust	Wang et al., 2013
StNLR1	Ubiquitin E3 ligase	Negative regulator of plant immunity	*Phytophthora infestans*	Biotrophic	Potato	Interacts with effector to promote immune suppression by degradation of PCD inducing SWAP70. StNLR1 deletion – stopped SWAP70 degradation by the effector Pi02860.	He et al., 2018

1.7 Gene silencing strategies in plants for functional genomic analysis and further applications

1.7.1 Gene silencing is mediated by RNA interference (RNAi)

Gene silencing mediated by RNA interference (RNAi) is a highly conserved phenomenon across eukaryotes, occurring at transcriptional, post-transcriptional or translational level. In eukaryotic cells, RNAi is conveyed by small interfering RNA (siRNA). This naturally occurring phenomenon has been exploited to develop robust functional genomic tools to address gene function by gene expression knock-down or gene silencing, for subsequent study of silenced gene phenotype.

RNAi-dependent gene silencing is triggered by the presence of double stranded (ds) RNA molecules. These are processed by a eukaryotic RNase III enzyme called DICER or DICER-like (DCL) proteins. The siRNA and DICER form RNA Induced Silencing Complex (RISC) in the presence of ARGONAUTE proteins. The recruitment of RISC complex results in siRNA unwinding and cleavage of sense strand. The antisense strand then forms a duplex with target mRNA which is cleaved by the catalytic domain of ARGONAUTE resulting in post-transcriptional gene silencing (Danehault, 2006) (Figure 1-15).

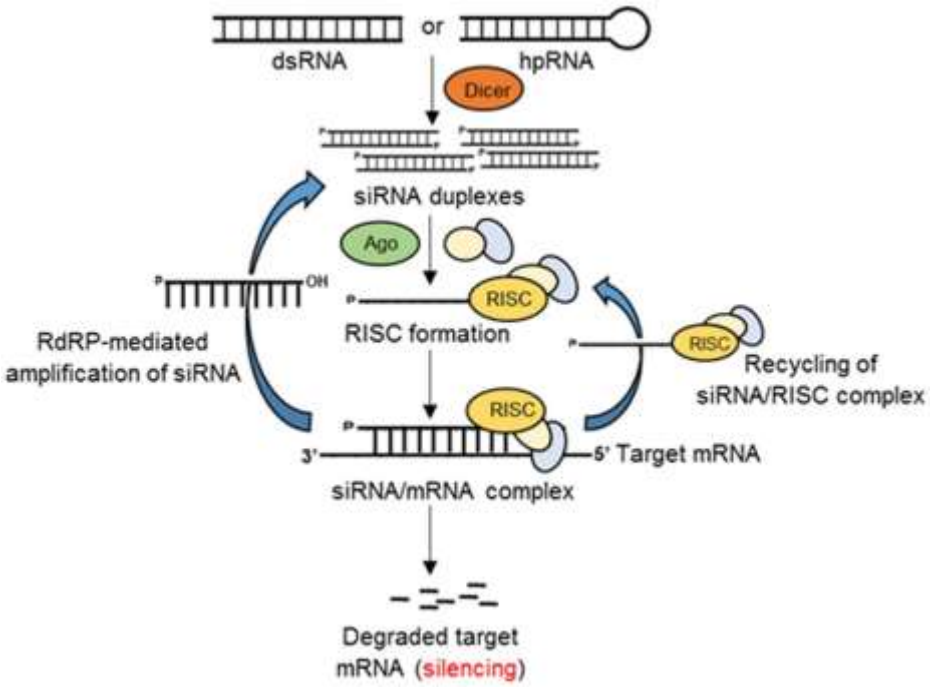


Figure 1-15: Mechanism of RNAi gene silencing

RNAi gene silencing mediated by long double stranded RNA (dsRNA) or hairpin RNA (hpRNA) occurs through post-transcriptional DICER mediated degradation of target mRNA by engaging the ARGONAUTE and RISC complex present in eukaryotic cells. Diagram obtained from Majumdar et.al., 2017.

RNAi silencing has been extensively utilised by researchers in functional genomics and in disease therapeutics in animal models (reviewed by Burnett et.al., 2011). In plant sciences, RNAi silencing has been successfully utilised for improving agronomic traits. Suppression of gene expression of rice GA 20 oxidase gene resulted in increased grain yield (Qia et. al. 2010). RNAi silencing has also been used to improve tomato fruit quality by enhancing flavonoids and carotenoids by silencing DET1 gene, which globally resulted in increased nutritional content (Davuluri et. al. 2005). Flavr Savr tomatoes were the first commercially available GM vegetables, approved by FDA in 1992, where polygalacturonase gene was silenced (Redenbaugh, 1992). Similarly, tomato shelf life has been increased by delayed senescence caused by RNA based silencing of ACC oxidase involved in ethylene production (Xiong et. al. 2005). Another important application of RNAi silencing has been in improving abiotic stress tolerance. Drought tolerance of rice plants has been improved by silencing of ubiquitin ligase gene encoding a RING-finger E3 ligase (Bae, 2011).

Biotic stress caused by phytopathogens is another important aspect of crop health, resulting in major yield and economic losses. Development of RNAi silencing based methods for understanding of plant-pathogen interactions and improving disease protection is therefore, of paramount importance in modern agricultural research.

1.7.2 RNAi gene silencing strategies for crop protection

In the recent years, RNAi silencing technology has progressively translated into strategies for crop protection against microbial pathogens, insects and nematode pests (Koch & Kogel 2014; Karthikeyan et al. 2013). The initial strategies involved delivery of dsRNA by viral vectors. VIGs used the mechanisms involved in plant's natural defence against viruses, resulting in post transcriptional gene silencing by degradation of target transcripts (Voinnet O., 2001). Binary vectors are created by cloning cDNAs of viral genomes minus the genes inducing viral symptoms under the CaMV35S promoter. Multiple cloning sites are introduced to insert target genes. Moreover, *Agrabacterium* left and right borders are also present to help in *Agrobacterium* mediated transformation of plants (Voinnet O., 2001). VIGS vectors contain usually 300–500-bp plant target gene fragment with efficient siRNA generation and no off-targets. A VIGS construct is introduced into plant cells via *Agrobacterium*-mediated transient expression. In the plant cells, the transgene is amplified along with the viral RNA by either an endogenous or a viral RNA-dependent RNA polymerase (RdRp) enzyme generating dsRNA molecules. These dsRNA intermediates are then recognized by DICER-like enzymes which cleave dsRNA into small interfering RNAs (siRNAs) of 21- to 25-nucleotides. The siRNA then binds to target mRNA. The resulting RNA dimer is recognised and degraded by the RISC complex, resulting is post transcriptional gene silencing (Ramegowda et al., 2014) (Figure 1-16A).

VIGs has been extensively used for both functional genomics and crop improvement (Karthikeyan et al., 2013). Recently, VIGs has been used to validate the role of protein ubiquitination in resistance against necrotrophic pathogen *Botrytis cinerea* (Zhang et al., 2016) and has been adapted as a functional genomics tool for studying STB disease development by *Zymoseptoria tritici* (Lee et.al., 2015). Nevertheless, a major shortcoming of VIGS is the use of vectors derived from pathogenic viruses like Barley Strip Mosaic Virus (BSMV) for delivery of silencing cassette that can activate or hinder with the plant immune system (Tufan et.al., 2011).

RNAi gene silencing in organisms unable to produce stable or transient transformants *in vitro* (e.g. obligate biotrophs) was only made possible by the development of host-induced gene silencing (HIGS). Nowara et. al. (2010) developed a new *in planta* transient assay to silence the fungal effector gene Avr_{a10} in *B. graminis* by delivering silencing plasmids expressing a corresponding hairpin dsRNA (hpRNA) into barley plants. The HIGS method was established by using a plasmid expressing the hairpin RNA (hpRNA) silencing construct, which was delivered alongside with a GUS gene reporter construct by biolistic bombardment. The gene specific hpRNA is degraded to siRNA by plant or fungal dicers. The siRNA binds to the target mRNA, forming a dimer which is recognised and degraded by the RISC complex, resulting in post transcriptional gene silencing. Disease scoring using microscopy showed a decreased haustorial index, which was supported by decreased expression of fungal genes (Pliego et al., 2013) (Figure 1-16B). This method was also used to silence genes in hemi-biotrophic phytopathogens including the *F. graminearum* (Cheng et al., 2015). Over the years, HIGS type approaches have been developed as a significant tool for functional analysis of effectors in biotrophic systems. HIGS-based silencing as initially described by Nowara et al (2010) helped in validating the virulence role of putative *Blumeria* Effector Candidates (BEC) in *B. graminis* (Pliego et al., 2013), including a potential universal fungal virulence factor BEC1019 (Whigham et al., 2015). In one these functional genomics studies, HIGs was achieved by biolistic delivery of hpRNA (Pliego et al., 2013). HIGs based strategies have also been used in stable transgenic plants to restore the susceptibility of the cotton bollworm to pyrethoid insecticides (Mao et al., 2007). This susceptibility is restored by the insects feeding on leaves expressing hpRNAs to silence the insect cytochrome P450 reductase. However, HIGS transient silencing assays are not restricted to biolistic delivery. For instance, an *Agrobacterium* based transformation system was exploited to silence and then identify three haustorial proteins in *Puccinia sp.* acting as putative virulence genes in all three species of rust fungi (Panwar et.al., 2013; Yin et al., 2015). Another major shortcoming of HIGs and VIGs is the need for cloning RNAi silencing cassettes and suitable transformation procedures. Therefore, more convenient strategies involving direct delivery of silencing molecules such as antisense

oligodeoxiribonucleotides (ASO) and endonuclease prepared small interfering RNA (esiRNA) have been explored for efficient and high-throughput gene silencing (Koch et al., 2013; Sun & Ho, 2005).

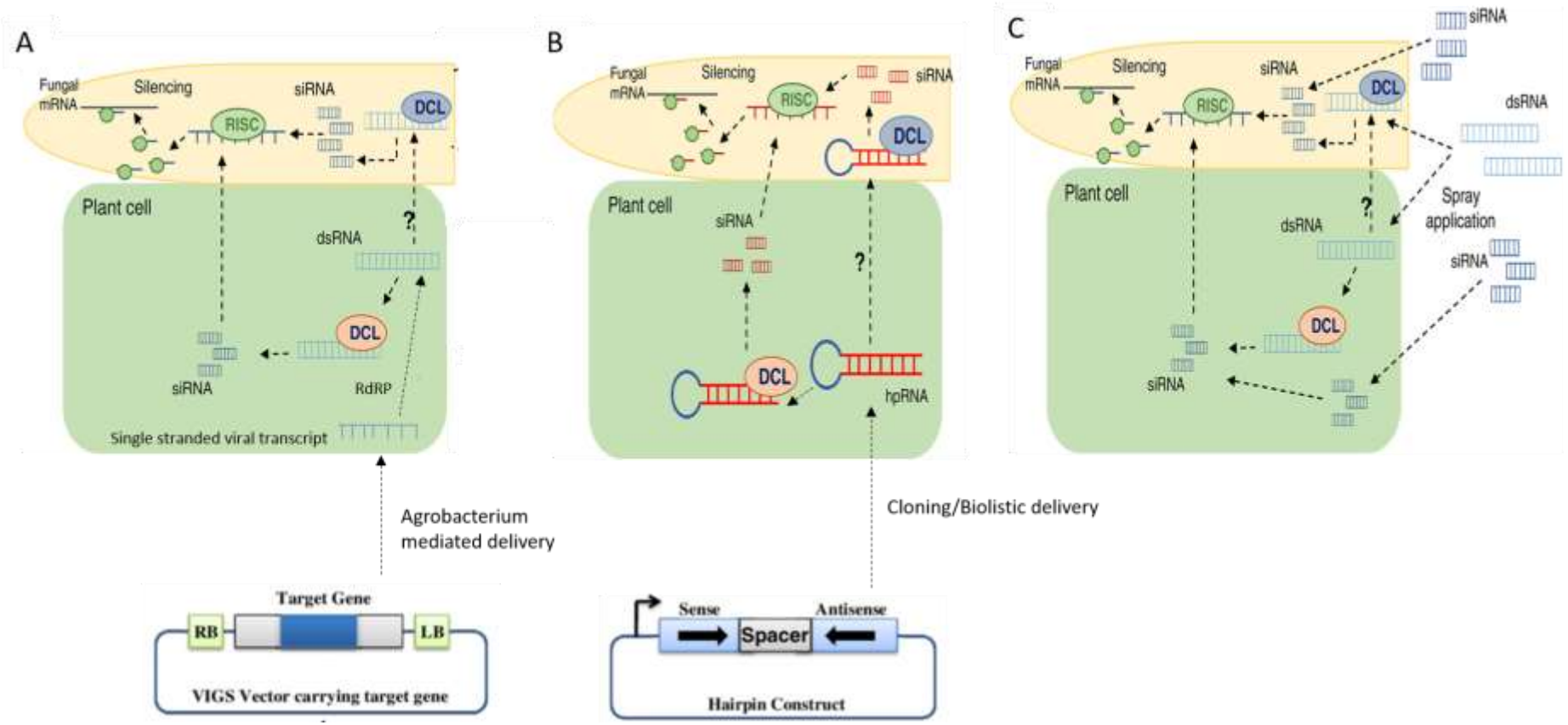


Figure 1-16: Comparison of silencing mechanisms of Virus induced gene silencing (VIGS), Host induced gene silencing (HIGS) and Spray induced gene silencing (SIGS).

A. VIGS: Transgenic plant with target RNA cloned into a binary viral-Agrobacterium vector and introduced into plants via Agrobacterium mediated transformation. A single stranded viral transcript is produced within the transgenic plant. Processed single stranded RNA (ssRNA) is converted into double stranded RNA (dsRNA) by the action of RNA-dependent RNA polymerase (RDRP). In the plant cell, the dsRNA processed by DCLs into several siRNA duplexes either in plants or fungi which act as guide molecule. Guide strand of siRNA then loaded into RISC and triggers target gene silencing via target transcript degradation.

B. HIGS: Transgenic introduction of hairpin RNA into the plants via either cloning or biolistic delivery. The transgenic plants produce long double-stranded RNAs (dsRNAs), which could be cleaved into small interfering RNAs (siRNAs) by either the plant Dicer-like proteins (DCL) or fungal DCL proteins. Once plant siRNAs enter the fungus (by still unknown mechanism), the guide siRNA strand binds with Argonaute and other proteins to form a RNA-induced silencing complex (RISC). The siRNA/RISC binds the complementary sequence of the target mRNA in the fungus, resulting in degradation of target mRNA.

C. SIGS: Ectopic spray application of silencing molecules resulting in non-transgenic organism. Sprayed long dsRNAs and siRNAs can be taken up by both plant and fungal cells. The long dsRNAs within the plant cell could be processed into siRNAs by the plant DCL proteins or taken up by the pathogenic fungi. Long dsRNAs within the fungi are processed into siRNA by the fungal DCL protein. The guide siRNA strand binds to Argonaute and other proteins to form a RISC. The siRNA/RISC binds the complementary sequence of the target mRNA in the fungi, resulting in target transcript degradation.

Diagram adapted from Machado et al., 2017.

1.7.3 Endonuclease prepared small interfering RNA (esiRNA) for gene silencing

Endonuclease-prepared small interfering RNA (esiRNA) are enzymatically prepared (*in vitro* or in *E.coli*) dsRNA of about 300 nt length used for gene silencing. The silencing mechanism is akin to that of other dsRNA mediated gene silencing. It acts at post-transcriptional level involving DICER, ARGONAUTE and the RISC complex, resulting in degradation of the target RNA. Double strand synthesised 300+ nts esiRNA is then processed by *E. coli* RNase III to shorter dsRNA ranging from 21-25 nt. Using longer dsRNA specific to the targeted gene often enhances the esiRNA molecule silencing efficiency but might decrease target specificity. In a recent study, direct uptake of long, unprocessed dsRNA targeting a gene of the ergosterol pathway resulted in its silencing and was accompanied with decreased *in vitro* growth of *Fusarium graminearum* (Koch et al., 2013). This was followed by multiple studies which have achieved HIGs of fungal genes by spraying the gene specific dsRNA. Spraying of 791 nt long dsRNA targeting the CYP genes in *F. graminearum* onto barley leaves, led to reduced lesion development post fungal inoculation. Silencing was believed to be mediated by siRNA formed by cleavage of the 791 nt long dsRNA by the plant dicer proteins (Koch et al., 2016). In another study, spray induced gene silencing (SIGS) of phenamacril fungicide target *Myo5*, reduced both fungal virulence and fungicide resistance in *Fusarium asiaticum* (Song et.al., 2018). Spraying of dsRNA mediated disease control was also achieved in *Botrytis cinerea*. Spraying of 20 ng/µl of *B. cinerea* dicer 1 and 2 targeting sRNAs and dsRNAs reduced brown rot development in fruits, vegetables and flowers (Wang et al., 2016). Interestingly, spraying of dsRNA targeting *F. asiaticum β-tubulin* gene, conferred plant resistance and increased MBC fungicide sensitivity in multiple phytopathogens like *F. asiaticum, B. cinereal, M. oryzae* and *C. truncatum*. These dsRNA molecules targeted 3 regions in the *F. asiaticum β-tubulin* gene which are highly conserved amongst the studied phytopathogens (Gu et al., 2019).

Once sprayed, the dsRNA is believed to be taken up by the plant or fungi by still unknown mechanism. Once in, the dsRNA is degraded to siRNA by dicer proteins. The siRNA bind target mRNA and cause post transcriptional gene silencing via target transcript degradation by the RISC complex (reviewed by Machado et al., 2017) (Figure 1-16C).

The biggest advantage of using dsRNA for gene silencing is the possibility of their direct delivery into plant cells, in the absence of a biological agent or biolistics, which eliminates the need for constructing cloning cassettes and elaborate transformation techniques. This technology is therefore, much simpler and less time consuming. Moreover, the direct delivery of the silencing molecules via the vasculature allows distribution to entire plant organs such as leaves, thus allowing for the global study of that organ, as potentially all cells have been treated for gene silencing. This is

an advantage over biolistic transformation which only allows individual single cell analysis. Therefore, dsRNA-based SIGs seems to be highly applicable in agriculture. However, their application was shown to be limited by the RNAi secondary amplification machinery in fungi. Spraying of *Myo5* targeting dsRNA could only confer resistance to multiple *Fusarium sp.* for up to 9 hours, requiring multiple applications for a long-lasting effect. While targeting plant genes, the silencing effect lasted much longer (Song et al., 2018). Therefore, dsRNA-based disease control has its limitations. This necessitated the exploration of novel silencing molecules like antisense oligodeoxynucleotides (ASOs), which can achieve HIGs by direct delivery into host plants (Dinc et.al., 2011).

A comparison of the silencing mechanisms via VIGs, HIGS and SIGS is discussed in Figure 1-16.

1.7.4 Antisense oligodeoxyribonucleotides (ASO) as alternative silencing molecules

The use of single stranded antisense deoxynucleotide (ASO/ODN) for gene silencing was first demonstrated by Zamecnik and Stephenson in 1978 in animal cells, when they reported growth inhibition 0f Roux Sarcoma virus in the infected cell lines on addition of 13-mer oligodeoxynucletiodes (Zamecnik & Stephenson, 1978). Antisense oligodeoxyribonucleotides (ASO) are single stranded 19-25 long nucleotides (nt) DNA molecules, which bind complementary RNA molecules, thus, resulting in gene silencing. However, after decades of ASO development, clinical applications were limited due to issues with intracellular stability and delivery of ASO into the animal cells. In the recent years, chemical modifications have been developed which have increased cellular stability and bioavailability of the ASOs (reviewed by Shen & Corey, 2018). The clinical trials of ASO based drugs started in the 1990s, with phase 1 trial of ASOs targeting p53 transcripts in patients with either relapsed or refractory acute myelogenous leukaemia or myelodysplastic syndrome (Bayever et.al., 1993). The first FDA approved ASO based drug was Fomiversen, where ASOs targeted cytomegalovirus (CMV) in HIV patients with CMV retinitis and was administered by intravitreous injections into the eye (Roeher, 1998). CpG ODNS, i.e. synthetically synthesised ODNs containing a cytosine residue linked to guanine residue by a phosphodiester bond, was first co-administered in a phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen, as an immunostimulatory ODN adjuvant (Halperin et. al. 2003). Similarly, synthetically produced G-rich ASOs, called aptamers, can form quadruplex that are known to have a high affinity to bind proteins via shape specific recognition. AS1411 aptamers have been developed for cancer therapy, where they are believed to bind nucleolin overexpressed in tumour cells, thereby interfering with nucleolin intracellular signalling, eventually leading to apoptosis (Bates et al., 2010). Lipid-micelle based ODN delivery has also been used for performing exon skipping for treatment of Duchene muscular dystrophy (DMD) (Cirak et al., 2011). ASOs since been used for gene therapy for many inherited

diseases (Reviewed by Martínez et al. 2013). Moreover, ASO mediated gene silencing based therapy is under clinical trials for cure of cancer (Moreno & Pêgo, 2014) and neurological disorders (Rinaldi and Wood, 2018).

In plant cells, applications of ASOs for gene silencing are comparatively limited, maybe because of the plant cell wall acting as a barrier for ASO delivery. Though direct uptake of ASO by barley aleurone cells from tissue culture media was first reported by Tsutsumi et. al. (1992). ASO delivery has also been achieved through uptake by pollen tubes leaf infiltration (Dinc et al. 2011; 2012) or direct uptake by plant vascular tissue by dipping cut leaves directly into the ASO solution (Sun & Ho, 2005; Dinc et al., 2011). These studies used ASO based gene silencing for functional genomic studies for many nuclear or chloroplastic genes in wheat, barley and *Arabidopsis* (Dinc et al. 2011; Sun & Ho 2005; Sun et al. 2007; Xie et al. 2014)

At the cellular level, ASO based gene silencing is thought to occur via different mechanisms. Firstly, ASOs may act at the transcriptional level, with ASO binding to the complementary matching pre-mRNA, and thus interfering with RNA maturation. At post-transcriptional level, gene silencing may occur by direct degradation of the ASO-mRNA heterodimers which are recognised and processed by RNase H, in a similar way to dsRNA molecules (Wu et al., 2004). ASOs also causes translational arrest by stearic hindrance resulting from prevention of mRNA binding to ribosome (Baker et al., 1997). Moreover, silencing may also occur by epigenetic phenomenon by modification of the chromatin at the DNA level causing changes in DNA methylation of the targeted gene (Baulcombe, 2004) (Figure 1-17).

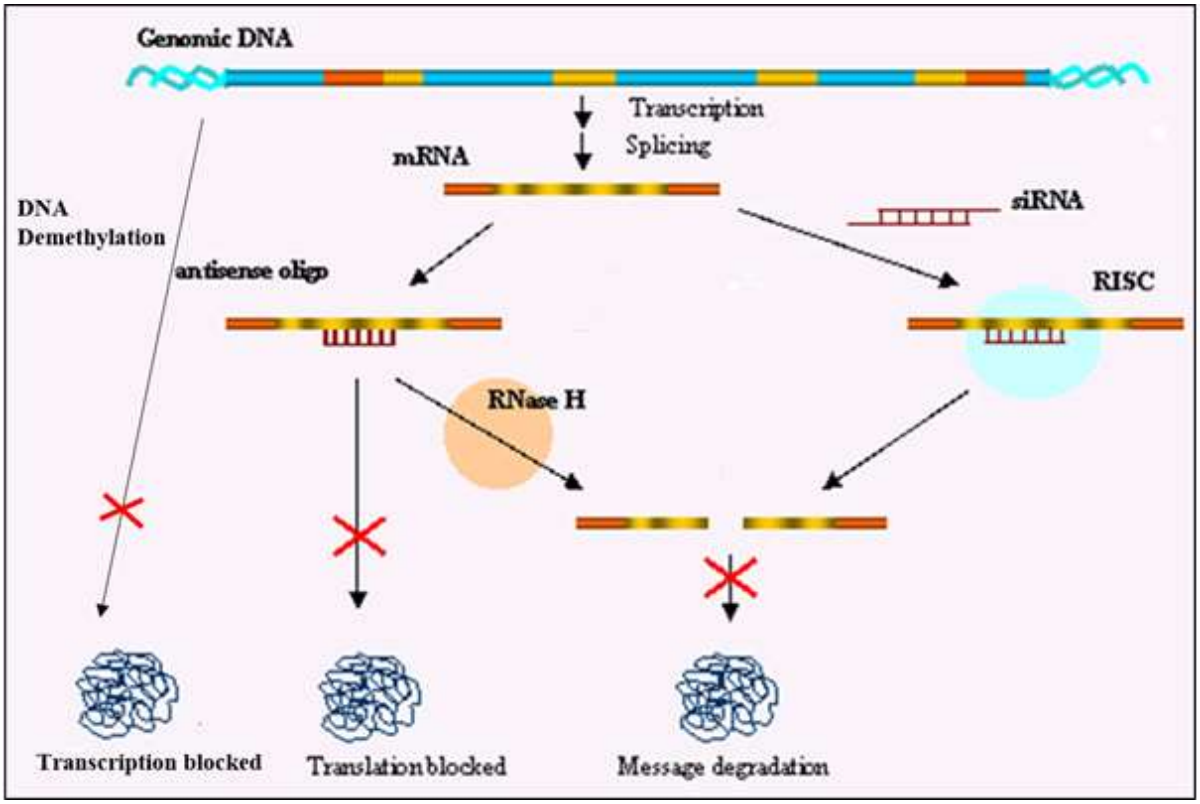


Figure 1-17: Comparison of silencing mechanism mediated by antisense oligodeoxynucleotides (ASO) and RNAi. *RNAi based gene silencing occurs at post-transcriptional level engaging the RISC complex, which degrades the siRNA-mRNA duplex to resulting in gene silencing. ASOs silence genes at transcriptonal levels by DNA demethylation, at post-transcirtional level by degradation of target mRNA by RNase H or through translational arrest mediated by stearic hinderance caused by its binding to the ribosomes. Diagram adapted from https://www.ncbi.nlm.nih.gov/probe/docs/applsilencing/.*

ASOs have limitations that affect drug development. For instance, they are vulnerable to degradation by host nucleases, have poor uptake by cell membranes, have sub-optimal binding to complementary sequences and can cometimes have poor pharmkokinetic properties (Shen & Corey, 2018). Therefore, chemical modifications have been explored to enhance cellular stability of ASOs. One of the first-generation modifications is the phosphorothioate (PTO) modification. In such a modification, the replacement of one of the non-bridging oxygen atoms of the phosphodiester bond by a sulphur atom in the DNA backbone (**Error! Reference source not found.**) increases ASO stability by making the ASOs for resistant to host nucleases (Stein et.al., 1988). PTO modifications also increase silencing efficiency by enhancing the bioavailability of the silencing molecules by promoting better uptake into the host systems (Koller et al., 2011). PTOs act as substrate for binding of RNase H enzyme that degrades the RNA moiety of the DNA-RNA heterodimer. Therefore, when PTOs are used for gene silencing, they act as a site for RNase H binding, resulting in gene silencing by degradation of the complimentary mRNA (Wu et al., 2004).

Second generation of ASO modification includes 2'-O-Methyl (2'-OMe) and 2'-O-Methoxyethyl (2'-MOE) modification of the ribose sugar in the DNA backbone. These modifications reduce the RNase H affinity for the DNA-RNA hetero-duplex resulting in suppression of RNase H mediated gene silencing. Therefore, silencing molecules consisting of 10 PTO modifications flanked by 5 OMe or MOE modified ASOs have been designed. Then these modifications still allow RNase H binding at the chimeric junctions supporting RNase H degradation along with stabilising the ASO against nucleases (Chan et.al., 2006). Alternative to gene silencing, these secondary modifications described above have been exploited for exon skipping based gene therapy of Duchene Muscular Dystrophy (DMD), a disease caused by mutation in the dystrophin gene (Cirak et al., 2011). Targeting secondary modified ASOs to intron-exon junctions of faulty exons can result in the deletion of the faulty exon in the mature mRNA. The translated protein though internally mutated, will be mostly functional. In DMD, exon skipping of the mutated regions of dystrophin gene results in expression of healthy, partially functional proteins (Cirak et al., 2011).

To further enhance ASO stability, target specificity and pharmacokinetics, tertiary modifications have also been introduced. These include furanose ring modifications. 2'- Fluoro (2'-F) modified ASOs were the earliest furanose ring modified ASOs. 2'F-ASO have been developed to target Amyotrophic lateral sclerosis (ALS) specific microRNAs (miRNAs) in the brain and neural tissue, resulting in increased survival of ALS mice model (Koval et al., 2013). However, 2'-Fluoro modified ASOs still are vulnerable to host nucleases (Koval et al., 2013). Another group of furanose ring modified ASOs are bridging nucleic acids (BNAs), where 2'-oxygen is linked to the 4' carbon of the ribose through bridging carbons. This group includes locked nucleic acids (LNA) and 2',4'-constrained ethyl nucleic acid ((S)-cET) (Braasch & Corey, 2001). Locked nucleic acid (LNA) is an RNA derivative in which the ribose ring is constrained by a methylene linkage between the 2'-oxygen and the 4'-carbon. The resulting conformation restriction increases binding affinity for complementarity sequences, increasing silencing efficiency of the LNA modified ASOs (Braasch & Corey, 2001). However, LNAs sometimes have high cytotoxicity, aspecific binding and a tendency for self-binding (Evers et al., 2015). Alternatively, 2',4'-constrained ethyl nucleic acid ((S)-cET) have the desired properties of strong binding capability and improved nuclease resistance without any cytotoxic (Evers et al., 2015). A (S)-cET-based ASO clinical application include targeting the STAT3 transcription factor in lymphoma and lung cancer (Hong et al., 2015). These tertiary modified ASOs are not a substrate for RNase H. Therefore, gene silencing occurs by translational arrest mediated by steric hindrance (Lebleu et al., 2008).

Phosphoroamidate morpholino oligomer (PMO) modified ASOs are another group of chemically modified ASOs (Lebleu et al., 2008). PMO modified ASOs have a backbone of morpholine rings

connected by phosphorodiamidate linkages. These are uncharged nucleic acid analogues that bind to complementary mRNA targets and block protein translation through steric hinderance. PMO modified ASOs have been developed as a cure for neurodegenerative diseases like Huntingdon's disease (Sun et al., 2014) and Spinal muscular atrophy (SMA) (Evers et al., 2015). Moreover, PMOs targeting exon-intron junction have been developed as agents for exon skipping to cure inherited diseases like Duchene muscular dystrophy (DMD) (Cirak et al., 2011). The different ASO chemistries are described in **Error! Reference source not found.**.

Despite lower stability against nucleases, PTO modified ASOs are more frequently used than other secondary or tertiary modifications, due to lower costs as compared to secondary and tertiary modifications and their compatibility to RNase H activity (Chan et al., 2006). They, therefore, constitute an attractive option for *in planta* gene silencing in this study, for silencing fungal virulence and barley or wheat susceptibility genes in some of the economically major pathosystems.

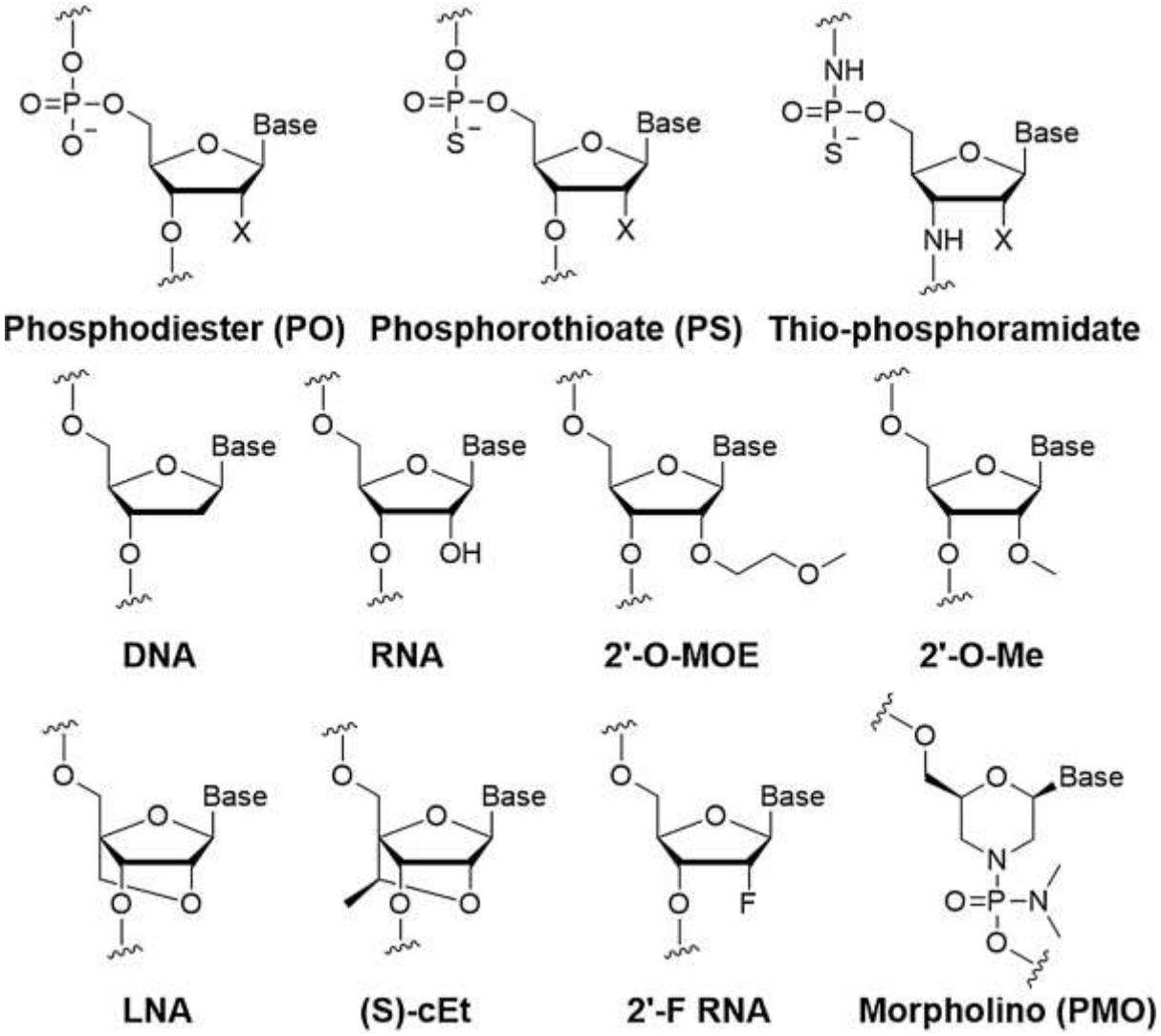


Figure 1-18: Antisense oligonucleotide chemical modifications used in clinical trials in animal systems for gene silencing. *O-ME: O-methoxy, O-MOE - O-methoxy ethyl, LNA: locked nucleotide (2',4'-methylene linkage), 2'F -2'- Fluoro,(S)-cEt- 2',4'-constrained ethyl nucleic acid. Daigram obtained from (Shen & Corey, 2018).*

1.7.5 Phosphorothioate modified antisense oligodeoxynucleotide based *in planta* gene silencing in barley-powdery mildew pathosystem

Phosphorothioate modified antisense oligodeoxynucleotide based *in planta* gene silencing is a novel tool developed in the Bindschedler laboratory for silencing of virulence and susceptibility genes in barley-powdery mildew pathosystem. This system initially used gene specific antisense oligodeoxynulceotides (ASOs) as the silencing molecule, which was later replaced by phosphorothioate modified antisense oligodeoxynucleotides, due to a higher silencing effect achieved by PTO modified ASOs as compared to unmodified ASOs (Orman,Das Gupta et.al., manuscript in prep). The gene specific PTOs is delivered by direct uptake into excised leaves. To

assess the effector efficiency, PTO treated leaves are then inoculated with *B. graminis* spores. The infection phenotype resulting from effector gene silencing is assessed microscopically at 48 hpi by observing the proportion of secondary hyphae formation as a proxy for the formation of functional haustoria. Alternatively, the relative fungal biomass in the barley host is estimated by comparing housekeeping gene expression ratio between the invading fungus and the plant host using qRT-PCR. So far, PTO mediated silencing of *B. graminis* f.sp. *hordei* effectors *BEC1011, BEC1019* and *BEC1054* and barley susceptibility gene blufensin 1 (*Bln1*) resulted in decrease in secondary hyphae development by 55%, 49%, 51% and 56% respectively (Orman,Das Gupta et.al., manuscript in prep). The phenotype associated with the silencing of *BEC1011* and *BEC1019* was further substantiated by showing a 33% decrease in fungal biomass as estimated by qRT-PCR (Orman,Das Gupta et.al., manuscript in prep). Therefore, anstisense PTO based silencing is a suitable functional tool for screening potential virulence and susceptibility genes in the barley –powdery mildew pathosystem.

Due to this success, this silencing methodology has the potential for further development as a gene silencing tool for functional genomics in other wheat pathosystems, and possibly might be further exploited for agricultural applications for fungal disease control.

1.8 Project aims and objectives

This project aims to translate antisense oligodeoxynucleotide (ASO) based silencing as an effective tool for *in planta* silencing of fungal effector genes during wheat and barley infection by a range of selected fungal pathogens, with the goal of controlling disease development. Virulence and housekeeping genes of the barley pathogen *Blumeria graminis f. sp. hordei* (*Bgh*), wheat pathogens *Blumeria graminis f. sp. tritici* (*Bgt*), *Fusarium graminearum* (*Fg*) and *Zymoseptoria tritici* (*Zt*) as well as wheat and barley susceptibility genes will be targeted for silencing using antisense phosphorothioate modified oligodeoxynucleotides (PTO modified ASO). Attempts will also be made to transfer the gene silencing technology from excised leaf to whole plant level.

The main objectives set for this project are as follows:

1.8.1 Transfer of PTO modified ASO based gene silencing methodology to silence genes in other wheat pathogens such as wheat powdery mildew, F. graminearum and Z. tritici.

1.8.2 To develop the PTO modified ASO based gene silencing method as tool for genetic studies and powdery mildew disease control in different barley cultivars

1.8.3 To use PTO modified ASO based gene silencing to discover new players in barley powdery mildew disease development

1.8.4 To investigate alternative methods for ASO delivery into whole plants resulting in successful *in planta* gene silencing

1.8.5 To investigate alternative ASO chemistries suitable for successful and more economical method of *in planta* gene silencing

1.8.6 Querying the universality of homologues of *BEC1019* as a virulence factor in wheat fungal pathogens such as wheat powdery mildew, *F. graminearum* and *Z. tritici*.

Chapter 2 : Materials and Methods

2.1 Fungal and plant growth and maintenance

2.1.1 Barley and wheat plant cultivation in soil

All barley (c.v. Golden Promise, Morex, Marys Otter) and wheat (c.v. Cerco, Riband, Bobwhite) seeds were stored in the cold room (4°C) for long term storage. Both barley and wheat were cultivated in pots containing John Innes no. 1 compost by placing 30-40 seeds in each pot and covering with a thin layer of soil (to encourage seed germination). The plants were grown at 20-22 °C and under 16-hours photoperiod for an age appropriate for the individual experiments.

2.1.2 Hydroponic growth of barley and wheat

The wheat and barley seeds were surface sterilised by soaking in 70% ethanol for 1 min, followed by 0.5% SDS for 15 min and subsequent washing in sterile distilled water thrice for 2 minutes each.

The sterile wheat and barley seeds were germinated by placing them on perlite saturated with ½ Hoagland's no. 2 basal salt solution (Sigma Aldrich) in closed tip boxes. The boxes were covered with tin foil and kept closed for 4 days. The plants were then cultivated at 20-22°C under 16-h photoperiod. The solution was replenished every 3-4 days to maintain nutrient availability for long-term growth.

2.1.3 *Blumeria graminis* growth and maintenance

Fully sequenced *Blumeria graminis* f.sp. *hordei* strain DH14 was used as wild type strain for all experiments. *B. graminis* susceptible barley variety Golden Promise were grown in John Innes no. 1 compost and used to propagate *Blumeria*. 7 days old barley plants were inoculated with *B. graminis* spores and maintained in a temperature control room, at 22°C and 16 h day (full spectrum day light fluorescent lights, Philips Sylvania Activa 172) and 8 h night cycle and 60-65% of relative humidity for propagation of the fungi.

Blumeria graminis f. sp. *tritici* (*Bgt*) Fielder isolate (NIAB, Cambridge) was used for all gene silencing experiments. *B. graminis* susceptible wheat variety CERCO was used as a host and cultivated on John Innes no. 1 compost. 10 days old wheat plants were inoculated with *B. graminis* f.sp. *tritici* spores and as described above for barley and *B. graminis* f.sp. *hordei*.

2.1.4 *Fusarium graminearum in vitro* growth

The fully sequenced *Fusarium graminearum* (*Fg*) strain PH1 (US) was used as wild type for gene knock out experiments based at Rothamstead Research, whereas the British strain 10*FG*131 (MU28), NRRL54110, UK Field isolate 2002 was used at RHUL for gene expression studies and *in planta* gene silencing experiments.

F. graminearum cultures were maintained as 15% glycerol stocks of spore suspensions at -80°C. To generate fresh spores, 100 µl of glycerol stock was plated on 12cm diameter petri dishes containing sterile Synthetic Nutrient Agar (SNA) (Minimal medium) (0.1% KH2PO4, 0.1% KNO3, 0.1% MgSO4 x 7 H2O, 0.05% KCl, 0.02% glucose, 0.02% saccharose, 2% bacto agar, pH 5.0). The plates were incubated at room temperature under near UV light for 7 days. The spores were then collected as spore suspension using 6 ml sterile distilled water.

For experimental work, freshly produced spores were resuspended in sterile distilled water in concentrations suitable for the experiment.

Alternatively, *F. graminearum* spores were plated on potato dextrose agar (Sigma Aldrich; Product code: P6685-250G) plates and incubated at room temperature under near UV light for 3 days. Agar plugs from the growing end of the hyphae were transferred to liquid cultures for acquiring fungal biomass for gene expression studies. The agar plugs were also transferred to SNA plates and incubated at room temperature under near UV light to generate fresh spores for infection and stock preparation.

The different growth media for *F. graminearum* growth are listed in supplementary table 1 (S1).

2.1.5 *Fusarium graminearum* infection of wheat heads

Wheat infection assays were carried out in wheat ears. Highly susceptible wheat (*Triticum aestivum L.*) cultivar Bobwhite was be grown until ear emergence and spikelets were infected by point inoculating 5 µl of 5 X 10^4 spores/ml *F. graminearum* spore suspension onto a wheat spikelet close to the top of the floret (Urban et. al. 2002). Infection was followed over a period of 1–20 days. Progression of the disease was recorded after 6 dpi, 9 dpi, 13 dpi and 18 dpi. Disease was scored as the number of visibly diseased spikelets divided by the number of inoculated spikelets per wheat ear below and at the inoculation point.

2.1.6 *Fusarium graminearum* infection of wheat coleoptiles

F. graminearum infection assays were also carried out on wheat coleoptiles. The wheat c.v. Bobwhite was cultivated hydroponically as described in section 2.1.2 for 4 days. On the fourth day, the 2-3 cm tip of the coleoptile was carefully removed using a blade. A 1 cm x 3 cm piece of filter paper was soaked in *F. graminearum* spore suspension (of 4 x 10^4 spores/ml concentration) and wrapped around the coleoptile by carefully tying with a thread. The seedlings were incubated with the filter paper for 3 days in a closed tip box covered with tin foil at 25°C. After 3 days, the tip box was opened, the filter paper was removed the plants were allowed to grow at 25°C, 16 h light period and 80% RH. Infection was assayed at 7 dpi by the length of the lesion developed on the leaf coleoptile.

2.1.7 *Fusarium graminearum* infection of barley attached leaves

F. graminearum infection assays were carried out on the 7 days old primary leaves of barley (c.v. Golden Promise) seedlings grown hydroponically as described in section 2.1.2. After 7 days, the barley seedlings were placed on 0.6% agar plates containing 20 mg/l benzimidazole such that the adaxial side of the primary leaf was up. The primary leaves were kept flat using a glass Pasteur pipette on the top and one just over the stems (without damaging the leaves). A small 1 mm X 1 mm piece of filter paper was placed each of the leaves to be inoculated. Each of the leaves were then inoculated with a single 20 µl droplet of *F. graminearum* spore suspension of 4 x 10^4 spores/ml concentration. A 20 µl droplet of sterile distilled water used as control. The lids of the plates were closed, and the plates were incubated at 25°C, 16 h light period for 4 days. The inoculations were done after placing the plates in the incubator to prevent disturbing the droplets. At 5 dpi, infection development was assayed by the length of the lesion developed on the leaf surface.

2.1.8 *Zymoseptoria tritici in vitro* growth

The fully sequenced *Zymoseptoria tritici* isolate IPO323 (Netherlands) was used as wild type strain for all experiments. Fungal strains were maintained as 20% v/v glycerol stocks at -80°C. Fungal

spores were revived by plating on Yeast Peptone Dextrose (YPD) agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 2% agar) and incubating at 18°C. Spores were harvested from 4-5 days old plates using sterile distilled water. The spores were recovered by filtration with four layers of sterile miracloth place in a funnel. For experimental work, spores were titrated (counting with haemocytometer plate) prior to dilution in sterile distilled water at concentrations suitable for the experiment (Keon et al., 2007). Alternatively, spores were stored in 20% glycerol stocks at -80°C.

The different growth media for *Z. tritici* growth are described is supplementary table 1 (S1).

2.1.9 *Zymoseptoria tritici* wheat leaf infection assay

Fourteen days old wheat plants of the STB-susceptible variety Riband were used for infection. A suspension of fungal spores at 10^5 spores/ml in 0.1% Tween-20 solution (in sterile water) was inoculated onto the secondary leaves as described by Keon et. al. (2007). Mock inoculated leaves with 0.1% Tween-20 were used as negative control. The infected plants were initially maintained at 100% relative humidity for 72 hours followed by incubation at 25°C and 16 h daylight cycle and 80 % relative humidity for 21 days (Keon et al., 2007).

Leaves were excised at 21 dpi for data collection. Infection was quantified by determination of biomass using qPCR at 21 dpi (Keon et al., 2007).

2.3 Bioinformatics analysis –

2.3.1 Plant and fungal genome databases

All gene, promoter, messenger RNA, coding and peptide sequences were obtained from either Ensembl Fungi (www.fungi.ensembl.org) for fungal pathogens and from Ensembl Plants (www.plants.ensembl.org) for the host plants. The genomic sequences of *B. graminis* f.sp. *tritici* genes were obtained from NCBI (www.ncbi.nlm.nih.gov).

2.3.2 Querying and identification of protein homologues

The homologues of different proteins were obtained by blasting the peptide sequence of the protein (using the BLAST tool) against the database of a particular fungal pathogen in Ensembl/NCBI. The proteins with the highest sequence identity were selected. Wherever applicable, known conserved domains were identified in these homologues. The genomic sequences were then acquired from Ensembl for further study.

2.3.3 Gene promoter analysis of CSEPs and other haustoria specific effectors to identify haustoria specific regulatory elements

For promoter analysis to identify motifs enriched in the promoters of haustoria only *B. graminis* effector candidates, the list of different candidate secretory effector proteins (CSEPs), CSEP like

proteins (CELPs) and hyphae only proteins were obtained. CSEPs were obtained from the transcriptomics study published in 2013 (C. Pedersen et al., 2012) and the list of 71 proteins obtained from the proteomics study of 2011 (Bindschedler et.al., 2011) were used as test subjects. A list of random 70 proteins expressed exclusively in *B. graminis* epiphytic hyphae was used as a negative control.

The 1000 kb upstream region including the promoter sequences for these genes were retrieved from Ensembl Fungi (www.ensembl.fungi.org) and grouped as CSEPs full list, CELPs full list, CSEPs + CELPs and hyphae only lists. The CSEPs with a haustoria/hyphae expression ratio of 50 or above (C. Pedersen et al., 2012) were also grouped in a separate list of "CSEPs with ratio above 50". For *de novo* discovery of promoter motifs enriched in haustoria specific *Blumeria* effectors, the list of promoter sequences was entered into Discriminative Regular Expression Motif Elicitation (DREME) tool (http://meme-suite.org/doc/dreme.html) in the Motif-based sequence analysis (MEME) suite (Bailey et al., 2009) along with the negative control list of hyphae only genes. The DREME tool used the discrete complementary model for *de novo* discovery of motifs (Bailey et al., 2009). The E-value was set to 0.05 as a threshold for positive motifs, motif count was set at default to not limit the number of motifs discovered and both the strands were searched for a motif length between 4-8 bases.

The motifs discovered through DREME were fed in to the motif identification tool TOMTOM within the MEME suite (Bailey et al., 2009). TOMTOM compared the motifs to known yeast transcription factor binding DNA motifs included in the JASPER 2016 database. TOMTOM aligns the *de novo* discovered motifs with the motifs from the database and rank them based on similarity using the Pearson coefficient as a metric. The Q value which denotes the minimum false discovery rate was set at 0.05 for identification of the *de novo* discovered motifs by TOMTOM.

A schematic diagram of the promoter analysis protocol is included in Figure 2-1.

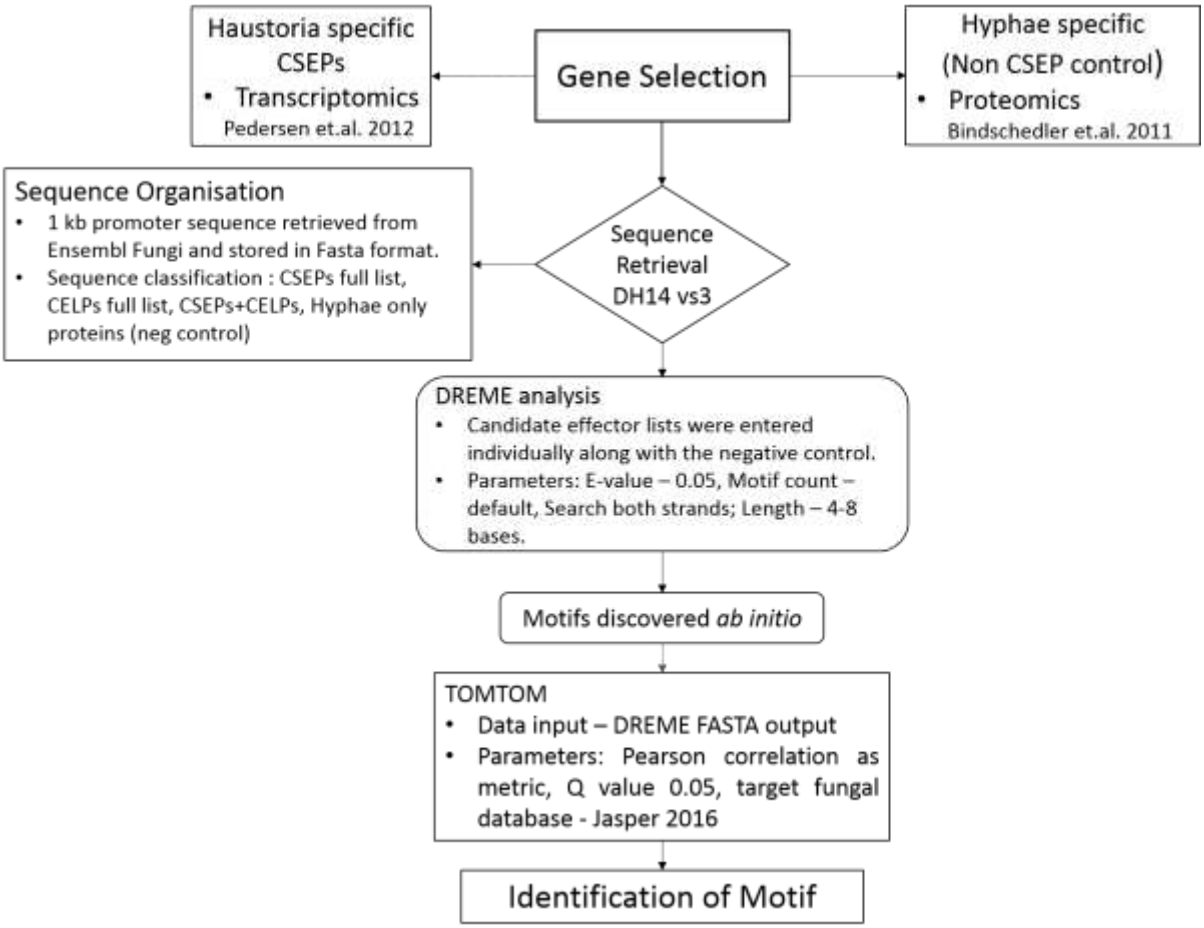


Figure 2-1: Ab-initio discovery of effector specific promoter motifs

Workflow summarising the steps involved in promoter analysis of haustoria specific effector candidates and the hyphae specific genes (negative control) for ab initio discovery of effector specific promoter motifs using the DREME tool and motif identification using TOMTOM tool of the MEME bioinformatics suite.

2.4 Silencing *Blumeria* genes *in planta*

2.4.1 Design of gene specific antisense oligodeoxynucleotide (asODN)

Nineteen nucleotides long (19-mer) ODNs were designed using OligoWalk software (http://rna.urmc.rochester.edu/cgibin/server_exe/ oligowalk/oligowalk_form.cgi, (Lu and Mathews 2008)). Gene ORFs were retrieved from Ensembl Fungi (http://fungi.ensembl.org/index.html) for all three fungi and from Plant Ensembl (http://plants.ensembl.org/index.html) for barley and wheat genes. ODNs were then selected from options provided based on probability of success by Oligowalk and position within the gene. The ASO sequences used are tabulated in Table 2-1.

Custom PTO modified ASOs were synthesised by Sigma Aldrich (1 µmol sample size; desalting purification).

Table 2-1: List of PTO modified ASO sequences used for silencing of various genes in different pathosystems.

Gene name	Gene reference	PTO modified ASO name	Antisense ODN sequence (5'- 3')	Length (X mer)	Reference
Wheat seed protein Z	Genbank: X97636.1	PTOZ	AAGCGGTTGAGCACTGAA	18	Sun & Ho, 2005
Bgh BEC1019	Ensembl: BLGH_04871	PTO 19.12	TTTGTCTGTGTAGCATTAC	19	Kate Orman
Bgh BEC1011	Ensembl: BLGH_07101	PTO 11.11	TATCTGGAACTCTATAATC	19	Kate Orman
Bgh Actin	Ensembl: BLGH_02125	PTO ACT1 PTO ACT2	TTCTCTCTGTTCGACTTGG TTGTCACATGAATACCACC	19	
Bgh GAPDH	Genbank: CCU80715.1	PTO GAPDH1 PTO GAPDH2	TAATTTCATCATAGGTAGC TATGTAATCTACTTGGAGC	19	
Zinc sensitive transcription factor – *Bgh ZAP1*	Ensembl: BLGH_03336	*Bgh* ZAP PTO1 *Bgh* ZAP PTO2	TATCTACTACTTCTTTCTC TATTAATGTGTCAATCGAC	19 19	
pH sensitive transcription factor – *Bgh PacC*	Ensembl: BLGH_01427	*Bgh PacC PTO1*	TTAATCATCAGAAACACGC	19	
Bgh* 2-Glycosyl transferase *(GT2)	Ensembl: BLGH_02326	*Bgh GT2* PTO1	TATTGACTCTCTAAGCTCC	19	
Bgt BEC1019	Genbank: EPQ66538.1	*Bgt*_B19_PTO 1 *Bgt*_B19_PTO 2	TTAAATACGTACTATGCTC TATTCCATTGTCAACTAGC	19 19	
Bgt ZAP1	Ensembl: BGT96224_3498	*Bgt ZAP1* PTO1 *Bgt ZAP1* PTO2	TTTGCTCGTTGTAATAAGC TAGAAAGTATGTCATCCGC	19	
Barley Pathogenesis related protein 5 *(PR5)*	Genbank: KP293850; Ensembl:	PTO PR5.1	TTGAAGAACATTGAGTAGT	19	James Fisher

	HORVU5HrG005180.6				
Barley Mildew Locus 1 (*MLO1*)	Genbank Z83834.1; Ensembl MLOC_70290.3	PTO MLO1	TAGTCAACGTACTTGCTGG	19	Moritz Bomer
Chlorophyll a/b binding protein *(Cab)*	Genbank: M10144.1	Cab ODN 1 Cab ODN 2	AGAGCACACGGTCAGAG GCGAGGCGGCCATTCTTG	17 18	Dinc et.al., 2011
Wheat negative control (no target)	No target in wheat	Wh_neg_control	GGCGGCTAACGCTTCGA	17	Dinc et al., 2011
***Fg GT2* (2-Glycosyl transferase)**	Ensembl: FGRAMPH1_01G01771	*Fg GT2* PTO1	TCTTCAAAACAATAGAAGG	19	
***Fg TRI5* (Trichodiene synthase)**	Ensembl: FGRAMPH1_01T13111	*Fg Tri5* PTO1	TAGTGCAAATTCTCGATGC	19	
***Zt GT2* (2-Glycosyl transferase)**	Genbank: XP_003857553.1	*Zt GT2* PTO1	TAAGATTCTTGACATTGGC	19	
***Zt* β-Tubulin**	Ensembl: Mycgr3G102950	*Zt Tub PTO1*	TTGAAGTAGACATTCATGC	19	
***Z. tritici* and wheat negative control**	No target in *Z. tritici* or wheat	Zt_Wh_neg_control	ACGGGGCACGTGAAACA	17	

2.4.2 Antisense phophorothioate modified oligodeoxynucleotide (PTO modified ASO) treatment of excised barley and wheat primary leaves for silencing *Blumeria* genes prior *Blumeria* infection

Antisense PTO modified ASO treatments were carried out by dipping three 8 cm long apical segment of barley primary leaves from 7 days old plants (*Hordeum vulgare* cultivar Golden Promise/ Morex/ Marys Otter) in 2 ml microfuge tubes containing 1 ml of 10 µM PTO solution. The leaves were cut under water to avoid air bubbles in the leaf vasculature. The leaves were incubated in continuous light for 24 hours. A sterile distilled water treatment was used as a negative control.

The 2 cm section at the base of the leaves, which were submerged in the ODN solution/ sterile distilled water, was discarded and the leaves were placed on 0.6% agar plates containing 20 mg/l benzimidazole (from 20 mg/ml stock solution in ethanol stored at -20°C). The treated leaves were arranged in a random order to randomise the infection. The leaves were then inoculated with *B. graminis* spores from barley golden promise plants infected for 7 days (7dpi). The spore density was monitored by placing a haemocytometer next to the agar plates and counting the number of spores per millimetre square using a haemocytometer. The inoculated leaves were incubated for 48 hours at 22°C and 16 h photoperiod before harvesting for infection assay and RNA extraction (Figure 2-2). For each gene target, 6 leaves were treated with a PTO for disease scoring and another 6 leaves were treated for RNA and genomic DNA extraction for analysis of gene transcript and fungal biomass levels.

The PTO modified ASO treatment of wheat (*Triticum aestivum* c.v. Cerco) leaves was carried using a similar protocol but using 9 primary leaves from 10 days old plants per gene target (Figure 2-2). The fungal inoculation was done using 14 dpi *B. graminis* f.sp. *tritici* infected plants.

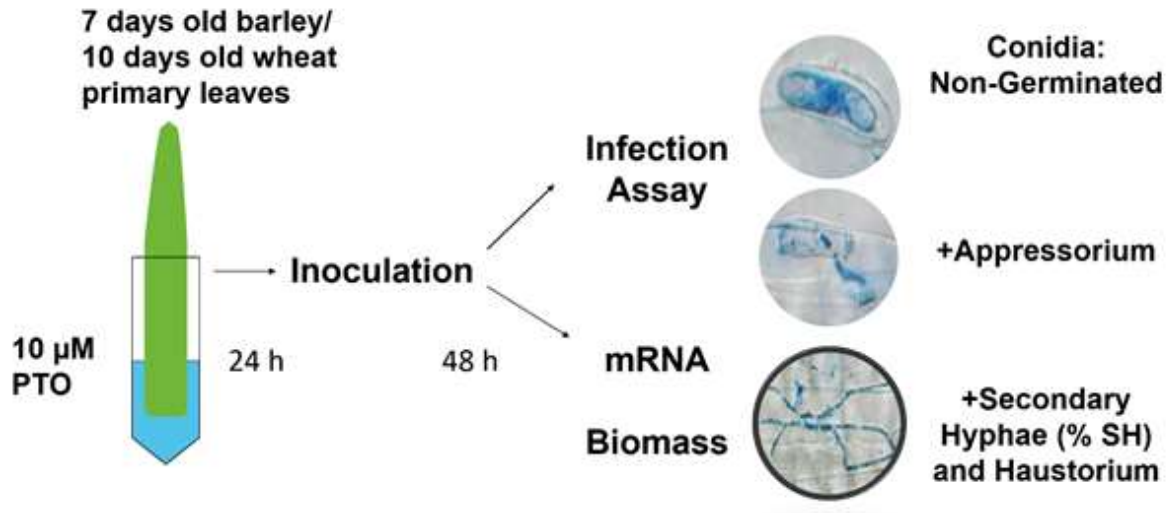


Figure 2-2 Workflow for PTO modified ASO based in planta gene silencing in detached leaves.

Fungal and plant genes were silenced by treating 7 days/10 days old barley/wheat primary leaves with gene specific PTO modified ASOs for 24 hours. The treated leaves were then inoculated with B. graminis spores from 7dpi barley powdery mildew/14 dpi wheat powdery mildew infected plants. Infection, transcript level and fungal biomass was analysed using microscopy and RNA respectively at 48 hpi. (Orman et. al. manuscript in prep).

2.4.3 *Blumeria* gene silencing by PTO modified ASO uptake through roots in barley and wheat seedlings grown hydroponically

The root delivery of PTO modified ASOs was carried out by using 7 days old barley (*Hordeum vulgare* c.v. Golden Promise) plants grown hydroponically as described in section 2.1.2. The seedlings were extracted from the perlite and the roots were gently washed with water to remove any residual perlite. PTO modified ASO treatment was carried out by submerging the roots of each of the seedlings in 2 ml Eppendorf tubes containing 1 ml of 10 µM PTO solution. The mouth of the tubes was sealed with parafilm before incubating the seedlings under continuous light for 24 hours for uptake of PTOs. After 24 hours, the seedlings were carefully placed on 0.6% agar plates containing 20 mg/L benzimidazole (Sigma). The fungal inoculation was carried out as previously described in section 2.4.2. Infection was assayed between 44-48 hours post inoculation by microscopy (Figure 2-3).

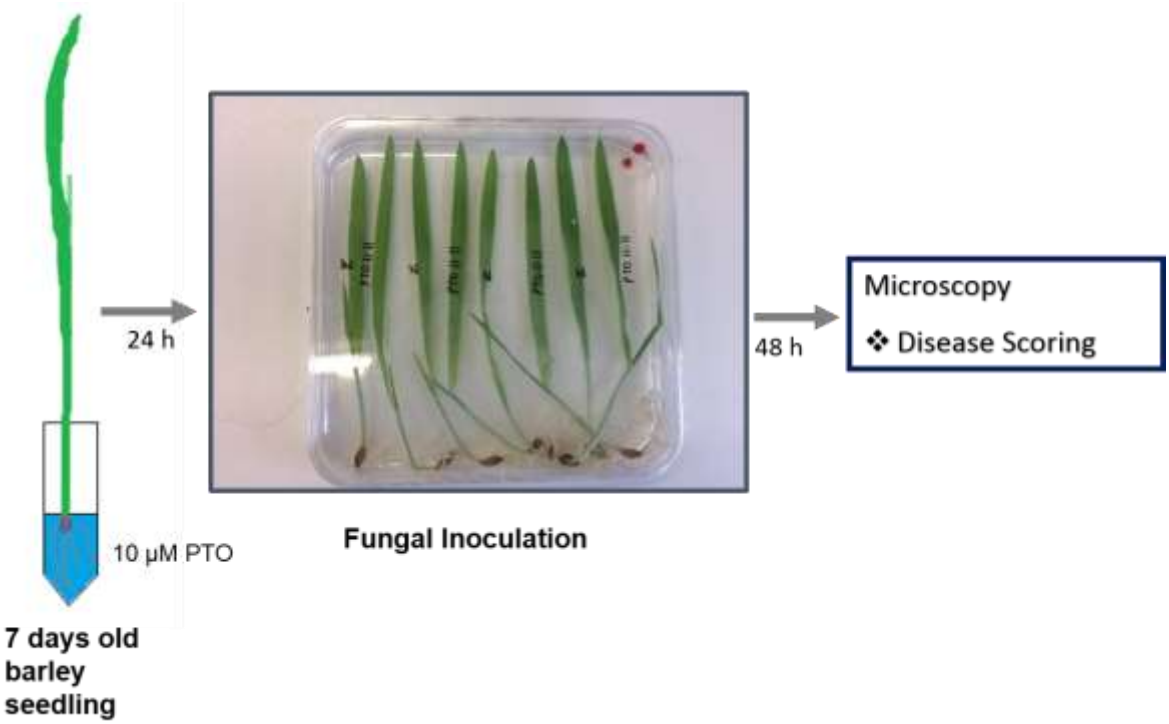


Figure 2-3: Workflow for in planta gene silencing in whole plants via root delivery of PTO modified ASOs.
Fungal genes were targeted by root delivery of PTO modified ASOs into 7 days old barley seedlings. The PTO treated seedlings were inoculated with B. graminis spores from 7 dpi infected barley powdery mildew plants and infection was assayed at 48 hpi by microscopy.

2.4.4 Powdery mildew disease scoring by microscopy

Between 44-48 hpi, the infected leaves were cut into 2 cm base and 2.5 cm apical sections, from the tips, discarding the most basal section (Figure 2-4). The leaves were transferred to 2 ml Eppendorf tubes (1 leaf/tube) and then stained in lactophenol blue (Sigma) for 20 minutes at 90°C. The leaves were destained in two changes of 3:1 ethanol: acetic acid mixture for 15 minutes at room temperature followed by overnight at 4°C. After destaining, the samples were stored at 4°C in 20% glycerol in the dark and disease scoring was performed within a week. Leaf samples were mounted in 20% glycerol and infection scoring was performed with a light transmission microscope at 250X magnification.

The number of non-germinated conidia, conidia forming appressoria and conidia forming secondary hyphae were counted. At least 200 conidia with appressoria were counted and the corresponding number of non-germinated conidia and hyphae were also noted. Disease scoring was done by calculating the number of secondary hyphae formed per conidia on the leaf surface. Secondary hyphae formation is an indicator of successful infection, as they are formed only after establishment of haustoria. Haustoria do not stain well, therefore, secondary hyphae are a good alternative

determinant of disease development as they reflect the presence of a functional haustorium (Figure 2-2).

The statistical significance of the effect of PTO modified treatments on disease development was determined using General Linear Model (GLM) (Ongoing) and one-sample t-test.

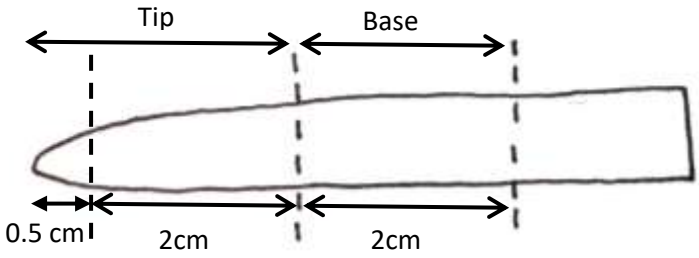


Figure 2-4: The pattern for cutting the treated leaves for staining.

2.4.5 Sample collection for gene expression studies post host induced gene silencing

The PTO modified ASO treated and *Blumeria* spore inoculated leaves were sampled between 44-48 hours for extraction of total RNA or genomic DNA. Six leaves per sample (containing both tip and base samples - Figure 2-4) were flash frozen in liquid nitrogen and stored at - 80°C for further grinding for RNA or genomic DNA extraction for all *B. graminis* silencing experiments. For PR5 transcript levels, only the 2 cm tip regions of six leave were frozen per treatment. Similarly, tip regions of nine leaves per treatment were sampled for RNA extraction for the *Bgt BEC1019* HIGS experiments.

2.5 Visualisation of fluorescent ODN uptake in barley and wheat leaves

2.5.1 Uptake of fluorescently labelled PTO by barley and wheat detached leaves

The uptake of ASOs by barley and wheat leaves was monitored by using a 6-FAM (6-fluorescene amidite) labelled PTO modified ASO targeting seed protein Z (Sigma). 6-FAM is a nucleotide labelling fluorescent dye with an absorption maximum at 492nm and an emission maximum at 517 nm. Leaves were treated with this PTO as previously described (section 92) but with 5 µM 6FAM-PTO solution and incubated under continuous white light for 24 hours to encourage transpiration and PTO uptake. The 1.5 ml tubes containing the 6-FAM PTOs were covered with aluminium foil to prevent photo-degradation. After incubation, the leaves were cut into small sections and mounted for microscopy.

2.5.2 6FAM-PTO uptake by epidermal cells and plasmolysis

Barley and onion epidermal strips were used to visualise 6FAM-PTO uptake by epidermal cells. The epidermal strips were placed on slide and immersed in a few drops of 5 µM 6FAM-PTO solution for 5 minutes. The cells were washed with water and then mounted for microscopic analysis.

2.5.3 Fluorescent microscopy

The fluorescent studies of 6FAM-PTO treated wheat and barley primary leaves was carried out using an Eclipse Ni fluorescent microscope (Nikon) fitted with a DS QiMc camera (make) and a UV lamp source Intensilight C-HGF1 (Nikon).

6FAM nucleotide labelling fluorescent dye has an excitation maximum at 492 nm (blue) and an emission maximum at 517 nm (green). Therefore, fluorescent microscopic analysis of treated primary leaves was carried out using the FITC channel (excitation max 494 nm/emission-max 520 nm).

Fluorescent micrographs were taken at 100 or 400X magnification with 100 ms exposure time.

2.6 Chlorophyll detection assay to assess *chlorophyll a,b binding (Cab)* protein silencing in wheat

Wheat gene chlorophyll a/b binding protein (*Cab*) was targeted using *Cab* specific ASOs, delivered through roots of wheat seedlings as previously described in (Dinc et.al. 2011). *Cab* silencing in excised wheat leaves has been shown to suppress greening after 8 hours of light exposure with a reduction in *Cab* RNA and protein levels along with reduction in chlorophyll b and total chlorophyll fluorescence (Emine Dinc et al., 2011). A modified version of this assay was repeated here as a starting trial to deliver ASO through roots, before transferring the protocols to silence genes relevant to wheat and barley pathosystems.

Sterile wheat seeds were germinated on water-soaked filter paper in complete darkness. After 3 days, germinated seeds were transferred to 35 ml petri dishes containing double filter papers (Thermo Fisher) soaked in 1 ml ODN solution or double distilled water. The transfer was carried out as much as possible in the dark protecting material from direct light with aluminium foil and 4 seeds were placed in each petri dish. The seeds were treated with *Cab* ODN1, *Cab* ODN2, double distilled water and a wheat negative control ODN with no known targets in wheat. The treatments were carried out in dark for 72 hours. The treated seedlings were then exposed to light for 8 hours and leaf greening was analysed by taking photographs of the seedling using an EZ4 HD microscope (Leica) magnification of 80X and by chlorophyll measurements.

Chlorophyll was extracted by incubating the seedlings in DMF in darkness and at 4°C for 2-3 days, until all the chlorophyll was obtained. Prior to incubation, the fresh weight of the seedings (minus the seed and the roots) was noted. The amount of chlorophyll extracted was determined by spectroscopic measurements at 673 nm (chlorophyll A) and 640 nm (chlorophyll B) (Inskeep & Bloom, 1985). Sometimes the extracted chlorophyll was diluted to get reliable spectroscopic measurements (OD between 0.2 - 1). Concentration of chlorophyll A, chlorophyll B and total cholophyll (in nmol/ml) was determined using Equation 2-1. The data was represented as chlorophyll A, chlorophyll B, total chlorophyll or ratio of chlorophyll A/chlorophyll B extracted per gram of the fresh weight of leaf.

$$Chl_a = 13.43\ A_{663.8} - 3.47\ A_{646.8}$$

$$Chl_b = 22.90\ A_{646.8} - 5.38\ A_{663.8}$$

$$Chl_{tot} = 19.43\ A_{646.8} + 8.05\ A_{663.8}$$

Equation 2-1: Formula for estimation of a chlorophyll A, chlorophyll B and total chlorophyll present in seedlings. (Porra et al., 1989).

Each biological repeat consisted of at least 6 seedlings treated with each of the PTOs. Statistical significance was determined by one sample t-test (Equation 2-3)

2.7 Gene expression studies

2.7.1 Sample collection

The qPCR samples for determination of gene transcript levels post HIGS in *Blumeria* was obtained by flash freezing PTO treated and *Blumeria* inoculated between 44- 48 hours as described in section 2.4.5 and stored at - 80°C for future RNA extractions.

For gene expression in *Blumeria* infection structures, 7 days old barley (c.v. Golden Promise) was inoculated with *B. graminsi* f.sp. *hordei* spores and incubated at 21°C, 60% RH and 16h photoperiod. For wheat powdery mildew, the 10 days old wheat plants (c.v. Cerco) were inoculated with *B. graminsi* f.sp. *tritici* spores and incubated at room temperature and natural daylight cycle. The leaves for haustoria and hyphae specific gene expression were sampled at 7 dpi for barley powdery mildew and 10 dpi for wheat powdery mildew.

Blumeria hyphae was separated by dipping the infected leaves in 5% cellulose acetate, allowing it to dry, before peeling and flash freezing the cellulose acetate layer enriched in *Blumeria* hyphae. The remaining leaf enriched in *Blumeria* haustoria was flash frozen for mRNA extraction. Ten leaves were sampled per timepoint for study of gene expression in *Blumeria* infection structures.

For time course experiments in *Blumeria*, the infected and maintained as described above. Three leaves were sampled per timepoint for RNA extraction. The timepoints varied from 0, 1, 2, 3, 4, 5, 6 and 7 dpi for PR5 expression and 0, 4, 8, 12, 16, 24, 48 and 72 hpi and *BEC1019* expression.

The samples for expression of fungal genes during *in vitro* growth conditions were collected by initiating the cultures in a suitable liquid medium. *F. graminearum* cultures were initiated by inoculating a 1 cm X 1 cm agar plug from a PDA plate into 100 ml of the SNA broth and incubating at RT for 7 days. *Z. tritici* cultures were initiated by inoculating *Z. tritici* spores from a 4-5 days old YEPD plate into the suitable media (PDB – Sigma Aldrich; Product code: P6685-250G, YEPD - 10 g/l Yeast Extract, 20 g/l Peptone, 20 g/l Glucose, Czapek dox – Sigma Aldrich; Product code: C1551-250G). The *Z. tritici* spores were harvested at 3 days from the rich media (PDB, YEPD) or at 5 dpi from the minimal media (Czapek dox). At a suitable timepoint, the spores were harvested by vacuum filtration. The spores were then collected in a 1.5 Eppendorf tube, flash frozen and stored at – 80°C for further grinding and RNA extraction. Alternatively, the freshly collected fungal spores were directly ground in liquid nitrogen and stored at – 80°C for RNA extraction.

2.7.2 Grinding of samples for DNA and RNA extraction

The harvested leaves and fungal spores were flash frozen in liquid nitrogen and stored at - 80°C to prevent DNA/RNA damage in the leaf samples.

The leaves/fungal spores were ground in liquid nitrogen in the presence of quartz using pre-cooled pestle and mortar sterilised using 70% ethanol. The biological material was kept frozen throughout the grinding process to avoid RNA/DNA damage. The ground samples were then transferred to precooled Eppendorf tubes as aliquots of 100 mg and flash frozen in liquid nitrogen. The samples were either directly used for extraction of DNA/RNA or stored at – 80°C for future use.

2.7.3 Extraction of genomic DNA

Genomic DNA was extracted from *Blumeria* infected leaves using Edward's method (Edwards et al. 1991). The frozen ground leaf samples were mixed with 300 µl ice cold Edward's buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). To extract the DNA, tubes were gently mixed and incubated at 65°C for 10 minutes, followed by 10 minutes' incubation on ice. The samples were then mixed well with 200 µl chloroform and centrifuged at 13,000 rpm for 3 minutes at 4°C. The supernatant was collected in a fresh Eppendorf tube and mixes with 1:1 ratio of ice-cold isopropanol. The samples were then centrifuged at 13,000 rpm for 5 minutes at 4°C. The pellet was washed by re-suspending it in 500 µl of 70% alcohol and centrifuged at 13,000 rpm for 5 minutes at room temperature. The pellet was air dried and re-suspended in 20 µl TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA) and stored at -20°C until further analysis.

Genomic DNA was also extracted from 100 mg of ground *Z. tritici* infected wheat samples and fungal spores (*Fg/Zt*) using DNeasy plant mini kit (Qiagen) as per manufacturer's protocol.

The concentration of DNA was determined using Nanodrop 1000.

2.7.4 Extraction of total RNA

RNA was extracted from frozen samples of 44-48 dpi powdery mildew infected leaves using RNeasy plant mini kit (Qiagen). RNA was extracted from 100 mg of ground leaf material as per the protocol provided by the kit. In brief, 10 µl of 14.3 M β-mercaptoethanol was added per 1 ml of RLT lysis buffer used to extract RNA. Frozen leaf samples were vortexed with 450 µl of RLT buffer (with β-mercaptoethanol), transferred to QIAshredder spin column and centrifuged for 2 min at 13,000 rpm. The supernatant was transferred into new tube, mixed with 0.5 volume of absolute alcohol by pipetting and transferred to the RNeasy mini spin column. The tubes were then centrifuged at 13,000 rpm for 15 seconds and the supernatant was discarded. The spin columns were then washed with 700 µl buffer RW1 once and 500 µl buffer RPE twice by centrifuging them at 13,000 rpm for 15 sec. The supernatant was discarded, and the spin column was dried by another centrifugation step at 13,000 rpm for 1 min. The RNA was collected by adding 30 µl RNase-free water and centrifuging at 13,000 rpm for 1 minute.

The concentration of RNA was determined using Nanodrop 1000. The extracted RNA was placed on ice and directly used for cDNA synthesis.

2.7.5 Determination of nucleic acid concentration by spectroscopy using a NanoDrop spectrophotometer

The concentration of extracted genomic DNA/RNA was estimated by analysing 2 µl of sample at 260 nm in a NanoDropTM ND-1000 spectrophotometer (NanoDrop Technologies, Inc). Concentration was determined using NanoDropTM 1000 operating software, version 3.8.1.

The concentration of DNA was determined using DNA-50 whereas concentration of RNA was determined using RNA-40 sample type in the software. The quality of nucleic acid was determined by ratio of sample absorbance at 260 and 280 nm. Pure DNA has 260/280 ratio of ~2.0 whereas pure RNA is indicated by 260/280 ratio of ~1.8. Low values for ratio of absorbance at 260 nm to 280 nm are indicative of contamination with protein, phenol other contaminants absorbing at wavelengths close to 280 nm.

2.7.6 cDNA synthesis by reverse transcription from total mRNA

cDNA was synthesised from 0.8 – 1 µg RNA using Quantitect Reverse Transcriptase kit (Qiagen) as per the protocol provided. Genomic DNA was first removed from the RNA sample before reverse

transcription was performed. 0.8 - 1 µg of RNA sample (up to a total volume of 12 µl) was mixed with 2 µl gDNA "wipeout" master mix an incubated at 42°C for 2 minutes to remove gDNA contaminations. The samples were then mixed with 4 µl Quantitect RT buffer, 1 µl RT primer mix and 1 µl Quantitect reverse transcriptase provided by the kit. Reverse transcription occurred by incubation at 42°C for 15 minutes followed by enzyme inactivation at 95°C for 3 minutes. The newly synthesised cDNA was placed on ice and directly used for performing qPCR, avoiding storage at -20˚ or -80˚C for better results and consistency.

2.7.7 Quantitative real time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed using synthesised cDNAs described in 2.7.6 to determine either relative gene transcript level or fungal biomass levels. Fungal biomass level was also determined using genomic DNA using quantitative PCR. Gene specific qPCR primers were designed for each of the gene investigated and reference genes which were either cDNA specific, genomic DNA specific or nucleic acid specific (supplementary table 3). The primers were designed with amplicon size between 75-150 bp, T_m~ 58-60°C and GC content of ~ 50%.

qPCR was performed using 400 ng of cDNA or 100 ng of gDNA in a 20 µl qPCR reaction. The 20 µl qPCR reaction mix was prepared by combining 5 µl of cDNA (400 ng) (assuming an equal reverse transcription efficiency in all the reactions) or 5 µl gDNA (100 ng) with 0.6 µl of each forward and reverse primers stock at 10 µM, 10 µl 2x Precision SY master mix (Primer Design, UK) and nuclease free water to make up the remaining volume. qPCR was performed using RotorGene Q qPCR machine (Qiagen). The PCR cycles included a hold step at 95℃ for 2 minutes followed by 40 cycles of 95°C denaturation for 14 seconds and a combined annealing and extension step at 60°C for 60 seconds as recommended by Precision SY master mix. SYBR green fluorescence data acquisition occurred during the combined annealing and extension step. A melting curve was acquired to determine the specificity of the primers. The curves were acquired by ramping the temperature from 65-95°C and fluorescence was measured at every temperature. At melting temperature, the maximum amplification occurs (visualised as a peak) and then the curve dips as the DNA is no longer double stranded. A single peak at the primer melting temperature was indicative of a single specific amplicon though small peaks at lower temperatures were also visible indicating primer dimer formations. The relative gene transcript levels were determined using Pfaffl ratio (Pfaffl, 2001). (Equation 2-2).

The qRT-PCR primer sequences used in this study are listed in

Table **2-2**.

$$\text{Ratio} = \frac{(E_{target})^{\Delta CTta(control-sample)}}{(E_{ref})^{\Delta C(control-sample)}}$$

Equation 2-2: Pfaffl ratio used for calculation of relative gene expression levels in qRT-PCR.

E refers to efficiency of the PCR; Target = Bgt BEC1019/ Bgt GAPDH; Ref= Bgt GAPDH/Wheat GAPDH. ΔCT refers to the difference in number of PCR cycles required for fluorescence between Bgt BEC1019 treated and PTOZ control samples.

Table 2-2: The list of qRT-PCR primers used is gene expression studies.

Primer name	Target gene	Primer sequence	Reference
Wheat_18srRNA_qPCR_F	Wheat *18s rRNA*	GTGACGGGTGACGGAGAATT	
Wheat_18srRNA_qPCR_R	Wheat *18s rRNA*	GACACTAATGCGCCCGGTAT	
Wheat_GAPDH_qPCR_F	Wheat *GAPDH*	TGTCCATGCCATGACTGCAA	
Wheat_GAPDH_qPCR_R	Wheat *GAPDH*	CCAAGTGCTGCTTGGAATGATG	Pennington et.al., 2016b
HvGAPDH F	Barley *GAPDH*	CTGATTGAGAAGGCTGATGGAT	Pennington et.al., 2016b
HvGAPDH R	Barley *GAPDH*	AGAGCAGGAGCGTCATTGA	Pennington et.al., 2016b
BghGAPDH F	*Bgh GAPDH*	GGAGCCGAGTACATAGTAGAGT	Pennington et.al., 2016b
BghGAPDH R	*Bgh GAPDH*	GGAGGGTGCCGAAATGATAAC	Pennington et.al., 2016b
BghACT F	*Bgh Actin*	CCCAATTTACGAAGGTTTCTCTC	Pennington et.al., 2016b
BghACT R	*Bgh Actin*	TCAGCGGTTGTGGAAAAAGT	Pennington et.al., 2016b
BEC1011 F new	*Bgh BEC1011*	TCATGGAGCATCTGCATTGTC	Kate Orman
*Bgh_BEC11*_qPCR_RT_R	*Bgh BEC1011*	CATGCTCTCCTTGCCAGTTT	Pliego et al., 2013
*Bgh_BEC19*_qPCR_RT_F	*Bgh BEC1019*	TCCTACGACTGGACAACACCT	Pliego et al., 2013
*Bgh_BEC19*_qPCR_RT_R	*Bgh BEC1019*	CATGCTGAGCAAGGGTTACA	Pliego et al., 2013
Bgh ZAP1 qPCR F	*Bgh ZAP1*	CGGTGCCCAAGTGAAGTTTG	
Bgh ZAP1 qPCR R	*Bgh ZAP1*	TTGAAGCTGCCCAGATCCAG	

Bgh PacC qPCR F1	*Bgh Pacc*	AATGCCTGGTAGTCACGCTC	
Bgh PacC qPCR R1	*Bgh PacC*	TTTGGGTAGTAAACGGGGCC	
*Bgt*_B19_qPCR_Fwd	*Bgt BEC1019*	TCCTATGACTGGACAGCACCT	
*Bgt*_B19_qPCR_Rev	*Bgt BEC1019*	CATGCTGAGCAAGGGTTACA	
*Bgt*GAPDH_qPCR_F	*Bgt GAPDH*	TCGTATAGGCCGCATTGTCT	
*Bgt*GAPDH_qPCR_R	*Bgt GAPDH*	CGCGGCATATTCTGGTTCAA	
Ta Tub F	Wheat *tubulin*	GCTCACATCTCGTGGGTCACAGA	
Ta Tub R	Wheat *tubulin*	CGCCAGTGTACCAATGCAAGAAA	
Zt Tub F	*Zt tubulin*	ATCACCAGCCCGCAAAGCTT	Marshall et al., 2011
Zt Tub R	*Zt tubulin*	ACGATCTTGTGTCCGAGTACCAGC	Marshall et al., 2011
Zt B19 F	*Zt BE1019*	TCCGACCTCATCCATCGTCT	
Zt B19 R	*Zt BEC1019*	TCGAATTCGGACTCCCCTCT	
Zt Zrt1 F	*Zt Zrt1*	GGCGGGTATTCTGATCTGGG	
Zt Zrt1 F	*Zt Zrt1*	CAAGAACACCATCGCCACG	
Zt PacC qPCR F1	*Zt Pacc*	CCGCTCAAACCACACAAGTG	
Zt PacC qPCR R1	*Zt PacC*	ATCGGCGTGTGTCTTGACAT	
Fg B19 qPCR F	*Fg BEC1019*	ATTGCTCTTGCCAAGTCCGA	
Fg B19 qPCR R	*Fg BEC1019*	TCAACCTCACCAGTGCATCC	
Fg PacC qPCR F1	*Fg Pac1*	TCTACTTCAACCAACCGGCC	
Fg PacC qPCR R1	*Fg Pac1*	TACGATCATAACCACCGCCG	
Fg EF1A qPCR F	*Fg EF1A*	GGCTTTCACCGACTACCCT	Lysøe et al., 2006
Fg EF1A qPCR R	*Fg EF1A*	ACTTCTCGACGGCCTTGATGACAC	Lysøe et al., 2006

2.7.8 Statistical analysis of gene expression studies

The statistical significance of the change in gene transcript levels due to gene specific PTO modified ASO treatment as compared to control treatment (PTOZ), was determined using one sample t-test. The relative fold change of the target gene compared to a housekeeping gene for each the test PTO modified ASO treatment was subtracted from the mean fold change of the target gene in the control treatment. The calculated difference was then checked for significant difference from 0 using one sample t-test (Equation 2-3).

$$t = \frac{\overline{x} - \mu_0}{s/\sqrt{n}}$$

$\overline{x}$ = sample mean of control treatments

μ_0 = sample (PTO treatments)

s = sample standard deviation

n = sample size

Equation 2-3: One sample t-test

Formula for one-sample t-test used for calculating the significance of the effect of gene specific PTO treatments as compared to control.

The one sample t-test was calculated by dividing the difference of the sample fold change value (gene specific PTO modified ASO treatment) from the mean of the sample values of control treatments (PTOZ) with the standard error of the mean of the sample values – calculated by dividing standard deviation of the samples with the square root of the number of samples (Equation 2-3). The P value obtained determined the significance which was denoted as * where, * P<0.05, ** P<0.01, *** P<0.001.

2.8 Gene silencing of *Z. tritici* genes using antisense oligonucleotides

PTO modified ASO based *in vitro* gene silencing was attempted in *Z. tritici* by silencing *β-tubulin*, the gene targeted by the MBC fungicide carbendazim. The objective was to silence *tubulin* gene and monitor the effect of the gene silencing on the carbendazim sensitivity of *Z. tritici*.

2.8.1 Growth of *Z. tritici* in the presence of fungicides

In vitro growth of *Z. tritici* (IPO323) was carried out in AE minimal media (Yeast extract 10g/L, $MgSO_4$ .7 H_2O – 0.5 g/L, $NaNO_3$ 0.6 g/L, KCl 0.5 g/L, KH_2PO_4 1.5 g/L, Glycerol 20 ml/L) supplemented with different concentrations of carbendazim fungicide in a 92-wells plate. *Z. tritici* spores were obtained from 5 days old cultures grown on V8 media (V8 juice 200 ml/L, $CaCO_3$ 3 g/L, Agar 2%) and set at a concentration of $5x10^5$ spores/ml in AE liquid media. The spore suspension was plated in a 92-wells plate supplemented with 100 mg/L, 10 mg/L, 1 mg/L, 10^{-1} mg/L, 10^{-2} mg/L and 10^{-3} mg/L of carbendazim to a final volume of 200 µl. Freshly prepared 100X stocks of the different carbendazim concentrations were used for ease of pipetting. All reactions were set in triplicate. The plates were sealed with parafilm and incubated at room temperature. Growth was monitored at 0, 1, 2, 3, 4, 5, 6, 7 dpi by spectroscopic measurements at 600 nm (A_{600}) using a multi-wells plate reader Spectra MAX 190 (Molecular Devices). A growth curve was obtained based on the OD measurements.

2.8.2 *In vitro* gene silencing of *Z. tritici* genes using PTO modified ASO

The *in vitro* silencing of *β-tubulin* was attempted by incubating *Z. tritici* spores in the presence of carbendazim fungicide and a PTO modified ASO targeting *Zt β-tubulin*. A PTO with no targets in *Z. tritici* was used as a negative control. A $5x10^5$ spores/ml suspension of *Z. tritici* in AE liquid media (Yeast extract 10g/L, MgSO4 .7 H2O – 0.5 g/L, NaNO3 0.6 g/L, KCl 0.5 g/L, KH2PO4 1.5 g/L, Glycerol 20 ml/L) was supplemented with 10^{-1} mg/L, 10^{-2} mg/L carbendazim and 10 µM PTO modified ASO to a final volume of 200 µl in a 92 wells plate. All reactions were plated in triplicate. The plates were sealed using parafilm and incubated at room temperature for fungal growth. The effect of PTO based targeting of *β-tubulin* on carbendazim sensitivity of *Z. tritici* was monitored at 0, 1, 2, 3, 4, 5, 6, 7 dpi by spectroscopic measurements at 600 nm (A_{600}) using a multi-wells plate reader Spectra MAX 190 (Molecular Devices).

2.9 Host induced gene silencing of *F. graminearum* genes using PTO modified ASO

The host induced gene silencing of *Fusarium graminearum* virulence and housekeeping genes was attempted using gene specific PTO modified ASOs.

2.9.1 PTO modified ASO treatments for *F. graminearum* gene silencing

The gene specific antisense PTO modified ASOs were either delivered by transpiration into detached leaves or by root delivery into seedlings of barley c.v. Golden Promise as described in sections 2.4.2 and 2.4.3 respectively. The treatments were carried out for 24 hours at 21°C and continuous light before *F. graminearum* inoculation.

Delivery of PTO modified ASOs (10 µM) was also attempted by painting on the surface of the 7 days old barley primary leaves attached to hydroponically grown seedlings and 14 days old barley secondary leaves detached from plants grown on soil. The leaves/seedlings were placed on 0.6% agar supplemented with 20 mg/L benzimidazole prior to painting. Once painted, the leaf surfaces were air dried and inoculated with *F. graminearum* by droplet inoculation.

Alternatively, PTO modified ASO treatments were performed simultaneously with the spore inoculation. A 20 µl droplet containing 10 µM PTOs and $4x10^4$ spores/ml *F. graminearum* was inoculated directly on the surface of a barley primary leaf (section 2.1.7).

2.9.2 *F. graminearum* infection of PTO modified ASO treated leaves

Once PTO modified ASO treated (either by transpiration or root delivery), the detached leaves/ barley seedlings were placed on 0.6% agar supplemented with 20 mg/L benzimidazole. The *F. graminearum* spores were inoculated as a 20 µl droplet of 10^4 spores/ml on the primary leaf surface

as described in section 2.1.7. The leaves were incubated at 25°C, 80% RH under 16h photoperiod and disease development was scored using the length of the lesion developed on the leaf surface.

2.10 Endoribonuclease prepared small interfering RNA (esiRNA) synthesis

Endoribonuclease prepared short interfering RNAs (siRNAs) were prepared for *Bgh BEC1019*. Primers were designed to amplify a fragment of 449 bp. The T7 promoter sequence (TAATACGACTCACTATAGGGAGA) was attached to the primers to amplify products suitable for subsequent *in vitro* transcription using the T7 MEGAshortscript kit (Ambion) (Table 2-3). A PCR was performed to amplify the desired region and the PCR products were used for *in vitro* transcription of long dsRNA. The dsRNA obtained was then purified by chloroform: isoamyl alcohol purification.

Table 2-3: Primers used for esiRNA synthesis targeting Bgh BEC1019.

	Primer name	**Sequence**
Forward Primer	BEC1019_T7_F1	TAATACGACTCACTATAGGGCTCTCTTTTGCAGAGAAAAGGC
Reverse Primer	BEC1019_T7_R1	TAATACGACTCACTATAGGGTTGCCAGATCCAACTGATGA

2.11 Gene deletion in *Fusarium graminearum*

The gene knock-out of BEC1019 homologue in *F. graminearum* was carried out by PCR based split-marker gene deletion method (Catlett, 2003). Spilt-marker method involves the replacement of a gene of interest with a marker gene (hygromycin resistance gene) during homologous recombination in *F. grminearum* (Figure 2-5).

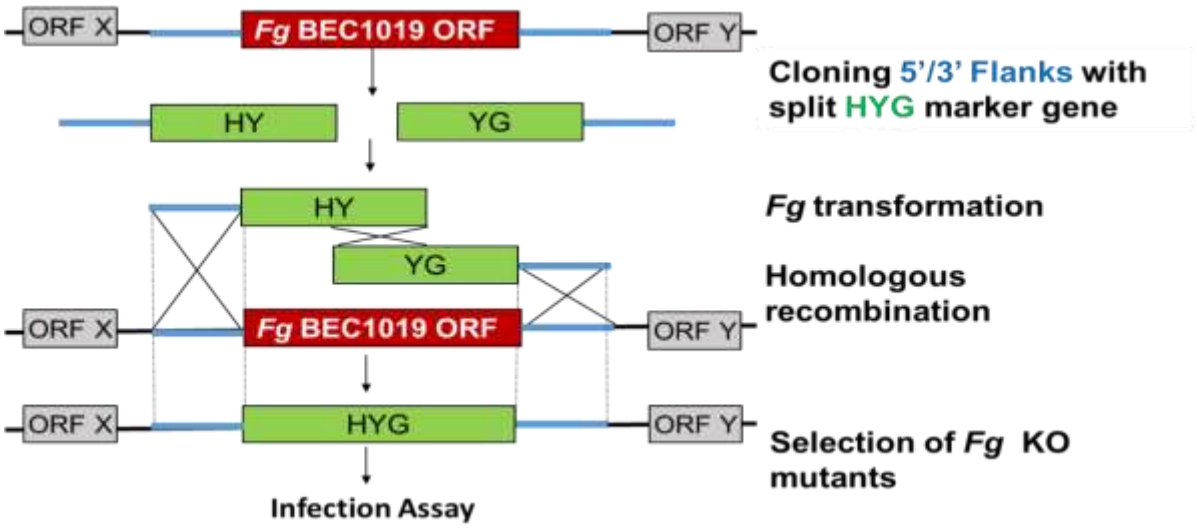


Figure 2-5: Split marker strategy for gene deletion of F. graminearum genes.

The flanking regions of Fg BEC1019 are amplified and attached to a hygromycin marker gene. This knock-out (KO) cassette is transformed into F. graminearum protoplasts for gene deletion following homologous recombination and gene replacement.

2.11.1 Construction of a gene deletion cassette

The gene deletion cassettes consisted of two parts of the split hygromycin gene attached to either 5' or 3' flank of *Fg* BEC1019 gene homologue. These cassettes were created by ligase independent cloning using the Gibson method (Gibson et al., 2009) . Gibson cloning efficiently assembles multiple fragments with overlapping sequences into a plasmid in a single step, irrespective of the lengths of the fragments. First the fragments were individually amplified using PCR and assembled in the right sequence and orientation, into a pUC19 plasmid using a commercial Gibson kit (New England Biolabs, Figure 2-6).

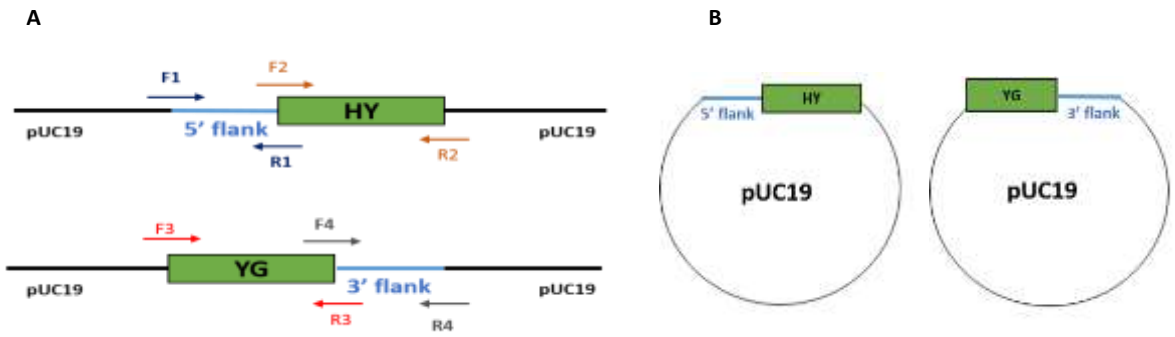


Figure 2-6: A: Diagram describing primer design strategy for constructing gene deletion cassette for split marker gene method of gene deletion. B: Gene deletion cassettes based on split-marker gene deletion method for replacement of Fg BEC1019 gene with a functional hygromycin gene by homologous recombination.

2.5.1.1 Linearization of pUC19 plasmid

The pUC19 plasmid was linearised by double restriction digestion with the enzymes BamH1 and HindIII for insertion of the gene knock-out cassette. A 20 µl reaction was set-up containing 2 µl 10X buffer E (Promega), 0.2 µl of acetylated BSA (10 µg/µl), 7 µl of pUC19 plasmid DNA (100 ng/µl), 9.8 µl nuclease free water and 5 units (U) of each of the enzymes. The digestion was carried out at 37°C for 1 hour followed by inactivation of the enzymes at 80°C for 20 min.

The digested products were analysed on a 1% agarose gel containing 1:10,000 SYBR safe stain (Thermo Fisher Scientific).

2.5.1.2 Primer design and amplification of fragments for the gene deletion cassette

The amplification primers were designed using the NEBuilder primer design software. Primers F1 and R1 were designed such that they contained a sequence specific to 5' flank of *Fg BEC1019* and a sequence overlapping with the pUC19 sequence adjacent to BamH1 restriction site or HY gene fragment respectively to amplify approximately 1 kb of the 5' flanking region. The primers F2 and R2 were designed with HY gene specific regions attached to 5' flank and region adjacent to pUC19 *HindIII* restriction site. Similarly, primers F3, R3 and F4, R4 were designed for amplifying YG and approximately 1 kb of 3' flank of *Fg BEC1019* homologue respectively with sequences specific to the gene of interest and the adjacent fragment in the gene deletion cassette (Figure 2-6A).

HY, YG fragments and 3' flanking regions were amplified using *Pfu* DNA polymerase (Thermo Scientific) whereas 5' flank was amplified using GoTaq DNA polymerase (Promega).

The primers are listed in Table 2-5, PCR reactions and PCR conditions are listed in

Table 2-4.

Table 2-4: Polymerase chain reaction (PCR) conditions
A. PCR reaction, B. PCR conditions used for Fg BEC1019 deletion using GoTaq DNA polymerase (Promega).

A

Components	Concentration
5X Go Taq buffer	1X
dNTPs (10mM each)	0.2 mM each dNTP
Fwd primer (10 µM)	1 µM
Rev primer (10 µM)	1 µM
$MgCl_2$	2 mM
GoTaq DNA polymerase	0.25 µl
gDNA/Plasmid	0.5 µg/ 100 ng
Nuclease free water	Up to 50 µl

B

Initial Denaturation	95°C	4 min	1 cycle
Denaturation	95°C	1 min	35 cycles
Annealing	Tm - 4°C	45 sec	35 cycles
Extension	72°C	1 min	35 cycles
Final Extension	72°C	5 min	1 cycle
Hold	4°C		

2.5.1.3 Gibson cloning

The gene deletion cassette was assembled into the linearised pUC19 plasmid using Gibson kit (NEB) based on the protocol provided. Gibson cloning assembles multiple overlapping fragments into a plasmid in a single reaction. The reaction is carried out using Gibson assembly master mix which is a combination of 3 different enzymes in a single buffer: exonuclease (to create 3' single-stranded overhangs for attachment of overlapping fragments), DNA polymerase (to fill in gaps between annealed sequences) and a DNA ligase (for sealing the nicks in the assembled DNA).

10 µl Gibson assembly mix was prepared incorporating 5 µl Gibson assembly master mix with 0.02-0.5 pmol of linearised pUC19 plasmid in combination with 2-3 folds excess of each of the fragments 1. HY and 5'flank or 2. YG and 3'flank.

The reaction was carried out at 50°C for 30 minutes and stored at -20°C until *E. coli* transformation.

Table 2-5: Gibson cloning primers for Fg BEC1019 gene deletion.
The sequence of primers used for Fg BEC1019 gene deletion cassette construction using Gibson cloning.

Primer number from Fig. 2.5	**Primer Name (Lab Reference)**	**Fragment Amplified (Target gene)**	**Overlapping Region (Adjacent region)**	**Primer Sequence**
F1	*Fg*_B19_5'FFwd	*Fg BEC1019* 5'Flank	pUC19 plasmid – BamH1 restriction site	AGCTCGGTACCCGGGATGGACTTGGTATTGACATTGACGAG
R1	*Fg*_B19_5'FRev	*Fg BEC1019* 5'Flank	HY fragment	GTTATCGAATCTTGAAGTGTTATTGGGGCTGAAAG
F2	*Fg*_B19_5'F_HY_Fwd	HY fragment	*Fg* BEC109 homologue 5'Flank	CACTTCAAGATTCGATAACTGATATTGAAGGAGCATTTTTTTGG
R2	HY_pUC19HindIII_Rev	HY fragment	pUC19 plasmid – HindIII restriction site	CCATGATTACGCCAGGATGCCTCCGCTCGAAGTAG
F3	pUC19BamH1_YG_Fwd	YG fragment	pUC19 plasmid – BamH1 restriction site	AGCTCGGTACCCGGGCGTTGCAAGACCTGCCTGAAAC

R3	YG_*Fg*_B19_3'F_Rev	YG fragment	*Fg* BEC109 homologue 3'Flank	TATTCAGCGCCTCGAGGTCGACGGTATCGATAAG
F4	*Fg*_B19_3'FFwd	*Fg BEC1019* 3'Flank	YG fragment	ACCTCGAGCGCTGAATACACCATGATAGATATGAC
R4	*Fg*_B19_3'FREv	*Fg BEC1019* 3'Flank	pUC19 plasmid – BamH1 restriction site	GACCATGATTACGCCACTCACGATCATCGTCGCTCTAC

2.5.1.4 Plasmid transformation into E.coli DH5-α competent cells

The Gibson reaction mix was transformed into commercially prepared DH5-α *E. coli* chemically competent cells using a protocol provided by the manufacturer (New England Biolabs, NEB). Two µl of Gibson reaction was mixed with 100 µl of *E. coli* competent cells and placed on ice for 30 minutes. Heat shock was performed at 42°C for 30 seconds and transferred on to ice for 2 minutes. 950 µl of pre-warmed SOC medium (NEB) was added to the reaction mix and incubated at 37°C for 1 hour with shaking at 250 rpm. The reactions were then placed on ice for transformation. An unaltered pUC19 plasmid was used as a positive control to check the transformation efficiency.

pUC19 plasmids have an ampicillin resistance gene and a β-galactosidase gene *lac Z* with multiple cloning sites (MCS). The deletion cassette was cloned into the MCS within the lac Z gene and therefore selection of transformants was done using blue/white colony assay. Selection plates containing LB agar (Sigma) and 100 µg/ml of ampicillin were plated with 100 µl of 20 mg/ml of β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-D-galactoside) followed by plating with 100 µl of 10 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) to induce β-galactosidase activity (LacZ). The colonies with insertion of gene deletion cassette outside the lac Z gene or no integration of the deletion cassette formed blue colonies due to cleavage of X-gal by β-galactosidase in the presence of IPTG to form a 5-bromo-4-chloro-indoxyl which spontaneously oxidises to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo. Colonies containing the gene deletion cassette ligated into lac Z gene formed white colonies due to the expression of a disrupted and inactive β-galactosidase protein.

2.5.1.5 Clone diagnostics by colony PCRs and sequencing

Colony PCRs were performed on up to 20 white colonies from the blue-white colony screens to confirm the correct assembly of the different fragments. Primers were designed to amplify the joints between the different fragments and are tabulated in Table 2-5. To extract DNA from the clones, a small colony was inoculated into 50 µl of sterile distilled water and boiled at 90°C for 3 minutes. PCRs were conducted using standard conditions (described in

Table 2-4) and using 2 µl of the supernatant from the boiled colony as a template. The correct size of the amplicons visualised on agarose gel confirmed the presence of the different fragments assembled. The diagnostic primers used are listed in Table 2-6.

The cloned plasmid was then sequenced to verify the correct insertion and orientation of both HYG fragments associated with the respective *Fg BEC1019* flanking regions for the construction of gene deletion cassette.

Table 2-6: Diagnostic PCRs for Fg BEC1019 deletion.

Primers used for confirming the correct assembly of the two Fg BEC1019 gene deletion cassettes in pUC19 plasmid.

Primer name	Region amplified	Sequence
pUC19-5'flank Fwd	Intersection of pUC19 and *Fg BEC1019* 5'Flank	CATTCAGGCTGCGCAACTG
pUC19-5'flank Rev	Intersection of pUC19 and *Fg BEC1019* 5'Flank	ACACCCATCAATGTCATCCCT
5'flank-HY Fwd	Intersection of *Fg BEC1019* 5'Flank and HY fragment	ACGCCTCCCACATTCCTATT
5' flank-HY Rev	Intersection of *Fg BEC1019* 5'Flank and HY fragment	GTCCTCGTTCCTGTCTGCTA
HY-pUC19 Fwd	Intersection of HY fragment and pUC19 plasmid	CGATTGCTGATCCCCATGTG
HY-pUC19 Rev	Intersection of HY fragment and pUC19 plasmid	CTTCCGGCTCGTATGTTGTG
pUC19-YG Fwd	Intersection of pUC19 and YG fragment	CTCTTCGCTATTACGCCAGC
pUC19-YG Rev	Intersection of pUC19 and YG fragment	TGTCGTCCATCACAGTTTGC
YG-3'flank Fwd	Intersection YG fragment and *Fg BEC1019* 3'Flank	ACGGCAATTTCGATGATGCA
YG-3'flank Rev	Intersection YG fragment and *Fg BEC1019* 3'Flank	AGACATCCTCCAGCACAACA
3'flank-pUC19 Fwd	Intersection *Fg BEC1019* 3'Flank and pUC19 plasmid	AGGCTTTCTGGTTGGTAGCT

3'flank-pUC19 Rev	Intersection *Fg BEC1019* 3'Flank and pUC19 plasmid	CTTCCGGCTCGTATGTTGTG

2.11.2 *Fusarium graminearum* transformation

F. graminearum conidia was plated on SNA agar (0.1% KH_2PO_4, 0.1% KNO_3, 0.1% $MgSO_4$ x 7 H_2O, 0.05% KCl, 0.02% glucose, 0.02% saccharose, 2% bacto agar) and incubated under near UV light for 8 days for conidiation. Conidia was collected using 8 ml of sterile distilled water and concentration of spores was determined.

For transformation, conidia germination was induced by inoculating 1-5 x 10^8 spores in 100 ml YEPD-2G (0.3% yeast extract, 1% bacto-peptone, 2% glucose) and incubated in a shaker incubator at 200 rpm and room temperature for 7-8.5 hours. Cultures containing germlings 3-10 conidial diameters in length were harvested by transferring the cultures into 50 ml falcon tubes and centrifuged at 3000 rpm for 10 minutes. The pellet was resuspended in 25 ml 1 M Sorbitol to rinse and centrifuged at 3000 rpm for 5 minutes. When required, hyphae were filtered out using sterile miracloth. The pellets were resuspended in 20 ml enzyme mixture (100 mg Sigma Lysing Enzyme, 500 mg Driselase, 1 mg Chitinase) and shaking at 80 rpm at 30° C for 1 hour. The protoplast development was then analysed, and the protoplasts were harvested by centrifuging at 3000 rpm for 5 minutes. The protoplasts were gently resuspended in 10 ml STC by rolling the tube between hands. Centrifugation was repeated at 3000 rpm for 5 minutes. The protoplasts were then washed twice in 1 ml STC and centrifuged at low speed. The protoplasts were finally resuspended in 200-500 µl STC and diluted to 1-2 x 10^8 protoplasts and placed at room temperature for transformation.

10 µl of DNA was mixed with 100 µl of STC and 100 µl of protoplast suspension. The mixtures were added to 50 µl of 30% PEG 8000 in a 15 ml conical tube, mixed carefully and incubated at room temperature for 20 minutes. 2 ml of 30% PEG 8000 was added, mixed thoroughly and incubated for 5 minutes at room temperature. Again 4 ml of STC was added and mixed thoroughly. 600 µl protoplasts were plated together with 20 ml of Sucrose medium bottom agar (1.6 M Sucrose medium + equal volume 2X Regeneration medium) and incubated overnight at room temperature. Next morning sucrose medium overlay agar containing hygromycin (150 ug/ml) was prepared. Transformants were visible after 5-7 days and was transferred on plates containing hygromycin for selection.

The gene deletion transformants were used to perform infection assay to investigate the role of *Fg BEC1019* on FHB disease development.

2.11.3 Diagnostics of *Fg BEC1019* deletion in *F. graminrearum* transformants

The replacement of *Fg BEC1019* by hygromycin resistance cassette was confirmed by screening the transformants using a diagnostic PCR. The amplicons for the diagnostic PCRs included a fragment spanning the joint between *Zt BEC1019* 5'Flank and HYG cassette (*Fg B19* 5'F P1-Hyg4 primers in Figure 2-7) , the HYG cassette and *Fg BEC1019* 3'Flank and a region in the *Zt BEC1019* gene cassette (Hyg4-*FgB19* 3'FP2 primers in Figure 2-7) and a region in the *Fg BEC1019* gene.

The correct insertion of the *Fg BEC1019* deletion cassette was confirmed by the correct amplicon lengths for PCR1 and PCR2 and the absence of the PCR3 amplicon, amplifying a region of the *Fg BEC1019* gene.

The PCRs were carried out using the standard PCR conditions described in

Table 2-4 and the diagnostic primers are listed in Table 2-7.

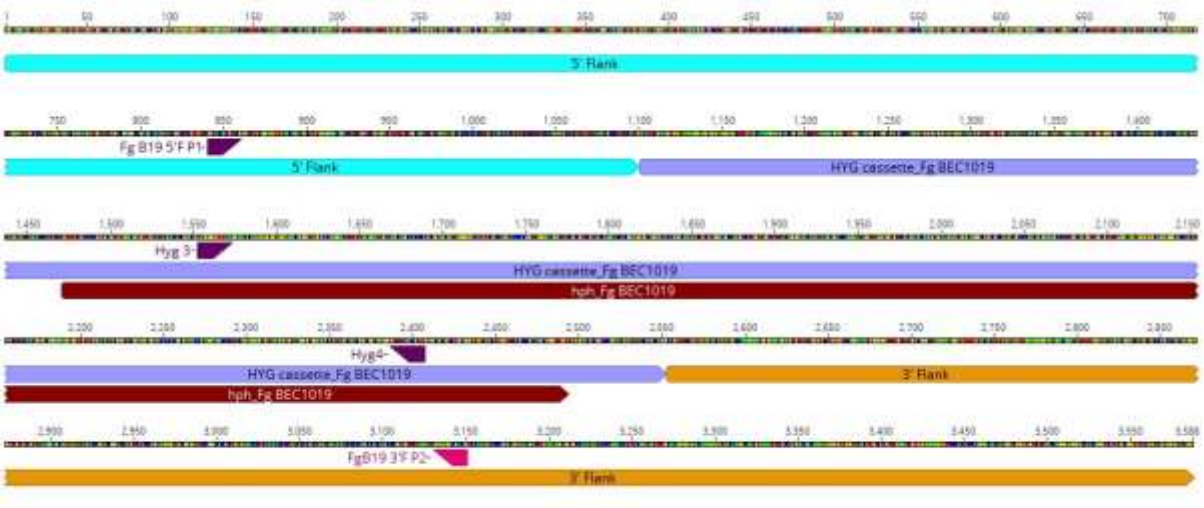


Figure 2-7: Strategy for diagnostic PCRs for Fg BEC1019 deletion.

Positions of diagnostic PCR primers for screening F. graminearum transformants with correct insertion of Zt BEC1019 gene deletion cassette. PCR1 - region between Fg B19 5'F P1- Hyg4 primers, PCR2 - region between Hyg3 - Fg B19 3'F P2 primers. PCR3 - region in the Fg BEC1019 region (not shown in the diagram).

Table 2-7: Diagnosis of Fg BEC1019 gene deletion in F. graminearum genome

Details of primers used for diagnosis of correct insertion of the Fg BEC1019 deletion cassette in the genome of the F. graminearum transformants.

Primer	Region amplified	Sequence
***Fg B19* 5'F P1**	Intersection of *Fg BEC1019* 5'Flank and HYG fragment	TGGACTACCTGCAGAAACCG
Hyg4	Intersection of *Fg BEC1019* 5'Flank and HYG fragment	TTCTGCGGGCGATTTGTGTAC
Hyg3	Intersection HYG fragment and *Fg BEC1019* 3'Flank	TCTCGGAGGGCGAAGAATCTC
***Fg B19* 3'F P2**	Intersection HYG fragment and *Fg BEC1019* 3'Flank	CAGGAGCGGACCAAAACTCT
***Fg B19* F**	Region within *Fg BEC1019*	TCCAAGACAAGTGAGAGCAG
***Fg B19* R**	Region within *Fg BEC1019*	AGATGATGGAGTTTAAATAGTTGGATT

2.12 Gene deletion in *Zymoseptoria tritici*

The genes were deleted in *Z. tritici* by replacing them with a hygromycin resistance cassette using *Agrobacterium* based transformation.

2.12.1 Construction of gene deletion cassette

The gene deletion cassette containing a hygromycin deletion cassette flanked by the 5' and 3' flanking regions of the gene of interest was assembled in pCHYG ti-plasmid (Figure 2-8) by Gibson

cloning as described in section 2.11.1. For *Zt ZAP1* gene, the assembly was directly done in the pCHYG plasmid. But, *Zt BEC1019* deletion cassette was first assembled in pUC19, then amplified and inserted into the pCHYG plasmid between the left and right borders of *Agrobacterium*.

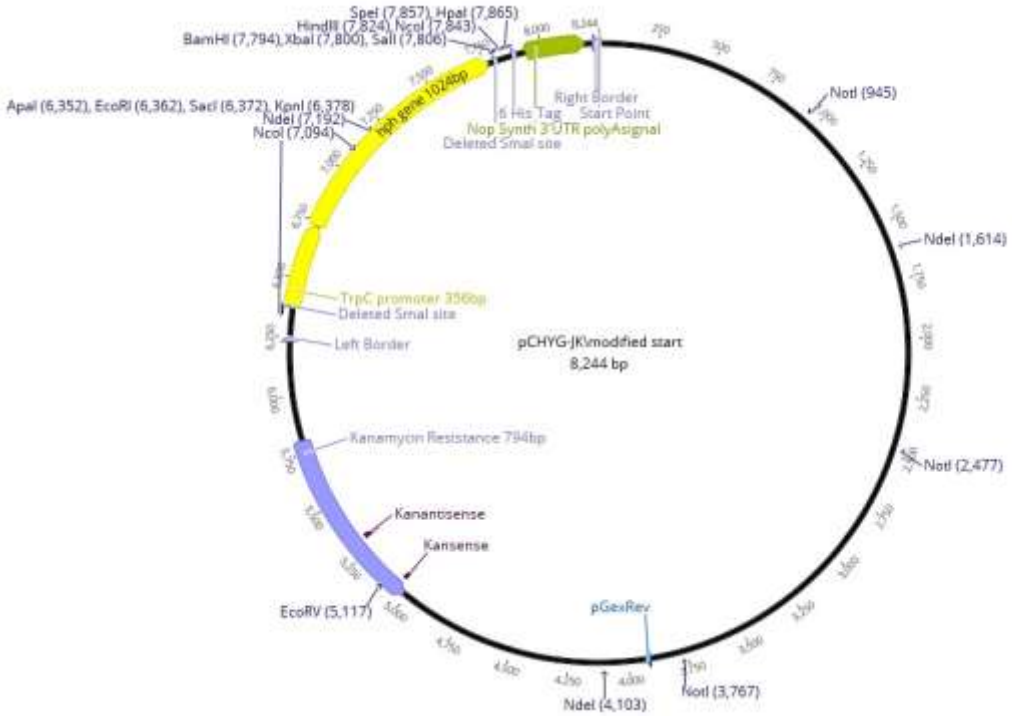


Figure 2-8: pCHYG plasmid

A diagram of the pCHYG plasmid used for assembly of Z. tritici gene deletion cassettes and Agrobacterium based transformation of Z. tritici.

Linearisation of pUC19 and pCHYG plasmid

The pUC19 plasmid was linearised for the initial assembly of the *Zt BEC1019* deletion cassette using the restriction enzymes *BamH1* and *HindIII* as described in section 2.5.1.1.

For transfer of the complete *Zt BEC1019* deletion cassette from the pUC19 plasmid to the pCHYG plasmid and for the assembly of the *Zt ZAP1* deletion cassette in pCHYG plasmid, the pCHYG plasmid was linearised using the *SacI* (Thermo Scientific) and *HpaI (KspaI)* (Thermo Scientific) restriction enzymes. A 20 µl reaction was set-up containing 2 µl 10X *SacI* buffer (Thermo Scientific), 0.75-1 µg of pCHYG plasmid DNA, 10 units (U) of *SacI* and 20 U of *HpaI* enzymes. The final volume was made up to 20 µl using nuclease free water. The digestion was carried out at 37°C for 1 hour followed by inactivation of the enzymes at 65°C for 20 min.

The digested products were analysed on a 1% agarose gel containing 1:10,000 SYBR safe stain (Thermo Fisher Scientific).

For assembly of the *Zt PacC* deletion mutant, the pCHYG plasmid was linearised using

2.4.1.2 Primer design and amplification of fragments for gene deletion cassette

The primers were designed to first amplify the different components of *Zt BEC1019* assembly in pUC19 and *Zt ZAP1* deletion cassette in pCHYG, such that the primers had a gene specific as well as an overhang region of the neighbouring fragment. These primers are listed in Table 2-8 and Table 2-10. Another set of primers was designed to amplify the whole *Zt BEC1019* deletion cassette from the pUC19 and t insert it into the pCHYG plasmid. These primers are listed in Table 2-9.

Table 2-8: Primers used to amplify different fragments of the Zt BEC1019 in the pUC19 plasmid.

The target region of the primer is in red and the overhang is in black.

Primer name	Amplicon	Primer sequence
pUC19 Zt B19 5'F F	pUC19 overhang + *Zt BEC1019* 5'Flank + HYG overhang	AGTGAATTCGAGCTCGGTACCCGGG TTCCATCAAGGTGTCCAAG
Zt B19 5F_HYG R	pUC19 overhang + *Zt BEC1019* 5'Flank + HYG overhang	CTCCTTCAATATCTTTGAGTGTGTGAATGGG
Zt B19 5F_HYG F	*Zt B19* 5'Flank overhang + HYG + *Zt B19* 3'Flank overhang	TCACACACTCAAA GATATTGAAGGAGCATTTTTTGG
HYG_Zt B19 3F R	*Zt B19* 5'Flank overhang + HYG + *Zt B19* 3'Flank overhang	TTCAATGAAAGCGCTATTCCTTTGCCCTCGG
HYG_Zt B19 3F F	HYG overhang+ *Zt B19* 3'Flank+pUC19 overhang	GGCAAAGGAATAGCGCTTTCATTGAAACGTC
Zt B19 3F_pUC19 R	HYG overhang+ *Zt B19* 3'Flank+pUC19 overhang	AACAGCTATGACCATGATTACGCCA GGTTGTTTGGCTTTGTGATC

Table 2-9: Primers for amplification of complete Zt BEC1019 deletion cassette

List of primers for amplification of Zt BEC1019 deletion cassette from pUC19 for insertion into linearised pCHYG plasmid.Primer sequence specific to target gene is in red and the overhangs are in black.

Primer name	Amplicon	Primer sequence
pCHYG SacI_Zt B19 5'F Fwd	pCHYG overhang at *SacI* site + *Zt BEC1019* deletion cassette + pCHYG overhang at *HpaI* site	GGGCCCGGCGCGCGCCGAATTCGAGCT TTCCATCAAGGTGTCCAAG
Zt B19 3'F_pCHYG Hpa1 R	pCHYG overhang at *SacI* site + *Zt BEC1019* deletion cassette + pCHYG overhang at *HpaI* site	CGTGGTGGTGGTGGTGGTGGCTAGCGTT GGTTGTTTGGCTTTGTGATC

Table 2-10: Primers for Zt Zap1 deletion cassette assembly.

Primers used for amplification of different components of the Zt ZAP1 deletion mutant for assembly in pCHYG plasmid. The target gene regions of the primer are in red and the overhangs are in black.

Primer name	Amplicon	Primer sequence
pCHYG_ Zt ZAP1 5'F F	pCHYG overhang + *Zt ZAP1* 5'Flank + HYG overhang	GGGCCCGGCGCGCCGAATTCGAGCT TTCCATCAAGGTGTCCAAG
Zt ZAP1 5F_HYG R	pCHYG overhang + *Zt ZAP1* 5'Flank + HYG overhang	CTCCTTCAATATCCTTGGCGATGGCTGTGCATG
Zt ZAP1 5F_HYG F	*Zt ZAP1* 5'Flank overhang + HYG + *Zt ZAP1* 3'Flank overhang	AGCCATCGCCAAGGATATTGAAGGAGCATTT TTTGG
HYG_Zt ZAP1 3F R	*Zt ZAP1* 5'Flank overhang + HYG + *Zt ZAP1* 3'Flank overhang	CTCTGCCTTCATTCTATTCCTTTGCCCTCGG
HYG_Zt ZAP1 3F F	HYG overhang + *Zt ZAP1* 3'Flank+ pCHYG overhang	GGCAAAGGAATAGAATGAAGGCAGAGGCAAG
Zt ZAP1 3F_pCHYG R	HYG overhang + *Zt ZAP1* 3'Flank+ pCHYG overhang	CGTGGTGGTGGTGGTGGTGGCTAGCGTT GCGACGTTTCGTTCCAAG

2.4.1.3 Gibson cloning

The Gibson cloning (NEB) was performed based on the manufacturer's instructions and as described in section 2.5.1.3**Error! Reference source not found.** and the reaction was stored at - 20°C for *E.coli* transformation.

2.4.1.4 Plasmid transformation into E. coli DH5-α competent cells

Once the plasmid was assembled, the Gibson mixture was transformed in to *E.coli* DH5α cells (New England Biolab), using manufacturer's instructions (also described in Section 2.4.1.4). The *E.coli* clones harbouring pUC19 assembly were screened by blue white colony screening as described in section 2.5.1.4 whereas pCHYG plasmid harbouring clones were screened using LB agar (Sigma) plates supplemented with kanamycin (50 µg/ml).

2.4.1.5 Colony PCRs and Sequencing

Colony PCRs and sequencing were performOed to confirm the correct assembly of the deletion cassette as described in section 2.5.1.5. The primers used for colony PCRs are tabulated in

Table 2-11 and the PCR conditions in

Table 2-4.

Table 2-11: Diagnostic primers for assembly of Zt BEC1019 and Zt ZAP1 deletion cassettes in the pCHYG plasmid.

Primer	Region amplified	Sequence
pCHYG_Zt B19 diag F1	Intersection of pCHYG and *Zt BEC1019* 5'Flank	AAGTCTGCCGCCTTACAACG
pCHYG_Zt B19 diag R1	Intersection of pCHYG and *Zt BEC1019* 5'Flank	CTTGCTTCTTGCGAATCGGC
pCHYG_Zt B19 diag F2	Intersection of *Zt BEC1019* 5'Flank + HYG + Intersection of *Zt BEC1019* 3'Flank	ATGACTTCGAAGCTTGGCCA
pCHYG_Zt B19 diag R2	Intersection of *Zt BEC1019* 5'Flank + HYG + Intersection of *Zt BEC1019* 3'Flank	GCTACACGATGGAGGCGAAT
pCHYG_Zt B19 diag F3	Intersection of HYG and *Zt BEC1019* 3'Flank	AATCAGCCTTCCAGTCCACG
pCHYG_Zt B19 diag R3	Intersection of HYG and *Zt BEC1019* 3'Flank	AGACCGGCAACAGGATTCAA

pCHYG_Zt ZAP1 diagnose F1	Intersection of pCHYG and *Zt ZAP1* 5'Flank	AAGTCTGCCGCCTTACAACG
Zt ZAP1 diagnose R1	Intersection of pUC19 and *Zt ZAP1* 5'Flank	AGAGAACTCGAGCCTGGACT
Zt ZAP1 diagnose F2	Intersection *Zt ZAP1 5*'Flank and HYG fragment	AGTTCATACTCCGCATCGCC
Zt ZAP1 diagnose R2	Intersection *Zt ZAP1 5*'Flank and HYG fragment	GCGGGAGATGCAATAGGTCA
Zt ZAP1 diagnose F3	Intersection of HYG fragment and *Zt ZAP1 3*'Flank	TGGCAAACTGTGATGGACGA
Zt ZAP1 diagnose R3	Intersection of HYG fragment and *Zt ZAP1 3*'Flank	TGGCAAGGCTGTGCAATTTC
Zt ZAP1 diagnose F4	Intersection of *Zt ZAP1 3*'Flank and pUC19	GGACTCCTCTCGTTGCTGAC
Zt ZAP1 diagnose R4	Intersection of *Zt ZAP1 3*'Flank and pUC19	TACCGCCTTTGAGTGAGCTG

2.12.2 *Agrobacterium* transformation

The assembled deletion cassette was transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation. 50 µl of electrocompetent *Agrobacterium* cells were four times diluted using sterile distilled water. 1 µl of the plasmid was added to 50 µl of the diluted cells, mixed by flicking and transferred to an ice-cold electroporation tube. Electroporation was performed by applying a pulse of 16.7 kv/cm for 6 mili seconds followed by subsequent addition of 1 ml LB broth to the cells, mixing (by pipetting) and transfer to a 15 ml falcon tube for incubation at 28°C, 150 rpm shaking for 1 hour. After 1 hour, 100 µl of the cell suspension was plated on LB agar plates supplemented with 50 µg/ml kanamycin and incubated at 28°C for 3 days. Liquid cultures were prepared of the individual transformed colonies in LB broth containing 50 µg/ml kanamycin. Glycerol stocks were prepared from these liquid cultures after 2 days growth at 28°C.

2.12.3 *Agrobacterium* based transformation of *Zymoseptoria tritici*

The *Agrobacterium* based transformation of *Z. tritici* was performed as described earlier by Zwiers & De Waard in 2001 with slight modifications using the *Z. tritici* KU70 strain. The *Agrobacterium* Agl-1 transformants containing *Zt BEC1019* gene deletion cassette was grown overnight in *Agrobacterium* induction medium (AIM) (10 mM KH2PO4, 10mM K2HPO4, 2.5 mM NaCl, 2 mM

MgSO4.7H2O, 0.7 mM CaCl2, 9 mM FeSO4, 4 mM (NH4)2SO4, 10 mM glucose, 0.5% glycerol, 40 mM MES buffer, 1 l H2O, pH 5.6, with Agar 20 g/L) supplemented with 50 µg/ml kanamycin at 28°C. *Z. tritici* KU70 spores derived from 5 days old YPD (10 g/l Yeast Extract, 20 g/l Peptone, 20 g/l Glucose, 2% Agar) plate were suspended in *Agrobacterium* induction medium and mixed in 1:1 ratio with *Agrobacterium* transformants growing in the same culture containing 200 µM acetosyringone (Sigma) and plated on a cellophane membrane mounted on IM plate supplemented with 200 µM acetosyringone. After incubating the plates at 16°C for 48 hours, the cellophane membranes were transferred onto *Aspergillus nidulans* minimal medium plates (AMM) (20X salt solution - $NaNO_3$ 120 g/L, KCl 10.4 g/L, $MgSO_4.7H_2O$ 10.4 g/L, KH_2PO_4 30.4 g/L; Trace elements - H_3BO_3 1.1%, $MnCl_2.4H_2O$ 0.5%, $FeSO_4.7H_2O$ 0.5 g/L, $CoCl_2.5H_2O$ 0.16 g/L, $CuSO_4.5H_2O$ 0.16 g/L, $(NH_4)6Mo_7O_{24}.4H_2O$ 0.11 g/L, Na_4EDTA 5 g/L; AMM agar – 20X Salts 50ml/L, Trace elements 1 ml/L, Glucose 10 g/L, pH 6.5-6.8; Agar 2%) containing hygromycin B (Calbiochem, San Diego, CA, U.S.A.) at 100 µg/ml and Timentin (ticarcillin disodium salt/potassium clavanulate) (Melford, Ipswich, U.K.) at 100 µg/ml. The plates were incubated at 16°C for 7 days prior to screening for putative hygromycin-resistant transformants. Independent fungal colonies of putative transformants were picked and sub-cultured on YEPD agar supplemented with hygromycin and timentin. The *Z. tritici* transformants were further sub-cultured twice to obtain material from a single yeast-like fungal colony and to kill off *Agrobacterium* cells.

Chapter 3 : Use of antisense phosphorothioate modified oligodeoxynucleotides mediated *in planta* gene silencing as a functional genomics tools for discovering new players in *Blumeria* virulence in different barley cultivars

3.1 Introduction

3.1.1 Disease control in fungi

Traditionally, fungal disease control has been achieved by resistance breeding (Burdon et al., 2014) and use of chemical fungicides (C. Yang et al., 2011). However, resistance breeding is not always very effective due to pathogen mutation leading to loss of resistance. Similarly, pathogens are also developing resistance to the traditional chemical fungicides, requiring the development of new tools for fungal disease control.

In the recent years, RNAi based gene silencing has been explored as a tool for controlling fungal diseases. RNAi based gene silencing is mediated by both small interfering RNA (siRNA) and hairpin RNA (hpRNA). In the initial studies, RNAi gene silencing was achieved by virus induced gene silencing where the gene specific hpRNA were cloned into viral vectors and introduced into the host by viral infection (Karthikeyan et.al., 2013). The VIGs approach was successfully used to query the virulence of candidate effectors in biotrophic pathogens like *B. graminis* (Aguilar et.al., 2015) and necrotrophic *B. cinerea* (Zhang et al., 2016). Disease control has also been successfully achieved by transforming host plants to express dsRNA targeting pathogen genes. For instance, pyrethroid fungicide susceptibility was restored in cotton bollworm larva by transforming cotton host plants to express dsRNa targeting the insect P450 monooxygenase gene. The larva feeding on the leaves of these transformed host plants demonstrated reduced growth and increased susceptibility to the pyrethroid fungicide gossypol (Mao et al., 2007). HIGS was also successfully mediated by biolistic delivery of gene specific hpRNA into barley cells to query the role of *Blumeria* effector candidates (Pliego et al., 2013). However, these strategies have limited use in agriculture due to complications involving the use of viral vectors (Tufan et.al., 2011) or use of genetically modified host plants. Therefore, more recent studies have explored direct delivery of dsRNA as a strategy for fungal disease control.

Direct delivery of dsRNA was successfully used to control fungal growth and virulence in fungal pathogens like *Fusarium sp.* (Gu et al., 2019; Koch et al., 2016; Koch et al., 2013; Song et.al., 2018) and *B. cinerea* (M. Wang, Weiberg, Lin, Thomma, Huang, et al., 2016). This was achieved by either

direct uptake of dsRNA from liquid media (Koch et al., 2013) or by spraying fungal gene specific dsRNA onto host plants (Gu et al., 2019; Koch, et al., 2016; Song, et. al., 2018; Wang et al., 2016). For instance, FHB development was reduced on wheat heads and wheat coleoptile by silencing *Fg Myo5* by spraying gene specific dsRNA on to the respective host tissues (Gu et al., 2019). Similarly, spraying of dsRNA targeting *B. cinearea* dicer enzyme onto fruits and vegetables reduced fruit rot development (Wang et.al., 2016). Nevertheless, the spraying of dsRNA also has its limitations as demonstrated by a maximum of 9 days of disease control post spraying of *Fg Myo5* dsRNA onto wheat heads (Song et al., 2018), indicating the requirement for multiple doses of dsRNA for long term disease control.

Therefore, novel silencing molecules that can be directly delivered into fungal and host cells for successful gene silencing and disease control need to be explored.

3.1.2 Phosphorothioate modified oligodeoxynucleotide (PTO modified ASO) based *in planta* gene silencing in *Blumeria graminis* f.sp. *hordei*

Phosphorothioate modified oligodeoxyribonucleotide (ASO modified PTO) based gene silencing is a novel tool recently developed in the Bindschedler lab for *in planta* silencing of virulence and susceptibility genes in the barley biotrophic pathogen *Blumeria graminis* f. sp*. hordei* (Orman et.al., manuscript in prep).

Antisense oligodeoxyribonucleotides (ASO) are single stranded oligomers with a length of 19-25 deoxynucleotides. ASO are designed as reverse complement sequences of the targeted mRNA in order to silence specifically the cognate gene (Dinc et.al., 2011). The silencing efficiencies of non-modified DNA oligos are compromised by ssDNA vulnerability to nuclease degradation in eukaryotic cells (Eder et. al., 1991). Therefore, chemical modifications have been explored to enhance cellular stability of ASOs. One of the first-generation of ASO modifications is the phosphorothioate (PTO) modification, where one of the two non-bridging oxygen atoms of the phosphodiester bond has been replaced by a sulphur atom in the DNA backbone of the ASO. Such PTO modifications increase the ASO stability because of its resistance to the organism's endogenic DNase activity (Stein et.al., 1988). Moreover, the PTO modification has been shown to enhance the biological uptake and bioavailability of these silencing molecules in the animal clinical studies (Khvorova & Watts, 2017; Koller et al., 2011). PTOs are substrate for the RNase H, an enzyme that degrades RNA-DNA heterodimers. Therefore PTO modified ASO mediate gene silencing by RNase H mediated mRNA degradation (Wu et al., 2004).

PTO modified ASO based *in planta* gene silencing in *B. graminis* has been developed to silence fungal and host genes by direct delivery of gene specific PTOs into excised barley leaves. Seven days old

barley leaves are dipped in 10 µM PTO modified ASO solutions for 24 hours and incubated under continuous light to encourage PTO modified ASO uptake by transpiration. To assess the role of the target gene in disease development, PTO treated leaves are then inoculated with *Blumeria* spores and infection was allowed to develop for 48 hours. The infection phenotype resulting from gene silencing is assessed microscopically at 48 hpi by observing the proportion of secondary hyphae formation as a proxy for the formation of functional haustoria. Alternatively, the relative fungal biomass in the barley host is estimated by comparing housekeeping gene expression ratio between the invading fungus and the plant host using qRT-PCR. Gene silencing is confirmed by change in target gene transcript abundance relative to a fungal housekeeping gene. So far, PTO modified ASO mediated silencing of *B. graminis* effectors *BEC1011, BEC1019* and *BEC1054* and barley susceptibility gene *blufensin 1* (*Bln1*) resulted in decrease in secondary hyphae development by 55%, 49%, 51% and 56%, respectively (Orman, Das Gupta et. al., manuscript in prep). The phenotype associated with the silencing of *Bgh BEC1011* and *BEC1019* was further substantiated by showing a 33% decrease in fungal biomass as estimated by qRT-PCR (Orman, Das Gupta et.al., manuscript in prep). Therefore, PTO based *in planta* gene silencing has been developed as a functional genomics tool for screening potential virulence and susceptibility genes in the barley – powdery mildew pathosystem.

In this study, this functional genomics tool was used to decipher the effect of *Blumeria* housekeeping gene silencing on barley powdery mildew infection development. This tool has been further used to discover new barley susceptibility factors. The strategy of silencing fungal housekeeping genes and host susceptibility genes for disease control in different barley cultivars.

3.1.3 Actin (ACT)

Actin is a cytoskeletal protein important for microtubule formation. Dynamic reorganisation of actin filaments plays an important role in fungal growth and morphogenesis (Bergs et. al., 2016). Moreover, it has a role in vital processes like cytokinesis, secretion, vesicular transport and organelle movement (reviewed by Berepiki et.al., 2011). Actin also plays a role in fungal virulence. The actin cytoskeleton mediated switch from yeast-like to hyphal form is essential for virulence in many fungi (Dagdas et al., 2012; González-Rodríguez et.al., 2016). For instance, in *B. cinerea,* deletion of genes coding for F-actin capping proteins prevented F-actin ring formation at the hyphal tips, resulting in mutants with hampered polarised growth and reduced virulence (González-Rodríguez et al., 2016). In the rice blast pathogen *M. oryzae,* during appressorium development, a doughnut-shaped filamentous actin network was formed inside the appressorium at the site of peg development. This network along with the colocalised septin ring were thought to be responsible for converting the high turgor pressure inside the appressorium into the mechanical energy of a penetration peg which

then penetrates the host (Dagdas et al., 2012). Therefore, actin was selected as one of the targets for silencing in *B. graminis* for validation of the PTO mediated *in planta* gene silencing methodology. In *B. graminis* f.sp. *hordei,* there are two actin isoforms, but only one is expressed in *Blumeria* haustorium (Bindschedler et.al., 2011). Since, haustoria is the interface of material exchange between host and the pathogen (Both et.al., 2005) and was assumed to be easily reachable by the PTOs, the actin isoform (Ensembl Gene Id: BLGH_02125) expressed in *Blumeria* haustoria was chosen as the suitable housekeeping gene target for validating the PTO based *in planta* gene silencing methodology.

3.1.4 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a classical glycolytic protein with a pivotal role in energy generation in all organisms, including fungi. It catalyses the important step of conversion of glyceraldehyde-3-phosphate to D-glycerate-1,2-bisphosphate for energy generation from glucose during glycolysis. Mammalian GAPDH also influences membrane fusion, microtubule bundling, phophotransferase activity, nuclear RNA transport, DNA replication and DNA repair (reviewed by Sirover, 1999). In entomopathogenic fungi like *Metarhizium anisopliae*, GAPDH is important for host adhesion and virulence, as demonstrated by the expression of GAPDH protein on the fungal surface and its capability to adhere to the wings of the insect hosts (Broetto et al., 2010). As *B. graminis* is an obligate biotroph, there is an overlap between factors required for fungal growth and virulence (Both et.al., 2005). Therefore, a housekeeping *gene Bgh GAPDH* was considered as a suitable target for validation of PTO mediated silencing and its role in *B. graminis* virulence was studied.

3.1.5 2-Glycosyl transferase (GT2)

Glycosyl transferases are biological enzymes that catalyse the transfer of sugar moieties to non-carbohydrate substrates like proteins and lipids and catalyse the formation of glycosidic linkages. These enzymes use an activated sugar donor and transfer the glycosyl group onto a nucleophile acceptor (Lairson et. al., 2008). They have been shown to be important for cell wall and capsule formation in both plant and human fungal pathogens (King et al., 2017; Klutts et. al., 2006). 2-Glycosyl transferase (GT2) was particularly shown to have a role in hyphae formation and virulence of important wheat pathogens like *Zymoseptoria tritici* and *Fusarium graminearum*. GT2 belongs to the family 2 of glycosyl transferases that use UDP-glucose, UDP-N-acetyl-galactosamine, GDP-mannose or CDP-abequose as a sugar donor and transfer the glycosyl group to a range of substrates including cellulose, dolichol phosphate and teichoic acids (InterPro id: IPR001173).

2-Glycosyl transferase (GT2) was shown to have a role in hyphae formation and virulence of important wheat pathogens like *Zymoseptoria tritici* and *Fusarium graminearum*. GT2 deletion resulted in inhibition of hyphae formation and complete loss of virulence in both the pathogens. In *Z. tritici, GT2* deletion mutants had cell wall abnormalities like bulges, protrusions or breakages when visualised under the transmission electron microscope. The lack of hyphenation on leaf surface was not due to abnormality in sensing the leaf surface. The ribosomal proteins were downregulated in the mutants as compared to wild type when growing on the leaf surface, and where similar in both the strains when growing in liquid culture. This suggested that GT2 was important for fungal growth on response to leaf-sensing. However, on getting in contact with the leaf, the pathogen constitutively overexpressed chitin-binding effectors and transmembrane proteins. Therefore, *Zt GT2* deletion triggered some early transcriptional changes that are normally induced in the later stages of the fungal infection of wheat (King et al., 2017). Amongst the over-expressed genes was a putative alpha-1,3-glucan synthase. Alpha-1,3-glucan is an important component of fungal cell wall and was shown to play a role in virulence of *A. fumigatus.* This suggests that the alpha-1,3-glucan synthase gene might be induced to compensate for the cell wall abnormalities caused by *Zt GT2* deletion. Therefore, it was suggested that GT2 may be important in synthesis of a yet unknown fungal outer cell wall or extracellular polysaccharide, which has a role in leaf sensing and fungal hyphenation on leaf surfaces (King et al., 2017).

Interestingly, GT2 is conserved in many fungi including *B.* graminis f.sp*. hordei* (King et al., 2017). Therefore, the role of *Bgh GT2* in fungal virulence was queried using the PTO based *in planta* gene silencing methodology, with the aim of discovering novel players in barley powdery mildew infection development.

3.1.6 Mildew locus O (MLO) – barley gene conferring susceptibility against powdery mildew

Mildew locus O (MLO) is a very well characterised barley susceptibility gene against the powdery mildews (Büschges et al., 1997). MLO deletion resulted in a recessively inherent (mlo), stable and durable mode of broad-spectrum resistance against *B. graminis* in barley, which has been successfully used in agriculture for the last four decades (JOrgensen, 1992). Structurally, barley MLO is a plasma membrane localised, seven–transmembrane domain protein with an extracellular amino terminus and an intracellular carboxy-tail (Devoto et al., 1999). But the role of this protein is not very well understood. Mlo-based resistance, additionally needs the expression of ROR-1 and ROR-2 genes (Freialdenhoven et.al., 1996), where the ROR1 gene is yet to be isolated (Acevedo-Garcia et al., 2013) and ROR-2 encodes for a member of the t-SNARE (Soluble N-ethylmalemide-sensitive factor Attachment protein REceptor) superfamily (Collins et al., 2003). The Mlo resistance is characterised by increased callose containing cell wall appositions (CWA) that block fungal penetration in a

manner resembling non-host resistance (Humphry et. al., 2006). Overaccumulation of defence-associated phenolics such as p-coumaroyl hydroxyagmatine (von Röpenack et. al., 1998) and hydrogen peroxide was also observed at the *Blumeria* infection sites (Huckelhoven et. al., 2000; Piffanelli et al., 2002), accompanied by a faster accumulation of defence-related transcripts in the mlo genotypes (Zierold et. al., 2005).

MLO activity in barley is dependent on cellular calcium levels. Mlo interaction with calmodulin (Ca^{2+} sensor) facilitates susceptibility to *B. graminis*, an interaction that increases during fungal entry and can be attenuated on addition of Ca^{2+} sequester EGTA. MLO based resistance is reduced by exogenous addition of Ca^{2+} and increased by overexpression of Ca^{2+} protein kinase *HvCDK1*. Moreover, expression of the programmed cell death (PCD) inhibitor BAX INHIBITOR 1 (BI1) in mlo lines restored powdery mildew susceptibility, suggesting a direct link between mlo resistance and PCD (Hückelhoven et. al., 2003). This was further supported by the study showing increased resistance to powdery mildew post BI1 silencing, a resistance that could be bypassed by overexpression of MLO (Eichmann et al., 2010). BI1 has also been shown to interact with calmodulin and need Ca^{2+} dependent ATPases to supress PCD (Ihara-Ohori et.al., 2007). Therefore, cumulatively, Mlo based resistance is grounded on expression of ROR1 and ROR2, callose synthesis, Ca^{2+} homeostasis and programmed cell death.

Mlo-based broad-spectrum resistance against powdery mildews has also been successfully established in other agriculturally and horticulturally important crops like grapevine, apple, cucumber and melon (reviewed by Kusch & Panstruga, 2017). However, there are reports of loss of Mlo based resistancce as a response to abiotic stress like drought and heat (Baker et al., 1998; Vallélian-Bindschedler et.al., 1998).

Therefore, MLO was selected as a suitable susceptibility gene for validation of PTO based *in planta* gene silencing methodology in barley-powdery mildew pathosystem.

3.1.7 Pathogenesis related protein 5 (PR5)

Pathogenesis related (PR) proteins are a group of diverse host proteins induced by pathogen attack and defence signalling (Van Loon & Van Kammen, 1970). They are presently grouped into 17 groups and have diverse functions such as α-1, 3-glucanase (PR2), chitinases (PR3), thaumatin like (PR5), peroxidases (PR9), plant defensins (PR12), thionins (PR13), amongst others (reviewed by Ali et al., 2018).

PR5 is classified as a thaumatin-like protein (TLP), due to its structural similarity to a sweet tasting thaumatin protein from the plant *Thaumatococcus daniellii* (Velazhahan et.al., 1999). Members of this super-family are present in plants, fungi, insects and nematodes (Liu et. al., 2010). First discovered as a protein induced in tobacco plants in response to viral attack (Van Loon & Van Kammen, 1970), PR5 activation has been associated with both biotic and abiotic stress response and tolerance (Misra et. al., 2016; Seo et.al., 2008; Singh et. al., 2013; van Loon et. al., 2006; Velazhahan et. al., 1999), indicating at its broad-spectrum role in plant defence (Figure 3-1).

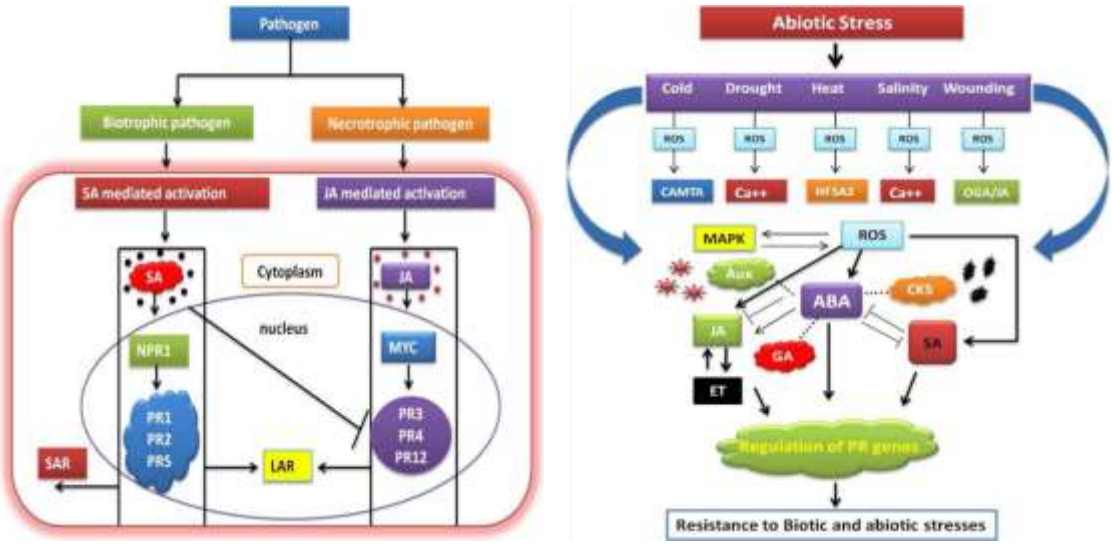


Figure 3-1: Pathways for PR protein activation as a response to biotic and abiotic stress in plants (
Diagram obtained from Ali et al., 2018.

Structurally, PR5 proteins have three domains with a cleft between the domains 1 and 2 predicted as the active site for ligand binding. This cleft is acidic in anti-fungal PR5s whereas basic in other PR5 isoforms facilitating binding of different ligand molecules with other functionality (Figure 3-2) (Batalia et.al., 1996; Koiwa et al., 1999; Liu et al., 2010).

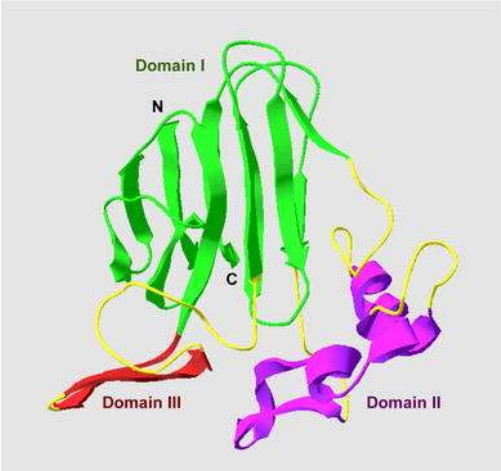


Figure 3-2: PR5 protein structure showing the three domains.
Diagram acquired from Liu et al., 2010.

The antifungal activity of certain PR5 isoforms (like TLP1) was demonstrated against several fungal pathogens and in several plant systems, where overexpression of host TLP1 protein reduced fungal infection (El-kereamy et al., 2011; Hejgaard et.al., 1991; Misra et al., 2016; Rout et.al., 2016; Zhang, et.al., 2017). PR5 isoforms are also induced during other plant fungal diseases like Fusarium head blight (Xiao et al., 2013). Therefore, it is not surprising that PR5 isoforms are targeted by fungal effectors of plant pathogens like *Botrytis cinerea* and *Verticilium dahliae*, to promote fungal virulence (González et. al., 2017; Zhang et al., 2019). PevD1, which is overexpressed during *V. dahliae* infection in multiple plant hosts, co-purified with a cotton PR5 isoform. This interaction was confirmed to be localised to the C-terminal end of PR5 by yeast-two-hybrid studies. Moreover, PevD1b (similar to PevD1) reduced *in vitro* anti-fungal activity of cotton PR5. Interestingly, PevD1 also interacts with the salicylic acid induced transcription factor NPR1 which regulates PR5 induction (Zhang et al., 2019).

During barley powdery mildew infection, PR5 isoforms are induced when barley is challenged with *B. graminis* (Bryngelsson & Gréen, 1989; Scheler et.al., 2016). Moreover, a barley TLP5 isoform of PR5 was shown to interact with the *Blumeria* effector BEC1054 (CSEP0064) , as demonstrated by pull down assay and yeast two hybrid studies (Pennington, Gheorghe, et al., 2016). But no information is available regarding the antifungal role of TLP5 against *B. graminis*.

Therefore, the barley PR5 isoform TLP5 was selected as a target for asPTO mediated silencing, with the goal of understanding its role in *B. graminis* virulence.

3.2 Objectives and experimental rationale

3.2.1 To study the role of B. graminis housekeeping genes in fungal virulence during barley powdery mildew development in different barley cultivars

After the earlier development of the antisense PTO based *in planta* gene silencing in barley-powdery mildew pathosystem in Bindschedler laboratory (Orman et. al., manuscript in prep), this project focussed on further validating this methodology by using fungal housekeeping and host susceptibility genes as targets for control of barley powdery mildew infection. With that focus, the *Blumeria* housekeeping genes *actin* and *GAPDH* and the barley susceptibility gene *MLO1* were silenced and their effect on powdery mildew development was studied in barley c.v. Golden Promise.

With the objective of developing a functional genomics and powdery mildew disease control tool in different barley cultivars, the *Blumeria* housekeeping gene *actin* and barley susceptibility gene *MLO1* were silenced and their effect on powdery mildew development was studied in barley c.v. Morex.

Bgh actin silencing was also performed in barley c.v. Maris Otter and its effect on powdery mildew development was studied.

With the aim of discovering novel players in barley powdery mildew infection development, a homologue of 2-glycosyl transferase (GT2), a known virulence factor in *Z. tritici* and *F. graminearum* (King et al., 2017) and a pathogenesis related protein 5 (PR5) isoform shown to be interacting with *Blumeria* effector BEC1054 (Pennington, Gheorghe, et al., 2016) were also silenced to investigate their effect on *Blumeria* virulence.

3.3 Results

3.3.1 Effect of silencing *Bgh* actin on *Blumeria* infection efficiency in various barley cultivars (c.v. Golden Promise, Morex, Maris Otter)

To investigate the role of fungal housekeeping genes in *B. graminis* virulence, the *B. graminis* housekeeping gene *actin* was silenced and its role in powdery mildew infection development was studied. The silencing was performed in multiple barley cultivars (c.v. Golden Promise, Morex, Maris Otter) to test the suitability of this assay as a functional genomics tool for barley powdery mildew infection in multiple barley cultivars.

Bgh Actin (Ensembl: BLGH_02125) silencing was attempted using two different PTOs in barley c.v. Golden Promise. Treatments with PTO ACT1 and PTO ACT2 led to reduced infection development (%Secondary hyphae/germinated conidia) by ~32% and 30% approximately as compared to control PTOZ, which is a PTO targeting a seed protein Z (Figure 3-3A). As PTO ACT1 had a stronger effect, quantitative RT-PCR was carried out using this treatment to determine the change in actin transcript and fungal biomass levels. The transcript levels were determined by normalising *ACT* expression with *Bgh GAPDH* expression levels. Relative fungal biomass was determined by normalising *Bgh GAPDH* expression levels with that of barley *GAPDH* levels. PTO ACT1 treatments reduced *Bgh actin* transcript levels by 34% and fungal biomass by 48% (Figure 3-3B). Based on these results, *Bgh actin* is important for *in planta* growth and barley PM disease development.

Barley c.v. Golden Promise

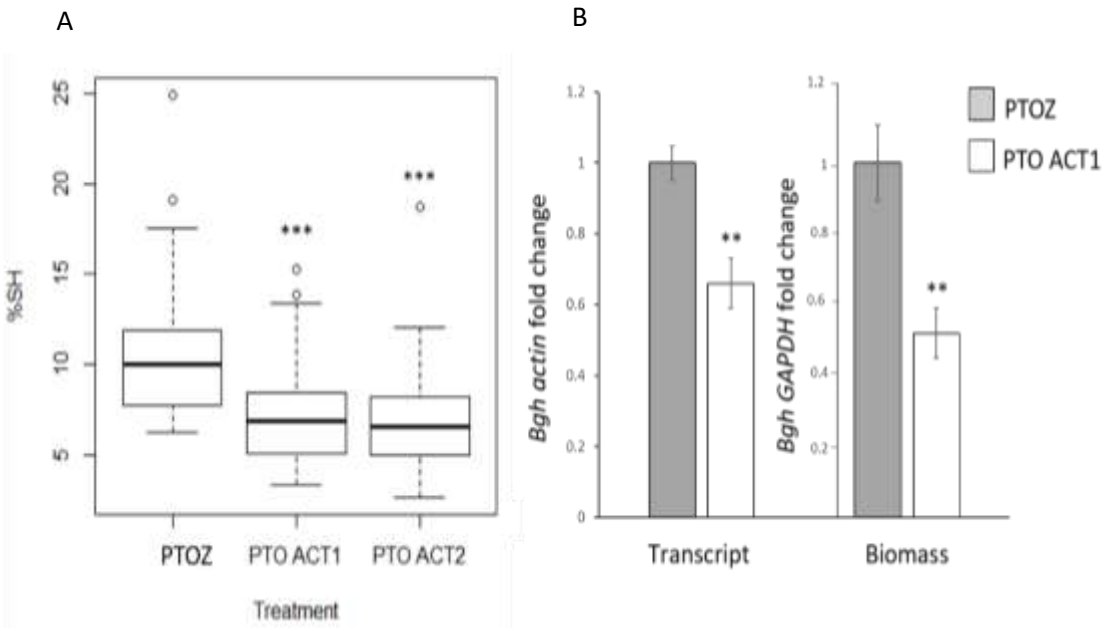


Figure 3-3: Bgh Actin silencing in barley c.v. Golden Promise.

*A. Disease scoring and B. Bgh ACT transcript and fungal biomass levels post Bgh ACT silencing in barley c.v. Golden Promise. PTO targeting seed protein Z was used as control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Disease was scored as % secondary hyphae formed per germinated conidia (%SH). ACT transcript was normalised against Bgh GAPDH expression and fungal biomass was determined by calculating Bgh GAPDH relative to HvGAPDH. Data was derived from 4 biological repeats for infection assay and 5 biological repeats for qPCRs, where each biological repeat consists of 6 leaves. Significance (GLM for infection data /1 sample t-test for qPCR) * P<0.05, ** P<0.01, *** P<0.001.*

Furthermore, silencing of *Bgh actin* in barley cv. Morex and c.v. Maris Otter reduced barley powdery mildew infection by ~59% and ~33% respectively (Figure 3-4). The data for Morex was derived from 3 biological repeats and for Maris Otter from 1 biological repeat, each consisting of 6 leaves per treatment. This confirmed that PTO based *in planta* gene silencing can be used gene function studies during barley mildew infection in different barley cultivars.

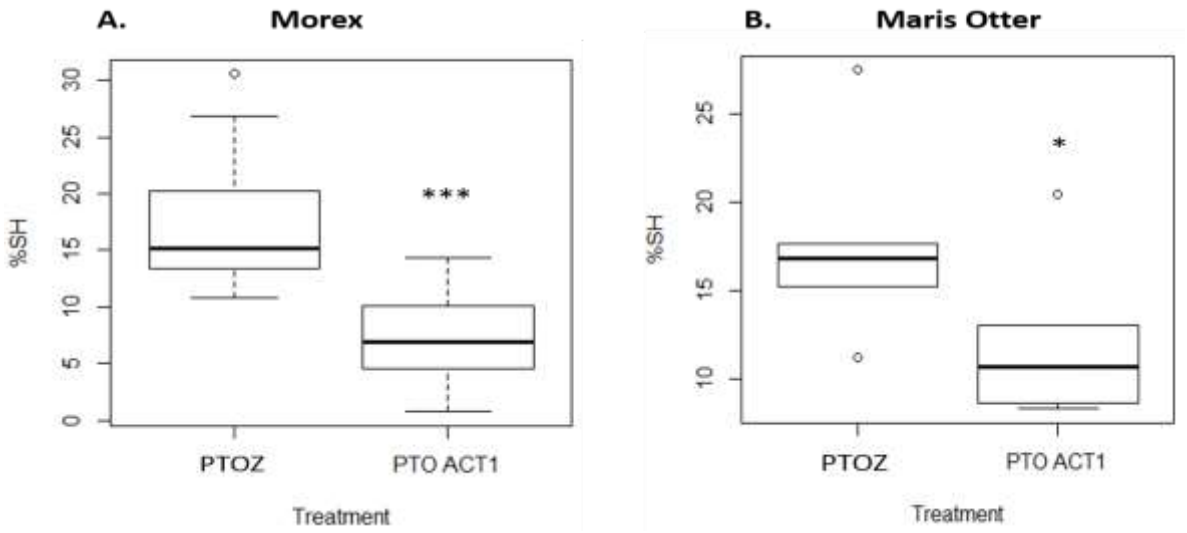


Figure 3-4: Bgh Actin silencing in barley c.v. Morex and Maris Otter.

*Infection data post PTO ACT1 mediated silencing of Bgh actin in barley A. c.v. Morex and b. c.v. Maris Otter PTO targeting seed protein Z was used as control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Infection was measured as % secondary hyphae (%SH) formed per germinated conidia. Data was derived from 3 independent biological repeats for Morex and one biological repeat for Maris Otter, where each repeat consisted of 5-6 leaves. Significance (GLM) * P<0.05, ** P<0.01, *** P<0.001.*

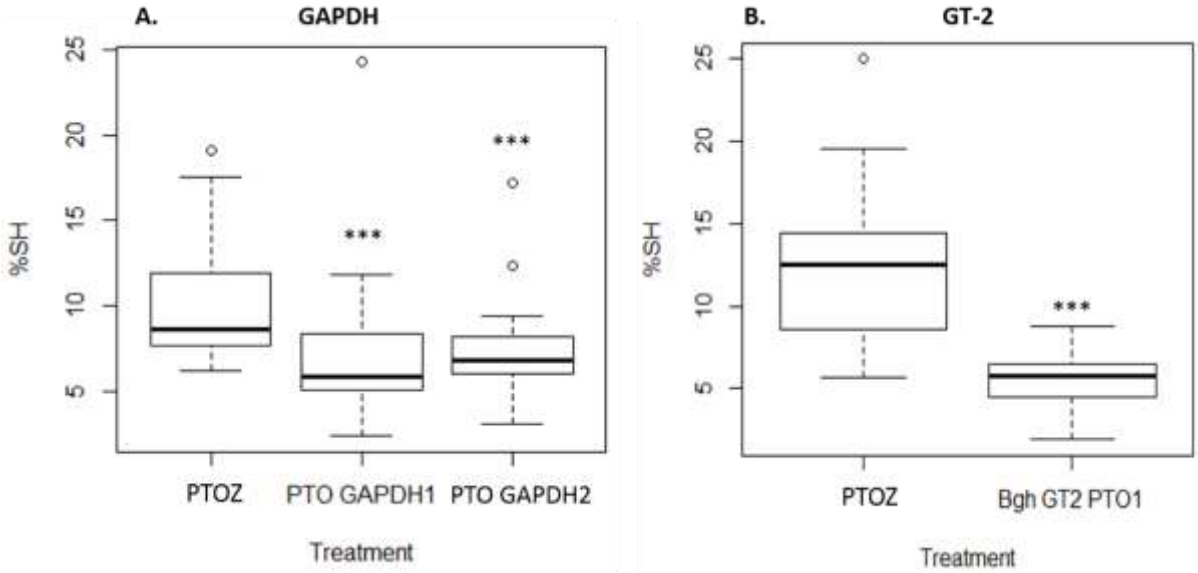
3.3.2 Effect of *Bgh GAPDH* silencing on *Blumeria* infection in barley

Since the fungal housekeeping gene *glyceraldehyde-3-dehydrogenase (GAPDH)* was shown to be important for virulence of other fungal pathogens (Broetto et al., 2010), *Bgh GAPDH* was chosen as a suitable target to query the role of housekeeping genes in *B. graminis* virulence. For this, *Bgh GAPDH* was silenced and its effect on powdery mildew disease development was studied.

Bgh GAPDH (Genbank Gene Id: CCU80715.1) was targeted using two gene specific PTOs – PTO GAPDH1 and PTO GAPDH2 – resulting in a corresponding reduction in barley powdery mildew infection by 27% and 26% as compared to a control PTO targeting a seed protein Z (Figure 3-5A). Infection was calculated as the % secondary hyphae formed per germinated conidia (%SH). This data needs to be correlated to change in *Bgh GAPDH* transcript levels to confirm that the infection reduction is due to gene silencing.

However, this data indicates that *B. graminis* housekeeping genes like *GAPDH* contribute to fungal virulence and can be used as targets for controlling barley powdery mildew infection development.

Figure 3-5: Bgh Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) GAPDH and 2-Glycosyltransferase (GT2) silencing in barley c.v. Golden Promise.



*Barley powdery mildew infection development measured post silencing of A. Bgh Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) B. Bgh 2-Glycosyl transferase (GT2). PTO targeting seed protein Z was used as control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Infection was measured by % secondary hyphae (%SH) formed per germinated conidia. Data was derived from 3 independent biological repeats, where each repeat consisted of 5-6 leaves. Significance (GLM) * P<0.05, ** P<0.01, *** P<0.001.*

3.3.3 Effect of *Bgh GT2* silencing on *Blumeria* virulence

With the aim of discoring novel players in *Blumeria* virulence, a homologues of 2-glycosyl transferase (GT2), a known virulence factor in *Z. tritici* and *F.graminearum* (King et al., 2017), was analysed for its role in *B. graminis* virulence.

This was achieved by targeting *Bgh GT2* using gene specific PTOs and studying its effect on barley powdery mildew infection development. Infection was scored as % secondary hyphae formed per germinated conidia (%SH). PTOZ, targeting a seed protein Z, was used as a control.

AsPTO based targeting of *Bgh GT2* resulted in 55% reduction in secondary hyphae formation, suggesting that GT2 may have a role in *Blumeria* virulence (Figure 3-5B), indicating at a probable role of *Bgh GT2* in fungal virulence during powdery mildew infection development. This data needs to be correlated to a reduced *Bgh GT2* transcript levels to confirm gene silencing.

3.3.4 Effect of silencing barley susceptibility factor *MLO1* on *Blumeria* infection in barley (c.v. Golden Promise and Morex)

With the aim of validating the asPTO based *in planta* gene silencing in barley-powdery mildew pathosystem, barley susceptibility factor MLO1 was targeted by Kate Orman in the barley cultivar Golden Promise and its effect was studied on *B. graminis* virulence.

Barley powdery mildew disease was scored by measuring the % secondary hyphae formed per germinated conidia (%SH). A PTO targeting the seed protein Z was used as a silencing control. Silencing was confirmed by studying *Hv MLO1* transcript levels relative to *HvGAPDH* (reference gene). Change in fungal biomass was determined as relative expression of fungal housekeeping gene *Bgh GAPDH* compared to barley housekeeping gene *HvGAPDH*.

Barley MLO1 targeting by gene specific PTOs reduced barley powdery mildew infection by 41% (Lambertucci et.al., 2019; Figure 3-6A). This project showed that the reduction in infection was accompanied by 47% reduction in MLO1 transcript levels and 54% reduction in fungal biomass (Figure 3-6B) (Lambertucci et. al., 2019), reconfirming the role of MLO1 as a susceptibility factor against powdery mildew. These results validate the suitability of asPTO based *in planta* gene silencing as a functional genomics tool for studying barley powdery infection in barley cultivar Golden Promise.

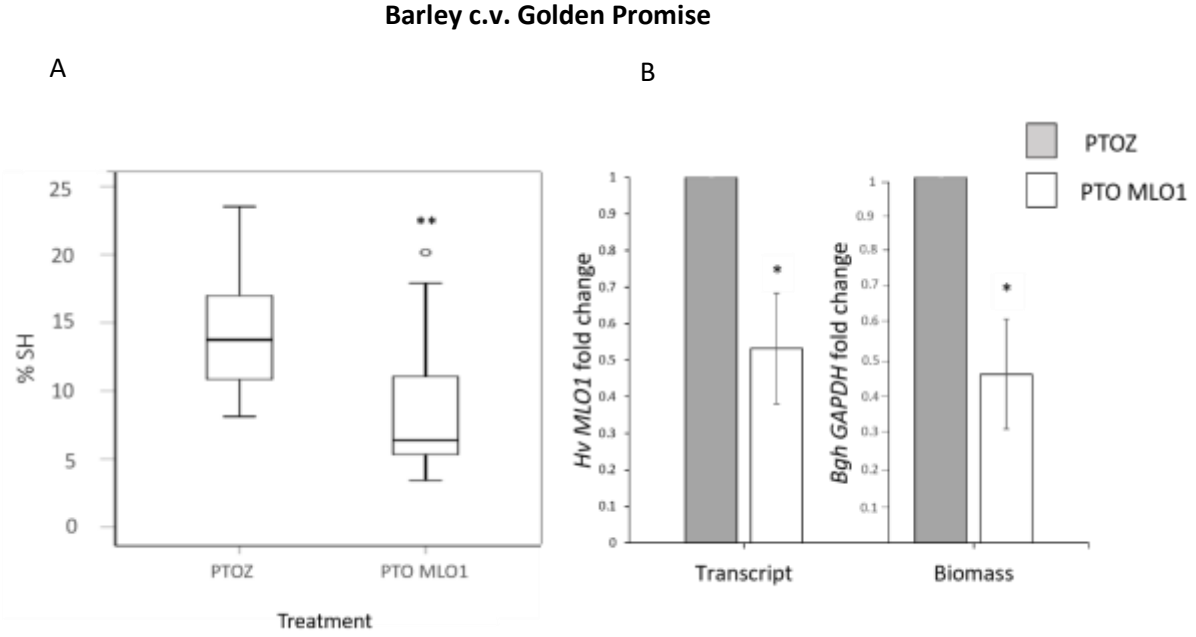


Figure 3-6: Hv MLO1 silencing in barley c.v. Golden Promise

*The effect of PTO mediated silencing of HvMLO1 in barley c.v. Golden Promise on A. barley powdery mildew infection development (%SH), B. MLO1 transcript levels and fungal biomass. PTO targeting seed protein Z was used as control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Infection was measured by % secondary hyphae (%SH) formed per germinated conidia. MLO transcript levels was normalised against barley GAPDH. Fungal biomass was measured as relative Bgh GAPDH expression compared to HvGAPDH. Data derived from 3 biological replicates for infection and 3 biological replicates for qRT-PCRs, where each independent replicate comprised of 6 leaves. Significance (GLM for infection data/1 sample t-test for qRT-PCR) * P<0.05, ** P<0.01, ***P<0.0001.*

To further analyse the suitability of this methodology as a functional genomics tool in other barley cultivars, barley *MLO1* homologue in the sequenced cultivar Morex was targeted using gene specific PTOs. *MLO1* targeting resulted in 63% reduction in barley powdery mildew secondary hyphae formation (Figure 3-7), confirming the suitability of asPTO based *in planta* gene silencing as a functional genomics tool for studying genes important for *Blumeria* virulence in different barley cultivars.

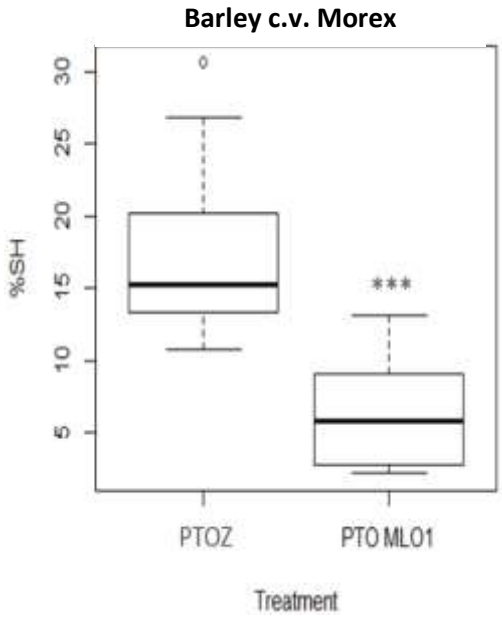


Figure 3-7: Hv MLO1 silencing in barley c.v. Morex.

Infection data for PTO mediated silencing of HvMLO1 in barley c.v. Morex. PTO targeting seed protein Z was used as control. PTO targeting seed protein Z was used as control. *Silencing was carried out on 7 days old barley (c.v. Morex) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Infection was measured by % secondary hyphae (%SH) formed per germinated conidia. Data derived from 3 biological repeats each comprising of 5 leaves. Significance (GLM) * P<0.05, ** P<0.01, *-** P<0.001.*

3.3.5 Role of the induced thaumatin like protein 5 *TLP5* (*PR5* isoform) on modulating resistance against barley powdery mildew infection

An isoform of pathogenesis related protein 5 (PR5), thaumatin-like protein 5 (*TLP5*) (Genbank: KP293850; Ensembl id: HORVU5HrG005180.6), was found to directly interact with *B. graminis* effector BEC1054 (Pennington, Gheorghe, et al., 2016). Therefore, it was hypothesised that TLP5 may be important for *B. graminis* virulence. To understand the role of barley *TLP5* in *Blumeria* virulence, the expression of *TLP5* was studied during barley powdery mildew infection development. The *TLP5* expression was studied at 0, 1, 2, 3, 4, 5, 6 and 7-days post *B. graminis* inoculation (dpi) on 7 days old barley leaves. Healthy barley leaves were used as a negative control. *TLP5* expression was

studied relative to barley *GAPDH* expression, used as a reference gene. The expression of *Bgh GAPDH* relative to barley *GAPDH* was also studied to confirm fungal biomass at different stages of *B. graminis* infection.

As expected, a steady increase in *Bgh GAPDH* expression was observed as infection progressed from 0 to 7 dpi in the infected leaves and was absent in the uninfected control (Figure 3-8B). Barley *TLP5* on the other hand was induced from 3 dpi, with an increase in expression as infection progressed from 4-7dpi as compared to uninfected control (Figure 3-8A). These results indicate that barley *TLP5* (PR5 isoform) is induced during powdery infection in compatible interactions, indicating at a possible role of TLP5 during disease development.

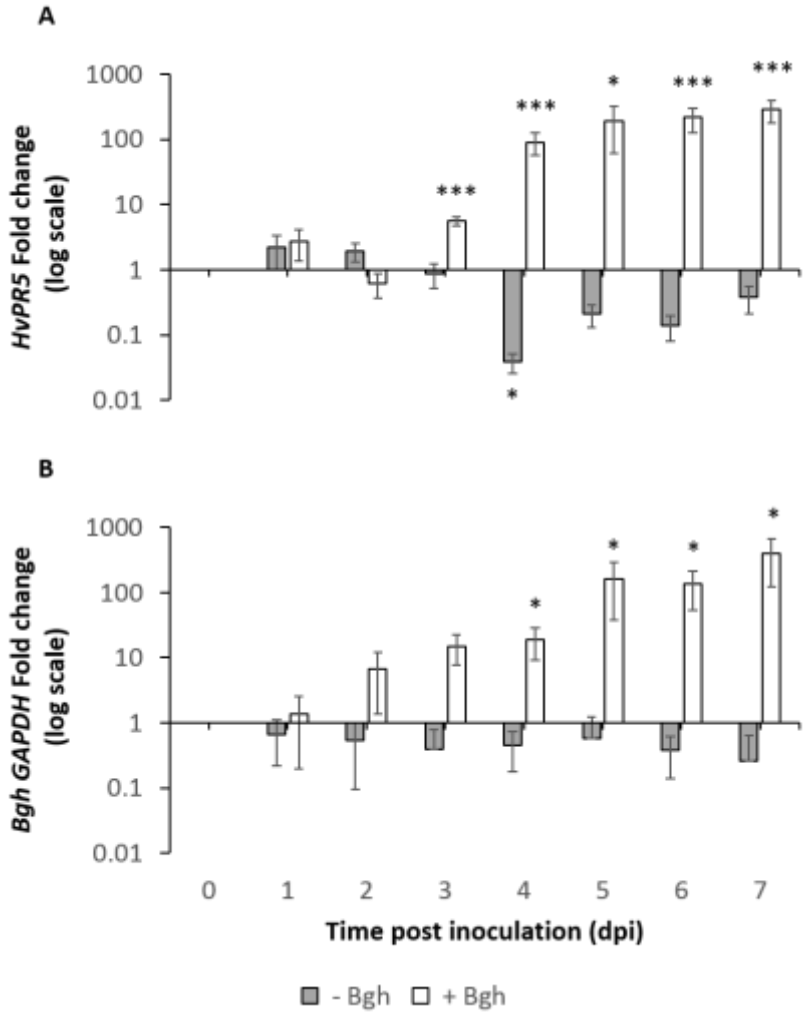


Figure 3-8: Hv PR5 and Bgh GAPDH expression time course during barley powdery mildew infection.

*Time course expression level of the A. PR5 isoform Thaumatin-like protein 5 TLP5 (KP293850.1/ HORVU5HrG005180.6) and B. Bgh GAPDH in uninfected control (grey) and Bgh infected (white) barley leaves. qRT-PCR was used to determine relative transcript levels using Hv GAPDH as the reference gene. Data were normalised to the 0dpi time point. Data derived from 3 independent biological replicates, where for each biological replicate 3 leaves were collected for each timepoint. Significance (One-sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

To query the role of barley TLP5 in *B. graminis* virulence, TLP5 was silenced using gene specific asPTOS (PTO PR5.1) and its effect on barley powdery infection development was monitored. PTOZ targeting a seed protein Z was used as a control treatment. Disease was scored as % secondary hyphae formed per germinated conidia (%SH). *TLP5* transcript levels were determined relative to barley *GAPDH* expression using qRT-PCR.

PTO PR5.1 treatment resulted in a 46% reduction in PR5/TLP5 transcripts (Figure 3-9B), confirming *TLP5* silencing. This was accompanied by a 54% reduction in barley powdery mildew infection (Figure 3-9A). These results suggest that barley PR5 isoform TLP5 is important for powdery mildew virulence, where it may act as a host susceptibility gene favouring infection development.

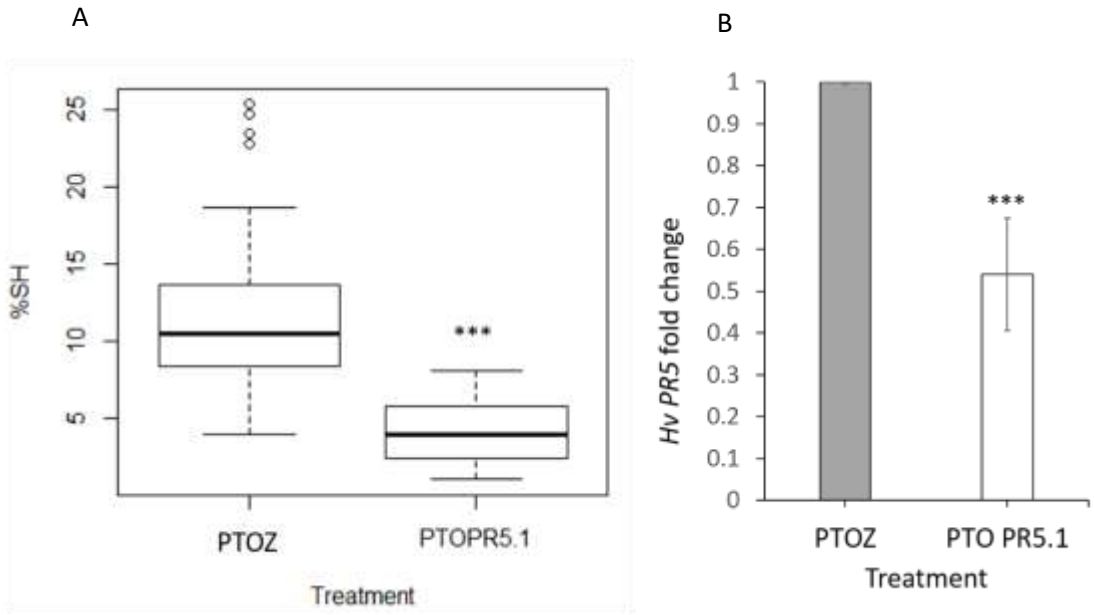


Figure 3-9: Hv PR5 gene silencing in barley c.v. Golden Promise.

*A. Powdery mildew infection development, B. Hv PR5 (TLP5) transcript levels post PTO mediated silencing of Hv PR5 (TLP5). PTO targeting seed protein Z was used as control. Infection was measured as % secondary hyphae (%SH) formed per germinated conidia. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Hv GAPDH was used as a reference gene for the qRT-PCR. Infection data and qRT-PCR data was derived from 4 independent biological replicates, where each repeat comprised of 5-6 leaves. Significance (GLM for infection data/1-sample t-test for qRT-PCR) * P<0.05, ** P<0.01, *** P<0.001.*

3.4 Discussion

3.4.1 Silencing of fungal housekeeping genes as a strategy for controlling barley powdery mildew infection

Initially, this asPTO based *in planta* gene silencing was developed as a host induced gene silencing (HIGS) tool to target *B. graminis* effectors as a strategy for disease control. But most of the fungicides target fungal housekeeping genes. For example, C14-demethylase is targeted by azole fungicides and succinate dehydrogenase is targeted by SDHI group of fungicides (FRAC.). Therefore, the strategy of silencing fungal housekeeping genes for disease control was explored.

The in planta silencing of both *Bgh actin* and *GAPDH* in barley c.v. Golden Promise was initially attempted with two different gene specific PTO modified ASOs. In both the cases, silencing efficiency was higher in PTO1 as compared to PTO2. For instance, infection reduced by 32% reduction after PTO ACT1 treatments as compared to 30% post PTO ACT2 treatment (Figure 3-3A). Similarly, during GAPDH silencing, 27% reduction of infection was observed after PTO GAPDH1 treatment as compared to 26% by PTO GAPDH2. (Figure 3-5A). These findings correlated with a higher silencing probability as predicted by the OligoWalk design software, where the PTO1 always had a higher probability than PTO2 for both the genes (Table 3-1). Both the PTOs for each of the genes target exons. However, the silencing efficiency is dependent on the RNA fold of the targeted region and the thermodynamic parameters of the DNA:RNA duplex formation (Lu & Mathews, 2008).

Table 3-1: P-values for silencing efficiency of gene specific PTOs targeting Bgh actin and GAPDH as generated by OligoWalk siRNA designing software.

Gene specific PTO	Probability of silencing effect (P-value)
PTO ACT1	0.944848
PTO ACT2	0.937736
PTO GAPDH1	0.955807
PTO GAPDH2	0.952026

As an obligate biotroph, in *B. graminis,* fungal growth and virulence are mutually inclusive (Both et al., 2005). So, it is not surprising that silencing of both *actin* and *GAPDH* led to reduced fungal infection. This confirmed the suitability of *Blumeria* housekeeping genes as targets for disease control. However, the infection reduction (26-33%) was lower than that achieved post silencing an effector (*Bgh BEC1011* – 53%, *BEC1019* – 40%) (Orman, Das Gupta et. al. manuscript in prep). Moreover, silencing of neither *actin* nor *GAPDH* completely stopped disease development. This

could be because silencing only knocks down the gene rather than completely knocking it out, which can result in a complete loss of expression. Therefore, if silencing of housekeeping genes is used as a method of disease control, a combination of genes will need to be silenced in order to completely inhibit disease development. Long term effect of gene silencing on *B. graminis* virulence will also need to be evaluated.

Gene silencing was confirmed by studying the transcript levels of the target gene. The transcript abundance was measured relative to the *Bgh GAPDH* housekeeping gene to confirm that the change was due to specific reduction in the target mRNA and not an artefact of the reduction in biomass caused by the PTO treatments. The reduction of transcript abundance post actin silencing, though significant, was quite nominal (~34%) (Figure 3-3B). This was however, greater than the reduction in transcript abundance observed post *Blumeria* effector silencing (~20%) (Orman, Das Gupta et al. manucript in prep). Such modest changes in mRNA levels may be due to the mediation of antisense oligodeoxynucleotide (ASO) based silencing by both post-transcriptional gene silencing (targeting mRNA) and translational arrest (affecting protein levels) (Chan et al., 2006). The post-transcriptional gene silencing happens when PTOs bind to complementary mRNA and trigger its RNase H mediated degradation. Similarly, PTOs also bind to ribosomes, causing stearic hinderance against mRNA binding resulting in translational arrest (Chan et al., 2006). Therefore, like animal models, both mRNA and protein levels may need to be studied to fully quantify the silencing effect mediated by asPTOs.

3.4.2 Antisense PTO based *in planta* gene silencing as a functional genomics tool for studying genes involved in barley powdery mildew development in various barley cultivars

With the aim of establishing a functional genomics tool for barley-powdery mildew pathosystem that is applicable to various barley cultivars, *Blumeria* housekeeping gene actin and barley susceptibility gene MLO1 were silenced during powdery mildew infection in barley cv. Golden Promise, Morex and Maris Otter.

Actin is a component of fungal cytoskeleton and is important for fungal growth and virulence (Dagdas et al., 2012; González-Rodríguez et.al., 2016). For instance, microscopic studies during *M. oryzae* infection of rice have confirmed the role of an f-actin network in concentration of appressorial turgor pressure into mechanical pressure of the penetration peg important for host penetration (Dagdas et al., 2012). Moreover, in *B. cinearea*, absence of f-actin ring at the hyphal tip resulted in mutants defective in polarised growth and virulence (González-Rodríguez et al., 2016).

MLO, on the other hand, is a well-established barley susceptibility gene against powdery mildews (Büschges et al., 1997). A recessive MLO allele has been used for barley powdery mildew resistance in the agriculture for the last four decades (reviewed by Kusch & Panstruga, 2017). Moreover, previous results from Bindschedler group showed a reduction in *B. graminis* virulence on silencing *Bgh actin* and barley *MLO1* during infection development in barley c.v. Golden Promise (Figure 3-3;

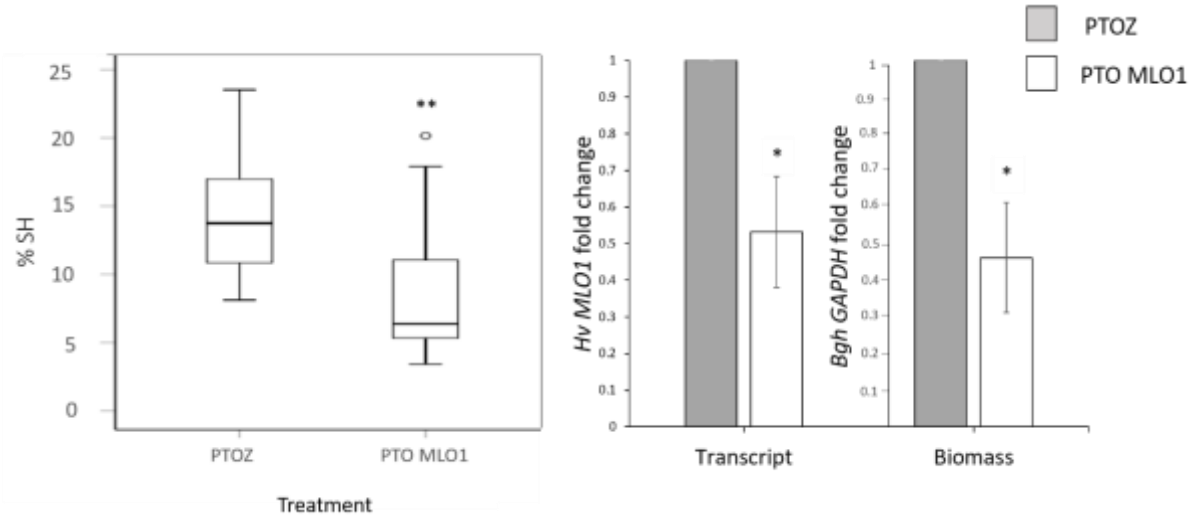


Figure 3-6). Therefore, *actin* was chosen as the fungal gene and *MLO1* as the plant susceptibility gene to be targeted for establishing asPTO based *in planta* gene silencing as a functional genomics tool for studying genes involved in powdery mildew infection development in different barley cultivars.

Actin silencing and its impact on *Blumeria* virulence was compared during infection development in three barley cultivars, Golden Promise, Morex and Maris Otter whereas MLO1 silencing was compared between Golden Promise and Morex. The secondary hyphae formation was most reduced during silencing in Morex as compared to the other two cultivars (Table 3-2). This also corresponded with a higher secondary hyphae formation during the control treatment (PTOZ) in Morex as compared to Golden Promise. The data for Maris Otter is not being taken into consideration for this comparison, as it is acquired from a single biological replicate (consisting of 6 leaves) as compared to 4 and 3 biological replicates for Golden Promise and Morex respectively. A similar trend was also seen during MLO1 silencing, where a higher %SH formation in the control treatment corresponded with a greater reduction in %SH post *MLO1* silencing in Morex as compared to Golden Promise (Table 3-2). Based on these observations, it may be concluded that a higher infection success in control conditions facilitates a more significant difference in infection development post gene silencing. In case of *MLO1*, a stronger silencing effect in Morex may also stem from the fact that the barley genome using which the gene specific PTOs were designed is based on Morex cultivar rather than Golden Promise.

Table 3-2: Percentage secondary hyphae development data for silencing Bgh actin and barley MLO1 during B. graminis infection in different barley cultivars.

Barley cultivar	%SH PTOZ (Control)	%SH PTO ACT1	% Reduction compared to control
Golden Promise (n=21)	11%	8%	30%

Morex (n = 15)	17%	7%	59%
Maris Otter (n = 6)	18%	12%	33%
Barley cultivar	**%SH PTOZ (Control)**	**%SH PTO MLO1**	**% Reduction compared to control**
Golden Promise (n=19)	14%	8%	41%
Morex (n=15)	17%	6%	63%

Overall, based on this study, we may now conclude that PTO modified ASO mediated *in planta* gene silencing is a suitable tool for genetic studies in the barley-powdery pathosystem in various susceptible barley cultivars. So far other silencing methods developed in the barley-powdery mildew pathosystem, used hpRNA to target fungal effector genes using either virus induced (Aguilar et.al., 2015; Ahmed et al., 2015) or particle bombardment (Pliego et al., 2013) induced gene silencing and were based on barley c.v. Golden Promise (Aguilar et.al., 2015; Ahmed et al., 2015; Pliego et al., 2013). For instance, the first HIGS experiments in *B. graminis* involved the silencing of silencing the effector gene *Avra10*, via BSMV viral vectors expressing gene specific hpRNA which was introduced into barley c.v. Golden Promise cells by particle bombardment (Nowara et al., 2010). Similarly, Pliego et. al. in 2013 delivered hpRNA targeting potential effectors like *Bgh BEC1011* into individual cells of barley cultivar Golden Promise via biolistic delivery and studied the effect of effector silencing on *B. graminis* haustoria count in individual barley cells (Pliego et al., 2013). In contrast, this method involves direct uptake of silencing molecules by barley leaves, not requiring any cloning, viral vector or particle bombardment (Pliego et al., 2013). Moreover, same PTO modified ASOs can be used to target genes in multiple barley cultivars and impact of silencing can be studied on barley powdery mildew development in whole leaves as compared to individual transformed cells (Pliego et al., 2013). Also, so far only potential effectors and virulence factors of *B. graminis* f.sp. *hordei* have been targeted in the previous silencing experiments (Aguilar et.al., 2015; Ahmed et al., 2015; Pliego et al., 2013).

Therefore, PTO modified ASO based silencing is the first gene silencing methodology in this pathosystem that can target both fungal (effector/housekeeping) and plant genes, involves direct uptake of the silencing molecule and is suitable for functional genomics studies in different barley cultivars.

3.4.3 *Blumeria 2-glycosyl transferase (GT2),* a novel virulence factor for barley powdery mildew infection development

This antisense PTO based gene silencing method was developed with the aim of discovering new *Blumeria* virulence factors. With that goal, the *B. graminis* homologue of 2-glycosyl transferase (GT2) enzyme was targeted. GT2 is responsible for transfer of sugar moieties onto non-carbohydrate molecules like protein and lipids, forming glycoproteins and glycolipids (Lairson et al., 2008). *GT2* deletion inhibited hyphae formation and infection development in the wheat pathogens *Fusarium graminearum* and *Zymoseptoria tritici* (King et al., 2017). This is not surprising as glycoproteins and glycolipids are integral part of fungal cell wall (Gow et.al., 2017) and hyphae formation is fundamental to host penetration and colonisation (Mendgen et.al., 1996). *Zt ΔGT2* mutants also possessed cell wall abnormalities and constitutively overexpressed LysM effectors and alpha-1,3-glucan synthase on contact with the leaf surface. Alpha-1,3-glucan synthase expression maybe as a response to cell wall damage, but LysM effector expression was clearly mistimed. So GT2 may be involved in triggering fungal gene transcription as a response to leaf sensing by *Z. tritici* (King et al., 2017). Therefore, GT2 is another structural gene that influences fungal virulence.

Bgh GT2 targeting reduced secondary hyphae formation, indicating at having a role in *Blumeria* virulence. This may be due to its impact on fungal cell wall and hyphae development, but this is yet to be confirmed. When compared to complete loss of hyphae formation post gene deletion (King et al., 2017), GT2 silencing only reduced %SH by ~50% (Figure 3-5B). Therefore, gene knock down by silencing may not give the full picture of the role of *Bgh* GT2 in fungal germination and hyphae formation.

3.4.4 Barley *TLP5*, a novel susceptibility factor against barley powdery mildew

Historically, pathogenesis-related protein 5 (PR5) has been shown to have antifungal effects against many fungal pathogens (El-kereamy et al., 2011; Hejgaard et al., 1991; Misra et al., 2016; Rout et al., 2016). Therefore, when Thaumatin-like protein 5 (TLP5), a PR5 isoform, was shown to interact with *B. graminis* f.sp. *hordei* effector BEC1054 (CSEP0064), it was postulated that BEC1054 may be targeting TLP5 to supress its antifungal activity. Other fungal effectors like PevD1 (*V. dahliae)* and elicitors like BClEB1 (*B. cinerea)* also target PR5 isoforms to promote fungal virulence by supressing their antifungal activity (González et al., 2017; Zhang et al., 2019). If this hypothesis was correct, *TLP5* silencing should have increased fungal virulence. However, *TLP5* silencing resulted in a significant reduction in *Blumeria* secondary hyphae formation post (Section 3.3.5; Figure 3-9) (Lambertucci et.al., 2019), suggesting that *TLP5* acts as a susceptibility factor for barley powdery mildew infection.

These results are further supported by the convergence of *TLP5* expression with increased fungal biomass during powdery mildew infection (Figure 3-8). TLP5 protein was also highly abundant in powdery mildew infected barley epidermis samples as compared to uninfected barley epidermis in a recent proteomics study undertaken in Bindschedler laboratory (Lambertucciet.al., 2019). Together, these findings suggest that TLP5 is important for *Blumeria* virulence. However, during powdery mildew infection, the *TLP5* induction happened 3 dpi onwards and not in the initial stages as seen in previous studies of *PR5* induction in *Blumeria* challenged barley (Scheler et al., 2016). In the same study carried out by Scheler et. al., PR5 induction was even faster in the resistance phenotype between 12-32 hpi. This maybe because the Scheler study looked at the expression of other PR5 isoforms (GenBank Accession no. AK371265, AJ001268) (Scheler et al., 2016) as compared to TLP5 (GenBank accession: KP293850) and TLP5 may have a different role in *B. graminis* virulence as compared to these two isoforms. However, TLP5 is unlikely to act alone. Barley has many more PR5 isoforms, are there is a possibility that some of these isoforms may have a similar role as TLP5. So, one cannot exclude that silencing of one isoform might trigger the induction of expression for other isoforms.

To conclude, based on the present study, the PR5 isoform TLP5 is a novel susceptibility factor for barley powdery mildew infection. But the mechanism of TLP5 based susceptibility is yet to be understood. TLP5 protein pull-down assays to discover potential interactors and *in planta* localisation during powdery mildew infection may shed some light on the mechanism and infection stage specific to PR5 activity during barley powdery infection. This will in turn, will enrich our knowledge of the complexity of the plant-pathogen interactions that lead to the induction of this heterogenous group of PR5s.

Chapter 4 Gene silencing using ASO with alternative PTO modifications and adapted delivery methods for gene silencing in whole plants

4.1 Introduction

4.1.1 Existing gene silencing methods: alternative *in planta* delivery of a range of silencing molecules

Host induced gene silencing (HIGS) of fungal genes has been successfully achieved by RNAi, mediated by both short dsRNA and hairpin-RNA (hp-RNA) in different fungal pathogens, where the host plant expressed the fungal gene specific hpRNA or dsRNA resulting in gene silencing (Gu et al., 2019; Koch et al., 2016; Nowara et al., 2010; Wang et al., 2016). Initially, this was achieved by virus mediated transformation of host plant to express gene specific hpRNAs targeting fungal genes (Nowara et al., 2010; Panwar et.al., 2013). HIGs was also achieved by biolistic delivery of hpRNA in some fungal pathogens (Pliego et al., 2013). But these delivery methods have the potential risks associated with triggering of host immune system by the viral pathogens (Tufan et.al., 2011) as well as the complications involved with genetic modification, which make them unsuitable for agricultural use. Therefore, recent studies have explored the direct delivery of small dsRNA as a method for control of phytopathogens like *Fusarium sp.* and *Botrytis cinerea* (Gu et al., 2019; Koch et al., 2016). This was achieved by spraying the gene specific small dsRNA onto the host plants, a method now coined as spray-induced gene silencing (SIGS) (Machado et.al., 2017). SIGS has a potential to be used in agriculture for fungal disease control. However, the secondary amplification of the dsRNA by the fungal siRNA machinery limits its application, as demonstrated by a maximum of 9 hours of silencing resulting from a single dose of dsRNA (Song et al., 2018). Therefore, improved methods for *in planta* fungal gene silencing by direct delivery of silencing molecules need to be explored.

4.1.2 Ectopic application of short phosphorothioate modified antisense oligodeoxynucleotides (PTO modified ASOs) for gene silencing for functional genomics studies during barley- powdery mildew interaction

Short antisense oligodeoxinucleotides (ASOs) have been widely used in animal systems and in clinical studies (reviewed by Martínez et.al., 2013; Shen and Corey 2018). The application of ASO based gene silencing in disease control was first demonstrated by Zamecnik and Stephenson in 1978, where they showed that the introduction of 13mer ASOs reduced infection Roux Sarcoma Virus infection in infected cell lines (Zamecnik & Stephenson, 1978). Clinical trials of ASO based drugs started in the 1990s, where ASO based drugs targeted the p53 transcripts in patients with either relapsed or

refractory acute myelogenous leukaemia or myelodysplastic syndrome (Bayever et.al., 1993). Fomivirsen was the first ASO based FDA approved drug used to cure CMV retinitis in HIV patients (Roeher, 1998). PTO modified ASOs based exon skipping is being used for cure of genetic diseases like Duchene muscular dystrophy (Cirak et al., 2011). Moreover, ASO mediated gene silencing based therapy is under clinical trials for cure of cancer (Moreno & Pêgo, 2014) and neurological disorders (Rinaldi and Wood, 2018).

In plants, the first direct uptake of ASOs was described in barley aleurone cells growing in cell culture (Tsutsumi et. al., 1992). Later, ASO was also used for silencing of plants genes by direct uptake into excised leaves in barley (C. Sun & Ho, 2005), excised leaves in wheat (Dinc et al., 2011) and more recently, direct uptake into *Arabidopsis* pollen tubes (Mizuta & Higashiyama, 2014). ASO was also delivered into by infiltration into tobacco leaves and *Arabidopsis* leaves (E. Dinc et al., 2011).

ASOs have limitations like vulnerability to degradation by intracellular nucleases, poor uptake through cell membranes, unfavourable biodistribution and pharmacokinetic properties and sub-optimal binding affinity for complementary sequence. Therefore, chemical modifications were introduced to the ASOs to mitigate these limitations (reviewed by Shen & Corey, 2018) In this silencing method the ASOs were modified with phosphorothioate (PTO) to the deoxynucleotide backbone, which increased their silencing potency by making them resistant to degradation by host nucleases as well as for facilitating their uptake and therefore increasing their bioavailability (reviewed by Khvorova & Watts, 2017). Alternative modifications have been explored in the animal systems resulting in successful gene silencing. Some studies use end-capped PTOs, which include introduction of PTO modification in the last two phosphosdiester bonds in both ends of the ASOs. Use of end-capped PTO modified ASOs resulted in increased silencing efficiency compared to fully modified ASO and also reduced the cytotoxic side effects of the fully PTO modified ASO (Hebb & Robertson, 1997). In this study, suppression of *c-fos* and *ngfi-a* gene was achieved *in vivo* in the striatum of adult rats. However, reduction in sulphur molecules due to reduced number of PTO modifications also resulted in reduced cytotoxicity (Hebb & Robertson, 1997). PTO is a primary modification of ASOs, involving modification of the ASO backbone, which acts as a substrate for RNase H, resulting in degradation of target mRNA (Wu et al., 2004). Secondary modifications such as o-methoxy ASOs have higher nuclease resistance and silence genes by translational arrest, therefore, have been used for clinical applications in animal systems including exon skipping to cure genetic diseases like DMD (Cirak et al., 2011). However, they do not act as a substrate for RNase H. Therefore gapmers containing O-methoxy and PTO modifications have been used for gene silencing, where RNase H binds to the PTOs resulting in target mRNA degradation (Chan et.al., 2006; Monia et. al. 1993).

There are multiple alternative ASO chemistries showing successful gene silencing. One of the earliest ribose ring modifications include 2'- Fluoro (2'-F) modified ASOs. 2'F-ASO have clinical applications, however, they are are still vulnerable to intracellular nuclease degradation (Koval et al., 2013). Other ribose ring modifications include bridging nucleic acids (BNAs), consisting of the locked nucleic acids (LNA) and 2',4'-constrained ethyl nucleic acid ((S)-cET), which are now undergoing clinical trials for gene silencing mediated therapy in animal systems (Braasch & Corey, 2001). LNAs modification increases binding affinity for complementarity sequences (Braasch & Corey, 2001). However, LNAs sometimes show higher toxicity than other modifications, higher probability of aspecific binding and a tendency for self-binding (Evers et al., 2015). Alternatively, 2',4'-constrained ethyl nucleic acid ((S)-cET) have the desired properties of strong binding capability and improved nuclease resistance without any cytotoxic effects (Evers et al., 2015). These are tertiary ASO modifications, involving the modification of the ribose sugar and cause silencing by causing stearic hinderance during mRNA binding of ribosome, resulting in translational arrest (Braasch & Corey, 2001; Hong et al., 2015). Other modifications include phosphorodiamidate morpholino oligomer (PMO) and peptide nucleic acid (PNA). PMO modified ASOs have a backbone of morpholine rings connected by phosphorodiamidate linkages. These are uncharged nucleic acid analogues that bind to complementary mRNA targets and block protein translation through steric hinderance (Shen & Corey, 2018). The different ASO chemistries and their clinical applications have been described in detail in section 1.7.4 and Figure 1-18.

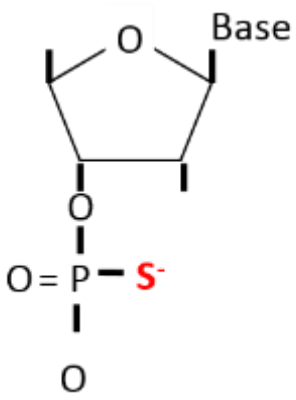


Figure 4-1: Phosphorothioate modification of oligodeoxynucleotide.

Phosphorothioate modification of oligodeoxynucleotide (PTO), where the bridging oxygen atom of the phosphodiester bond is replaced by a sulphur molecule.

Direct uptake of ASOs in a saline preparation, demonstrated first by Stein et. al. (2009) is extensively used for clinical studies in animal systems. They have a clear advantage on the use of dsRNA which

require additional carriers like nanoparticles of carrier ligands like N-acetyl glucosamine for successful uptake into animal cells (reviewed by Shen & Corey, 2018).

Inspired by these publications, a gene silencing method was devised in the Bindschedler laboratory involving gene specific PTO modified ASOs as silencing molecules for functional genomics in the barley powdery mildew pathosystem, querying barley and *B. graminis* f.sp. *hordei* genes (*Bgh*). The PTO modified ASOs were delivered *in planta* to barley excised leaves, where they were taken up by transpiration via the leaf vascular tissue. For proof of concept, this approach was applied for *in planta* gene silencing of known *B. graminis* effectors and known barley susceptibility genes. Such approach was successful, as it resulted in the reduction of powdery mildew disease development (Orman et. al. manuscript in prep). The silencing effect was highly sequence specific, as one mis-match in the ASO sequence, reduced the silencing efficiency and two mismatched did not result in any significant change in infection development post gene silencing (Orman et. al. manuscript in prep).

This included, for instance, the silencing of known effectors such as *BEC1011, BEC1054* and *BEC1019* (Pliego et al., 2013), as well as the known barley susceptibility genes *Blufensin 1* and *MLO1* (Orman et.al. manuscript in prep, Lambertucci et al, 2019), confirming the suitability of this assay for functional genomics studies in barley-powdery mildew pathosystem. However, the assay was devised for excised leaves, which is not convenient to study long term effect, or for its translation to crop protection strategies. Therefore, gene silencing assay in whole, integral barley seedlings are required and alternative methods of PTO delivery need to be trialled to silence genes in whole barley seedlings rather than excised leaves. Similarly, the assay needs to be adapted to wheat, to study wheat powdery mildew and other wheat pathosystems. Biolistic HIGS is difficult to achieve in wheat leaves due to collapse of wheat cells due to mechanical stress caused by biolistic delivery of silencing molecule (Personal communication, Patrick Schweizer). Therefore, it is not surprising that the current gene silencing assays in wheat powdery mildew pathosystem include the production of transgenic lines by VIGS mediated expression pathogen gene specific hpRNA (Várallyay et.al., 2012; Zou et.al., 2018). Therefore, ASO based gene silencing assays involving direct uptake of PTO modified ASOs will also benefit functional studies and disease control in the wheat powdery mildew pathosystem.

In the original assay devised by Orman et al., the silencing molecules were designed as 19 nucleotides long antisense single stranded DNA with phosphorothioate modification in each of the phosphodiester bonds. The short ASOs increase specificity of the silencing method by reducing off-targets (Orman et. al., manuscipt in prep). As mentioned above, in the phosphorothioate

modification one of the non-bridging oxygen atoms in the phosphodiester bond is replaced with a sulphur. Such modification prevents nuclease degradation of the antisense DNA, while keeping the PTO modified ASOs silencing properties (Monia et.al., 1996). PTO modification also increase bioavailability of these silencing molecules by facilitating uptake into host cells (Koller et al., 2011). This is a rather expensive DNA modification and has also been shown to be cytotoxic in certain animal models (Morassutti et al., 1999). Therefore, alternative chemistries to fully substituted PTO modified ASOs will be tested for suitability for silencing of fungal and plant genes in the barley-powdery mildew pathosystems, in order to reduce costs and possible side effects, such as reduced transpiration rates when comparing excised barley leaves treated either with PTO modified or non-modified ASO.

4.1.3 Simple assays as a tool to evaluate gene silencing effects: Silencing of *chlorophyll A, B binding protein (Cab)*

The Chlorophyll a,b, binding protein (Cab) is a component the light harvesting complex (LHC), the light receptor responsible for capturing and delivering excitation energy to closely associated photosystems I and II. Under variable light conditions, chlorophyll a/b binding proteins balances the excitation energy between the two photosystems (Liu & Shen, 2004).

The wheat gene encoding for the *chlorophyll a,b, binding protein (Cab)* was one of the first plant genes to be silenced by direct uptake of gene specific antisense oligodeoxynucleotides (ODN/ASO) by excised leaves (Dinc et al., 2011). The silencing treatment for *cab* gene was carried out in the dark by placing the excised wheat leaves in ODN solutions for 12 hours. Post silencing treatment, the excised leaves were exposed to light for 8 and 12 hours. Silencing effect was assessed by studying leaf greening, chlorophyll content and LHCII protein levels (Dinc et al., 2011).

Because it is easy to evaluate the impact of silencing the *Cab* gene, by monitoring leaf greening or quantify the chlorophyll amount, and the proportion of Chlorophyll a and chlorophyll b, this *Cab* silencing assay was modified to evaluate the silencing efficiency, when delivering PTO modified ASOs via the root system in order to adapt this method for *in planta* gene silencing in whole wheat seedlings.

4.2 Objectives and experimental rationale

4.2.1 Developing an alternative gene silencing workflow for gene silencing in whole intact seedling via root delivery of PTO modified ASOs to silence host and pathogen genes in barley and wheat leaves

One of the objectives of this project was to develop a system to silence both host and pathogen genes in whole seedlings rather than in excised leaves. With that aim, the assay designed for excised leaves was adapted to whole seedlings. Wheat *chlorophyll a,b protein* (*Cab)* was targeted using gene specific PTO MODIFIED ASOs described in DInc et al, (2011) delivered through roots into germinating wheat seedlings in the dark. The silencing effect was assayed by change in leaf greening and chlorophyll content (amount and quality) in the emerging leaves post light exposure.

Next, the *B. graminis* effector *BEC1011* was targeted to validate the PTO modified ASOs root delivery method for *Bgh in planta* gene silencing in barley-powdery mildew pathosystem.

The gene silencing workflow, derived from the excised leaf assay, was performed on 7 days old barley seedlings grown in hydroponics. These were transferred in PTO modified ASOs solutions for 24 h under continuous light to promote root uptake of PTO modified ASOs. The whole treated seedlings (root and shoots) were then placed on agar plates and inoculated with *Blumeria* spores. The silencing effect was assayed by microscopic disease scoring at 48 hpi.

4.2.2 Alternatives PTO chemistries for efficient ASO delivery and *Blumeria* gene silencing

Since PTO modifications are added cost when purchasing ASO, and PTO modified ASOs have been reported to be cytotoxic in animal systems, the silencing efficiency of 1-in-3 PTO modified and end-capped PTO modified ASOs were compared to all-PTO modified ASOs in the detached leaf silencing protocol. End-capping involves introducing two PTO modifications (EC-PTO) on the phosphodiester bonds on each end of the silencing oligomer. EC-PTO modified ASOs have been shown to effectively silence genes in animal models (Hebb & Robertson, 1997). To access the EC-PTO and 1-in-3-PTO ASO, *Blumeria* genes 2-glycosyl transferase (GT2) and BEC1011 were silenced using 1-in-3 and end-capped PTO ASOs, and their effect on barley powdery mildew infection development was studied.

4.3 Results

4.3.1 Effect of root delivery of ASO and PTO modified ASOs targeting *chlorophyll a,b (Cab)* on greening and chlorophyll content of emerging leaves of wheat seedlings

With the aim of adapting the *in planta* gene silencing protocol from excised leaves to whole plants, ASO uptake by the root system in germinating seedling was attempted. For this, the wheat *Cab* gene

described above (GenBank id: M10144.1) was targeted, since targeting of *Cab* gene by direct uptake of unmodified ASOs (oligodeoxynucleotide: ODN) into cut wheat leaves slow down the onset of the greening process of leaves transferred from darkness to light. It correlated with reduced amounts of chlorophyll a, b and total chlorophyll in wheat leaves (Dinç et.al., 2012; Dinc et al., 2011).

The initial experiments involved root uptake of *Cab* ODNs by 3 days old wheat seedlings germinated in the dark in petri dishes containing filter papers soaked in 10µM *Cab* ODN1 and *Cab* ODN2 solutions, with followed by 24 hours exposure to light. Water and an ODN/ASO with no targets in wheat genome (Dinc et al., 2011) were used as negative controls. After 24 hours, the *Cab* ODN treated seedlings looked much less green when compared to water and an no target ASO negative control (Figure 4-2).

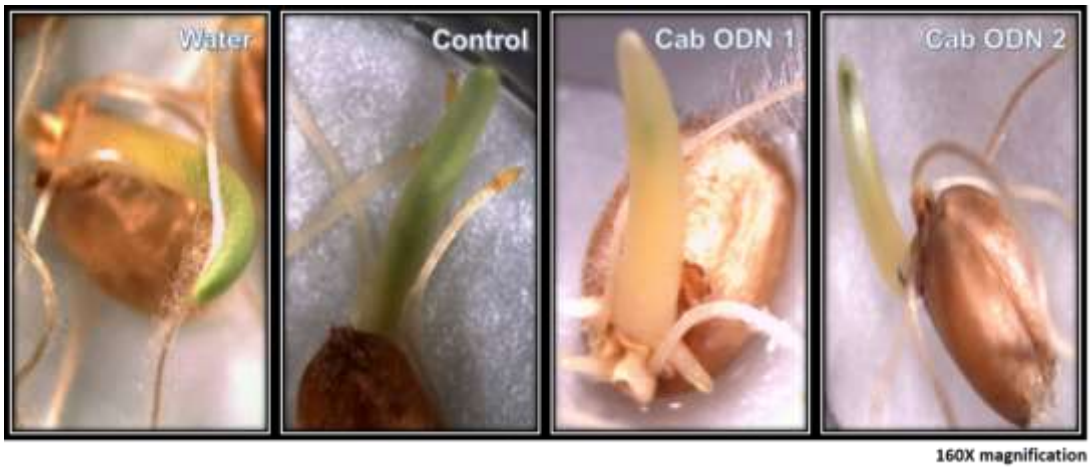


Figure 4-2: Chlorophyll a,b binding protein (Cab) silencing using gene specific ASOs.

Pictures showing typical greening of emerging wheat leaves after 24h ODN based silencing targeting of chlorophyll a,b binding protein (Cab) by root delivery into wheat seedlings. No target ODN and water treatments were used as controls. (N=6, for each treatment).

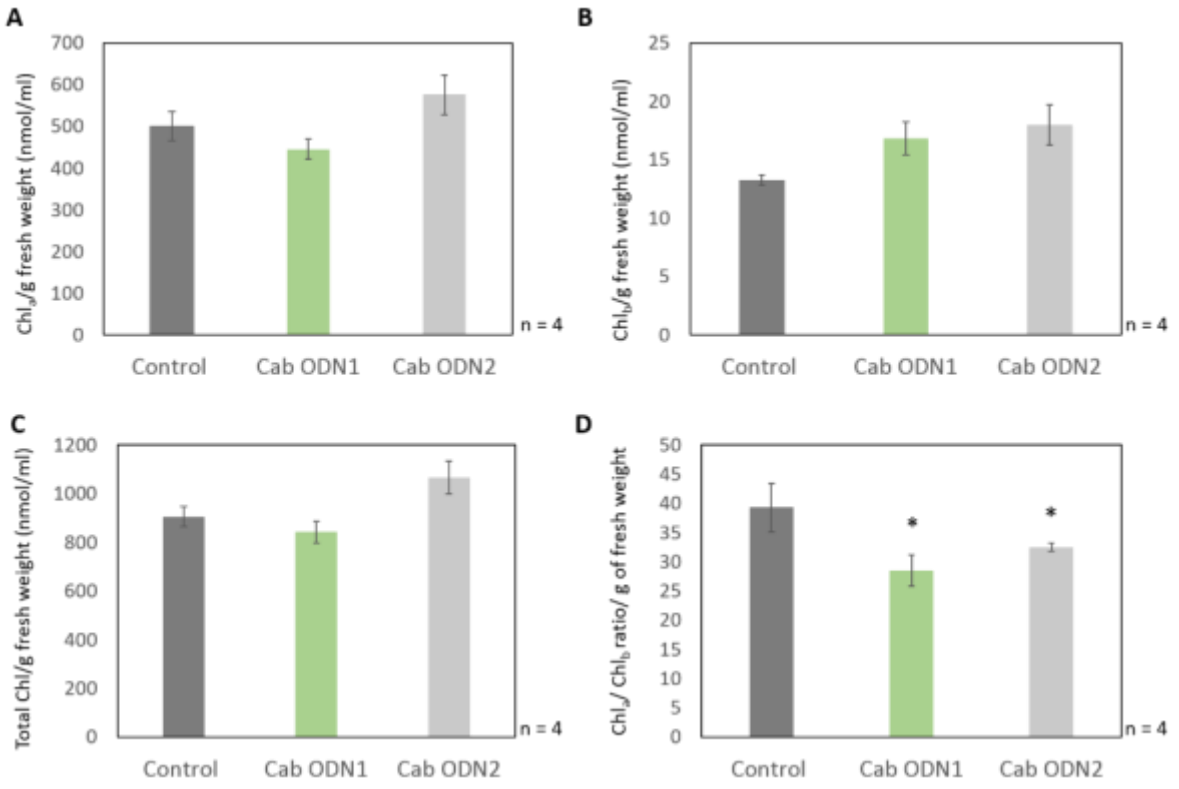


Figure 4-3:Chlorophyll levels post chloropyhyll a,b binding protein silencing in wheat seedling via root delivery of ASOs. Chlorophyll amount extractable from greening emerging wheat leaves, after 24h treatment with Cab ODN1 and Cab ODN2 via root delivery in whole seedlings, for the silencing of the wheat Cab gene, followed by transfer of seedlings from dark to light. A. Chlorophyll A, B. chlorophyll B, C. total chlorophyll, D. chlA/chlB ratio per gram of fresh weight and monitored in emerging leaves. An ODN with no target in wheat was used as a negative control. N=4. Significance (1 sample t-test) was shown as * P>0.05 **P>0.01 ***P>0.001.

Cab gene silencing was repeated in whole wheat plantlets and its impact on chlorophyll content of leaves post treatment was studied. This was compared to the ODN control treatment. Four seedlings were included for each type of ASO treatment. The results showed a significant reduction in the chlorophyll a/ chlorophyll b ratio in *Cab* ODN treatments as compared to the negative control ASO treatment (Figure 4-3**Error! Reference source not found.**). However, chlorophyll a, chlorophyll b or total chlorophyll amount did not show any significant difference between control and ODN treatment to silence *Cab* gene.

Since previous data in Bindschedler lab had confirmed a higher silencing efficiency of PTOs over non-modified ASOs (ODNs) (Orman, Das Gupta et. al. manuscript in prep), *Cab* silencing was attempted by root uptake of *Cab* PTO2 ASOs (corresponding to the sequence of *Cab* ODN 2) and a no-target negative PTO modified ASO as control. Like the above experiment, seeds were germinated for 3 days in the dark, on filter paper, saturated with the 10µM PTO solution. Quantitative chlorophyll measurements were performed after 24 hours of light exposure of the PTO treated seedlings. Eight

seedlings were treated in each treatment. The results showed a significant reduction in chlorophyll a, b, total chlorophyll and chlorophyll a/chlorophyll b ratio in *Cab* PTO treated seedlings as compared to negative control Figure 4-4.

Based on these findings, it was concluded that root delivery of PTO modified ASOs was effective for silencing host genes *in planta*.

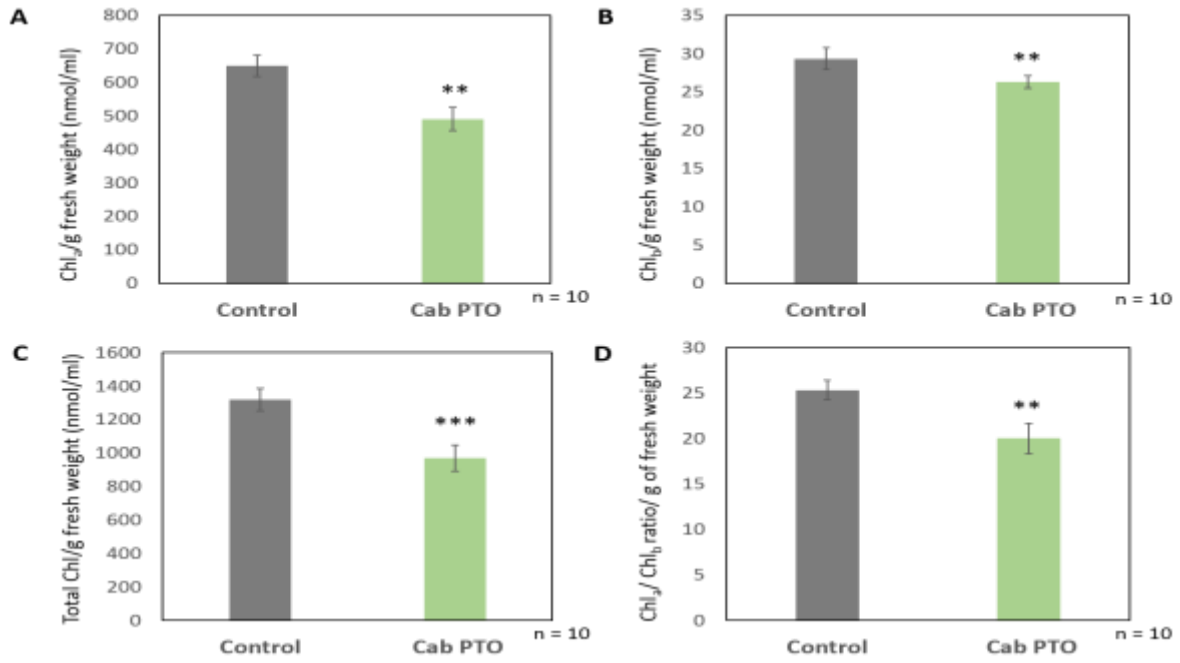


Figure 4-4: Chlorophyll a,b protein(Cab) silencing via root delivery of PTO modified ASOs.

*Chlorophyll amount extractable from greening emerging wheat leaves, after 24h treatment with Cab PTO via root delivery in whole seedlings, for the silencing of the wheat Cab gene, followed by transfer of seedlings from dark to light. A. Chlorophyll A, B. chlorophyll B, C. total chlorophyll, D. chlA/chlB ratio per gram of fresh weight and monitored in emerging leaves. An ODN with no target in wheat was used as a negative control. N=4. Significance (1 sample t-test) was shown as * P>0.05 **P>0.01 ***P>0.001.*

4.3.2 Powdery mildew infection impact by root delivery of PTO modified ASOs to whole barley seedlings for silencing of *Bgh BEC1011*

Since it was possible to silence the Cab gene in whole barley seedlings via root delivery of PTO modified ASOs, the aim was extended to silencing *Blumeria* genes in whole barley seedlings rather than excised leaves. For this, the *Blumeria* effector *Bgh BEC1011* was targeted by root delivery of the gene specific PTO 11.11 ASO. PTO 11.11 effect on powdery mildew infection of barley seedlings was monitored.

Root based ASO treatments were carried out on 7 days old barley seedlings grown hydroponically. Each seedling was then transferred to a tube containing 1 ml of PTO modified ASO solution. As previously, PTOZ, with no express target in barley leaves was used as a negative control. Barley

seedlings were then transferred to agar plates and inoculated with *Bgh*. As previously, infection success was scored as % secondary hyphae formed per germinated conidia (%SH) at 48 hpi. Seedlings treated with *Bgh BEC1011* by root delivery of PTO 11.11 ASO prior *Blumeria* infection, showed a 70% reduction of secondary hyphae formed (Figure 4-5), confirming the suitability of the root delivery of PTO modified ASOS as a method for *in planta* silencing of fungal genes in whole seedlings.

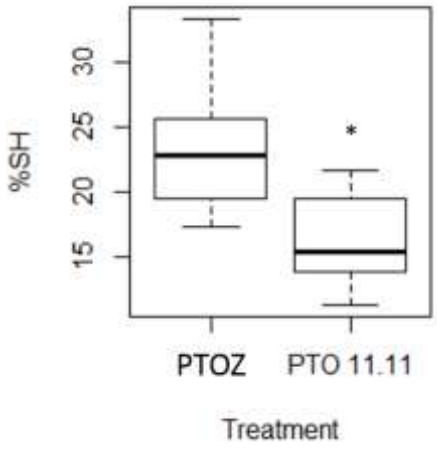


Figure 4-5: Bgh BEC1011 silencing via root delivery of PTO modified ASOs into barley seedlings.

*Box plot graph showing % secondary hyphae formed /germinated conidia) in barley seedlings treated with PTO 11.11 ASO root delivery mediated targeting of Bgh BEC1011. PTO with no expressed target in barley leaves (PTOZ) was used as a negative control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) seedlings. For infection assay, at least 250 appressoria forming conidia were counted, monitoring the number of non-germinated conidia, conidia with apressoria, and conidia with appressoria and a developing hyphae. Data are shown as box plot with median value, the box representing the upper and lower quartile, the whiskers the maximum and minmal values. Data were derived from 3 independent biological replicates, each of the treatment in each repeat comprising of 4 barley seedlings. Significance (1 sample t-test) *P<0.05, **P<0.01, ***P<0.001.*

4.3.3 Efficiency comparison of 1-in-3 PTO and end-capped PTO to fully PTO modified ASO on silencing *Blumeria* genes

Three type of PTO modified ASO of otherwise identical sequence were compared to each other: the fully PTO modified ASO used so far, ASO, in which only 1 in 3 phosphate bridge were substituted with PTOs and end capped PTOs, where PTO modifications were introduced in the last two phosphodiester bonds in each end of the ASO. For the comparison *Bgh GT2* and *Bgh BEC1011* were targeted using the excised leaf assay. For the negative controls, the corresponding modified PTO ASO for the previously described PTOZ were included, as fully modified, 1-in-3 or end capped PTOs. As previously, the infection success rate was scored as % secondary hyphae formed per germinated conidia (%SH).

In these experiments, *Bgh GT2* targeted by fully modified, 1-in-3 and end-capped PTOs reduced *Blumeria* % secondary hyphae formation by 26%, 31% and 45% respectively (Figure 4-6) as compared to their corresponding controls. These data suggest that the end-capped PTO (EC-PTO modified ASOs had the highest silencing effect when compared to the other PTO ASOs.

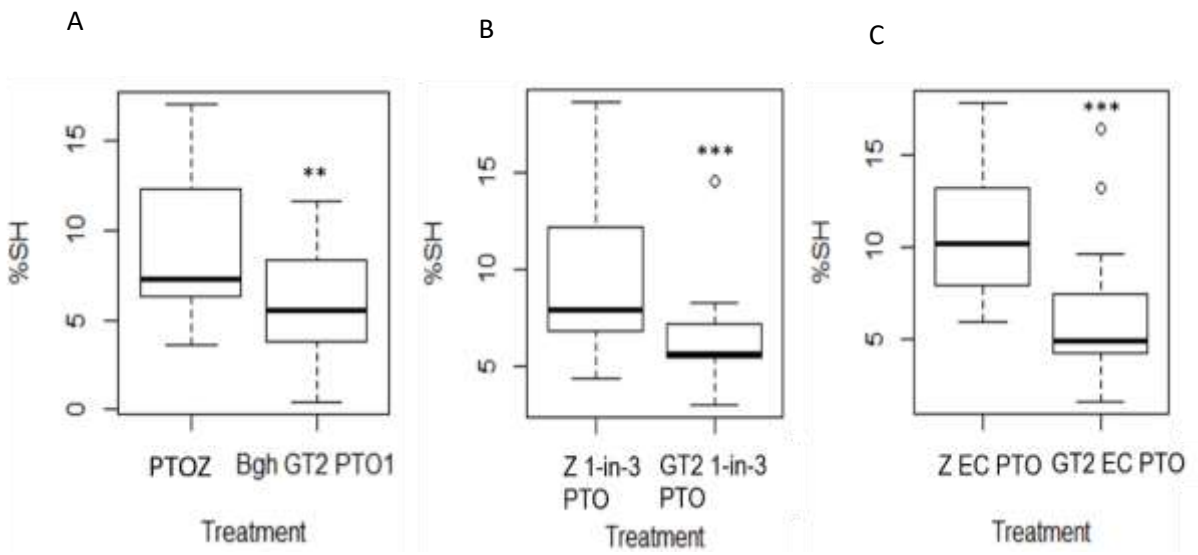


Figure 4-6: Trial of different PTO chemistry during gene silencing of Bgh 2-glycosyl transferase (GT2) in barley variety c.v. Golden promise.

*Barley powdery mildew infection success following ASO treatment of excised barley leaves. The Bgh 2-glycosyl transferase (GT2) was targeted for silencing using a GT2 specific ASO with different PTO modification A. fully modified PTO, B. 1-in-3 PTO modification and C. end-capped PTO in excised barley leaves. All were compared to their corresponding PTO Z controls. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For the infection assay, at least 250 appressoria forming conidia were observed, counting the number of non-germinated conidia and hyphae. Infection was assayed as %secondry hyphae formed per germinated conidia (%SH). Data are shown as box plot with median value, the box representing the upper and lower quartile, the whiskers the maximum and minmal values. Data acquired from 4 independent biological repeats, each comprising of 5-6 leaves per treatment. Significance (1 sample t-test) *P<0.05, **P<0.01, ***<0.0001.*

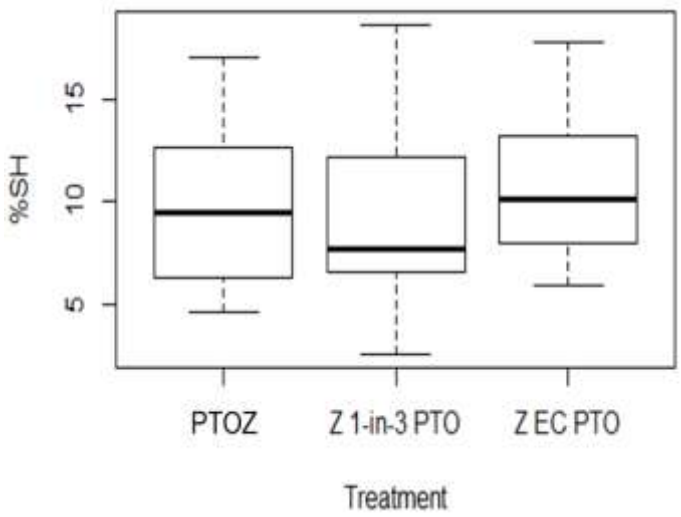


Figure 4-7: Comparison of negative control ASOs (PTOZ) with different PTO modifications during in planta gene silencing in barley excised leaves.

*Comparison of negative control ASOs (PTOZ) with different PTO modifications (all modified PTOs, 1-in-3 PTO modified and end-capped PTO) on the infection success rate during barley powdery mildew disease (as monitored by the proportion of spores forming secondary hyphae per total germinated conidia (%SH)) . PTO treatments were carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were observed on each leaves, noting the number of non-germinated conidia with appressoria, conidia with appressoria and hyphae. Data were acquired from 4 independent biological repeats, each comprising of 5-6 leaves per treatment. Significance (1 sample t-test) *P<0.05, **P<0.01, ***<0.0001*

To monitor whether the alternative PTO modified ASO had an effect on *Blumeria* hyphae development the three negative controls with the three type of modified PTO, i.e. the fully modified PTOZ, the 1-in-3 PTOZ and the end-capped PTOZ were compared to each other to assess any effect of these PTO chemistries on *B. graminis* virulence. No significant difference was seen in %SH between the various controls (Figure 4-7). Therefore, reducing the number of PTO modifications did not affect *B. graminis* virulence.

The effect of the different PTO chemistries was also checked for treatments silencing the *Blumeria* effector *Bgh BEC1011*. *Bgh BEC1011* was targeted treating excised barley leaves with 1-in-3 and end-capped PTO molecules. Then the silencing efficiency was assessed by comparing barley powdery mildew infection success rate for the various treatments and the corresponding PTO Z treatments.as measured as the % secondary hyphae formed per germinated conidia (%SH).

Results were acquired from two biological repeats, each repeat with 5-6 leaves per treatment. It showed a significant reduction of 31% in %SH post *BEC1011* targeting by end-capped PTOs (Figure 4-8B). However, no significant difference in infection was observed when *BEC1011* was targeted

using 1-in-3 modified PTOs (Figure 4-8A). However, these experiments would need to be further repeated to increase confidence in their reproducibility.

Nevertheless, cumulatively, our findings suggest that end-capped PTO may be the most efficient PTO chemistry for *in planta* silencing of *Blumeria* genes.

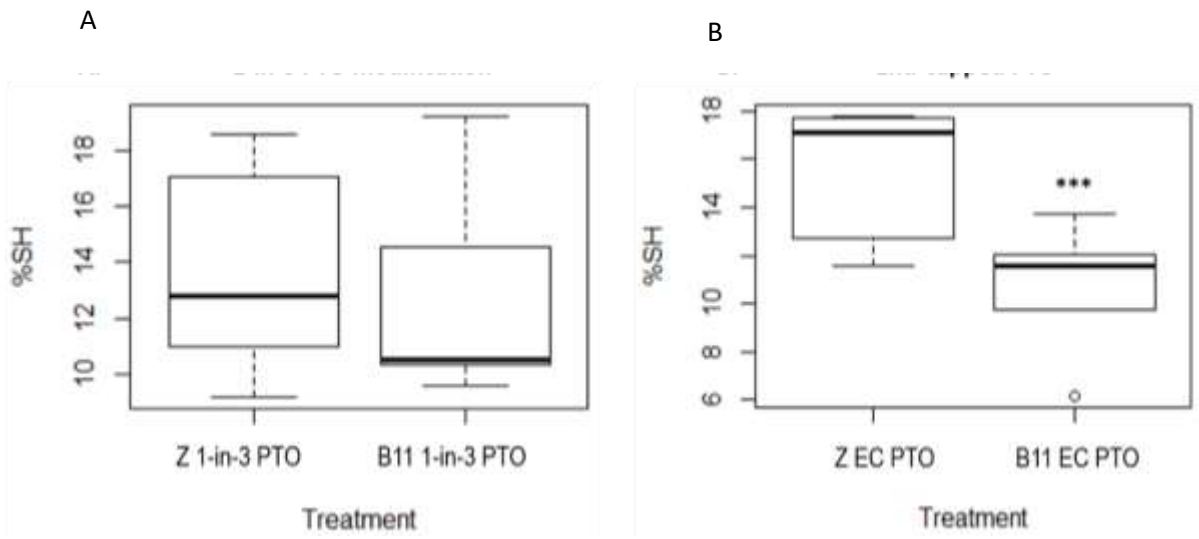


Figure 4-8: Trial of alternative PTO chemistries during silencing of Bgh BEC1011 in excised leaves of barley c.v. Golden Promise.

*Barley powdery mildew infection success following ASO treatment of excised barley leaves. The Bgh BEC1011 was targeted for silencing using a gene specific ASO with different PTO modifications. Barley powdery mildew infection development post Bgh BEC1011 targeting using a gene specific A. 1-in-3 PTO modified and B. end-capped PTO in excised barley leaves. 1-in-3 and end-capped PTOs targeting seed protein Z were used as control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Infection was assayed as % secondary hyphae formed per germinated conidia (%SH). Data acquired from 4 independent biological repeats, each comprising of 5-6 leaves. Significance (1 sample t-test) *P<0.05, **P<0.01, ***<0.0001.*

4.4 Discussion

4.4.1 *In planta* gene silencing of plant genes by root delivery of gene specific PTOs

One of the objectives of this project was to develop an *in planta* gene silencing assay that could move away from strictly functional genomics analysis to crop protection strategies, in particular for powdery mildew disease control in barley seedlings. This was attempted by silencing plant and fungal genes by root delivery of gene specific PTOs. The chlorophyll a,b binding protein was considered a good silencing target for establishing an *in planta* gene silencing assay by root uptake of PTO modified ASOs, as it can be easily monitored by observing the delay of the greening of the

leaves of *Cab* silenced plants transferred from darkness to light (E. Dinc et al., 2011), a phenotype easily measurable either visually or by spectrophotometric quantification of the chlorophyll content. A protocol was developed for silencing of wheat *Cab* gene and chlorophyll measurement in young leaves post gene silencing. Gene specific PTO modified ASOs seemed to have a more significant effect on chlorophyll development as compared to unmodified ASOs. These results are akin to previous results in Bindschedler lab which showed greater reduction in *Blumeria* infection on silencing of *BEC1011* effector using PTO modified ASOs as compared to unmodified ASOs (Orman, Das Gupta et.al. manuscript in prep).

For the chlorophyll silencing experiment, the age of the leaves was an important factor when determining chlorophyll levels, where, younger leaves have lower chlorophyll levels than older leaves. In the absence of synchronised seed germination, care needed to be taken to use homogenised seedling size for the treatments to avoid the developmental age effect on chlorophyll measurements. However, the presence or absence of the coleoptile did not affect the efficiency of chlorophyll extraction, therefore, for simplification, the coleoptile was not removed during chlorophyll extraction.

These initial findings confirmed the possibility of silencing plant genes by root delivery of PTO modified PTOs. This is the first reported assay where plant genes could be silenced in whole seedlings by root delivery of silencing molecule. This could be further developed to target host susceptibility genes to control fungal diseases.

4.4.2 Antisense PTO modified ASOs for *in planta* gene silencing can be delivered in whole plants for functional genomics studies in the barley-powdery mildew pathosystem

With the aim of developing an *in planta* gene silencing methodology for targeting fungal genes in whole plants rather than excised leaves, the root delivery of gene specific PTO modified ASOs was attempted. For this, firstly, a protocol for hydroponic growth of wheat and barley seedlings was also developed for *in planta* targeting of fungal genes by root delivery of silencing molecule (Section 2.1.2). As targets, the well-established *B. graminis* effector *BEC1011* (Pliego et al., 2013) were chosen.

Table 4-1: Comparison of gene silencing via biolistic HIGS and PTO modified ASO based in planta silencing in whole plants.

Comparison of % infection reduction post gene silencing using biolistic delivery of hpRNA, asPTO delivery into excised leaves and barley seedlings.

Gene targeted	Reduction in haustoria formation by HIGS (Pliego et al., 2013)	Reduction in %SH/germinated conidia in excised leaves treated with full-PTO ASO	Reduction in %SH/germinated conidia in whole barley seedlings treated with full-PTO ASO
BEC1011	70%	53%	70%
PacC	-	52%	67%

Table 4-2: Comparison of Bgh BEC1011 silencing in excised leaves and whole barley seedlings.

Comparison of %SH and % infection reduction during BEC1011 targeting in barley seedlings and excised leaves.

	%SH in PTOZ treatments	% SH in PTO BEC1011 treatments (% Reduction in infection compared to PTOZ control)
Root delivery of PTOs by seedlings	23%	6.9% (70% reduction)
Uptake of PTOs by excised leaves (K. Orman)	14%	6.6% (53% reduction)

Bgh BEC1011 silencing attempt by root delivery of PTO modified ASOs into barley seedlings, reduced *Bgh* %SH formation by 70% (Figure 4-5). This reduction in infection was similar to the reduction in haustoria formation observed in *BEC1011* HIGS assay where a plasmid carrying a construct to express hairpin RNA (hpRNA) is biolistically introduced by nanoparticle cargoes (Pliego et al., 2013). This reduction is 17% higher than the reduction observed when silencing PTO modified ASOs were delivered to excised leaves, using a protocol which was previously developed and routinely used in the Bindschedler laboratory (Orman, Das Gupta et.al., manuscript in prep) (Table 4-1). Therefore, PTO modified ASOs triggered gene silencing seemed more efficient when the silencing molecules were delivered via the roots into barley seedlings as compared to direct delivery through the vascular tissue into excised leaves. A similar trend was also observed when targeting the transcription factor *Bgh PacC* using full-PTO modified ASOs (Table 4-1). However, it needs to be noted that the infection data for *PacC* targeting in barley seedlings was only obtained from a single biological repeat and further repeats are required to check reproducibility of the results. Therefore, it was not included in the main result section of this chapter. Nevertheless, in the case of the difference in silencing effect for *Bgh BEC1011* PTO modified ASOs delivered to whole seedlings via

the root system was also associated with a higher infection success of the corresponding negative control treatment with the PTOZ modified ASO, since a higher %SH of 23 %SH was obtained for this control when compared to 14 %SH obtained when PTOZ modified ASO was delivered into excised leaves. To some extent, this might explain for a greater relative reduction in infection (Table 4-2). As an obligate biotroph, the global lower infection success of *B. graminis* in excised leaves is likely due to accelerated senescence and wounding stress of the excised primary leaves, when compared to whole barley seedlings. Therefore, a silencing workflow involving whole barley seedlings rather than detached leaves reflects better what would happen in the field. It will also facilitate the use of this seedling based silencing protocol for evaluating the long-term gene silencing effect of PTO modified ASOs targeting of *B. graminis* virulence genes.

Uptake assays were performed in the Bindschedler lab to visualise the uptake of fluorescently labelled PTO modified into the barley cells via transpiration. The fluorescence from the ASOs was shown to be strongest in the vascular tissue, with subsequent reduction in the fluorescent in the cytoplasm of epithelial and mesophyll cells These observations indicated that the uptake of PTO modified ASOs in the excised leaves occurred through the vasculature which then load the ASOs into the epithelial cells, the site of *B. graminis* infection. The uptake of ASOs by the epithelial cells maybe via sugar transporters as shown by studies carried out by Sun et. al. (2007). During root delivery of PTO modified ASOs, the uptake maybe through the root hairs along with the water.

However, while developing such an assay, the economic aspects also need to be considered, when planning large scale screening. In the excised leaves assay, three excised leaves could be treated with 1 ml of 10 µM PTO ASO whereas for root delivery into whole barley seedlings, only one seedling could be treated with the same amount. Therefore, the PTO ASO delivery into whole seedling via roots is less cost effective than the direct uptake of PTO ASO into excised leaves. Nevertheless, the exploration of alternative suitable ASO with partial PTO modified chemistries are likely to reduce the experimental price and help to exploit the advantages of the root delivery assay.

4.4.3 End-capped PTOs - an efficient silencing molecule for host induced gene silencing of fungal genes

Antisense oligodeoxynucleotide (ASO) based gene silencing has been widely used in animal systems for both functional genomics and clinical applications (Gewirtz et.al., 2013). One of the primary widely used modifications of ASOs is the phosphorothioate modification (PTO) i.e. replacement of one of the non-bridging oxygen with a sulphur atom, in the phosphodiester bond (Monia et al., 1996). PTO modifications increase the silencing efficiency by preventing nuclease degradation of the ASO, as well as increasing its bioavailability by enhancing its uptake by the host (Chan et.al., 2006).

For gene silencing in the barley-powdery mildew pathosystem, PTO modified ASOs worked better than siRNA in the Bindschedler laboratory. In some preliminary experiments, direct uptake of 10 and 1 µM siRNA resulted in shrivelled barley leaves. Moreover, no change in barley powdery mildew infection was observed when the siRNA concentration was lowered to 0.1 µM, even though the barley leaves looked still healthy after siRNA treatment.

These fully PTO modified ASOs were previously used for *in planta* silencing of both host and pathogen genes (Orman, Das Gupta et.al., manuscript in prep). However, each PTO modification included increased the cost of the synthetic ASO by ca £3 per modification. Typically, a 19 mer ASO, fully modified with PTO costed ca. £80. when using a 1µM synthesis scale. This amount was sufficient for the treatment of just 45 excised leaves or 15 seedlings. Moreover, fully PTO modified ASO has also been shown to have cytotoxic effects in animal models (Morassutti et al., 1999). Therefore, for cost effectiveness, and to address the putative cytotoxicity, the effect of reducing the number of PTO modifications on silencing efficiency was trialled by using 1-in-3 and end-capped PTOs for silencing *Blumeria* genes and studying their role on powdery mildew disease development. These type of PTO modified ASOs have already been shown to successfully silence genes in animal systems, with higher silencing efficiency and reduced cytotoxicity compared to a fully PTO modified ASO (Hebb & Robertson, 1997).

Two *Blumeria* genes, the vital gene 2-glycosyl transferase (GT2) and the effector BEC1011, were chosen as targets. *GT2* is important for virulence in fungal pathogens (King et al., 2017), and in this study it was also shown to be required for *B. graminis* f.sp. *hordei* virulence, when silenced using fully PTO modified ASO (Section 3.3.2). Similarly, silencing of *Bgh BEC1011* with a fully PTO modified ASO reduced fungal virulence and disease spread in multiple studies (Orman, Das Gupta, et.al., manuscipt in prep) as well when silenced by HIGS using biolistic delivery (Pliego et al., 2013).

Table 4-3: Relation between control infection and silencing effect during Bgh GT2 infection using different PTO chemistries.

Comparison of %SH in control treatments and silencing effect during GT2 silencing in experiments to compare different PTO chemistries and previous GT2 silencing experiments (Section 5.3.2) using fully PT modified GT2 PTO1.

Treatments	Avg. %SH/germinated conidia in control treatment (PTO Z)	% Infection reduction	No. of PTO modifications
Full PTO modified ASO *Bgh GT2* PTO1	8.4%	26%	18
1-in-3 PTO *Bgh GT2* PTO1	9.4%	31%	7
End-capped PTO *Bgh GT2* PTO1	11.1%	45%	4
Bgh GT2 PTO1 (fully modified PTO) (previous experiments - Section 5.3.2)	12.4%	55%	18

In the experimental set presented in this chapter, silencing of *Bgh GT2* with *Bgh GT2* PTO1 reduced *B. graminis* infection by 26% using fully modified PTOs, 31% using 1-in-3 PTOs and 45% using end-capped PTOs (Figure 4-6; Table 4-3). The reduction in infection following the silencing treatment with the fully PTO modified ASO was 55% lower than the reduction observed in a previous set of experiments (Section 3.3.2; Table 4-3). This is likely due to lower *B. graminis* infection success in leaves treated with the negative PTOZ control (Table 4-1). Therefore, spore quality and quantity which determine disease pressure, as well as some of the experimental conditions, which cannot fully be controlled, are important factors influencing *Blumeria graminis* aggressiveness and host resistance. All this has a likely impact on the relative infection success following the silencing of *B. graminis* genes.

Interestingly, when experiments were run in parallel under the same experimental conditions for direct comparison, the number of PTO modifications present in the ASO was inversely correlated to the silencing efficiency as estimated by the reduction of %SH: The end-capped PTOs containing just 4 PTO modifications showed the greatest reduction in infection following *GT2* silencing (Table 4-3). These results matched the studies in animal models where end-capped PTOs proved to be more efficient at silencing genes with reduced cytotoxic effects (Hebb & Robertson, 1997). Lower silencing efficiency in fully modified PTOs could be due to stereochemical bias introduced during its synthesis. The phosphorothioate group is a chiral molecule, with either Rp or Sp configuration at the phosphorous (Jahns et al., 2015). A fully modified PTO is usually a mixture of these diastereomers, where the population with Rp centres being more resistant against nuclease effect, has a higher silencing potential than the Sp diastereomers as was shown in the animal system (Jahns et al., 2015).

Hence, only a fraction of the PTO molecules used for silencing are effective. This ratio between Rp/Sp diastereomers can be altered depending on the synthesis method (Jahns et al., 2015). Reduction in PTO modifications may relatively reduce this stereochemical bias, as fewer types of diastereomers are created. Therefore, this may increase the silencing effect, since a higher proportion of "efficient" diastereomer are present, therefore in a higher concentration.

Lower number of PTO modifications also correlated with higher %SH in the control treatments. However, this was not statistically significant

Figure **4-7**; Table 4-3).

Financially, a tube of 19 mer end-capped PTO modified ASO (EC-PTO modified ASO with 4 PTO modifications) typically costs around £40 (1µM synthesis scale) which is half of the value for the equivalent fully modified PTO modified ASO. Therefore, the use of end-capped PTOs is an experimental improvement as EC PTOs increase both efficiency and cost effectiveness of the silencing assay.

The use of other chemistries could also be trialled for gene silencing in the barley-powdery mildew pathosystem, including commercially available linked nucleic acid ASOs. As mentioned earlier, LNA modified ASOs are being widely used in the animal studies and are undergoing clinical studies gene silencing based therapies (Shen & Corey, 2018; Swayze et al., 2007). It is important to note that LNA modified ASOs mediate gene silencing by translational arrest. Therefore, gene silencing will need to be confirmed by monitoring target protein levels. Protein levels could be monitors using Multiple Reaction Monitoring (MRM), a protein quantification which monitors protein levels by identifying multiple peptides specific for a single protein. However, silencing effect could also be monitored by barley powdery mildew disease scoring. Morpholino modified ASOs (PMOs) are another alternative chemistry that has been successfully used to silencing genes in animal systems are now undergoing clinical trials (Lebleu et al., 2008; Shen & Corey, 2018). However, LNAs and PMOs are more expensive modifications as compared to PTO modifications.

Chapter 5 : Role of *Blumeria* metallo-protease like effector (BEC1019) homologues in fungal zinc sequestration and virulence of *Blumeria graminis, Fusarium graminearum* and *Zymoseptoria tritici*

5.1 Introduction

5.1.1 *Blumeria* metallo-protease like effector (BEC1019), a *B. graminis* virulence factor

The *Blumeria* Effector Candidate 1019 (BEC1019) is a metallo-protease-like haustoria specific protein (Bindschedler et. al., 2011) involved in the virulence of *Blumeria graminis f. sp. hordei* (Whigham et al., 2015, Pliego et al., 2013). Discovered as a haustoria specific protein in a proteo-genomics study, *Bgh* BEC1019 is a small protein, possessing a signal peptide that showed a high haustoria to hyphae expression ratio, leading to its classification as a *Blumeria* effector candidate (BEC) (Bindschedler et al., 2011). However, it was not classified as a candidate secreted effector protein (CSEP) by another transcriptomics study (C. Pedersen et al., 2012), due to the presence of BEC1019 homologues in other fungal species (Whigham et al., 2015). BEC1019 possesses a HRXXH similar to a HEXXH domain found in M35 family of metallo-proteases, but no protease activity has yet been reported (Whigham et al., 2015). Hence, the name metallo-protease like effector.

BEC1019's initial contribution to *B. graminis* virulence was evidenced by hpRNA based HIGS experiments which led to reduced haustoria and hyphae formation. Following delivery of gene specific hpRNA by biolistic bombardment, the % of success haustoria formed (haustoria index), a marker of successful powdery mildew infection, was reduced (Pliego et al., 2013). Another study silenced *Bgh BEC1019* by virus induced gene silencing using a BSMV vector construct containing the hpRNA, resulting in a reduction in fungal biomass and powdery mildew symptom development (Whigham et al., 2015). Therefore, BEC1019 acts as a virulence factor in barley powdery mildew.

To understand the mode of BEC1019 mediated virulence, BEC1019 was introduced into barley plants, resulting in suppression of hypersensitive reaction (HR) triggered by bacterial pathogens. But this was later retrieved, as the results were believed to be artefacts caused by a faulty bacterial effector delivery system used (Whigham et al., 2015). Therefore, the role of BEC1019 in *Blumeria* virulence is yet to be understood.

5.1.2 BEC1019 homologues and their role in zinc sequestration and virulence of fungal pathogens

A recent study has highlighted the presence of BEC1019 homologs in 96 out of 241 sequenced fungal genomes, including human pathogens, plant pathogens and non-pathogenic fungi belonging to both ascomycetes and basidiomycetes (Figure 5-1) (Whigham et al., 2015). These include known virulence

factors in human pathogens like PRA1 (pH Regulated Antigen 1) in *Candida albicans* and the allergen Aspf2 in *Aspergillus fumigatus* (Whigham et al., 2015). Aspergillosis and candidiasis development by *A. fumigatus* and *C. albicans* in humans necessitate the pathogens to survive in zinc limiting alkaline conditions. PRA1 and AspF2 deletion reduced fungal growth under alkaline zinc limiting conditions, confirming their role as virulence factors in *C. albicans* and *A. fumigatus* (Amich et.al., 2010; Citiulo et al., 2012). ZPS1, the BEC1019 homologue in *Saccharomyces cerevisiae*, is a zinc and pH regulated membrane-bound putative metallo-protease protein, whose role in *S. cerevisiae* growth is still unknown (Mira et.al., 2009; Wu et al., 2008). Another BEC1019 homologue VdAspF2 in *Verticilium dahliae* was shown to play a role in microsclerotia (fruiting body) formation, important for prolonged survival under adverse conditions. *VdAspF2* deletion in *V. dahliae* did not impact verticillium wilt development, but significantly delayed the development of melanised microschlerotia formation in microschlerotia-inducing medium, confirming its role in long term survival of the fungus (Xie et.al., 2017).

These proteins are characterised by a conserved HRXXH domain, closely related to the M35 family of zinc binding proteases, containing a zinc binding HEXXH catalytic domain (Figure 5-2) (Whigham et al., 2015).

PRA1 is important for host zinc sequestration and utilisation needed for host endothelial cell colonisation by *Candida*. *Candida* was shown to sequester host zinc by microscopic studies that labelled the zinc using the zinquin fluorescent dye during endothelial infection. PRA1 which could bind zinc *in vitro,* when deleted, inhibited fungal growth under zinc-limiting conditions, confirming its role as a zinc sequester in *C. albicans* (Citiulo et al., 2012). In *C. albicans* this zinc sequestration is governed by successful PRA1- ZRT1 interactions, where ZRT1 is a predicted zinc transporter protein (Citiulo et al., 2012). Aspf2, on the other hand, works in association with zinc transporter ZrfC (Zrt1 homologue) to maintain zinc homeostasis for the survival of *Aspergillus fumigatus* in zinc-limiting conditions (Amich et. al., 2010). Deletion of either *AspF2* or *ZrfC* prevented *A. fumigatus* survival under zinc limiting conditions (Amich et al., 2010). PRA1 and Aspf2 sequester zinc under neutral-alkaline conditions and are highly repressed under acidic conditions (Amich et. al., 2010; Citiulo et al., 2012). Interestingly, PRA1-ZRT1 and Aspf2-ZrfC are genetically linked sharing an intergenic promoter sequence containing zinc sensitive and pH sensitive elements (Amich et al., 2010; Citiulo et al., 2012). This gene pair together form a zinc acquisition locus which is evolutionarily conserved in some fungi belonging to both ascomycetes (*Aspergillus sp.* few species of *Candida, Coccidiodes immitis)* and basidiomycetes (*Ustilago maydis, Sporisorium reylanum),* but not in others like *F. graminerum* and *M. oryzae* (Citiulo et al., 2012).

The expression of genes of this zinc acquisition locus is regulated by a zinc sensitive transcription factor (ZAP1 homologue) and a pH sensitive transcription factor (PacC/Rim101 homologue) in both *C. albicans* and *A. fumigatus.* Deletion of *ZAP1* (also called *CSR1)* in *C. albicans* and *ZafA* (ZAP1 homologue) in *A. fumigatus* had reduced disease development (Moreno et al., 2007; Nobile et al., 2008). Similarly, deletion mutants of pH sensitive transcription factor, *PacC* in *A. fumigatus* and *RIM101* in *C. albicans),* also had reduced virulence (Bignell et al., 2005; Nobile et al., 2008), confirming that zinc acquisition under neutral and alkaline pH plays an important role in disease development by these fungal pathogens.

Though both PRA1 and Aspf2 bind zinc, no protease activity was noticed in PRA1 or Aspf2 in the presence of metallo-protease substrate casein (Amich et al., 2010; Citiulo et al., 2012). This observation is further supported by a similar lack of protease activity in BEC1019 (Whigham et al., 2015). This could be due to the glutamic acid to arginine substitution in the HRXXH domain of these proteins as compared to HEXXH domain in M35 metallo-protease family, resulting in the loss of the protease catalytic site (Citiulo et al., 2012).

The BEC1019 homologues also share a conserved ETVIC domain (Figure 5-2), which was shown to mediate the suppression of HR in barley, though later retracted (Whigham et al., 2015). Therefore, these findings suggest a putatively conserved universal virulence function of BEC1019 homologues in pathogenic fungi. However, this hypothesis needs to be further strengthened with experimental proof and the BEC1019 mode of action remains to be elucidated.

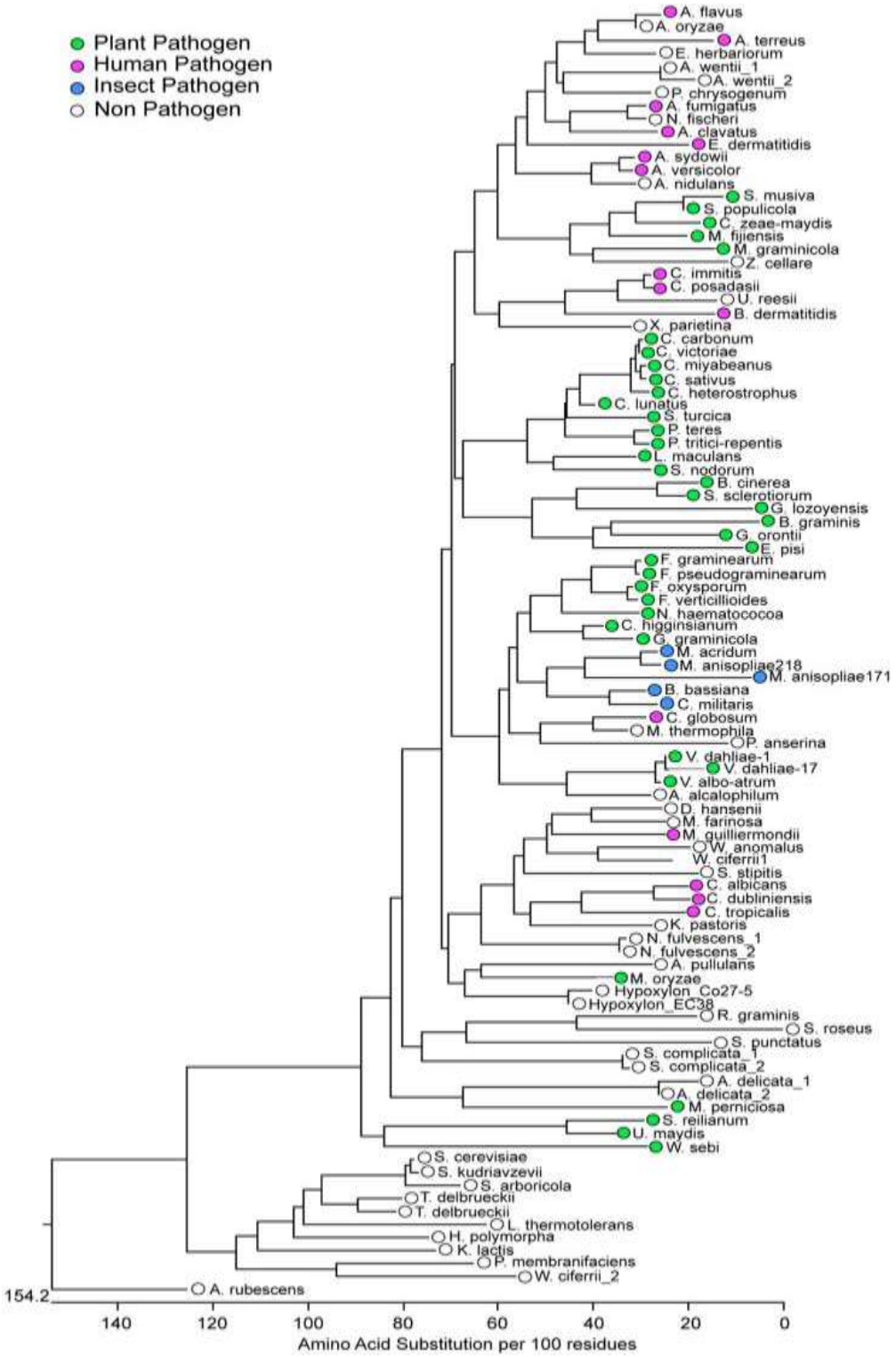


Figure 5-1: BEC1019 homologues in kingdom fungi.

Phylogenetic tree confirming presence of BEC1019 homologues in numerous pathogenic, non-pathogenic and free-living fungal species. The tree shows 95 different BEC1019 orthologues identified by a blastp query in the NCBI redundant database. The tree was generated based on a multiple alignment generated using MegaAlign. (Whigham et al., 2015)

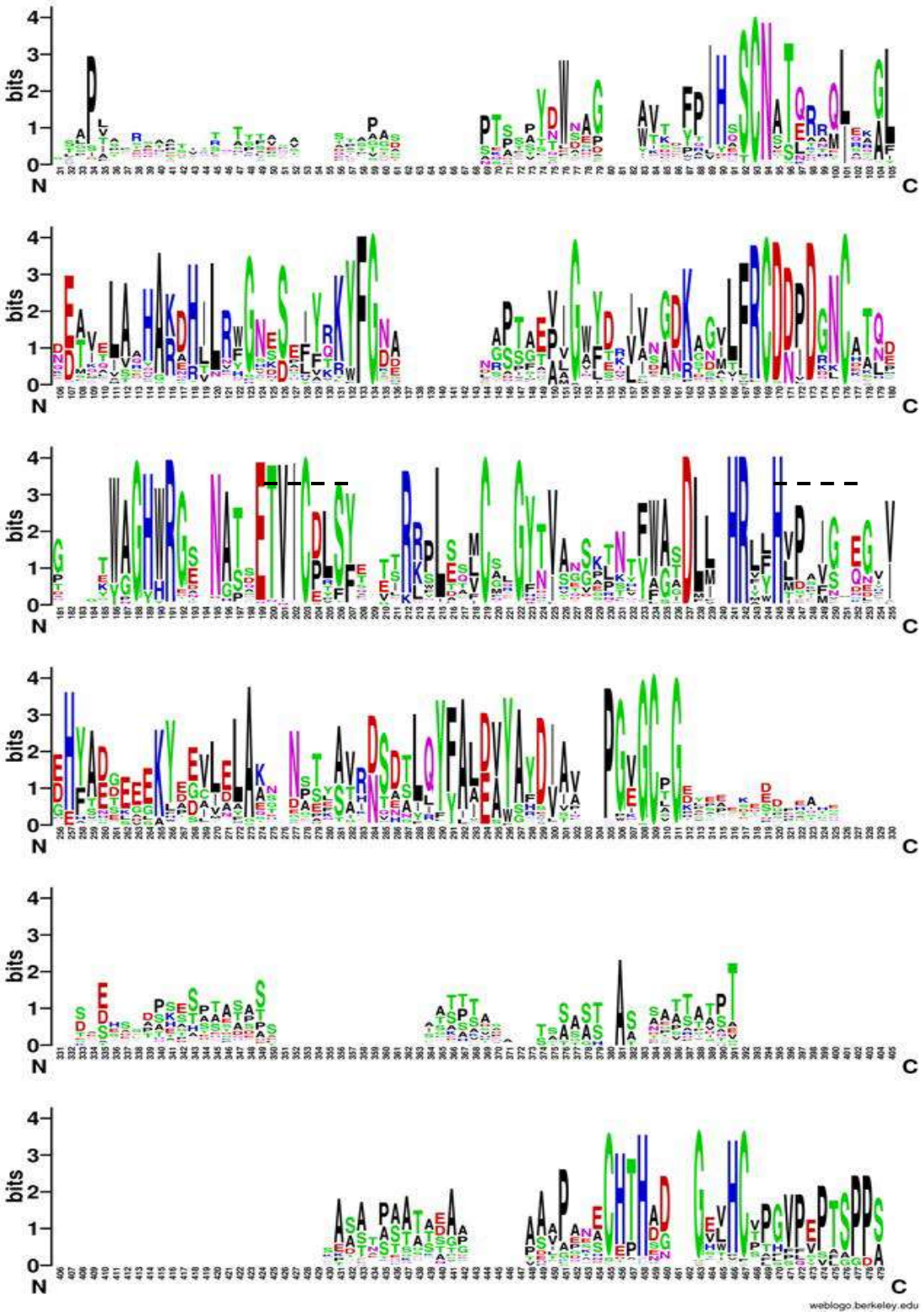


Figure 5-2: Sequence logo of BEC1019 homologues.

A partial sequence logo generated using WebLogo illustrating the position of the conserved amino acid residues in the different BEC1019 homologues. The sequence logo was generated based on multiple alignment data generated through MegaAlign. The height of the letters corresponds to the degree of conservation. A conserved HRXXH and ETVIC domains are marked using dotted lines.(Whigham et al., 2015)

5.1.3 Role of zinc as a trace element in fungi

BEC1019 homologue PRA1 in *C. albicans* and AspF2 in *A. fumigatus* were shown to be important for sequestration of host zinc facilitating fungal survival under zinc limiting conditions (Amich et al., 2010; Citiulo et al., 2012). *C. albicans* was microscopically shown to sequester host zinc, which is dependent on the zinc-binding PRA1 protein, in the absence of which the fungus is unable to grow under zinc limiting conditions (Citiulo et al., 2012). Therefore, zinc availability is an important factor governing fungal growth and virulence. This necessitates the understanding of the importance of zinc during fungal growth to better understand the putative role of BEC1019 homologues in fungal zinc sequestration.

Zinc is an essential trace element in fungi, which impacts both fungal growth and virulence (Staats et.al., 2013). Fungal zinc is mostly available bound to zinc binding proteins, which account for approximately 5% of the predicted proteome of *S. cerevisiae* (Cherry et al., 2012). Twenty five percent of these zinc binding proteins are zinc-finger containing transcription factors which have been shown to regulate essential processes like amino acid metabolism, nitrogen assimilation, mitosis and meiosis in *S. cerevisiae* (MacPherson et. al., 2006). Zinc binding proteins also include metabolic enzymes such as DNA/RNA polymerases, alcohol dehydrogenase and superoxide dismutase, which are most abundant zinc binding proteins in *S. cerevisiae* (Staats et.al., 2013). Superoxide dismutase neutralises host-induced ROS and therefore protects the fungal pathogen against host immune response (Huang et. al. 2009). SOD1 deletion mutants in *C. albicans* showed reduced virulence and increased susceptibility to host induced ROS (Kang et al., 2015). Although direct effect of zinc deprivation in fungi is not very well understood, zinc deficiency in *S. cerevisiae* impacted lipid metabolism, methionine and sulphate metabolism and stress tolerance (Iwanyshyn et.al., 2004; Wu et.al., 2007; Wu et al., 2009). Therefore, zinc availability plays an important role in fungal growth and pathogenicity.

5.1.4 Role of zinc as a trace element in plants

As mentioned earlier, fungal pathogens like *C. albicans* and *A. fumiagtus* compete for the available zinc with the human host to ensure pathogen survival under zinc limiting conditions (Amich et al., 2010; Citiulo et al., 2012). The human host counters this by expressing a zinc-binding calprotectin protein, which sequesters zinc away from the pathogens, limiting their growth and infection spread. Calprotectin was shown to be associated with *Aspergillus*-induced neutrophil traps in humans, which limits the fungal infection spread (McCormick et al., 2010). Therefore, zinc homeostasis is an important factor governing host-pathogen interactions. This necessitates the need to understanding the role of zinc in plant hosts to better understand the putative role of BEC1019 in fungal zinc sequestration and virulence.

The importance of zinc in plant growth was first demonstrated in maize, followed by barley and dwarf sunflower (reviewed by Broadley et.al., 2007). In plants, zinc plays a part in function of enzymes (hydrogenases, carbonic anhydrases), stability of ribosomes and synthesis of cytochromes (Tisdale et.al., 1985). Therefore, it influences carbohydrate metabolism, protein synthesis, cell membrane integrity, regulation of growth hormones like auxin, pollen development and environmental stress tolerance (Marschner & Marschner, 2012). Its role in root water uptake and transport in plants influences drought tolerance (Disante et.al., 2011). Zinc availability is also known to alleviate salt and heat stress (Peck & McDonald, 2010; Tavallali et.al., 2010). In salt-stressed pistachio leaves, exogenous zinc application alleviated stress symptoms by increasing the activity of antioxidant enzymes such as superoxide dismutase, catalase and ascorbate peroxidase (Tavallali et al., 2010). Similarly, in zinc deficient wheat, the heat stress symptoms were more pronounced than in wheat supplied with adequate amounts of zinc in their nutrition (Peck & McDonald, 2010). Zinc is essential for tryptophan synthesis, a precursor for the auxin IAA, therefore impacting growth hormone regulation in plants (Cheng Tsui, 1948; Nason, 1934). Moreover, zinc interacts with phospholipids and sulphydryl groups of membrane proteins, facilitating membrane stability (Cakmak & Marschner, 1988).

Zinc deficiency is the most common micronutrient deficiency in plants, especially in high pH soils of India, China, Turkey and Western Australia (Alloway, 2002). It has a direct impact on plant growth and on human nutrition (Welch & Graham, 2004). Symptoms include stunted growth, reduced yield (Fageria, 2004), poorer harvested product quality and increased susceptibility to biotic and abiotic stress (reviewed by Broadley et. al., 2007). Conversely, excessive zinc causes phytotoxocity, resulting in reduced photosynthesis and yield (Chaney, 1993). Therefore, zinc plays a significant role in growth and stress tolerance in plants.

5.1.5 Zinc based immune response in humans, plants, bacteria and fungi

Competition for zinc plays an important role in host-pathogen interactions in human fungal pathogens (reviewed by Staats et. al., 2013) The mammalian immune system uses both zinc starvation and zinc overdose as a method of limiting pathogen growth. Macrophages use zinc toxicity induced by zinc overload to induce microbial cell lysis (Botella et al., 2011). The human immune system also produces the zinc-binding protein calprotectin to limit the available zinc in the local vicinity of the fungal pathogens (Corbin et. al. 2008). For instance, neutrophils secrete calprotectin to restrict the growth of *A. fumigatus* (McCormick et al., 2010)*, C. albicans* (Urban et al., 2009) and *Cryptococcus neoformans* (Mambula et.al., 2000). Moreover, zinc internalisation and compartmentalisation by cytokine granulocyte macrophage colony stimulating factor (GM-CSF) is also used to restrict growth and mitigation of fungal pathogens like *Histoplasma capsulatum*

(Vignesh et.al., 2013). Zinc starvation is believed to interfere with the functioning of important fungal transcription factors and enzymes (Staats et al., 2013). Zinc acts as a cofactor for superoxide dismutase (SOD), the enzyme that converts O_2^- to H_2O_2. O_2^- is an important form of host generated ROS, which when produced in excess neutralises the pathogen. Fungal superoxide dismutase detoxifies the host generated ROS, to facilitate fungal virulence (Huang et. al. 2009). Therefore, pathogen zinc starvation induces ROS -mediated pathogen neutralisation. This mechanism of host mediated modulation of micronutrient availability for restricting pathogenic growth is called nutritional immunity (Hood & Skaar, 2012). Apart from direct use of excess zinc as an anti-microbial agent, zinc modulation also plays a part in activation of defence mechanisms, e.g. activation of mycroglia (Kauppinen et al., 2008) and dendritic cells (Kitamura et al., 2006) which are important for pathogen neutralisation.

Nutritional immunity in plants is not very well understood. However, zinc/manganese binding protein metallothionine is upregulated in *Arabidopsis*, tobacco and the velvet leaf during infection (Dauch & Jabaji-Hare, 2006). Similarly zinc-binding phytochelatins have been linked to basal immune responses, where phytochelatins were shown to be important for callose deposition during non-host resistance in *Arabidopsis* against bacterial pathogens (Clemens & Peršoh, 2009).

Human fungal pathogens have similarly developed a zinc sequestration mechanism to cope with nutritional immune responses. Zinc sensitive transcription master regulators (ZAP1 homologues), zinc scavengers (*Aspf2, PRA1)* and zinc transporters (ZIP proteins) are activated to enhance zinc sequestration by the pathogen and subsequent fungal virulence of *A. fumigatus and C. albicans* (reviewed by Staats et al., 2013). In the bacterial phytopathogens, a zinc sensitive global transcription regulator Zur regulates bacterial zinc homeostasis preventing zinc uptake and inducing zinc efflux (Haatke et. al. 2001, 2005; Hung et. al. 2008). *Zur* deletion mutants in *Xanthomonas oryzae* and *X. campestris* have increased zinc sensitivity and reduced virulence (Yang et. al. 2007; Tang et. al. 2005). The virulence effect in this instance might be due to reduced extracellular polymeric substance (EPS) production by the mutant (Yang et. al. 2007). Zur regulates hypersensitive reaction and pathogenicity related genes (*hrp* genes) in *X. campestris* (Huang et. al. 2009). The *Hrp* gene, hypersensitivity response conserved (*hrc*) encodes a type 3 secretion system (T3SS) important for effector delivery into host cells. Therefore, Zur has a direct impact on *X. campestris* pathogenicity, by regulating the expression of the effector delivery system. Zur protein also regulates the expression of T3SS genes in the animal pathogen *Salmonella enterica* (Ellermeir and Slauch, 2008).

5.1.6 ZAP1, a zinc-sensitive transcription factor

During fungal growth and virulence, zinc is essential as a structural and functional component of important proteins as described in section 5.1.3. Therefore, the "zinc quota" or cellular zinc level needs to be tightly regulated. Fungal pathogens have a remarkably similar and divergent method of maintaining zinc homeostasis. ZAP1, a zinc finger containing transcription factor first discovered in *S. cerevisiae,* plays an important role in maintaining zinc quota in cells (Hui Zhao et al., 1998). In *S. cerevisiae* ZAP1 contains 7 zinc-finger domains and 2 activation domains, which are believed to be important for ZAP1 activity (Figure 5-4) (Bird et al., 2003; Moreno et al., 2007). During zinc limitation, ZAP1 is activated by positive autoregulation and induces a zinc uptake system encoded by ZIP transporter genes *Zrt1, Zrt2* and *Fet4* (Waters & Eide, 2002; H Zhao & Eide, 1996a, 1996b) and vacuolar zinc influx and efflux transporters encoded by the *ZRC1* and *ZRT3* genes, respectively in *S. cerevisiae* (MacDiarmidet. al., 2000; MacDiarmid et.al., 2002). Subsequently, ZAP1 orthologues were identified and studied in other fungal pathogens like *Candida albicans (Csr1), Candida dubliniensis (Csr1), Aspergillus fumigatus (ZafA), and Cryptococcus deuterogatti (ZAP1),* where they play a role in fungal virulence by regulating expression of zinc transporters (*ZIP* genes) and zinc scavengers *(PRA1/AspF2*) (Böttcher et.al., 2015; Schneider et al., 2012; Kim et.al. 2008; Moreno et al., 2007; Amich et al., 2010; Citiulo et al., 2012).

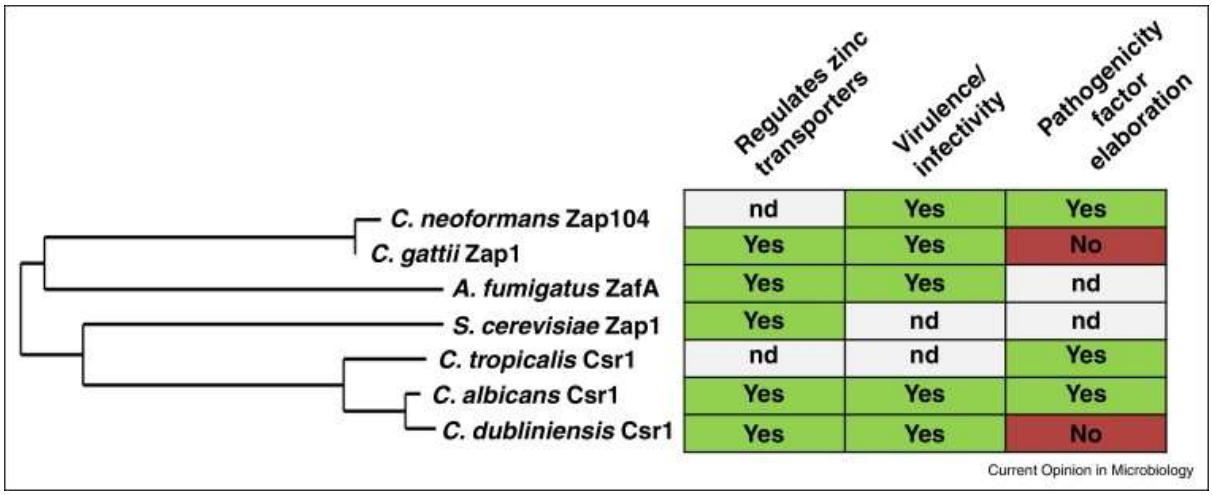


Figure 5-3: Phylogenetic and functional relationship between ZAP1 orthologues in fungi *(Ballou & Wilson, 2016).*

Despite such conservation in its activity as a regulator of zinc assimilation and virulence, ZAP1 orthologues in certain related fungal pathogens have different roles for their regulated genes. Two related fungal pathogens *C. neoformis* and *C. deuterogatti* have three virulence factors with roles in infectivity, capsule formation and melanin production. Deletion mutants of ZAP1 orthologue in *C. neoformis* lack capsules and melanin (Jung et al., 2015; Liu et al., 2008), whereas *C. deuterogatti*

ZAP1 orthologue is dispensable for capsule and melanin synthesis (Schneider et al., 2012). Therefore, these studies show a variance in the impact of ZAP1 on virulence factors (involved in capsule formation and melanin pigmentation) in related fungal pathogens. Similarly, despite diverging from Basidiomycetes about 500 mya (Taylor & Berbee, 2006), ZAP1 orthologues in *C. albicans* and *C. neoformis* influence virulence attributes such as hyphal morphogenesis, adhesion and biofilm maturation (Kim et.al., 2008), which is not regulated by ZAP1 in other *Candida sp* (Q. Zhang et al., 2016). Therefore, fungal ZAP1 orthologues have a conserved, likely main role in regulation of zinc homeostasis leading to fungal virulence, with an additional role influencing other virulence factors in some fungal pathogens. The phylogenetic and functional relationship between fungal ZAP1 orthologues in described in Figure 5-3.

5.1.7 Zinc responsive elements (ZREs) and regulation by ZAP1

ZAP1 regulates zinc sensitive genes, including itself, by binding to Zinc Responsive Elements (ZREs) in their promoter regions (Wu et al., 2008; Zhao et al., 1998). In *S. cerevisiae,* ZREs have a core sequence of 5'- ACCTTNAAGGT-3' (Zhao et al., 1998). ZAP1 autoregulates itself by binding to ZREs present in its promoter region, a phenomenon regulated by zinc availability (Frey et.al., 2011; Zhao et al., 1998). The expression of zinc sensitive genes is dependent on the nature of the ZREs present in their promoter region i.e. ZREs of high or low sensitivity. ZAP1 activated by zinc deprivation, first activates the genes encoding proteins needed for zinc homeostasis, which are expressed under low to medium zinc levels and have highly sensitive ZREs. Genes expressed under severe zinc starvation have less sensitive ZRES and are activated after rapid amplification of ZAP1 caused by severe zinc limitation (Wu et al., 2008).

ZAP1 homologues are characterised by the presence of five or more ZRE/DNA binding C_2H_2 type zinc finger domains (ZF) and two N-terminal trans-activation domains (AD) (Figure 5-4) (M. Á. Moreno et al., 2007; Hui Zhao et al., 1998). Both the ADs and ZFs are zinc sensitive and play a part in activation of ZAP1 by binding to ZREs (Bird et al., 2000; Bird et al., 2003; Frey et. al., 2011; Herbig et al., 2005).

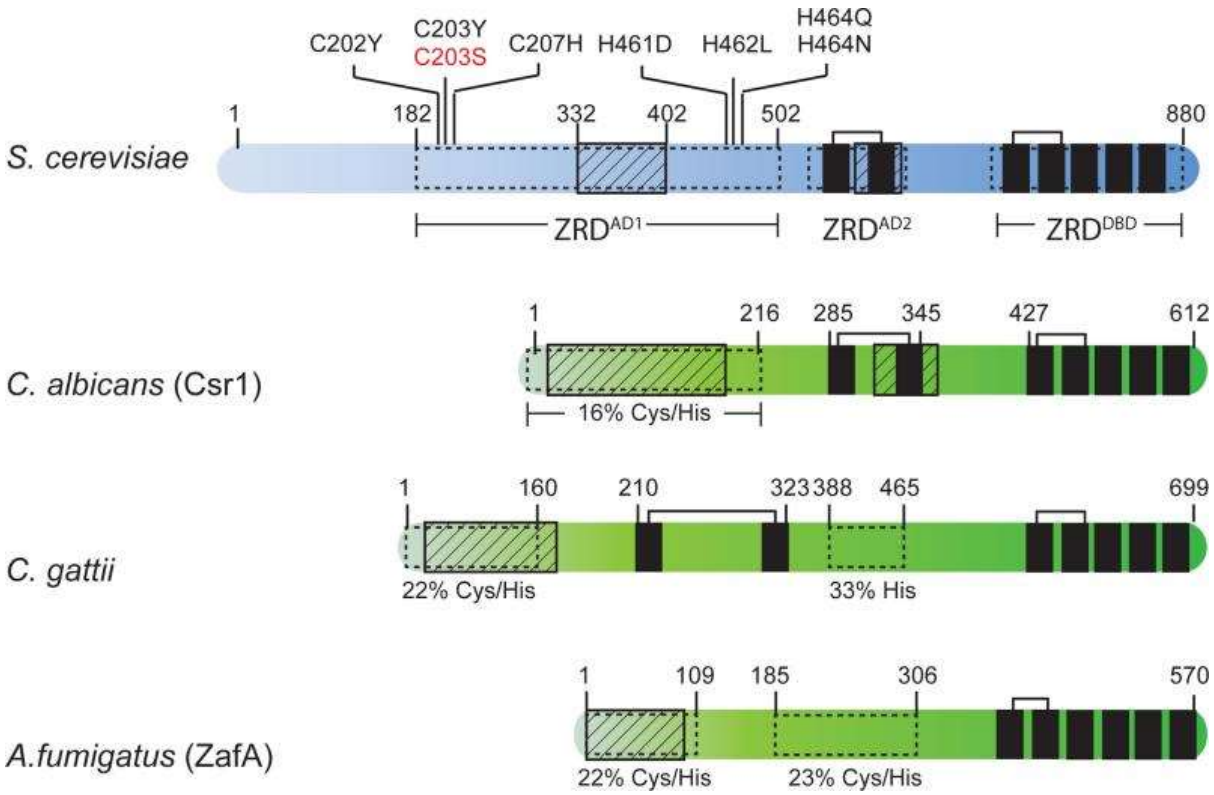


Figure 5-4: Gene structure with domain positions in fungal ZAP1 homologues.

Activation domains (patterned box) and zinc finger domains (solid black boxes) needed for zinc mediated activation and ZRE binding. (Wilson & Bird, 2016)

5.1.8 Role of pH sensitive transcription factor (PacC) in fungal zinc sequestration

PRA1/AspF2 zinc binding BEC1019 homologues are regulated by ambient pH, where they are activated under neutral to alkaline pH conditions. In fungi, the expression of alkaline/neutral activated genes is mainly regulated by the transcription factor PacC/RIM101.

PacC in *Aspergillus sp.* or RIM101 in *S. cerevisiae* is a pH sensitive C_2H_2 zinc finger containing transcription factor which is conserved in the kingdom fungi. *PacC* is activated under neutral to alkaline conditions and induces genes required for fungal growth under neutral/alkaline pH, while supressing genes needed under acidic conditions (Tilburn et al., 1995). In human pathogens like *A. fumigatus* and *C. albicans,* PacC/RIM101 is essential for fungal virulence under alkaline conditions (Bignell et al., 2005; Nobile et al., 2008). It facilitates virulence by regulating zinc transporters (*ZrfC/Zrt1*) and zinc scavengers (*AspF2/PRA1*) required for host zinc sequestration under alkaline conditions (Amich et al., 2010; Citiulo et al., 2012), while supressing the expression of zinc transporters active under acidic conditions (*ZrfA, ZrfB* in *A .fumigatus*) (Amich et.al., 2009). No effect of PacC deletion was seen on the expression of zinc sensitive transcription factor *ZafA* (ZAP1 homologue) in *A. fumigatus,* suggesting that the expression of *ZafA* is not dependent on pH or PacC (Amich et al., 2009).

PacC regulates gene expression by binding to conserved pH regulatory elements (PREs) in the promoter region of the genes (Espeso et al., 1997). PREs have a conserved 5'-GCCARG-3' sequence, where 'R' stands for either A or G (Espeso et al., 1997).

The role of PacC in fungal growth and virulence in different fungal pathogens is discussed in detail in chapter 6.

The mechanism of fungal zinc sequestration under alkaline conditions is described in the Figure 5-5 based on the studies in *C. albicans* and *A. fumigatus.*

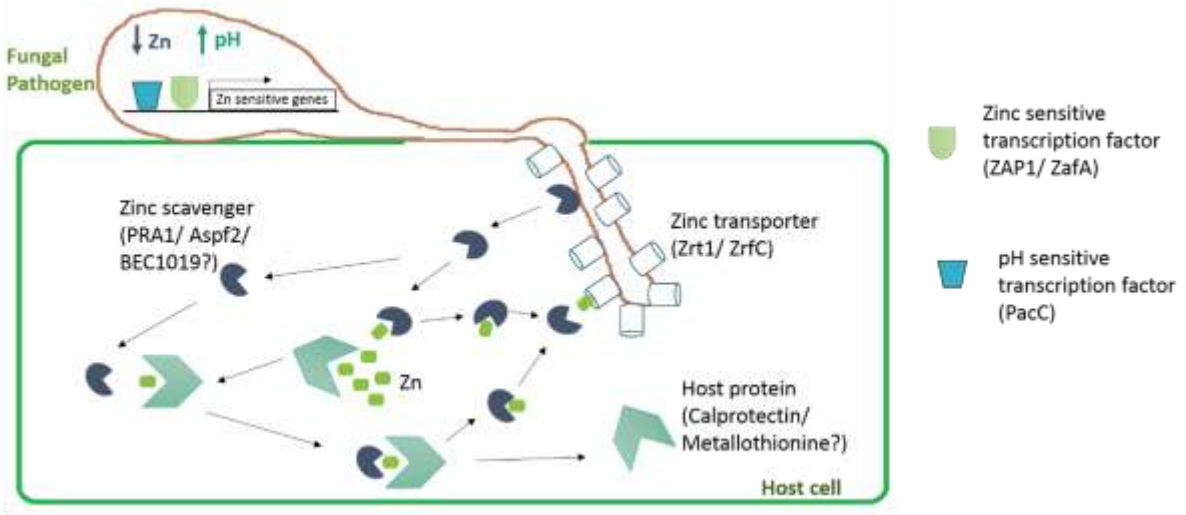


Figure 5-5: Zinc sequestration model in C. albicans

Under neutral to alkaline pH and zinc starvation, PRA1/AspF2 (a BEC1019 homologue) expression is induced by pH responsive (PacC/RIM101) and zinc responsive (ZafA/ZAP1) transcription factors to compete for available zinc in the host cell. PRA1/AspF2 sequester the zinc and transport it into the fungal cells by zinc transporter (Zrt1), to favour fungal growth and virulence. Human hosts produce zinc-binding calprotectin protein to limit the availability of zinc to the fungal pathogen to limit disease spread. Diagram adapted from Citiulo et al., 2012.

5.2 Objectives and experimental rationale

5.2.1 BEC1019, ZAP1, Zrt1 and PacC expression studies during growth and infection of *Blumeria, Fusarium, Zymoseptoria*

With the aim of querying the role of BEC1019 homologues in *Blumeria graminis, Zymoseptoria tritici and Fusarium graminearum* genes involved in fungal virulence and zinc homeostasis the homologues of the putative zinc scavenger BEC1019, the putative zinc transporter Zrt1 and the transcription factors PacC and ZAP1 were identified in the fungal species under study. Their *in-planta* expression during fungal infection was studied either experimentally or by analysing available RNA seq/ microarray data from the literature, to understand their role in fungal virulence. The *in vitro*

expression of BEC1019 homologues in *Z. tritici* and *F. graminearum* will also be investigated to study their regulation by zinc and alkaline pH.

5.2.2 *Blumeria* gene silencing workflow to query virulence role of effectors such as BEC1019 in wheat and barley powdery mildews

In the obligate biotrophic pathogen *B. graminis* f.sp. *tritici,* in the absence of transformation protocols, an antisense phosphorothioate modified oligodeoxynucleotide (PTO) based host induced gene silencing (HIGS) method was adapted to study the virulence role of *Bgt BEC1019* during wheat powdery mildew infection. This method was translated from an already established *in-planta* gene silencing methodology previously developed in our lab to silence *B. graminis* f.sp. *hordei* effectors and virulence factor such as *Bgh BEC1019* (Orman et. al., manuscript in prep).

5.2.3 Gene silencing of ZAP1 and PacC transcription factor to elucidate BEC1019 gene expression regulation.

In order to understand the role of BEC1019 for zinc homeostasis in *Blumeria*, we hypothesised that BEC1019 may be regulated by the homologues of zinc sensitive transcription factor ZAP1 and pH sensitive transcription factor PacC. This was queried by monitoring the change in *BEC1019* transcript levels post silencing of *Bgh* ZAP1 and *Bgh PacC* using an established PTO based HIGS method in barley-powdery mildew pathosystem. This allowed us to investigate whether BEC1019 expression is influenced by zinc availability and ambient pH.

5.2.4 Deletion of BEC1019, ZAP1 to query their virulence role in *Fusarium* and *Zymoseptoria* infection

With the objective of analysing the role of BEC1019 homologues during *F. graminearum* and *Z. tritici* virulence, BEC1019 deletion mutants were created in these fungal pathogens to query the impact of deletion of *Fg BEC1019* and *Zt BEC1019* on the virulence of these two fungal pathogens. Similarly, the role of ZAP1 in *Z. tritici* virulence and in regulating the expression of *Zt BEC1019* were queried by creating *Zt ZAP1* deletion mutants and analysing the impact of the deletion on infection development and *Zt BEC1019* expression, both *in vitro* and *in planta*.

5.3 Results

5.3.1 Identification of BEC1019 paralogues in *B. graminis f.sp. tritici, F. graminearum* and *Z. tritici*

The paralogues of *Bgh BEC1019* in *B. graminis* f.sp. *tritici*, *F. graminearum* and *Z. tritici* in were identified by sequence similarity using the BLAST algorithm in the Ensembl Fungi database. The BEC1019 paralogues of barley and wheat powdery mildew share a very high identity of 94% in their

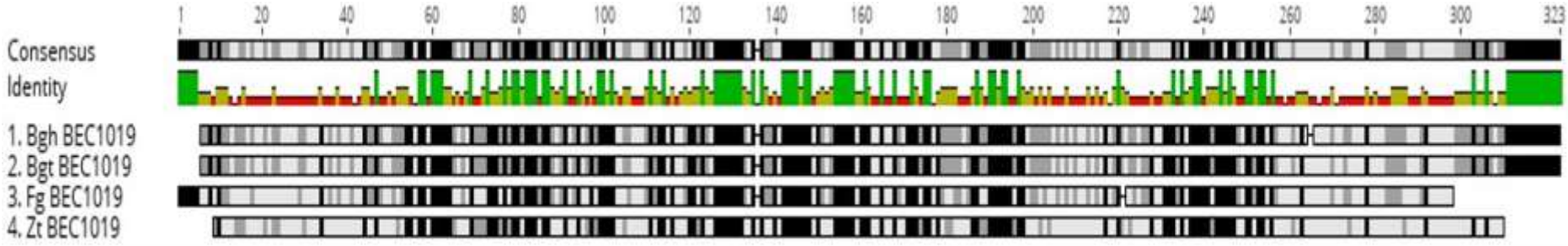
peptide sequences, but the peptide sequence identity of *Zt BEC1019* and *Fg BEC1019* ranged between 35-36% when compared to *Bgh BEC1019 (*Figure 5-6*)*. However, a conserved HRXXH domain was identified in all the homologues (Figure 5-7), which is closely related to the HEXXH domain of the zinc binding M35 superfamily of peptidases. The catalytic site E (glutamic acid) was replaced by a R (arginine) residue (Citiulo et al., 2012; Whigham et al., 2015). The homologues also share a conserved ETVIC domain (Figure 5-7), which was suggested to be important for suppression of host HR in barley but was later retracted (Whigham et al., 2015).

The list of homologues in *B. graminis* f.sp. *tritici*, *F. graminearum* and *Z. tritici* are tabulated in Table 5-1 and the alignments are shown in Figure 5-7. The nucleotide sequence alignments have been included in supplementary Figure 2 (S2).

Table 5-1: Details of the homologues of BEC1019 in B. graminis f. sp. tritici, F. graminearum and Z. tritici , showing the gene id, gene location, protein and nucleotide sequence identity to Bgh BEC1019.The peptide sequences were obtained using BLAST tool in Ensembl Fungi and multiple sequence alignment was performed based on identity using Geneious bioinformatics software.

Pathogen	Gene Id	Locus	Identity in Protein Sequences to *Bgh BEC1019*	Identity in Nucleotide Sequences to *Bgh BEC1019*
Blumeria graminis f. sp. tritici	EPQ66538.1 (GenBank)	Not available	94%	91%
Fusarium graminearum	FGRAMPH1_01G14115 (Ensembl Fungi)	Chr2: 6,487,541-6,488,482	36%	50%
Zymoseptoria tritici	Mycgr3T42164 (Ensembl Fungi)	Chr5:224006-225198	37%	49%

A



B

	Bgh BEC1019	Bgt BEC1019	Fg BEC1019	Zt BEC1019
Bgh BEC1019		94%	38%	32%
Bgt BEC1019	94%		38%	32%
Fg BEC1019	38%	38%		35%
Zt BEC1019	32%	32%	35%	

Figure 5-6:Multiple amino acid sequence alignment of Bgh BEC1019, Bgt BEC1019, Fg BEC109 and Zt BEC1019.

A. Multiple amino acid sequence alignment of Bgh BEC1019, Bgt BEC1019, Fg BEC109 and Zt BEC1019.The green blocks on the top and the black in the individual protein sequences denote regions of identity.

B. Values of percentage of identity between the peptide sequences of Bgh BEC1019, Bgt BEC1019, Fg BEC1019 and Zt BEC1019 when compared to each other.

```
Consensus    MQSVLLLTXMXQSFIATASPLVERATPX----LSFAEKRPQXVSYDWTAXYVKDFXIHGS   56
Bgh BEC1019  MQSVLLLTVLTQSFIATASPLVERSTP-----LSFAEKRPQKVSYDWTTPYVKDFTIHGS   55
Bgt BEC1019  MQSVLLLTIVTQSFIATASPLVERATP-----LSFAEKRPQRVSYDWTAPYVKDFIIHGS   55
Fg BEC1019   MMFKSTTAAMLLFGAATATPLFGRAEASQTKSASQSSKTSESSSYNWSEGWTKDYPIHQS   60
Zt BEC1019   ---------MLTSLLLTAIGASAAALPSVPSLETRQAPFEVLQAAPWDAGAVTEWQIHPS   51

Consensus    CNATQXNXLRRGLXXAVTLAQHAKEHILXHGKKSXIYQKYXGXLPTGAVIGWXDTIAXAB  116
Bgh BEC1019  CNATQTNVLRRGLEDAVTLAQHAKEHILVHGKESPIYQKYYGALPTGAVIGWFDTIATAN  115
Bgt BEC1019  CNATQTNVLRRGLEDAVTLAQHAKEHILVHGKKSPIYQKYYGALPTGAVIGWFDTIATAN  115
Fg BEC1019   CNATLRHQLSSALDETVQLAQHAKDHILRHGHKSEFFTKYFGNASTSQPIGWYDRVVNAD  120
Zt BEC1019   CNSSERLQLQQGLDEAVTLATHAKAHINRWGNSSEVYRKYFGNSPPFEAAGAYDIIINGD  111

Consensus    RAGVXFRCDDPDKXCAT-EBRWAGHWRGKBAXSETVICXXSFFERLPLEDLCSRGYXXAT  175
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Bgh BEC1019 RAGVTFRCDDPDKKCAT-ENRWAGHWRGKDAPSETVICDVSFFERLPLEDLCSRGYKIAT  174
Bgt BEC1019 RAGVTFRCDDPDKKCAT-ENRWAGHWRGKDAPSETVICDVSFFERLPLEDLCSRGYRIAT  174
Fg BEC1019  KTGVLFRCDDPDKNCAT-QDAWAGHWRGDNATSETVICPLSFEIRRNLDSVCNLGYTVAN  179
Zt BEC1019  RAGALFRCDDPDGNCAAFPDEWAGHYRGPNGTHETVICPLSYTSRQHLSQICSQGYDVRT  171

Consensus   GKVYSYWGADLIHRMFHVXIVGQNA-IXHAXXGYQDALXLAXXQNYTQXATBXDSLXYFA  234
Bgh BEC1019 GKVYSYWGADLIHRMFHVDIVGQNA-ITHASHGYQDALNLAAGQNYTQTATNTDSLIYFA  233
Bgt BEC1019 GKVYSYWGADLIHRMFHVDIVGQNA-ITHASHGYQDTLKLAAGQNYTQTATNTDSLIYFA  233
Fg BEC1019  SKLNTFWATDLLHRVLHVPIISEKT-VDHFAENYTDAIALAK-SDPSKSVIDSDALQYFA  237
Zt BEC1019  GSRTLYWGSDLIHRLYHMPAYGANGYIDHYAEGWAGIVTAAK-EGSPNSTHDSDGLQFFA  230

Consensus   VEAYAXDISVPGEGCAGQAXSIXXXEKXTPTXPNALPDSIXIPSPKEPEKKDXPEKKAEP  294
Bgh BEC1019 VEAYAFDISVPGEGCAGQASSIPDT-KVTPTDPNALPDSIVIPSPKEPEKKDEPEKKAEP  292
Bgt BEC1019 VEAYAFDISVPGEGCAGQAPSIPDTIAIPPTAPNTLPDSIVIPSPKEPEKKDEPEKKAEP  293
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Fg BEC1019   IDVWAYDIAAPGEGCTGEVEDETEEEKPTATKSDSSKPSATKEAPKECHTHDDGVVHCS-   297

Zt BEC1019   LEAYAYDVIYPGVGCPGSNAPVTEEEMEGSGGHGAAAAATTTSMAAAPAAVTDAPA----   286


Consensus    EKKAEPEAXGKNCHTHDDGEVHCV                                      318

Bgh BEC1019  EKKAEPEALGKNCHTHDDGEVHCV                                      316

Bgt BEC1019  EKKAEPEAPGKNCHTHDDGEVHCV                                      317

Fg BEC1019   ------------------------                                      296

Zt BEC1019   ---------GQNCHTHANGELHCT                                      301
```

Figure 5-7: Sequence view of amino acid sequence alignment of Bgh BEC1019, with Bgt BEC1019, Fg BEC1019 and C. Zt BEC1019.

The conserved zinc binding HRXXH domain and an ETVIC domain are highlighted in each alignment.

5.3.2 Identification of homologues of zinc sensitive transcription factor ZAP1 and zinc transporter Zrt1 in *B. graminis*, *F. graminearum* and *Z. tritici.*

Since BEC1019 homologues PRA1 was shown to physically interact with high affinity zinc transporter Zrt1 in *C. albicans*, to sequester zinc and contribute towards fungal virulence, the homologues of zinc transporter Zrt1 were identified in *B. graminis* f.sp. *tritici*, *F. graminearum* and *Z. tritici.* The expression of PRA1 and Zrt1 was coregulated by zinc through zinc sensitive transcription factor ZAP1 (also called Csr1) in *C. albicans* (Citiulo et al., 2012), therefore, ZAP1 homologues were also identified in the fungal pathogens under study.

Zrt1 homologues were identified by sequence similarity using the BLAST algorithm in the Ensembl Fungi database whereas ZAP1 homologues were identified by sequence similarity and the presence of 5 or more zinc finger domain (M. Á. Moreno et al., 2007). The ZAP1 homologues showed a very low identity in their peptide sequences, but possessed at least 5 zinc-finger domains (Table 5-2).The homologues are tabulated in Table 5-2 and Table 5-3.

Table 5-2: Homologues of zinc sensitive transcription factor (ZAP1)

Details of the homologues of zinc sensitive transcription factor ZAP1 in B. graminis f.sp. *hordei, B. graminis* f.sp. *tritici, F. graminearum and Z. tritici, including the gene id, gene position, number of zinc finger domains and the peptide sequence identity to A. nidulans ZAP1 homologue.*

Pathogen	Gene Id	Locus	No. of zinc finger domains	Identity in Protein Sequences
Blumeria graminis f. sp. hordei	BGHDH14_bgh03555 (Ensembl Fungi)	SuperContig HF943534: 483,381-485,735 reverse strand	6	14 %
Blumeria graminis f. sp. tritici	BGT96224_3498 (Ensembl Fungi)	SuperContig Scaffold-636: 344,517-347,218 reverse strand.	6	14 %
Fusarium graminearum	FGRAMPH1_01T22911 (Ensembl Fungi)	Chromosome 4: 855,785-857,656 forward strand	7	10 %
Zymoseptoria tritici	Mycgr3T89374 (Ensembl Fungi)	Chromosome 5:224006-225198	5	8 %

In *A. fumigatus* and *C. albicans*, the zincophores PRA1 and AspF2 (BEC1019 homologue) were genetically linked with a neighbouring zinc transporter which is a homologue of ZRT1 (Amich et al., 2010; Citiulo et al., 2012). The shared promoter region contains zinc sensitive as well as pH sensitive elements (Amich et al., 2010). Such genetic linkage was only found between *Zt* BEC1019 and *Zt Zrt1* (Table 5-1; Table 5-3).

Table 5-3: Homologues of zinc sensitive transporter (Zrt1)

Gene accession numbers and loci of zinc transporter *Zrt1 paralogues* in *B. graminis, F. graminearum* and *Z. tritici.*

Pathogen	Gene Id (Ensembl Fungi)	Locus
Blumeria graminis f. sp. *hordei*	BLGH_04848	SuperContig scaffold_41: 307,837-310,021 reverse strand.
Blumeria graminis f. sp. *tritici*	BGT96224_A21343	SuperContig Scaffold-153: 537,629-541,141 reverse strand.
Fusarium graminearum Zrt1 Paralogue 1	FGRRES_02248_M	Chromosome 1: 7,301,811-7,303,814 reverse strand.
Fusarium graminearum Zrt1 Paralogue 2	FGRRES_16634	Chromosome 3: 5,035,263-5,037,088 forward strand.
Zymoseptoria tritici	Mycgr3G100094	Chromosome 5: 227,144-228,308 forward strand.

5.3.3 Identification of homologues of pH sensitive transcription factor PacC in *B. graminis* f.sp. *hordei, B. graminis* f.sp. *tritici, F. graminearum* and *Z. tritici*

Homologues of pH sensitive transcription factor PacC have been shown to promote fungal growth under alkaline conditions and virulence in many fungal species like *Aspergilus sp., Candida sp.* (Bignell et al., 2005; Davis et. al., 2000) and is a negative regulator of virulence in *Fusarium oxysporum* (Caracuel et al., 2003). Since PacC regulates components of zinc homeostasis mechanism in certain fungi (Moreno et al., 2007), PacC homologues were identified in in *B. graminis* f.sp. *hordei*,

B. graminis f.sp. *tritici*, *F. graminearum* and *Z. tritici* (

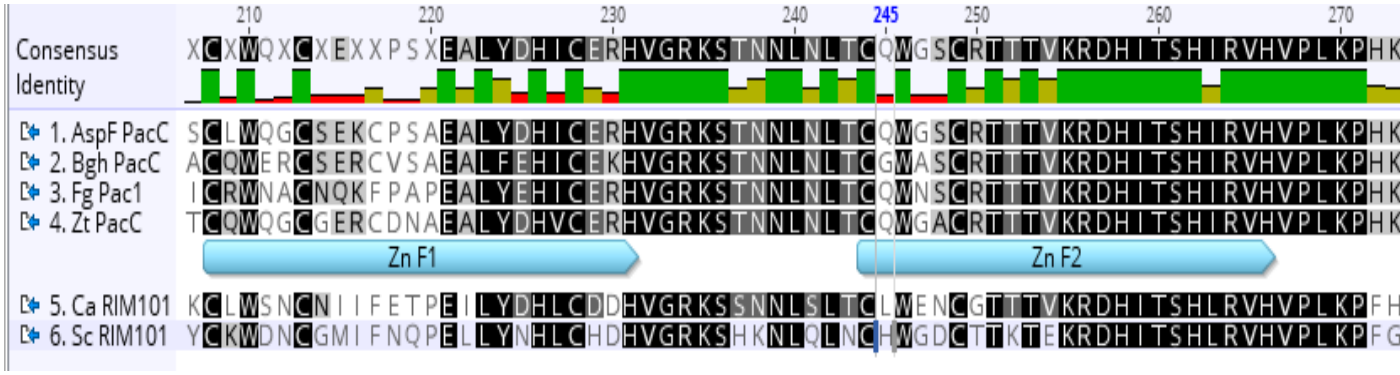

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Table 5-4: Homologues of pH sensitive transcription factor (PacC)

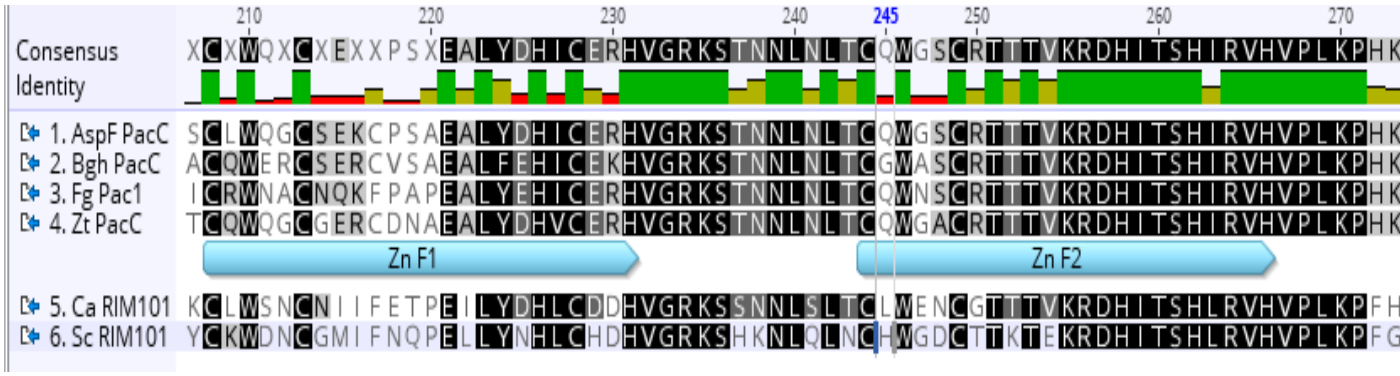
Gene accession numbers and loci of homologues of pH sensitive transcription factor PacC in B. graminis f.sp. hordei, B. graminis f.gp. tritici, F. graminearum and Z. tritici.

Pathogen	Gene Id (Ensembl Fungi)	Locus
Blumeria graminis f.sp. *hordei*	BLGH_01427	SuperContig scaffold_18: 1,632,864-1,635,098 forward strand.
Blumeria graminis f.sp. *tritici*	BGT96224_1512	SuperContig KE375045: 27,848-29,744 forward strand.
Fusarium graminearum	FGRAMPH1_01G22981	Chromosome 4: 946,184-948,800 reverse strand.
Zymoseptoria tritici	Mycgr3T40797	Chromosome 4:380661-381253forward strand.

The protein sequences of these homologues were aligned with PacC homologues in *Aspergillus sp., Candida sp.* and *S. cerevisiae* and the three C2H2 type zinc fingers were identified in each of these homologs (Table 5-5**: Amino acid sequence alignment of PacC homologues**.

A matrix showing the % of peptide sequence identity between PacC homologues of various fungi studied.

). Even though the overall identity of sequences varied between 15% to 35%, the homologues shared two highly conserved zinc fingers (Zinc fingers 2 and 3) (



), which have been shown to be involved in DNA binding (Espeso et al., 1997).

Therefore, this study successfully identified PacC homologues in *B. graminis* f.sp. *hordei, B. graminis* f.sp. *tritici, F. graminearum* and *Z. tritici.*

Table 5-5: Amino acid sequence alignment of PacC homologues.

A matrix showing the % of peptide sequence identity between PacC homologues of various fungi studied.

	A.nidulans P...	A.niger PacC	AspF PacC	Bgh PacC	Bgt PacC	Ca PacC	Fg Pac1_PH1	Sc PacC	Zt PacC
A.nidulans PacC		52%	44%	32%	32%	17%	20%	15%	23%
A.niger PacC	52%		54%	31%	32%	18%	22%	17%	24%
AspF PacC	44%	54%		32%	32%	17%	22%	17%	24%
Bgh PacC	32%	31%	32%		98%	15%	18%	15%	23%
Bgt PacC	32%	32%	32%	98%		16%	18%	16%	23%
Ca PacC	17%	18%	17%	15%	16%		17%	16%	18%
Fg Pac1_PH1	20%	22%	22%	18%	18%	17%		16%	23%
Sc PacC	15%	17%	17%	15%	16%	16%	16%		17%
Zt PacC	23%	24%	24%	23%	23%	18%	23%	17%	

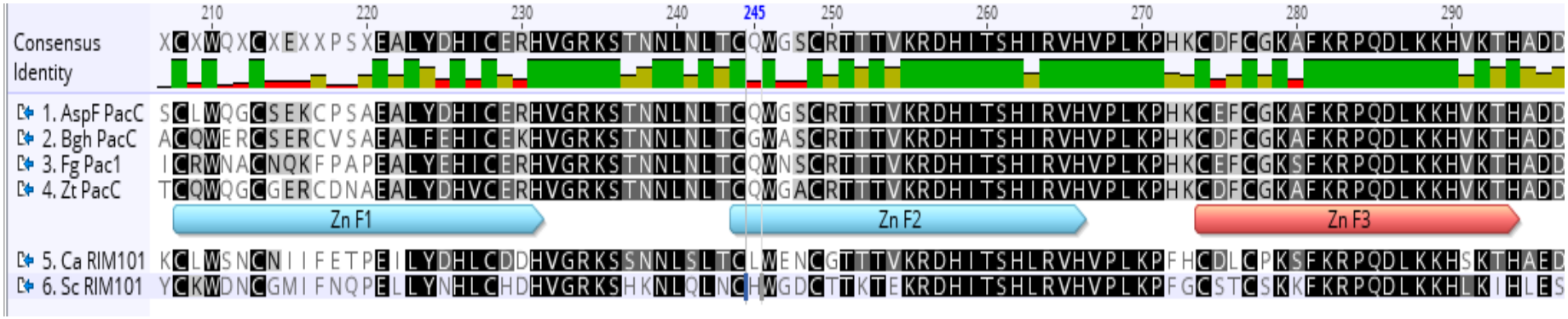


Figure 5-8: Multiple alignment of the three zinc finger domains present in the PacC homologues.

Multiple alignment of the three zinc finger domains present in the PacC homologues. of Aspergillus sp, Candida sp., S. cerevisiae, B. graminis f.sp. *hordei, B. graminis* f.sp. *tritici, F. graminearum and Z. tritici. The regions of identity are denoted as green blocks on the top of the sequences and black blocks in the actual peptide sequences. The zinc fingers are denoted as ZnF1, 2 and 3*

5.3.4 Identification of zinc and pH regulatory elements in the promoter regions of BEC1019 homologues in *B. graminis* f.sp. *hordei, B. graminis* f.sp. *tritici, F. graminearum* and *Z. tritici*

To investigate if BEC1019 homologues in *Blumeria, F. graminearum* and *Z. tritici* may be regulated by ZAP1 and PacC, the presence of zinc (ZRE)s and pH responsive elements (PREs) was queried in the promoter regions of these genes.

Both ZREs and PREs were identified in the promoter regions of *Bgh, Fg* and *Zt* BEC1019 homologues (Figure 5-9), suggesting that these genes may be regulated by both these transcription factors. *Bgt* BEC1019 promoter studies could not be done due to unavailability of reliable sequence data. In *Z. tritici,* these regulatory elements were identified in the intergenic region between *BEC1019* and *Zrt1* (Figure 5-9), suggesting that both the potential zinc scavenger (BEC1019) and zinc transporter (Zrt1) belong to a conserved zinc sequestration locus (Citiulo et al., 2012) and may be co-regulated by ZAP1 and PacC.

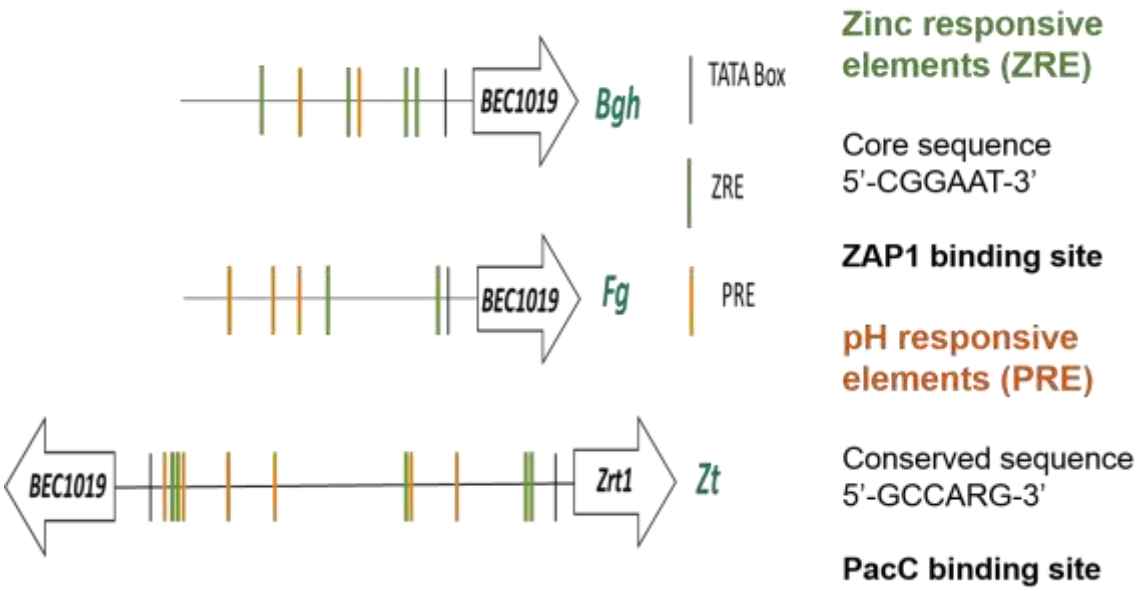


Figure 5-9: Identification of zinc (ZRE) and pH responsive elements (PRE) in BEC1019 promoters.

Prediction of zinc (ZRE) and pH regulatory elements (PRE) known to bind ZAP1 and PacC transcription factors respectively in the promoter regions of BEC1019 homologues in B. graminis f.sp. *hordei, F.graminarum and Z. tritici. Promoter scale – 1 cm = 100 bp.*

5.3.5 Study of *Bgt BEC1019* expression during wheat powdery mildew infection development

The expression of *Bgt BEC1019* (GenBank id: EPQ66538.1) was measured during the wheat powdery mildew infection cycle in wheat. A time course experiment was set-up and samples were collected at early time points of 0, 2 and 4 hpi, prior to appressoria formation. The time-points of 8 and 12 hpi, correspond to times when appressoria formation is being completed, whereas 16, 20 and 24 hpi correspond the window when primary haustoria are developed (Both, Eckert, et al., 2005). Some later time-points of fungal development (48 and 72 hpi) were also studied. The expression of *Bgt BEC1019* was normalised against *Bgt GAPDH* expression levels at each of the timepoints.

BEC1019 was expressed during wheat powdery mildew infection, implying its importance in *Blumeria* infection of wheat. The expression peaked between 16- 24 hpi when the fungal biomass is primarily formed of haustoria. As the infection progressed resulting in increased hyphal biomass, the expression of *BEC1019* decreased (48 and 72 hpi) (Figure 5-10***Figure 5-10***). This implied that *BEC1019* expression maybe localised in *B. graminis* f.sp. *tritici* haustoria.

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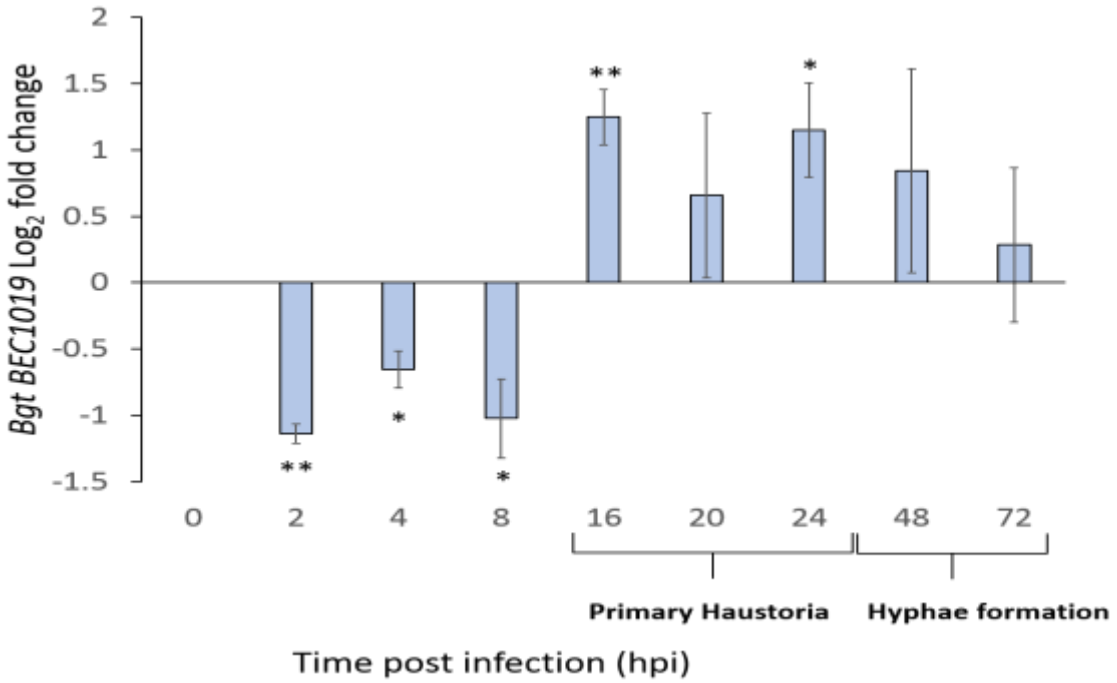


Figure 5-10: Bgt BEC1019 expression time-course at various timepoints during wheat powdery mildew infection. *0-2 hpi correspond to a period prior to appressoria development, 4-8 hpi is the time when appressoria development is complete, primary haustoria is formed in the window between 16-24 hi and at 48-72 hpi, fungal biomass is primarily composed of hyphae. Bgt GAPDH was used as a reference gene for the qRT-PCR. The data is derived from 4 independent biological repeats, where each repeat is an average of 3 leaves for a given time-point. Significance (1 sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

To further investigate this hypothesis, the relative expression of *BEC1019* was measured separately at 7 dpi in isolated *B. graminis* f.sp. *tritici* epiphytic hyphae and infected leaf tissues containing only haustoria (after the epiphytic hyphae and conidia were removed). Infected leaf samples were used as reference values. *Bgt* GAPDH was used as a reference gene for the gene expression studies. The *Bgt BEC1019* expression was ~5-fold higher in *B. graminis* f.sp. *tritici* haustoria as compared to hyphae (Figure 5-11B), confirming its localisation in the fungal haustoria. These findings corroborated with the localisation of *Bgh BEC1019* expression in the haustoria of the biotrophic pathogen (Figure 5-11A) and its discovery as a haustoria-specific protein (Bindschedler et al., 2011). This further supports the hypothesis that BEC1019 may be a secreted virulence factor for *B. graminis* f.sp. *tritici*, as haustoria is the main site of material exchange between the pathogen and the host.

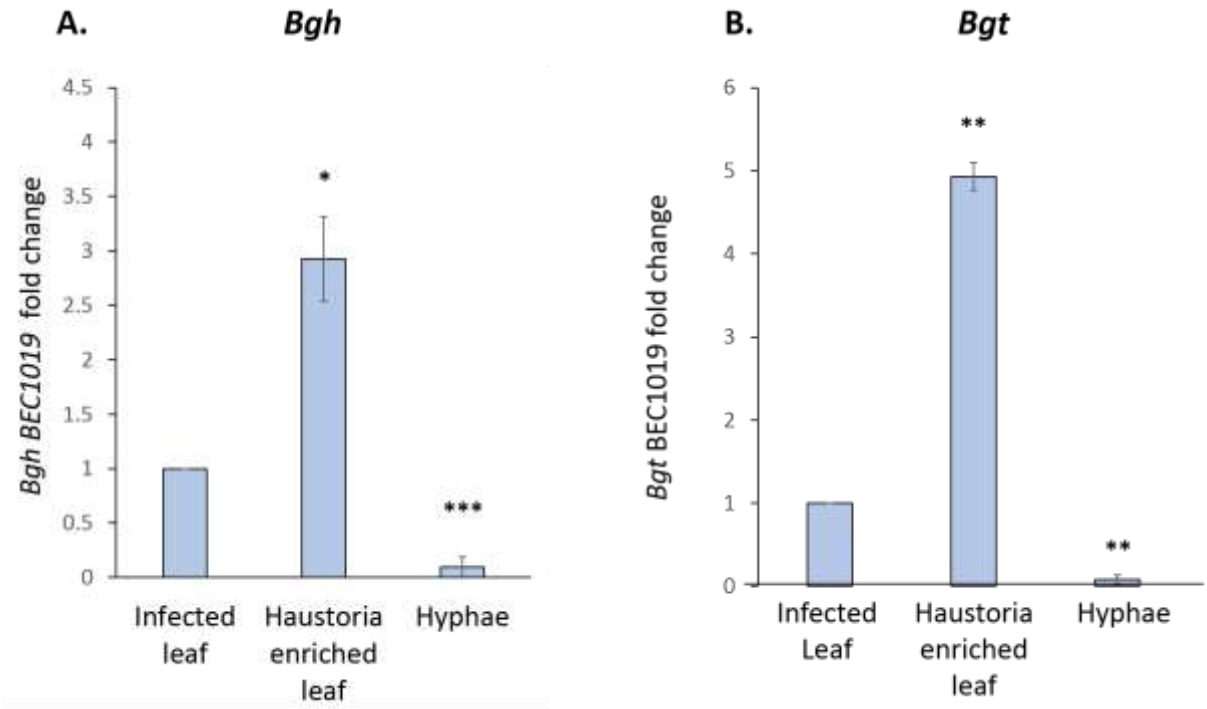


Figure 5-11: BEC1019 expression in various B. graminis tissue during powdery mildew infection.

*A. Bgh BEC1019 and B. Bgt BEC1019 expression studies in different Blumeria tissues during barley and wheat powdery mildew infection. Expression levels were compared in 7dpi whole leaf tissues (containing haustoria and hyphae, taken as a reference) to B. graminis haustoria (whole leaves without epiphytic hyphae from which hyphae were removed) and hyphae samples. Bgh/Bgt GAPDH was used as reference genes for the qRT-PCRs. Data derived from 3 independent biological replicates, where each repeat was an average of 10 leaves. Significance (1 sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

5.3.6 Study of *BEC1019* expression during *F. graminearum* wheat ear infection

The expression of BEC1019 homologue of *F. graminearum* was investigated to determine its putative importance during FHB development in wheat, as well as during *in vitro* growth in complete medium (potato dextrose broth), carbon and nitrogen deficient minimal medium. *Fg BEC1019* (Ensembl id -

FGSG_03954) was one of the proteins mined from the list of proteins predicted in the *F. graminearum* secretome by Brown et. al., 2012. Published affymetrix microarray data (Brown et.al. 2017) were also queried to compare *FgBEC1019* gene expression *in vitro* and *in vivo* during *F. graminearum* growth and during wheat ear infection. It also revealed a clear induction of *BEC1019* in both symptomatic and asymptomatic FHB infected wheat head tissues at 7 dpi as compared to *in vitro* growth in complete and minimal media (Figure 5-12).

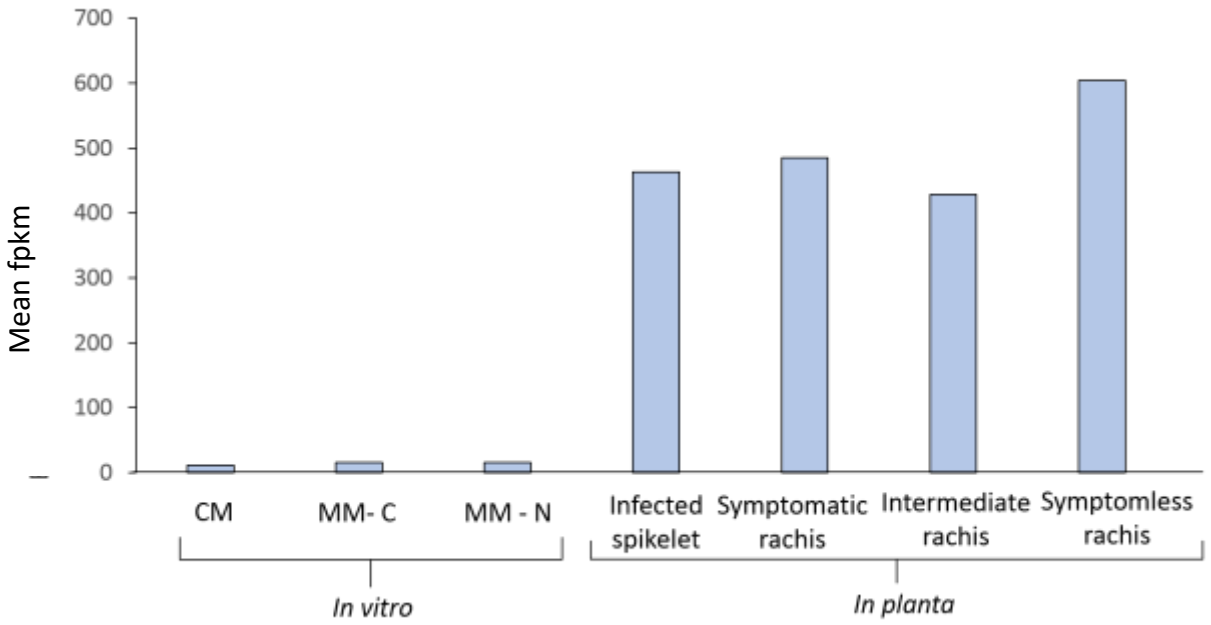


Figure 5-12: Fg BEC1019 homologue expression during in vitro growth and F. graminearum infection in wheat heads. *CM – Complete medium, MM-C = carbon deficient medium, MM-N = nitrogen deficient medium. Data mined from bioinformatics data obtained from Neil Brown (Rothamsted Research). The original data is an average of 3 biological replicates. Significance values were not available.*

5.3.7 *In vitro and in planta expression of Zt BEC1019 and Zrt1* homologues of Z. *tritici*

RNA sequencing data for genes expressed during *in vitro* and *in planta* growth of *Z. tritici* were published by Rudd et al. in 2015. These were mined to extract the expression data for the *Zt BEC1019* homologue (Ensembl gene id: Mycgr3T42164) , which is 32% identity to *Bgh BEC1019* peptide sequence (Figure 5-7; Table 5-1). Gene expression was reported for different *in vitro* growth conditions in either potato dextrose broth (PDB) (rich medium) or Czapek dox medium (CPD) (zinc deficient minimal medium). In this same study (Rudd et al., 2015), gene expression data *in planta* was collected following wheat infection at an early time point (1 dpi), during asymptomatic growth (4 dpi), during the switch from asymptomatic to symptomatic (9 dpi) and during late necrotic growth (14 and 21 dpi). *Zt BEC1019* expression was induced during late necrotrophic phase of STB disease cycle (14 and 21 dpi), when compared to the early time points reflecting the asymptomatic phase.

These results indicate that *Zt BEC1019* might be required for the late stages of STB disease development, or for the asymptomatic to symptomatic lifestyle switch and for the formation of asexual spores. When *Z. tritici* was grown in vitro *Zt BEC1019* was induced during *Z. tritici* growth in CPD, a minimal medium not containing zinc, while expression was much lower when the fungus was grown in a rich medium such as PDB (Figure 5-13). Interestingly, when comparing gene expression between both media, *Zt BEC1019* was the 7th most induced gene during *in vitro* growth of *Z. tritici* in the CPD medium. This might be explained by the absence of zinc in the CPD medium. Other BEC1019 homologues, such as *PRA1* in *Candida albicans* (Citiulo et al., 2012) and *Aspf2* in *Aspergillus fumigatus* (Amich et al., 2010) were also shown to be induced by absence of zinc in the growth conditions.

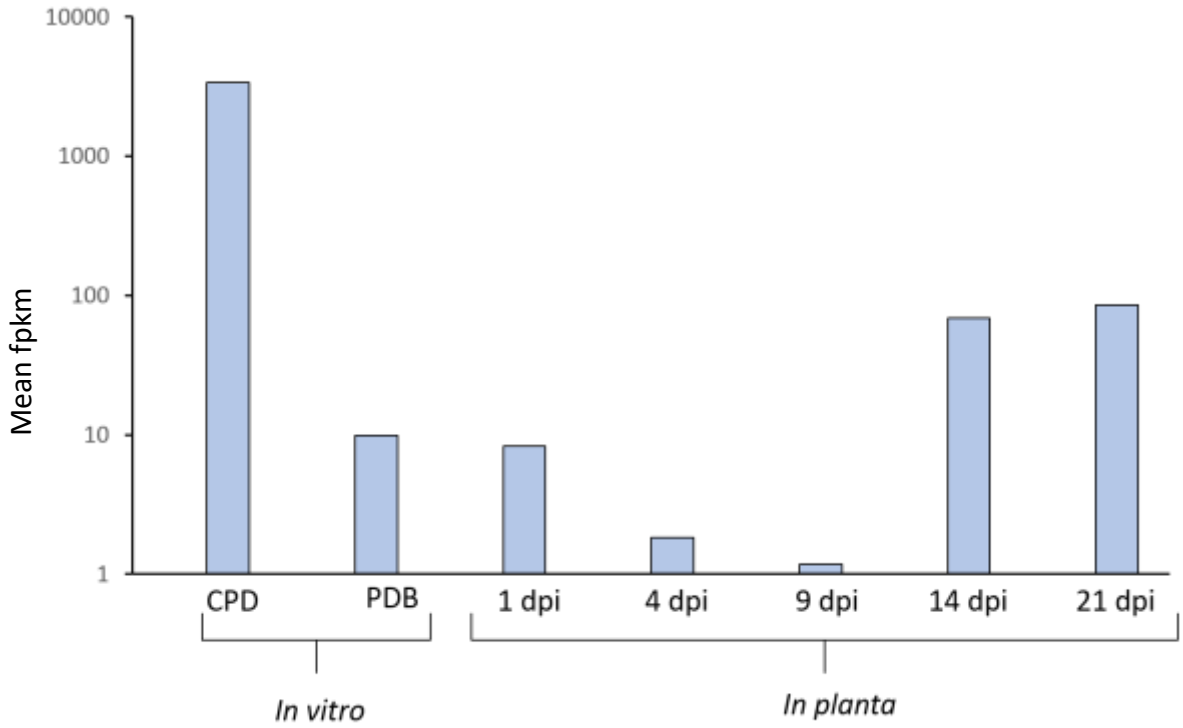


Figure 5-13: Zt BEC1019 expression during Z. tritici vitro growth and infection

RNA sequencing data were derived from Rudd et. al. 2015 to estimate mRNA expression levels of Zt BEC1019 (Ensembl gene id: Mycgr3T42164) in plant and in vitro. For the in vitro study, Z. tritici was grown in a rich (potato dextrose broth, PDB) or poor medium depleted in zinc (Czapek dox broth, CPD). For the in planta study, gene expression was monitored at non-symptomatic phases (1,4 dpi) during the symptomatic switch (9dpi) and at later necrotic phases (14, 21 dpi) following leaf infection leading to Septoria tritici blotch.

Similarly, the expression of *Zt Zrt1* (putative zinc transporter) homologue was also extracted from the transcriptomics data (Rudd et al., 2015) to query whether its expression is corelated with *Zt BEC1019,* as described above, to confirm Zt Zrt1 potential regulation by zinc and STB disease stage. *Zt Zrt1* (Ensembl id: Mycgr3G100094)was highly expressed in CPD (-Zn; pH 7.5) as compared to PDB

and induced *in planta* at 14 and 21 dpi (necrotic phase) of *Z. tritici* infection of wheat (Figure 5-14), confirming a similar expression pattern to that of *Zt BEC1019*. These findings indicate that *BEC1019* and *Zrt1* may be co-regulated in *Z. tritici.*

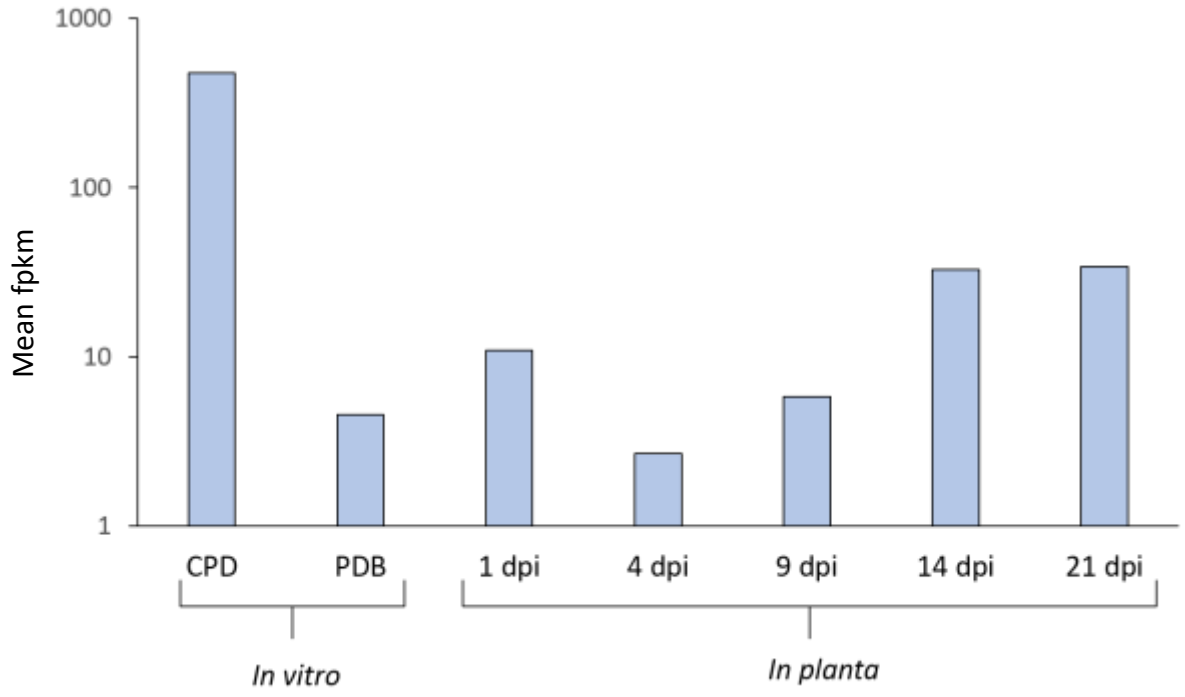


Figure 5-14: Zt Zrt1 expression during Z. tritici vitro growth and infection

RNA sequencing data were derived from Rudd et. al. 2015 to estimate mRNA expression levels of Zt Zrt1 (Ensembl gene id: Mycgr3G100094) in plant and in vitro. The gene expression was depicted as mean fpkm. For the in vitro study, Z. tritici was grown in a rich (potato dextrose broth, PDB) or poor medium (Czapek dox medium, CPD: -Zn/pH 7.5). For the in planta study, gene expression was monitored at non-symptomatic phases (1,4 dpi) during the symptomatic switch (9dpi) and at later necrotic phases (14, 21 dpi) following leaf infection leading to Septoria tritici blotch.

5.3.8 Gene silencing of *Bgt BEC1019* to show its implication as a virulence factor during wheat powdery mildew infection

BEC1019 homologues are known virulence factors in both plant and animal fungal pathogens including *B. graminis* f.sp. *hordei* (Amich et al., 2010; Citiulo et al., 2012; Pliego et al., 2013; Whigham et al., 2015). Wheat powdery mildew also has a homologue of BEC1019 which has 94% amino acid sequence identity with the known virulence factor *Bgh BEC1019* (Table 5-1). Additionally, kin to other BEC1019 homologues, they share two conserved domains – a putative zinc binding HRXXH domain and an ETVIC domain (Whigham et al., 2015). Therefore, it was speculated that *Bgt BC1019* may contribute to wheat powdery mildew virulence.

To study the role of *Bgt BEC1019* (GenBank id: EPQ66538.1) in wheat powdery mildew virulence, *Bgt BEC1019* was silenced and its impact on infection development was monitored. *Bgt BEC1019*

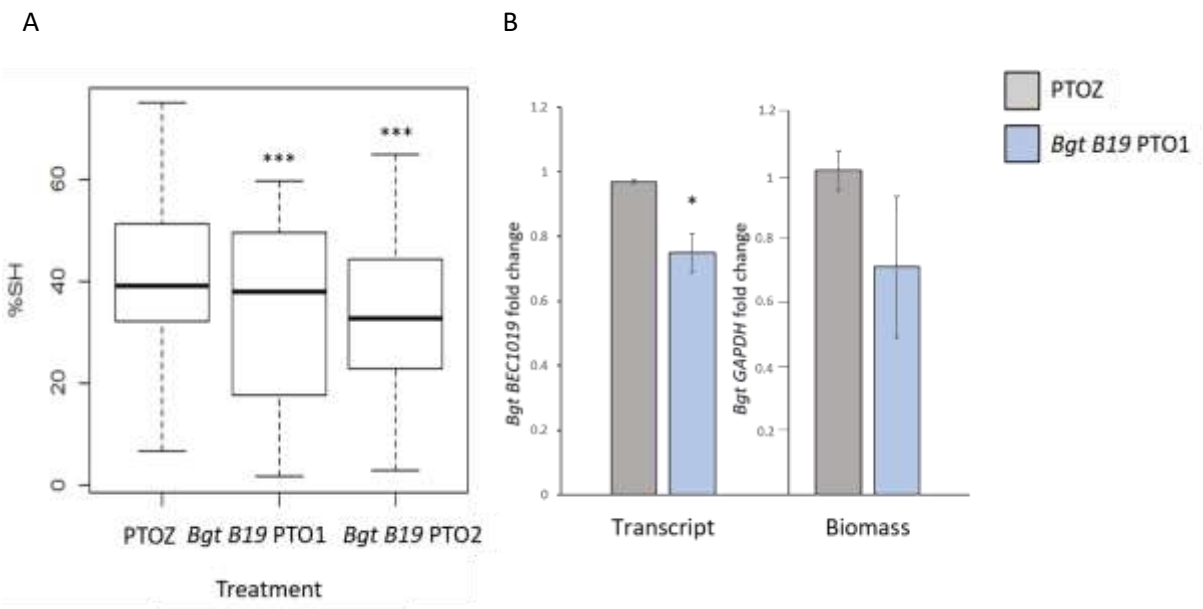
silencing was attempted using two different PTOs, *Bgt BEC1019 PTO1 and PTO2*. A PTO targeting seed protein Z was used a control (PTOZ) (C. Sun & Ho, 2005). Infection success was calculated as a percentage of secondary hyphae formed per germinated conidia observed (%SH). Secondary hyphae are reliable determinants of successful infection as they are formed subsequent to haustoria development. qRT-PCR was used to determine both change in transcript levels and fungal biomass levels post *BEC1019* targeting. *Bgt GAPDH* was used a reference gene to study *BEC1019* transcript levels. Fungal biomass was determined by measuring *Bgt GAPDH* expression relative to the expression of wheat reference gene 18srRNA.

Targeting of *Bgt BEC1019* reduced %SH by approx. 37% when targeted with *Bgt BEC1019* PTO1 as compared to 23% when targeted *with Bgt BEC1019* PTO2 (

Figure **5-15**A). The data was derived from four biological repeats each consisting of nine leaves. The data showed significant reduction in infection for each of these biological repeats. Therefore, PTO1 was used to monitor the impact of HIGS on the amount of mRNA present in treated plants post *Bgt BEC1019* gene silencing. Targeting of *Bgt BEC1019* by PTO1 resulted in 23% reduction in *BEC1019* transcript levels, confirming gene silencing. The change in fungal biomass was more variable (

Figure **5-15**B). The variation in fungal biomass maybe due to less sensitivity of qRT-PCR to detect the fungus at 48 hpi in a big background of plant material, when the fungal biomass is mostly concentrated as haustoria and conidia as compared to hyphae (Both et al., 2005).

However, these findings prove that *Bgt BEC1019* plays a role in fungal virulence during wheat powdery mildew disease development.



Treatment	Avg. disease scoring (%SH/germinated conidia)	%Reduction compared to control	P-value
PTOZ	43%		
Bgt B19 PTO1	27%	37%	<0.001
Bgt B19 PTO2	33%	23%	<0.001

Figure 5-15: Bgt BEC1019 silencing during wheat powdery mildew infection.

*A. Disease scoring (%SH/germinated conidia), B. gene transcript and fungal biomass levels post Bgt BEC1019 silencing using antisense PTO. PTOZ targeting a wheat seed protein Z was used as control. Silencing was carried out on 10 days old wheat (c.v. Cerco) leaves. Wheat powdery mildew spores were derived from 14 dpi infected wheat plants. For infection, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Expression of Bgt BEC1019 was normalised against Bgt GAPDH expression. Fungal biomass was determined by evaluating the expression of Bgt housekeeping gene GAPDH relative to wheat housekeeping gene 18srRNA.The table included contains the average %SH post Bgt BEC1019 PTO treatments. Data derived from 4 biological replicates for infection and qRT-PCR, where each biological replicate was an average of 9 leaves. Significance (GLM/1 sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

5.3.9 To study the putative role of *Bgh ZAP1* in controlling *Bgh BEC1019* expression and consequently barley powdery mildew virulence

Homologues of zinc sensitive transcription factor ZAP1 in *A. fumigatus* (ZafA) and *C. albicans* (ZAP1) have been shown to regulate both zinc scavengers (BEC1019 homologues) and zinc transporters (ZRT1 homologues) (Amich et al., 2010; Citiulo et al., 2012). Also, ZAP1 homologues have been shown to be important for virulence of these fungal pathogens (M. Á. Moreno et al., 2007; Nobile et

al., 2009). In *Blumeria*, BEC1019 is a known virulence factor important for barley powdery mildew disease development (Pliego et al., 2013; Whigham et al., 2015). Therefore, it was speculated that ZAP1 may indirectly influence barley infection development by regulating expression of virulence factor like *Bgh BEC1019*.

To check this hypothesis, a homologue of zinc sensitive transcription factor ZAP1 was identified in *B. graminis* f.sp. *hordei* genome (Ensembl id: BGHDH14_bgh03555).

If *Bgh ZAP1* regulates *BEC1019* expression, it should have a similar expression pattern to *Bgh BEC1019.* With this idea, *Bgh ZAP1* expression was studied in different *B. graminis* tissue during infection development. At 7-9 dpi, *Bgh ZAP1* expression was observed in both *Bgh* haustoria and hyphae (Figure 5-16). This is in contrast with the haustoria-specific expression of *Bgh BEC1019* at 7 dpi (Figure 5-11A). However, the presence of *Bgh ZAP1* in *Blumeria* haustoria at 7 dpi suggests that *Bgh ZAP1* may regulate *Bgh BEC1019* expression, but it cannot be the only factor responsible for the expression of *BEC1019*.

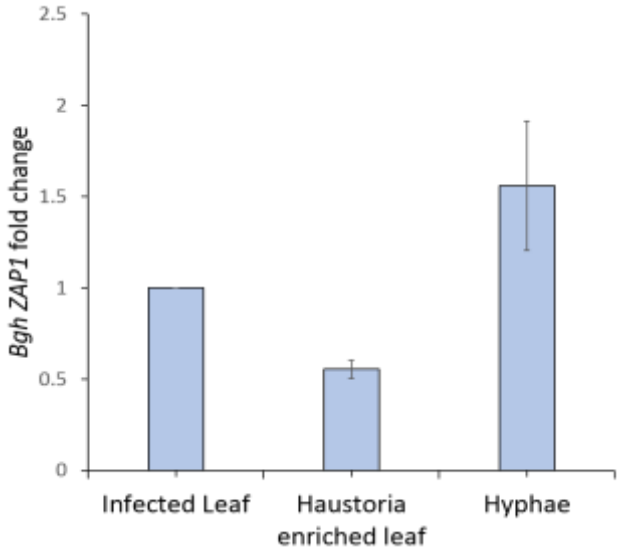


Figure 5-16: Bgh ZAP1 expression during barley powdery mildew infection.

*Expression study of the homologue of zinc sensitive transcription factor ZAP1 in different Blumeria tissues during powdery mildew infection. Expression levels were compared in 7-9dpi whole leaf tissues (containing haustoria and hyphae, taken as reference) to B. graminis haustoria (whole leaves without epiphytic hyphae from which hyphae were removed) and hyphae samples. Bgh GAPDH was used as the reference gene for the relative quantification by qRT-PCRs. Data represent results from 2 independent biological replicates (N=3), where each repeat is an average of 10 leaves. Significance (1 sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

Next, the putative role of *Bgh ZAP1* in barley mildew infection development was queried by studying the effect of *Bgh ZAP1* silencing on *B. graminis* virulence.

Two antisense PTOs targeting *Bgh* ZAP1 were used for *in planta* silencing of ZAP1. PTOZ targeting seed protein Z was used as control. Significant reduction in barley powdery disease (scored as % secondary hyphae formation per germinated conidia (%SH)) was observed post treatment with both the PTOs (Figure 5-17A). However, infection development post *Bgh ZAP* PTO1 treatment (37% reduced from control) was significantly lower in all the three biological replicates, as compared to only two out of three biological replicates post *Bgh ZAP* PTO 2 treatments (23% reduced from control). This is supported by the fact that during PTO design by OligoWalk software, PTO 1 was shown to have a higher probability of causing silencing than PTO 2. Therefore, future experiments were carried out using PTO 1. Reduction in infection post *ZAP* PTO1 treatment was accompanied by reduced *ZAP1* transcripts, (Figure 5-17B), confirming that *ZAP1* has been silenced. Therefore, *Bgh ZAP1* has a role in *B. graminis* virulence.

To investigate if *Bgh ZAP1* regulates *Bgh BEC1019* expression, the effect of silencing of the zinc sensitive transcription factor *ZAP1* on the *Bgh BEC1019* expression level was checked. For this, the *BEC1019* transcript levels were monitored following *Bgh ZAP1* PTO 1 treatments. Treatments with *Bgh ZAP1* PTO 1 resulted in reduction in *ZAP1* transcript levels by 20% confirming *Bgh ZAP1* silencing (Figure 5-17B). This reduction in *ZAP1* transcript levels was also accompanied by 20% reduction in *Bgh BEC1019* transcript levels (Figure 5-17B). These findings indicate that the expression of *Blumeria* virulence factor *BEC1019* maybe be regulated by a zinc-sensitive transcription factor *ZAP1.* This in turn supports the hypothesis that BEC1019 may be also be regulated by zinc availability via ZAP1 transcription factor.

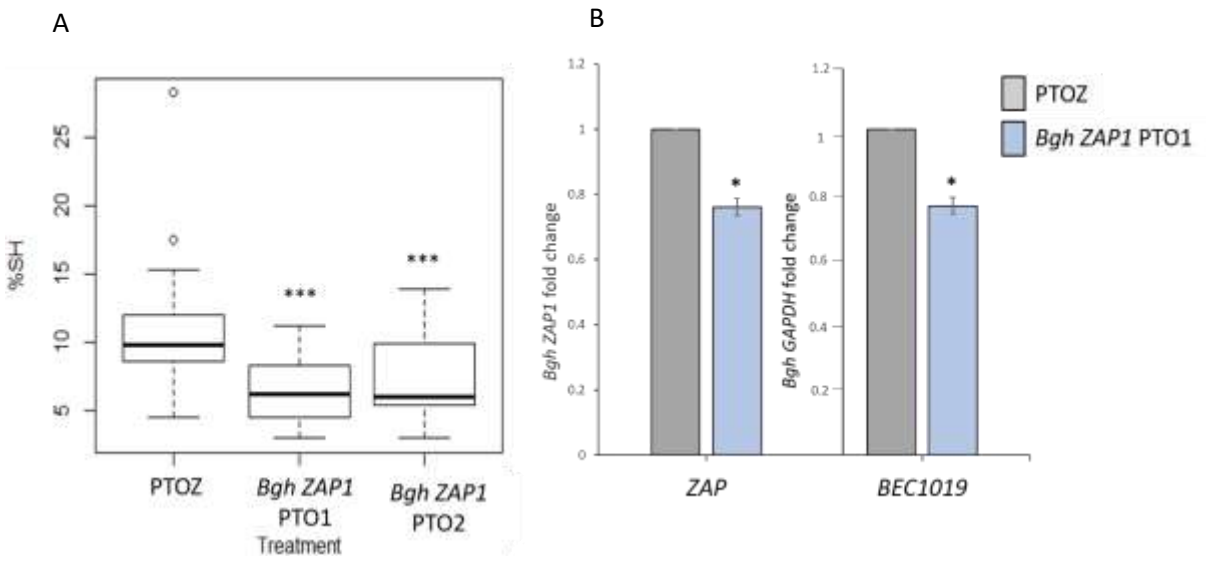


Figure 5-17: In planta gene silencing of Bgh ZAP1.

A. Disease scoring (%SH/germinated conidia) post *Bgh ZAP1* targeting using PTO1 and PTO2. *B. Bgh ZAP1 and BEC1019 transcript levels post Bgh ZAP1 PTO1 treatment. PTOZ targeting a seed protein Z was used as a control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Bgh GAPDH was used as a reference gene for qRT-PCR. Data derived from 3 biological repeats for infection and 4 biological repeats for qRT-PCR, where each biological repeat consisted of 6 leaves. Significance (GLM/1 sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

5.3.10 To query the role of zinc sensitive transcription factor *ZAP1* in wheat powdery mildew infection development

As *B. graminis* f.sp. *hordei* and *tritici* have very similar genomes and life cycles, it was hypothesised that akin to *Bgh ZAP1, Bgt ZAP1* may also be important for wheat powdery mildew virulence.

To query this, silencing of *Bgt ZAP1* (Ensembl id: BGT96224_3498) was attempted by treatments with two different PTOs, *Bgt ZAP1* PTO1 and 2 and their impact of wheat powdery mildew development was studied. PTOZ targeting a wheat seed protein Z was used as a control. Infection development was determined microscopically as %secondary hyphae formed per germinated conidia. As haustoria are is difficult to visualise, secondary hyphae that is formed only subsequent to haustoria establishment is a good marker of successful infection. The experiments were carried out using fungal spores from 10 dpi and 14 dpi wheat powdery mildew infected plants.

The two biological replicates involving inoculum from 10 dpi infected plants, did not show any significant difference in infection development between the different PTO treatments (data not shown). However, initial results from another two biological replicates using fungal inoculum from 14 pi infected plants showed a significant reduction of 37% in wheat powdery mildew development (%SH developed per germinated conidia) post *Bgt ZAP1* PTO2 treatment (Figure 5-18). No significant

change in infection was observed post *Bgt ZAP1* PTO1 treatment (data not shown). Therefore, all future experiments will be carried out using *Bgt ZAP1* PTO2 and 14 dpi wheat powdery mildew spores. These experimental conditions match the ones used for silencing *Bgt BEC1019* (chapter 2).The 37% wheat powdery mildew infection reduction post *Bgt ZAP1* PTO2 was equivalent to barley powdery mildew infection reduction observed post *Bgh ZAP1* silencing (Figure 5-17A). Therefore, these preliminary findings indicate towards a role of zinc sensitive transcription factor ZAP1 in *Bgt* virulence, to a similar level to *Bgh BEC1019* during barley powdery mildew infection (Section 5.3.9), though more repeats need to be done to check reproducibility of the data.

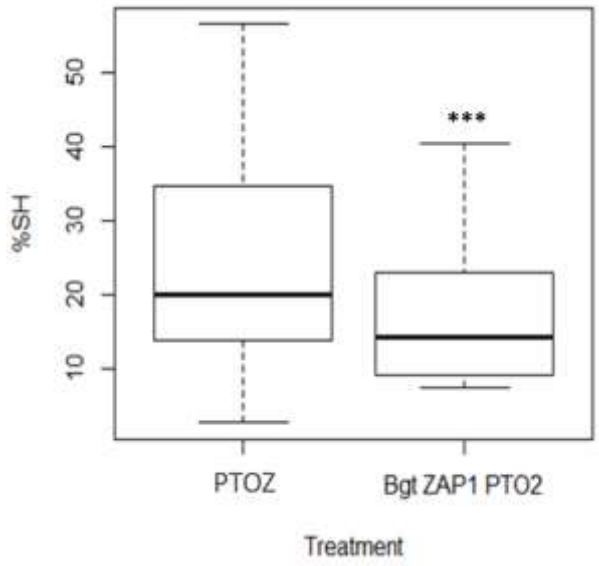


Figure 5-18: In planta gene silencing of Bgt ZAP1..

*Graph showing wheat powdery mildew disease development (%SH/germinated conidia) post Bgt ZAP1 PTO2 treatment. PTOZ targeting a wheat seed protein Z was used as a control. Silencing was carried out on 10 days old wheat (c.v. Cerco) leaves. For disease scoring, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Infection was carried out using 14 dpi wheat powdery mildew infected plants. Data derived from 2 independent biological replicates, where each repeat consists of 9 leaves. Significance (1 sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

5.3.11 To study the expression of *B. graminis* f.sp. *hordei* homologue of the pH sensitive transcription factor PacC during barley powdery mildew infection development

Since pH regulatory elements (PREs) were identified in the promoter region of *Bgh BEC1019*, it was hypothesised that *BEC1019* expression may be regulated by pH sensitive transcription factor PacC (Section 5.3.4). To query this, the expression of *Bgh PacC* was studied in *Blumeria* hyphae and haustoria and was compared to the expression pattern of *Bgh BEC1019* in these *Blumeria* tissues during powdery mildew infection.

Bgh PacC (Ensembl id: BLGH_01427) expression was compared between barley leaf samples enriched in *Blumeria* haustoria and separated *Blumeria* epiphytic hyphae. Whole powdery mildew infected leaves were used for reference values. *Bgh GAPDH* was used as a reference gene for qRT-PCR.

At 7 dpi, *Bgh PacC* homologue was dominantly expressed in *Blumeria* haustoria as compared to hyphae (Figure 5-19), matching the localisation of *BEC1019* expression in *B. graminis* haustoria at the same time point during barley powdery mildew infection (Figure 5-11A). Since, both *Bgh Pacc* and *BEC1019* expression was localised in the same tissue at 7 dpi, it was concluded that PacC may regulate the expression of *Bgh BEC1019*.

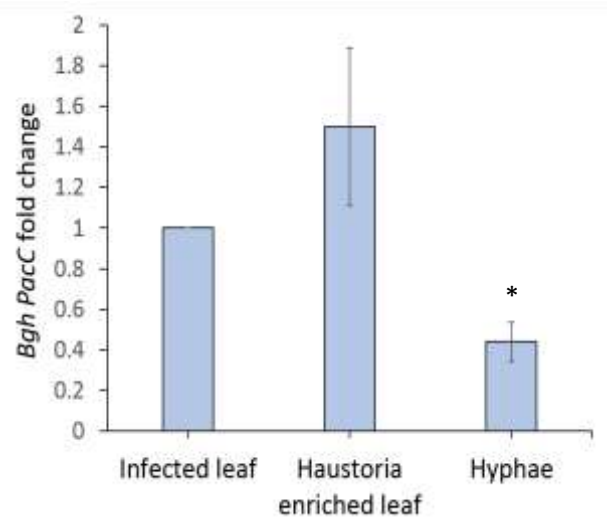


Figure 5-19: Bgh PacC expression during barley powdery mildew infection.

*Expression study of the homologue of pH sensitive transcription factor PacC in different Bgh tissues during powdery mildew infection. Expression levels were compared in 7dpi infected whole leaf tissues (containing haustoria and hyphae, taken as reference) to B. graminis haustoria enriched barley leaves (whole leaves without epiphytic hyphae from which hyphae was removed using cellulose acetate peeling) and hyphae samples harvested from cellulose acetate peels obtained from infected leaves. Bgh GAPDH was used as the reference gene for the relative quantification by qRT-PCRs. Data represent results from 3 independent biological replicates (N=3), where each repeat is an average of 10 leaves. Significance (1 sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

5.3.12 To study the importance of pH sensitive transcription factor PacC in *B. graminis* f.sp. *hordei* virulence and expression regulation of *Bgh BEC1019* virulence factor

Since PacC was shown to impact fungal virulence by regulating the expression of BEC1019 homologues PRA1 and AspF2 (Amich et al., 2010; Citiulo et al., 2012), the role of *Bgh PacC* in barley powdery mildew virulence and regulation of *Bgh BEC1019* expression was investigated.

Bgh PacC (Ensembl id: BLGH_01427) silencing was attempted by treatment with gene specific *Bgh PacC* PTO1. A PTO targeting a seed protein Z was used as a control. Disease scoring was done microscopically to determine the change in % secondary hyphae formation per germinated conidia post *Bgh PacC* targeting, a good indicator of successful infection. qRT-PCRs were used to study change in *Bgh PacC* and *BEC1019* transcript levels post *PacC* targeting. *Bgh GAPDH* was used as a reference gene for the gene expression studies.

Targeting of *Bgh PacC* by gene specific PTO reduced *PacC* transcript levels by 58% as compared to the control (Figure 5-20B), confirming the silencing of the gene. Disease scoring confirmed an accompanying reduction in barley powdery mildew infection development by 48% (Figure 5-20A), confirming a role of PacC in *B. graminis* virulence.

To query the regulation of *Bgh BEC1019* expression by PacC transcription factor, *BEC1019* transcript levels were studied post *PacC* silencing. Results confirmed a 51% reduction in *BEC1019* transcript levels post *PacC* silencing (Figure 5-20B), indicating that PacC may be one of the factors regulating *BEC1019* expression during barley mildew infection development.

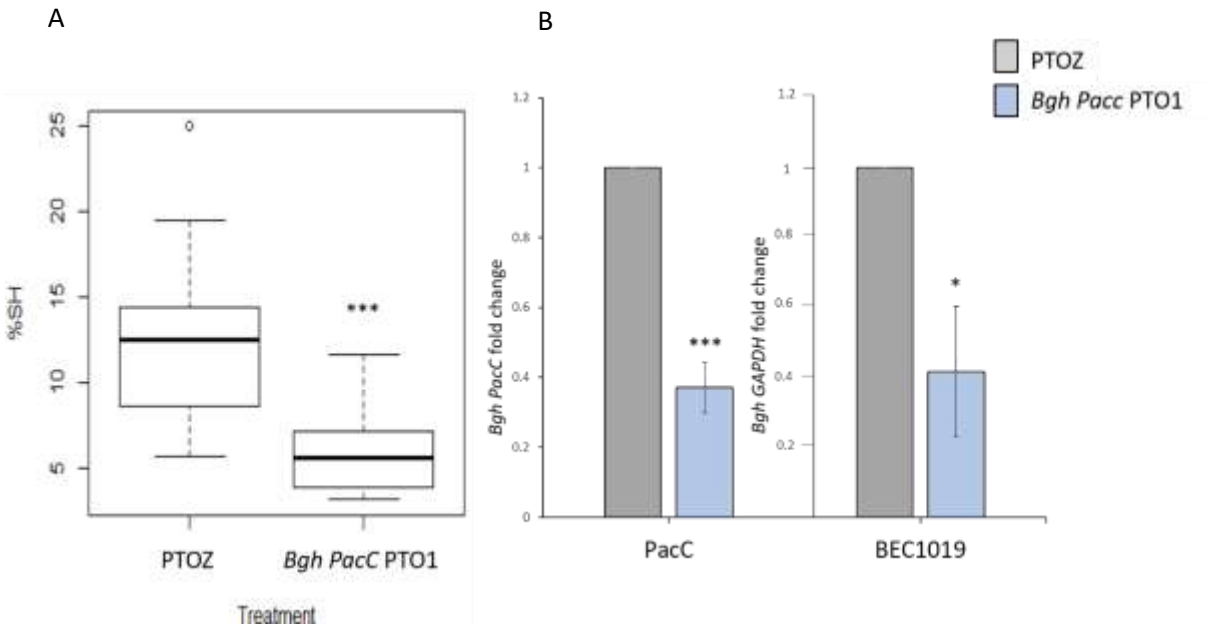


Figure 5-20: In planta gene silencing of Bgh PacC

*The effect of silencing Bgh PacC on A. barley powdery mildew infection, as measured via the proportion of secondary hyphae (%SH) formed per germinated conidia, B. PacC and BEC1019 transcript levels. PTOZ targeting a seed protein Z was used as a control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Bgh GAPDH was used as a reference gene for qRT-PCR. Data derived from 3 biological replicates for infection and 4 biological replicates for qRT-PCR, where each repeat consisted of 6 leaves. Significance was calculated in R using the general linear model for (A) and 1 sample t-test for (B) * P<0.05, ** P<0.01, *** P<0.001.*

5.3.13 Preparation of *Fusarium graminearum BEC1019* deletion mutants using a gene replacement strategy

Since BEC1019 homologues act as virulence factors in other fungal pathogens (Amich et al., 2010; Citiulo et al., 2012; Pliego et al., 2013; Whigham et al., 2015) (Section 5.3.8), *Fg BEC1019* deletion mutants were obtained with the intension of studying the influence on *BEC1019* deletion on FHB development in wheat heads.

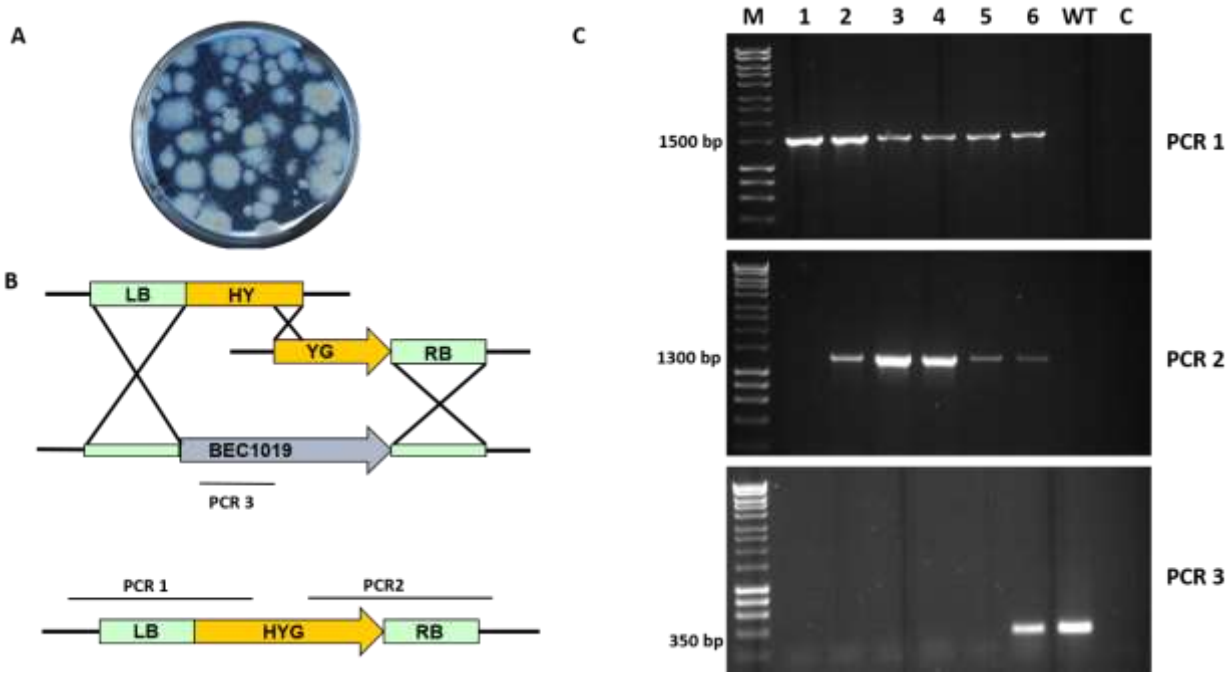


Figure 5-21: Fg BEC1019 gene deletion.

A. Fg ΔBEC1019 mutants obtained post F. graminearum (PH1) protoplast transformation with the BEC1019 deletion cassettes. B. A split-marker method of gene replacement was used. The position of the diagnostic PCR primers used to confirm the replacement of BEC1019 by hygromycin resistance gene (HYG) are also showed. PCR1 and 2 diagnosed the insertion of HYG next to BEC1019 leaf and right borders (LB and RB) respectively, whereas PCR3 confirmed the absence of BEC1019 in the deletion mutants. C. Diagnostic PCRs for Fg ΔBEC1019 mutants 1- 6. A wild type strain (WT) and water were used as controls. The correct insertion of HYG in the correct direction and position was diagnosed by a 1500 bp product in PCR 1, a 1300 bp product in PCR 2 and the absence of a BEC1019 band in PCR 3.

The deletion mutants of *Fg BEC1019* (Ensembl id: FGRAMPH1_01G14115) were obtained using a combination of Gibson cloning and split marker method of gene deletion to allow the replacement of *Fg BEC1019* by a hygromycin resistance cassette. The gene deletion cassette was obtained by ligase-free Gibson cloning, where two deletion cassettes were created each containing a part of the hygromycin resistance cassette flanked by either left or right flanking region of *Fg BEC1019* (Figure **2-5**). The linearised deletion cassettes were then purified and transformed into protoplasts of *F. graminearum* PH1 wild type strain. An average of 45 colonies/ plate of transformants were obtained on regeneration agar plates containing 75 µg/ml hygromycin (

Figure 5-21A). These colonies were sub-cultures in hygromycin containing media to obtain stable transformants and diagnostic PCRs were performed to obtain four deletion mutants with correct insertion of the hygromycin cassette. The details of the method are described in section 2.11.

Three PCR reactions were designed for diagnostics. PCR 1 amplified a 1500 bp amplicon spanning the left border of *Fg BEC1019* and a part of the neighbouring HYG fragment. Similarly, PCR 2 amplified a 1300 bp amplicon beginning from the HYG fragment and ending beyond the selected *BEC1019* left border region in the *F. graminearum* genome. The PCR 3 on the other hand amplified a 350 bp region with the *Fg BEC1019* gene (

Figure 5-21B).

Mutants 1-4 (also called B1- B4) were obtained which showed the right sized amplicon for PCR 1 and 2 confirming the correct insertion of the HYG cassette in the correct position and orientation. Furthermore, each transformant lacked the *BEC1019* amplicon in PCR 3 confirming the deletion of *BEC1019* (
Figure 5-21C). These mutants were then used for infection assay for evaluating the role of *BEC1019* in *Fg* virulence and for *in vitro* growth assays.

5.3.14 Characterisation of *Fg BEC1019* deletion mutants

5.3.14.1 Fg BEC1019 deletion in vitro phenotype

Since BEC1019 homologues PRA1 and AspF2 play a role in zinc sequestration (Amich et al., 2010; Citiulo et al., 2012) and VdAspF2 contributes to fruiting body formation (C. Xie et al., 2017), the *Fg ΔBEC1019* mutants were further investigated for their role in fungal growth under zinc limiting conditions and for their capability for forming fruiting bodies (perithecia) under perithecia inducing media.

To investigate if *Fg BEC1019* is required for fungal growth in the absence of zinc under different pH conditions, the growth of *Fg BEC1019* deletion mutants was studied in zinc deficient slightly acidic synthetic nutrient agar (SNA) (-Zn minimal media; pH 5.8) and zinc deficient slightly alkaline Czapek dox medium (-Zn minimal medium, pH 7.5). Growth in of rich medium, potato dextrose agar (PDA), was used as a control. Growth was measured after 6 days post inoculation. The effect of BEC1019 deletion on perithecia (sexual fruiting body) formation and ascospore formation was also studied by growing the wild type and mutant stains on carrot agar for 21 days.

No discernible difference was seen in either hyphal growth or perithecia formation in different media conditions described above (Figure 5-22), confirming BEC1019 to be dispensable for fungal growth under zinc deficient conditions and for perithecia formation in *F. graminearum.*

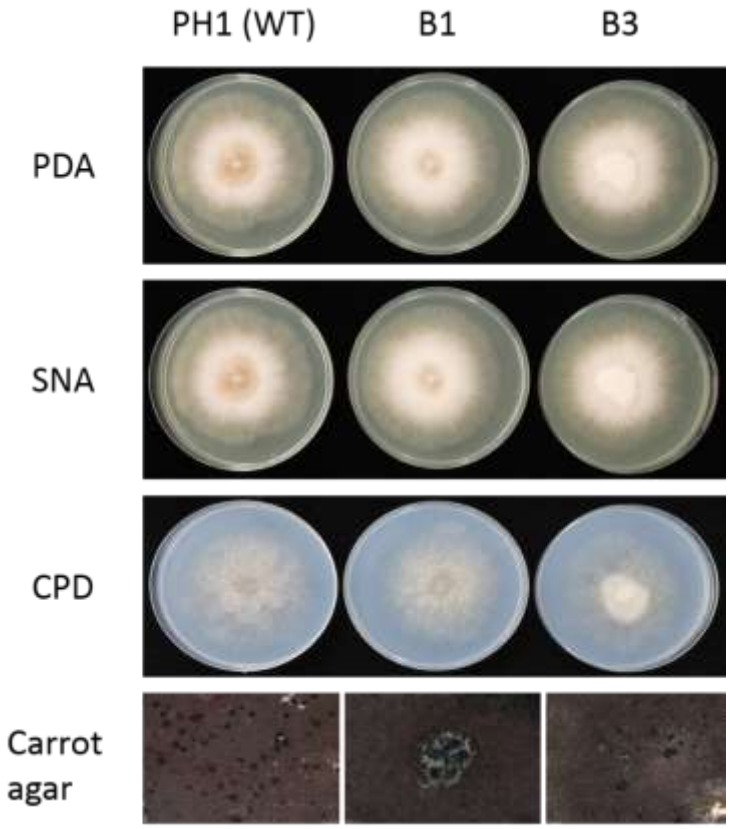


Figure 5-22: In vitro growth of Fg BEC1019 deletion mutants.

Analysis of invitro growth of wild type F. graminearum (PH1) and BEC1019 deletion mutants (B1, B3) in PDA (rich), SNA (-Zn, minimal, pH 5.8), CPD (-Zn, pH7.5) and carrot agar (perithecia formation).

5.3.14.2 FHB infection development by Fg ΔBEC1019 mutants

BEC1019 homologues are important virulence factors in both plant and human pathogens (Amich et al., 2010; Citiulo et al., 2012; Pliego et al., 2013). Therefore, the role of *Fg BEC1019* during FHB development was queried by deleting *Fg BEC1019* and scoring the disease development by the deletion mutants.

Just as anthesis commenced, wheat florets were infected by point inoculation of 5 µl of 5 X 10^4 spores/ml *F. graminearum* spore suspension onto a wheat spikelet close to the top of the floret. Progression of the disease was recorded after 6 dpi, 9 dpi, 13 dpi and 18 dpi. Infection development was calculated as a percentage of spikelets infected in each inoculated wheat head at 18 dpi and is expressed as percentage disease rating.

At 18 dpi, no significant change in disease rating was seen in wheat heads infected with all the four Δ*BEC1019* mutants as compared to the wild type isolate (Figure 5-23A). similarly, at 13 dpi, the amount of discolouration due to FHB development was comparable in both wild type and *BEC1019*

deletion mutants (Figure 5-23B). Therefore, it is unlikely that *BEC1019* acts as a virulence factor for *F. graminearum* during wheat floral infection.

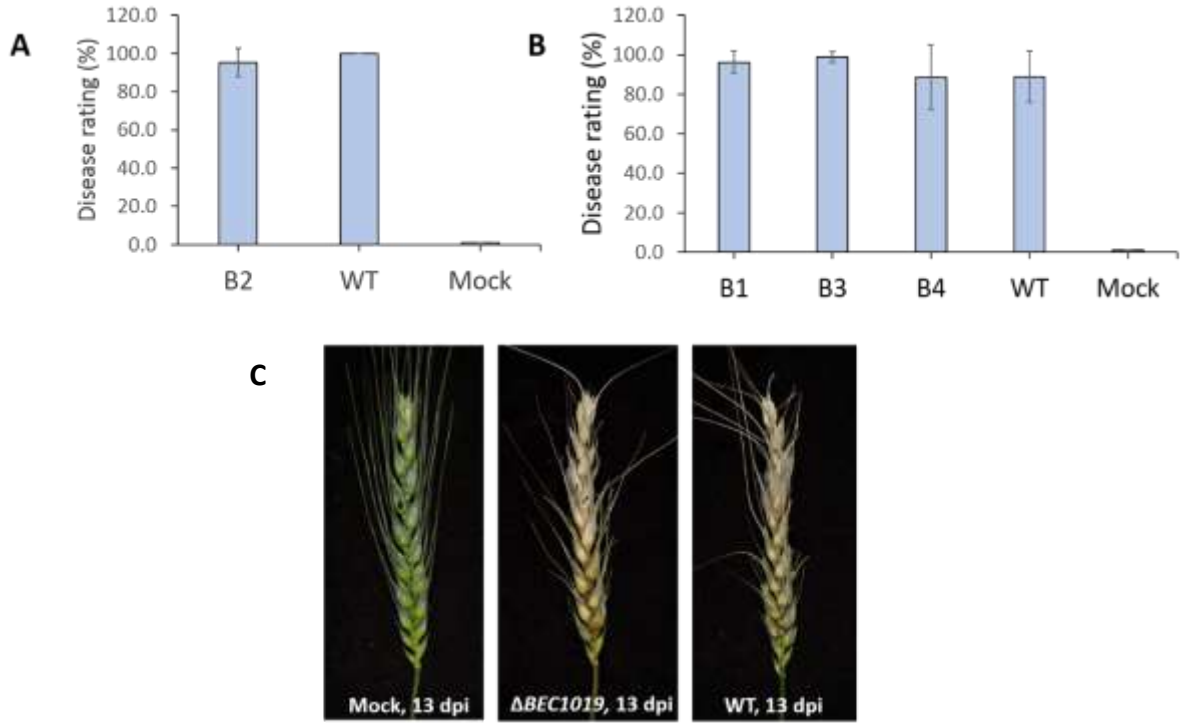


Figure 5-23: FHB infection assay using FgΔBEC1019 mutants

A, B. Wheat spikelet infection assay using Fg BEC1019 deletion mutants (B1- 4), wild type (WT) F. graminearum (PH1 strain) and mock control (water). One of the spikelets in each wheat head was inoculated with F. graminearum spores and infection development (denoted by discolouration of spikelets) was monitored up to 18 dpi. Disease rate denotes the percentage of spikelets infected in a wheat head at 18 dpi. C. 13 dpi pictures of FHB development by wild type (WT), Fg BEC1019 deletion mutant (B4) and mock control (water). The black mark designates the point of inoculation.

5.3.15 To study the regulation of *Fg BEC1019* by pH sensitive transcription factor Pac1 (PacC homologue)

BEC1019 homologues have been shown to be regulated by homologues of pH sensitive transcription factor PacC in other fungal pathogens like *C. albicans* and *A. fumigatus* (Amich et al., 2010; Citiulo et al., 2012). Recent data from our laboratory also suggested that *Bgh BEC1019* is regulated by *Bgh PacC* (Section 5.3.12). Therefore, it is postulated that *Fg BEC1019* expression maybe regulated by *Fg Pac1*, the homologue of the pH sensitive transcription factor PacC in *F. graminearum*.

To investigate the regulation of *Fg BEC1019* (Ensembl id: FGRAMPH1_01G14115) by the Pac1 transcription factor (PacC homologue) in *F. graminearum*, the expression of *BEC1019* was studied in *Pac1* deletion mutants (*ΔPac1)* as well as in *pac1* deleted mutants complemented with *Fg Pac1*

(pac1/Pac1 complemented mutants). These mutants were obtained from J. Merhej (Merhej et.al., 2011). The expression levels were studied by growing the *F. graminearum* fungus *in vitro* in a zinc deficient SNA medium with the pH adjusted to be slightly alkaline (pH 8.0), to ensure conditions likely to induce the expression of both *PacC* and *BEC1019*.

Fg BEC1019 expression was noticeably lower in the *Pac1* deleted mutant , when grown in the zinc deficient slightly alkaline medium , as compared to the wild type strain of *F. graminearum* (Figure 5-24). However, in the *pac1/Pac1* complemented mutant, *BEC1019* expression levels were similar to those of the wild type strain (Figure 5-24), confirming a role of Pac1 (PacC homologue) to promote and control the expression of *Fg BEC1019*.

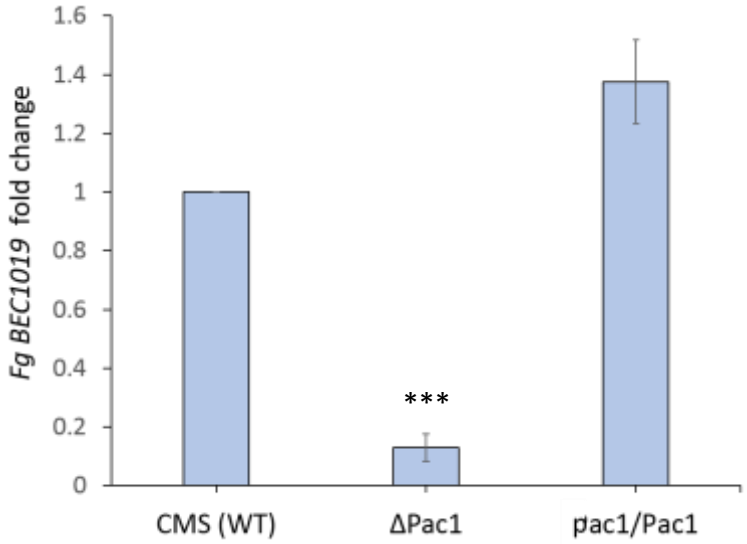


Figure 5-24: Fg BEC1019 expression during in vitro growth of Fg PacC deletion mutants.

*Expression study of Fg BEC1019 in F. graminearum wild type strain (CMS), Fg Pac1 (PacC homologue) deletion mutant (ΔPac1) and Pac1 complemented mutant (pac1/Pac1) while growing in zinc deficient slightly alkaline medium (SNA medium; -Zn, pH 8.0). Fg EF1A was used as reference gene for qRT-PCR. N=3. Significance (1 sample t-test): *** P<0.001.*

5.3.16 To investigate the role of exogenous zinc in the regulation of *Zt BEC1019* and *Zt Zrt1* expression

The expression of BEC1019 homologues has been shown to be regulated by exogenous zinc in other fungal pathogens like *C. albicans (PRA1)* and *A. fumigatus (AspF2),* where these homologues act as zinc sequesters important for fungal virulence (Amich et al., 2010; Citiulo et al., 2012). In these fungal pathogens, the BEC1019 homologue has a neighbouring zinc transporter gene (Zrt1 homologue) and they share a common intergenic sequence containing zinc regulatory elements where zinc binds to co-regulate their expression (Citiulo et al., 2012). Together the zinc sequester and the transporter form a conserved zinc sequestration locus (Citiulo et al., 2012) which is also present in *Z. tritici* (Figure 5-9). *Zt BEC1019* and *Zt Zrt1* share an intergenic sequence containing zinc

binding zinc responsive elements (Figure 5-9). Moreover, *Zt BEC1019* and *Zrt1* have a similar expression pattern *in planta* and are induced in zinc deficient slightly alkaline Czapek dox medium, (Figure 5-13; Figure 5-14; Section 5.3.7) (Rudd et al., 2015). Therefore, it was postulated that zinc availability may co-regulate the expression of *Zt BEC1019* and *Zt Zrt1*.

The role of zinc in expression regulation of *Zt BEC1019* (Ensembl id: Mycgr3T42164) and *Zt Zrt1* (Ensembl id: Mycgr3G100094) was investigated by studying the *BEC1019* and *Zrt1* expression in *Z. tritici* growing in zinc deficient slightly alkaline Czapek dox medium (CPD; pH7.5) and comparing it to their expression in *Z. tritici* grown in CPD complemented with 100 µM zinc chloride. *Z. tritici* grown in the rich medium, potato dextrose broth (PDB), was used as a control. Expression studies were performed on 5 days old CPD/CPD+Zn cultures and 3 days old PDB cultures to ensure logarithmic phase of fungal growth (Rudd et al., 2015).

Both *Zt BEC1019* and *Zrt1* were upregulated in Czapek dox medium (-Zn, pH 7.5) as compared to rich medium (PDB) (Figure 5-25). These results match the *in vitro* expression patterns seen in transcriptomics data published by Rudd et al., 2015 (Figure 5-13, Figure 5-14). This induction was suppressed on addition of 100 µM exogenous zinc to the Czapek dox medium (CPD+Zn) (Figure 5-25). These findings confirm that *Zt BEC1019* and *Zrt1* expression are co-regulated by availability of zinc in *Z. tritici.*

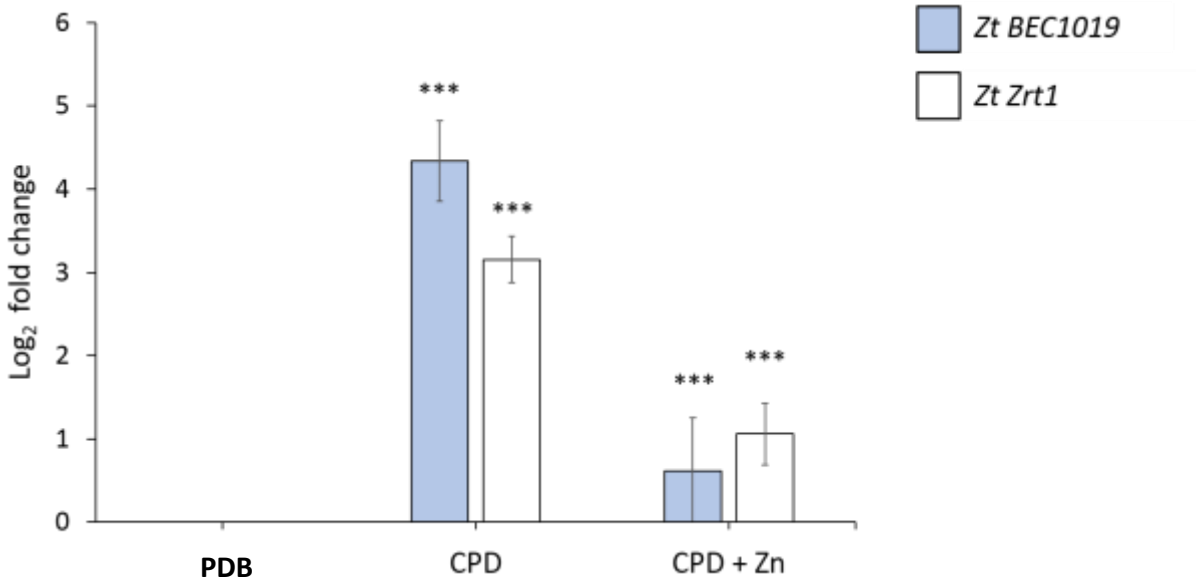


Figure 5-25: Expression studies of Zt BEC1019 and Zt Zrt1 during in vitro growth of Z.tritic under variable zinc conditions. *Expression studies of Zt BEC1019 and Zt Zrt1 during in vitro growth of Z.tritici in the slightly alkaline Czapek dox medium in the presence (CPD + 100µM Zn; pH 7.5) and absence (CPD, -Zn ; pH 7.5) of exogenous zinc Z. tritici growing in rich potato dextrose broth (PDB) was used for reference measurements. Zt tubulin was used as a reference gene for qRT-PCR. N = 3. Significance (1 sample t-test): *** P<0.001.*

5.3.17 To study the role of pH in regulation of *Zt BEC1019* expression

Homologues of BEC1019 in *C. albicans (PRA1)* and *A. fumigatus (AspF2)* are induced under alkaline conditions and their expression is regulated by pH sensitive transcription factor PacC/RIM101 (Amich et al., 2010; Citiulo et al., 2012). PacC binds to the pH responsive elements (PREs) present in their promoter region to regulate their expression (Espeso et al., 1997). In *Z. tritici*, *BEC1019* promoter region also contains these PREs (Figure 5-9; Section 5.3.4), indicating that *Zt BEC1019* expression maybe regulated by ambient pH and *Zt PacC,* the homologue of pH sensitive transcription factor.

To test this, the expression of *Zt BEC1019* (Ensembl id: Mycgr3T42164) and *Zt PacC* (Ensembl gene id: Mycgr3T40797) were studied during *Z. tritici* growth in zinc deficient Czapek dox medium set at acidic (pH 4.0) and slightly alkaline (pH 8.0) pH, to ensure conditions suitable for induction of both *Zt BEC1019* and *Zt PacC. Z. tritici* grown in potato dextrose broth (PDB) rich medium was used as a control. Expression studies were performed on 5 days old CPD and 3 days old PDB cultures to ensure a logarithmic phase of fungal growth (Rudd et al., 2015).

Zt BEC1019 expression was upregulated at pH 8.0 as compared to pH 4.0. This was accompanied by an induction of *Zt PacC* expression at pH 8.0, which was suppressed under pH 4.0 (Figure 5-26). These findings confirm a role of ambient alkaline pH in the expression of *Zt BEC1019*. The co-induction of *Zt PacC and Zt BEC1019* at alkaline pH and the presence of PREs in *Zt BEC1019* promoter (described in section 5.3.4) support our hypothesis that PacC may be one of the factors regulating *Zt BEC1019* expression, but this needs to be experimentally proved. Interestingly, *BEC1019* expression is not completely supressed at pH 4.0, as seen in PacC (Figure 5-26). This could be because of the absence of zinc in the Czapek dox medium, indicating that induction of *Zt BEC1019* is equally dependent on both zinc availability and ambient pH.

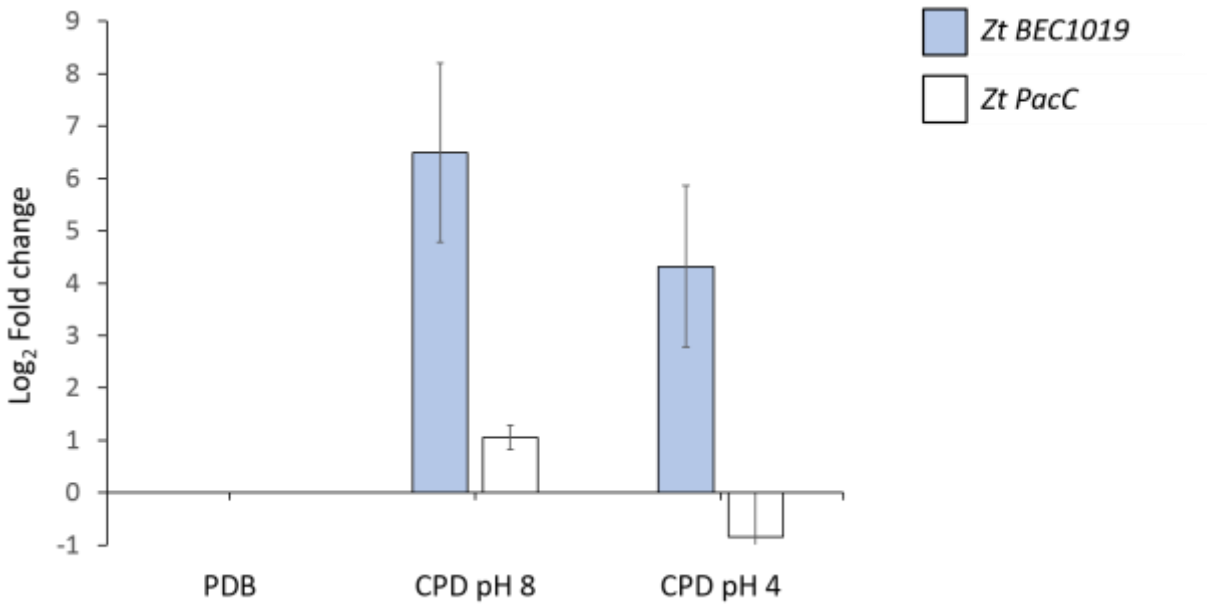


Figure 5-26: Expression studies of Zt BEC1019 and Zt PacC during in vitro growth of Z.tritic under variable ambient pH. *Expression studies of Zt BEC1019 and Zt PacC in Z. tritici growing in zinc deficient Czapek dox medium (CPD; -Zn) set at slightly alkaline (ph 8) and acidic pH (pH 4). Z. tritici growing in potato dextrose broth (PDB) rich medium was used for reference measurements. Zt tubulin was used as reference genes for qRT-PCR. N=2. Significance could not be measured due to lack of three biological replicates.*

5.3.18 *Zt BEC1019* and *ZAP1* deletion mutants could not be obtained.

BEC1019 homologues are known virulence factors in other fungal pathogens and their expression has been shown to be regulated by zinc sensitive transcription factor ZAP1 (Amich et al., 2010; Citiulo et al., 2012; Section 5.3.9). Therefore, it was postulated that *Zt BEC1019* may be important for *Z. tritici* virulence and its expression may be regulated by *Zt ZAP1*. To investigate this deletion mutants of *Zt BEC1019* (Ensembl id: Mycgr3T42164) and *Zt ZAP1* (Ensembl id: Mycgr3T89374) were to be obtained. Disease development by *Zt BEC1019* deletion mutant was to be studied to determine its role in fungal virulence. The expression of *Zt BEC1019* was to be determined in the *Zt ZAP1* deletion mutant, to investigate the role of *ZAP1* in regulation of *BEC1019* expression.

Deletion cassettes for deleting *Zt BEC1019* and *ZAP1* genes were successfully assembled in *E. coli* using Gibson cloning (Figure 5-27). But no deletion mutants could be obtained after multiple *Zt* transformation attempts by *Agrobacterium* co-cultivation. Therefore, role of *BEC1019* in *Zt* virulence and its regulation by zinc sensitive transcription factor ZAP1 could not be further investigated.

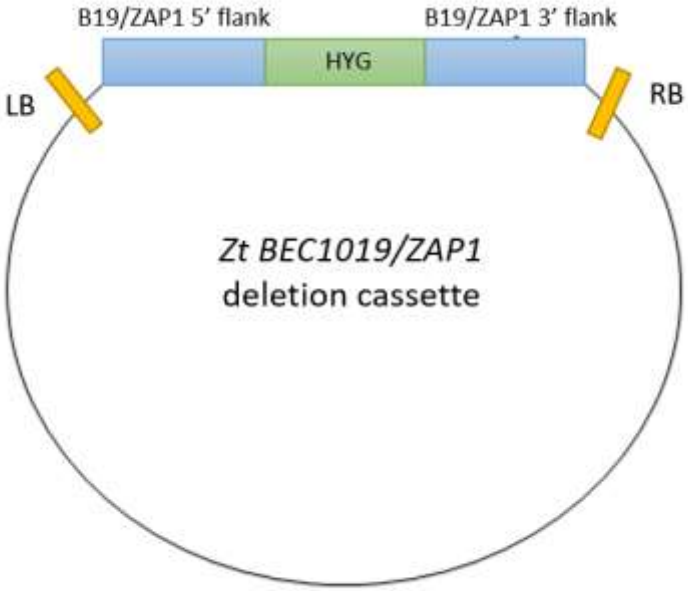


Figure 5-27: Deletion cassette for Zt BEC1019 and Zt ZAP1 deletion.

LB: Agrobacterium left border. RB: Agrobacterium right border. HYG: Hygromycin resistance gene. B19: Zt BEC1019. ZAP1: Zt ZAP1. 5'Flank/3'flank: Gene flanking regions.

5.4 Discussion

Zinc is an important micronutrient affecting growth in different organisms (Broadley et al., 2007; Staats et.al., 2013). Zinc is the second most abundant metal in organisms and present in all six enzyme classes including oxidoreductases, transferases, lyases, isomerases and ligases. Zinc binds to proteins at either catalytic, structural or co-catalytic sites. For instance, in the catalytic sites of the enzyme carbonic dehydrogenase, zinc binds to histidine residues and complexes with water and have three S, N, O donors. Similarly, in the structural zinc binding sites in enzymes like alcohol dehydrogenases, zinc binding site consists of four cysteine residues and no bound water molecules. In co-catalytic sites of enzymes like superoxide dismutase, zinc binding contributes to enzyme structure, catalysis and regulation. These sites consist of two or three zinc ions separated by amino acid resides like histidine/aspartic acid/glutamic acid. Another kind of zinc binding site involves the binding of ligands from two proteins to a single zinc atom as seen in nitric oxide synthases (Broadley et al., 2007). The biggest group of zinc binding proteins are transcription factors that regulate gene transcription by DNA/RNA binding, RNA metabolism, site-specific modifications and chromatin modification (Englbrecht et.al., 2004; Klug, 1999).

Zinc has an equal role to play in both host and pathogens. For instance, in *Arabidopsis*, 2042 zinc binding proteins were discovered by literature review, bioinformatics and *in silico* studies, which

included transcription factors, enzymes, transporters, signalling molecules, translation and enzyme regulators (Broadley et al., 2007). Another study showed that approximately 9% of eukaryotic proteome and 5-6% of prokaryotic proteome binds zinc. (Andreini et.al., 2005). Therefore, competition for zinc is an important aspect of host-microbe interaction (Staats et al., 2013).

Some fungal pathogens have virulence factors that play a role in sequestering zinc away from the host, like BEC1019 homologues Aspf2 and PRA1, which have been shown to act as zinc scavengers important for virulence in *A. fumigatus* and *C. albicans* (Amich et al., 2010; Citiulo et al., 2012). *In vivo* zinc binding capability of PRA1 was confirmed by staining the PRA1 bound zinc using the stain zinquin (Citiulo et al., 2012). These protein homologues are characterised by a conserved zinc binding HRXXH domain and an ETVIC domain of yet unknown role (Whigham et al., 2015). *AspF2* and *PRA1* are expressed during fungal growth under alkaline conditions in the human host during disease development by these fungal pathogens (Amich et al., 2010; Citiulo et al., 2012). This study showed the *BEC1019* homologues are also expressed during the infection cycle of plant fungal pathogen *B. graminis* f. sp. *tritici* (Section 5.3.5). Interestingly, the expression is upregulated between 16-24 hpi (Section 5.3.5), which is the period for primary haustoria development during powdery mildew infection (Both et al., 2005). This is also similar to the expression pattern of haustoria specific *Bgh BEC1019* during barley powdery mildew infection (Bindschedler et al., 2011; Pliego et al., 2013). Our data further confirmed the localisation of *BEC1019* expression in the *Blumeria* haustoria during both barley and wheat powdery mildew infection (Figure 5-11). Moreover, *Bgt BEC1019* silencing led to reduced wheat powdery mildew infection (Figure **5-15**). Therefore, alike *Bgh BEC1019*, *Bgt BEC1019* is also a haustoria specific virulence factor during wheat powdery mildew infection. In biotrophic plant pathogens like *Blumeria,* nutritional uptake from host is directly linked to *in planta* growth and disease development (Both et al., 2005). As zinc is an important trace element required as a cofactor in a third of proteins, including transcription factors, DNA/RNA polymerases, with a role in ribosomal functions (Fones & Preston, 2013) and defence against host induced ROS (Huang et al., 2009), therefore, it is not surprising that a potential zinc scavenger may act as a virulence factor during powdery mildew development in wheat and barley. However, the zinc binding capability of both *Bgh* and *Bgt* BEC1019 is yet to be proved. This could be investigated by expressing BEC1019 in barley leaves and staining the *in planta* BEC1019-bound zinc using zinquin dye (Citiulo et al., 2012).

Similarly, analysis of previously published bioinformatics studies showed the expression of *BEC1019* homologues during the infection cycle of other wheat pathogens like *F. graminearum* (Section 5.3.6) and *Z. tritici* (Section 5.3.7). However, deletion of *Fg BEC1019* did not affect *F. graminearum* virulence (Section 5.3.14.2), proving BEC1019 to be to dispensable for FHB development. No data could be collected for the effect of *Zt BEC1019* on STB development, as *BEC1019* deletion mutants could not be generated.

In *C. albicans* and *A. fumigatus*, the zinc scavenger is genetically linked to a zinc transporter (ZRT1 homologue) i.e., they are neighbouring genes sharing a common intergenic sequence and form a

conserved zinc sequestration locus (Citiulo et al., 2012). The genes of the zinc sequestration locus are co-regulated by availability of zinc through a zinc dependent (ZafA/ZAP1) transcription factors and by pH through a pH sensitive transcription factor (PacC), which are themselves important for fungal virulence (Bignell et al., 2005; Moreno et al., 2007; Nobile et al., 2008, 2009). Therefore, homologues of zinc transporter Zrt1 and the transcription factors ZAP1 and PacC were identified in *B. graminis* f.sp. *hordei*, *B. graminis* f.sp. *tritici*, *F. graminearum and Z. tritici* (Sections 5.3.2 and 5.3.3). However, the zinc transporter Zrt1 was genetically linked to the potential zinc scavenger BEC1019 only in *Z. tritici* and not in the other fungal pathogens under study. In *F. graminearum*, this could be explained by the *in vitro* growth studies of *ΔBEC1019* in a zinc deficient alkaline media, where absence of *BEC1019* did not impact fungal growth under zinc deficient alkaline conditions (Figure 5-22). Maybe, the dispensability of *BEC1019* for zinc homeostasis in *F. graminearum* may have led to an evolutionary loss of the zinc acquisition locus in this fungus. Moreover, wheat head is a nutritionally rich plant organ, therefore, availability of zinc might not be a limiting factor for Fusarium head blight (FHB) development. Although, in *B. graminis,* the loss of this evolutionarily conserved zinc acquisition locus can't be explained, as our data confirms a role of *BEC1019* in *Blumeria* virulence (Section 5.3.8) and its regulation by both ZAP1 and PacC transcription factors (Sections 5.3.9 and 5.3.12), increasing its probability of acting as a zinc scavenger. Saying that, the BEC1019 homologue ZPS1 in *S. cerevisiae* is also not genetically linked to a zinc transporter (Citiulo et al., 2012). However, ZPS1 is believed to be a membrane-bound protein regulated by zinc and alkaline pH with yet unknown role (Mira et.al., 2009; Wu et al., 2008). So maybe being membrane-bound themselves, they do not need a transporter. As it was reported that in *C. albicans* PRA1 associates with the fungal membrane by binding to Zrt1, enabling zinc uptake (Citiulo et al., 2012). However, it is also interesting that the synteny between the zinc scavenger and transporter was shown to be broken in another *Candida* species *C. guilliermondii,* the causative agent of a rare form of invasive candidiasis (Citiulo et al., 2012). Therefore, it is a possibility that the synteny between the zinc scavenger and the zinc transporter is not always conserved in all fungal pathogens where zinc in important for infection.

As *B. graminis* is an obligate biotroph, the role of *BEC1019* in fungal zinc acquisition under alkaline conditions was studied in *Z. tritici, where in vitro* studies were possible. Our data showed that *Zt BEC1019* (potential zinc scavenger) and *Zt Zrt1* (zinc transporter) are upregulated in the absence of zinc and under alkaline conditions, a phenomena that was reversed on addition of zinc or acidification of the medium (Sections 5.3.16 and 5.3.17). This data was supported by a previous transcriptomics study which showed upregulation of *Zt BEC1019* and *Zt Zrt1* in Czapek dox medium (a zinc deficient alkaline medium) (Rudd et al., 2015), however, the reason for this upregulation was

explained in this study. This further supported the hypothesis that *BEC1019* along with *Zrt1* may have a role in fungal zinc acquisition. The co-regulation of *Zt BEC1019* and *Zt Zrt1* was also supported by the same transcriptomics dataset , where both these genes are upregulated *in planta* during the necrotic phase of STB development (Rudd et al., 2015). Interestingly, supplementation of Czapek dox medium (-Zn; pH 7.5) with exogenous zinc almost supressed the expression BEC1019 whereas it's expression was 2-fold when the Czapek dox medium was acidified (Sections 5.3.16 and 5.3.17). Therefore, there is a possibility that the expression of BEC1019 maybe more tightly regulated by zinc availability than ambient pH. This is unlike the data acquired in *C. albicans*, where no PRA1 was expressed even in the absence of zinc at pH 5.5 (Citiulo et al., 2012). This data can be further validated by expressing a GFP protein under the *Zt BEC1019* promoter and studying its expression during *Z. tritici* growth under different zinc and pH conditions (Citiulo et al., 2012). However, in *B. graminis*, the co-regulation of *BEC1019* and *Zrt1* is yet to be investigated. This could be done by studying the time-course of *Zrt1* gene expression and comparing it with that of *BEC1019*. The expression patten of these genes during barley powdery mildew infection can also be acquired from previously published *B. graminis* f. sp. *hordei* transcriptomics data.

This study also confirmed that *Bgh ZAP1* and *Bgh PacC* influence barley powdery mildew infection development by regulating the expression of *Bgh BEC1019* virulence factor (Sections 5.3.9 and 5.3.11). These observations reciprocate previous studies which show a role of ZAP1 and PacC homologues in the virulence of other fungal pathogens like *C. albicans* and *A. fumigatus* (Bignell et al., 2005; Kim et al., 2008; Moreno et al., 2007; Nobile et al., 2009). Similarly, wheat powdery mildew infection was reduced on silencing of *Bgt* ZAP1, confirming its role in wheat powdery mildew disease development (Figure 5-18). However, the role of *Bgt ZAP1* is yet to be investigated. Alike barley powdery mildew, this can be done by studying the expression of *Bgt BEC1019* expression post *Bgt* ZAP1 silencing. Similarly, the role of *Bgt PacC* during wheat powdery mildew needs to be studied. Moreover, to better understand the role of Zrt1 in *B. graminis* zinc transport, its regulation by *B. graminis ZAP1* and *PacC* also needs to be studied.

In *F.graminearum*, *ΔPac1* (PacC homologue) mutants did not express any *Fg BEC1019*, confirming the role of PacC in *BEC1019* regulation (Figure 5-24). Interestingly, a recent study showed that PacC negatively regulates the expression of *TRI* genes needed for synthesis of DON toxin, an important virulence factor for FHB development (Merhej et al., 2011). So potentially, PacC may be a negative regulator of *F. graminearum* virulence, where it prevents virulence by supressing essential virulence genes. As, *Fg BEC1019* is positively regulated by PacC, this might explain why deletion of *Fg BEC1019* did not affect *F. graminearum* virulence and FHB development in wheat heads. These findings match previous studies in another *Fusarium sp*. For instance, in *F. oxysporum*, PacC was shown to be a

negative regulator of infection, where PacC+/- mutants were more virulent than the wild-type strain in root infection assays with tomato plants and the pacCc mutants were less virulent. This was explained by the fact that fungal entry into host roots is accompanied by acidification of the host environment. It was speculated that under those acidic conditions, PacC acted as a negative regulator of virulence to plants, possibly by preventing transcription of acid-expressed genes important for infection (Caracuel et al., 2003; Personal communication, Prof Di Pietro).
However, in *Z. tritici*, the role of BEC1019, Zrt1, ZAP1 and PacC are yet to be investigated. During this study, despite multiple attempts, deletion mutants of *Zt BEC1019, Zt PacC* and *Zt ZAP1* could not be obtained. These deletion mutants need to be obtained and their influence on *Zt* virulence needs to be studied. Moreover, expression of *Zt BEC1019* in *Zt PacC* and *Zt ZAP1* deletion mutants need to be studied to investigate their role in regulation of *Zt BEC1019.*

Chapter 6 : Regulation of *Blumeria* effector candidates by the pH dependent transcription factor homologue *Bgh* PacC

6.1 Introduction

6.1.1 Role of pH in fungal growth and virulence

This study aims to assess whether the pH sensitive transcription factor homologue *Bgh PacC* plays a role in regulating the expression of *B. graminis* effectors and virulence factors in haustoria, and therefore indirectly contribute to powdery mildew virulence during barley infection.

The ambient pH has a direct influence on fungal growth *in vitro* and *in vivo*, as well as for virulence in certain fungi (Alkan et. al., 2013; Fernandes et.al., 2017; Vylkova, 2017). This is especially important in fungal pathogens which grow in different host environments such as in highly acidic stomach at pH 2.0, moderately acidic skin and plant surfaces, which usually range from pH at 4.5 to 6.5; while other pathogens grow in a neutral to slightly basic environment such as blood at a pH of 7.4. Some fungal pathogens are confronted to alkaline parts of the intestine at pH <8.5 (Vylkova, 2017). Therefore, most, or all fungi have developed mechanisms sensing and modulating the ambient pH.

Fungal pathogens have been classified into two groups, either acidifying or alkalising, based on how they manipulate the ambient pH for growth and virulence (Alkan et. al., 2013). Acidifying fungi such as *Aspergillus sp., Colletotrichum sp.* have been known to produce organic acids to modify their micro-environment. This acid production is dependent on availability of production of organic acids, capability of these fungi to remove ammonium ions from ammonium salts and the secretion of H^+ on ammonium assimilation (Bi et al., 2016; Prusky et. al., 2001). Acid production has been shown to assist host invasion by necrotrophic pathogens such as *Botrytis sp.* For instance, *Botrytis cinerea* acidifies their environment by producing oxalic acid, and this acidification of the medium activates aspartic acid proteases which facilitate host invasion and necrotrophy (Manteau et. al., 2003). In certain fungi, like *Colletotrichum sp.,* acidification of the environment was also shown the inhibit the host ROS production (C. Chen & Dickman, 2005). Moreover, pathogen acid production allows for the activation of H^+-ATPase enzymes, which in turn leads to the activation of proteases and host invasion in *Fusarium oxysporum* (Brandao et. al., 1992) as well as the activation of virulence factors such as aspartyl proteases in *Candida albicans* (Naglik et. al., 2003).

On the other hand, host alkalinisation supports appressoria formation as well as activation of virulence factors like pectin lyases in *Colletotrichum sp.* (Shnaiderman et. al., 2013)*.*, a fungus initially classified as an acidifying fungus. Host alkalisation is generally mediated by ammonia production (Prusky et al., 2001; Vylkova et al., 2011), though fungal pathogens such as *F. oxysporum* have also been shown to secrete alkalising peptides into host cells to facilitate virulence (Masachis et al.,

2016). Nevertheless, alkaline conditions can also be detrimental for fungal growth, as they negatively impact the availability of nutrients and micronutrients such as zinc, copper and iron (Cyert & Philpott, 2013). This is countered by secretion of pH sensitive virulence factors involved in zinc sequestration under alkaline pH by *C. albicans* (PRA1) and *A. fumigatus* (AspF2) (Amich et.al., 2010; Citiulo et al., 2012).

Therefore, it is not appropriate to classify the phytopathogens simply into acidifying and alkalising fungi (Alkan et al., 2013) , as the fungal manipulation of ambient pH and its effect on fungal growth is much more complicated. In fact, it does vary depending on the stage of the life cycle or growth conditions of the fungus, especially during infection. For example, for *M. oryzae*, alkaline conditions induce spore germination whereas mildly acidic conditions (pH 6.5) facilitate host invasion and colonisation (Landraud et. al., 2013). Sometimes availability of a nutrient in the medium may be enough of a trigger for a fungus to switch between acidifying or alkalising its environment. For instance, in *Colletrichum sp.,* acidification was induced by carbon excess whereas carbon deprivation induced alkalisation of the medium (Bi et al., 2016). These findings are biologically relevant as fungal pathogens encounter varied levels of carbon availability based on the nature of the host tissue infected as well as the stage of infection (Fernandes et al., 2017). Therefore, fungi have developed a sophisticated ambient pH sensing and response mechanism, which has a direct impact on their growth, morphology, development and virulence. So, in this context, it is expected that fungi have a tight regulatory expression system that will depend on ambient pH or on pH switch. Therefore, fungal pH responsive transcription factors like PacC are likely to be involved in the regulation of effectors and virulence factors as a response ambient pH or a switch in the ambient pH.

6.1.2 Alkali pH sensing and adaptation in fungi

Fungal pH sensing and adaptation has been very well studied in the model organisms *S. cerevisiae* and *A. nidulans.* Sensing and response to high pH (alkaline) conditions is governed by the activation of Pal/Rim pathway, which is highly conserved in both ascomycetes and basidiomycetes (Peñalva & Arst, 2002; Peñalva et. al., 2014). The PalH receptor (also called RIM21 in *S. cerevisiae)* initiates the pH responsive signalling after sensing pH changes through alkali induced alterations in the lipid asymmetry of the plasma membrane. The C-terminal end of PalH senses the extracellular pH change, resulting in its displacement from the plasma membrane (Nishino et. al., 2015). The relocation of PalH onto the plasma membrane is dependent on PalI (also called RIM9 in *S. cerevisiae)*, which forms a membrane bound complex with PalH at the plasma membrane (Calcagno-Pizarelli et al., 2007). PalH then mediates the ubiquitination and phosphorylation of its an α-arrestin interacting partner PalF (also called RIM8 in *S. cerevisiae)* (Herranz et al., 2005). This in turn, results in the endocytosis of the membrane receptor complex formed of PalH and PalI, and the recruitment of the endosomal

sorting complexes (ESCRT) (Xu et.al., 2004). Finally, PalA (also called RIM20 in *S. cerevisiae)* presents the yet inactive transcription factor PacC (called RIM101 in *S. cerevisiae*) as a substrate to the signal protease PalB (also called RIM13p in *S. cerevisiae)*, resulting in its activation via double proteolytic cleavage mediated by both PalB and the proteasome (Denison et. al., 1995; W Xu & Mitchell, 2001). Once active, the PacC transcription factor moves into the nucleus where it mediates the activation of alkali-expressed genes and supresses acid-expressed genes (Tilburn et al., 1995) (Figure 6-1; Table 6-1). Therefore, PacC plays a pivotal role in fungal growth under different environmental pH conditions.

Table 6-1: Pal pathway components and their role in fungal pH response.

Pal pathway components in *A. nidulans*	**Role**	**Name in *S. cerevisiae* and *C. albicans***
PalH	Receptor	RIM21
PalI	Part of receptor complex	RIM9
PalF	α-arrestin	RIM8
PallA	Recruits PacC for proteolysis	RIM20
PalB	Signal protease	RIM13
PacC	pH sensitive transcription factor	RIM101

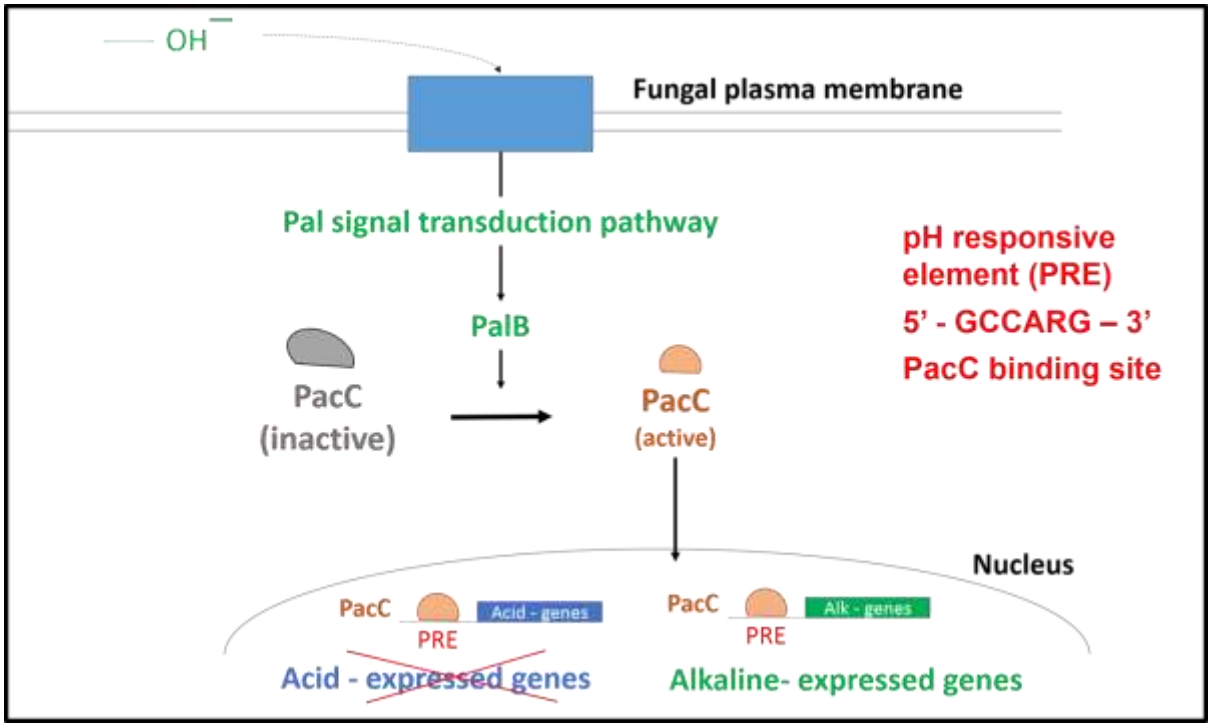


Figure 6-1: Role of PacC during fungal growth.

Under neutral to alkaline pH, PacC (RIM101 in yeast) is activated by proteolytic cleavage by PalB from Pal pathway and proteasome. Active PacC binds to pH regulatory elements (PREs) of alkaline/acid expressed genes, resulting in their activation/ suppression. Model adapted from Hua et.al., 2010.

6.1.3 The pH regulated transcription factor PacC

The pH responsive transcription factor PacC is the master regulator of genes needed for fungal adaptive responses to environmental pH. PacC is activated by two C-terminal proteolytic cleavages. The first alkaline pH-dependent proteolytic cleavage is catalysed by the signalling protease PalB described above. A second pH-independent cleavage is proteasome mediated (Díez et al., 2002; Peñas et al., 2007). The first PalB mediated cleavage converts the 72 kDa (PacC72) inactive PacC protein into an intermediate 53 kDa (PacC53) form, which is further cleaved to an active 27 kDa (PacC27) form by the proteasome. The PacC72 avoids proteasome mediated proteolytic cleavage under acidic conditions due to intra-molecular interactions, although a portion of PacC72 have a proteasome accessible conformation, which results in its activation under mildly acidic pH of 6.5. Most of the PacC activity is carried out by PacC27 (Peñas et al., 2007). Once activated, PacC, in the 27kDa form induces alkali-expressed and represses acid-expressed genes (Tilburn et al., 1995) by binding to highly conserved pH responsive elements in the promoter region of these alkali or acid expressed genes (Espeso et al., 1997) (Figure 6-1).

Through its activity in modulating pH responsive genes, PacC has also been shown to be important for the virulence of various fungal pathogens such as *C. albicans* (Davis et.al., 2000)*, A. fumigatus*

(Bignell et al., 2005), *M. oryzae* (Landraud et al., 2013), *where PacC* deletion reduced fungal virulence and increased susceptibility to ROS damage under alkaline conditions. PacC also negatively regulates *F. oxysporum* virulence during root infection (Caracuel et.al., 2003) . *PacC* deletion mutants in *F. oxysporum* had growth defects under alkaline conditions but were more virulent than wild type strain during root infection. *PacC* complementation restored the virulence in these deletion mutants, confirming that PacC is a negative regulator of *F. oxysporum* virulence (Caracuel et.al., 2003) . Since the pH of the host tissue infected by *F. oxysporum* was between 5.5-6.0, it was speculated that PacC might negatively impact on infection by negatively regulating and repressing acidic genes like pectinases which are important for *F. oxysporum* virulence (Caracuel et al., 2003). Interestingly, during apple cranker disease, caused by *Valsa mali*, PacC positively regulates virulence by producing citric acid that supports virulence by acidifying the host environment (Wu et.al., 2018). *PacC* deletion mutants of *V. mali* have reduced virulence and the ambient pH in the host during lesion development is noticeably higher (Wu et.al., 2018). But in most cases, PacC promotes the virulence of fungal pathogens by regulating the expression of virulence factors such as *AspF2* in *A. fumigatus* (Amich et.al., 2010), *PRA1* in *C. albicans* (Citiulo et al., 2012), secreted lytic enzymes in *M. oryzae* (Landraud et al., 2013) and genes involved in DON toxin production in *F. graminearum* (Merhej et.al., 2011).

In this research task, the role of the *Bgh* PacC homologous to other fungal PacC was investigated, to evaluate its role in virulence via regulation of effector expression during barley powdery mildew infection development.

6.1.4 *Blumeria* effector candidates as potential regulatory targets of *Bgh* PacC?

Effectors are defined as usually species-specific secreted proteins that assist with host colonisation and infection development. These effectors are predicted to interfere with the host processes that support host immunity (Jones & Dangl, 2006).

After the sequencing of the *B. graminis* genome in 2010 (Spanu et. al., 2010), a proteo-genomics approach identified several *Blumeria* effector candidates (BECs). These were small proteins with a predicted signal peptide and a high haustoria to hyphae expression ratio (Bindschedler et al., 2009; Bindschedler et. al., 2011). Another transcriptomic study identified a set of candidate secreted effector proteins (CSEPs) which were characterised as small mildew specific proteins lacking a transmembrane domain and possessing a signal peptide (Pedersen et al., 2012; Spanu et. al., 2010). There is a large overlap between BECs and CSEPs, but some BECs were not included in CSEPs due to presence of homologues in other fungi, making these exceptions virulence factors rather than species-specific effectors (Aguilar et. al., 2015; Bindschedler et al., 2011).

These signal-peptide-containing CSEPs were highly upregulated in the haustoria, indicating that they might be secreted into the host through the haustorium to assist with *B. graminis* virulence. Therefore, the obligate biotrophic pathogen *Blumeria graminis* f.sp. *hordei* has a battery of predicted effectors and virulence factors that are secreted, species specific and might act as virulence factor to assist barley powdery mildew infection and establishment in the host. (Aguilar et. al., 2015; Ahmed et al., 2015; Orman et. al., manuscript in prep; Pliego et al., 2013; Whigham et al., 2015). *B. graminis* also possesses transposon associated family of avirulence genes (EKA family) that compose the third group of effectors (Amselem et al., 2011). These are all listed in Table 1.2.

During barley powdery mildew infection, *Blumeria* effector candidate 1011 (BEC1011/CSEP0264), is a haustoria specific effector (Bindschedler et al., 2011), as demonstrated by reduction in *B. graminis* virulence and infection spread post BEC1011 silencing (Pliego et al., 2013b). BEC1011 belongs to CSEP family 21, that are characterised by a conserved RNase like fold structure and are called RNAse like proteins in haustoria (RALPHs) (Pedersen et al., 2012). Family 21 also consists of other known *Blumeria* effectors like BEC1054 (CSEP0064) (Pliego et al., 2013b), CSEP0065 and CSEP0066 (Orman, Das Gupta et.al., manuscript in prep).

Similarly, *Blumeria* metallo-protease like effector (BEC1019) is a haustoria specific protein, as confirmed by high haustoria to hyphae expression ratio of the protein (Bindschedler et al., 2011). It was also subsequently shown to play a role in *Blumeria* virulence, since BEC1019 silencing reduced fungal virulence and disease spread (Pliego et al, 2013; Whigham et al., 2015). This data was also reproduced by Kate Orman in the Bindschedler lab using PTO modified ASO based *in planta* silencing of *Bgh BEC1019* (Orman, Das Gupta et.al., manuscript in prep). As described in chapter 5, in more details, it has homologues in a third of all sequenced fungal species, which include known virulence factors like PRA1 in *C. albicans* and AspF2 in *A. fumigatus* (Whigham et al., 2015b). Rather than acting as proteases, PRA1 and AspF2 were shown to be a zinc sequesters that influences fungal virulence by scavenging host zinc under alkaline conditions. Both *AspF2* and *PRA1* genes are regulated by the pH sensitive transcription factor PacC (Amich et al., 2010; Citiulo et al., 2012).

6.2 Objectives and experimental rationale

6.2.0 Aim: Investigating whether haustoria specificity of effectors and virulence factors such as BEC1019 and CSEPs from family 21, is governed by the transcription factor PacC, as well as discovery of promoter elements that are specific to effectors and virulence factors, highly expressed in haustoria

6.2.1 Objective 1: Promoter analysis of *Blumeria* effector candidate genes highly expressed in haustoria relatively to hyphae, to identify specific regulatory elements that can explain haustoria specificity

As mentioned above, most CSEPs/BECs have a high haustoria to epiphytic hyphae expression ratio (Pedersen et al., 2012; Spanu et.al., 2010; Bindschedler et al., 2011). In order to query the regulation of haustoria specific expression of *Blumeria* effector candidates, a promoter analysis was carried out using the Discriminative Regular Expression Motif Elicitation (DREME) (http://meme-suite.org/doc/dreme.html) in the Motif-based sequence analysis (MEME) suite (Bailey et al., 2009). This analysis aimed at *ab initio* discovery of any motifs enriched or specific to the promoters of the CSEPs with higher haustoria expression relative to hyphae, as compared to a list of genes which coded proteins expressed exclusively in *Blumeria* hyphae. The candidate effector data was obtained from both transcriptomics (C. Pedersen et al., 2012) and proteomics studies (Bindschedler et al., 2011). The full list of CSEPs was with high haustoria to hyphae ratio was obtained from the transcriptomics data (C. Pedersen et al., 2012). A list of 71 haustoria-only proteins identified by the shot-gun proteomics study (Bindschedler et al., 2011) were included in the study to cover both CSEPs and CSEP like proteins (CELPs), which were not mildew-specific and hence not included in the CSEP list. For negative control, another list of genes obtained from a random selection of 70 proteins identified in *Blumeria* epiphytic hyphae during the proteomics study was used. The promoter elements enriched in the haustoria specific effector candidates were then identified using the motif comparing TOMTOM tool in the Motif-based sequence analysis (MEME) suite (http://meme-suite.org/doc/tomtom.html) (Gupta et.al., 2007), which compares the motifs to the Jasper database of known yeast promoter motifs that bind specific transcription factors.

6.2.2 Objective 2: To query the virulence role of the pH sensitive transcription factor (PacC)

Since PacC transcription factor binding pH responsive elements (PREs) were shown to be enriched in the promoters of CSEPs or *Blumeria* effector candidates, the role of PacC in *B. graminis* virulence will be investigates by silencing *Bgh PacC* and studying its impact on powdery mildew infection development.

6.2.3 Objective 3: To investigate if *Bgh PacC* regulates effector expression in *B. graminis. f.sp. hordei*

Since PacC influences fungal virulence by regulating the expression of virulence factors in other fungal pathogens like *C. albicans* and *A. fumigatus* (Amich et al., 2010; Citiulo et al., 2012), the role of *Bgh PacC* in regulation of known *Blumeria* effectors/virulence factors BEC1011 and BEC1019 will be queried. To achieve this, the effect of *Bgh PacC* silencing on *BEC1011* and *BEC1019* transcript levels will be monitored.

6.3 Results

6.3.1 Promoter studies for *ab initio* discovery of motifs enriched in promoters of haustoria-specific *Blumeria* effector candidates

The *Blumeria* effector candidates are haustoria-specific proteins (Bindschedler et al., 2011), many of which were identified as CSEPs (Pedersen et al , 2012). To understand the factors influencing their haustoria-specific expression, the promoter regions of effector candidates with a high haustoria to epiphytic hyphae expression ratio were queried using the DREME software tool within the MEME suite for the *ab initio* discovery of any effector specific promoter elements. The full CSEPs list of mildew-specific effector candidates identified based on their high haustoria to hyphae expression ratio during a transcriptomics study was obtained (C. Pedersen et al., 2012). Along with the complete list of CSEPs, a group of CSEPs with haustoria/hyphae expression ratio above 50 was included as a separate dataset, to analyse the CSEPs with higher expression in haustoria (C. Pedersen et al., 2012). Another list of 71 genes encoding for haustoria-only proteins identified during a shotgun proteomics study (Bindschedler et al., 2011) were also included in the analysis to cover the *Blumeria* effector candidates that were not mildew-specific and hence were not classified as CSEPs (Pedersen et al., 2012; Spanu et.al., 2010). This group was called CSEP like proteins (CELPs). CELPs also included known *Blumeria* virulence factors like BEC1019. A random list of promoters from 70 hyphae specific genes generated from proteins identified exclusively in hyphae (Bindschedler et al., 2011) was used as negative control. The full list of datasets is included in Table 6-2. The full list of CSEPs are included in the supplementary table 3 (S3).

Table 6-2: Details of data sets used for the promoter analysis.

Effector candidates	Classification of data	Source of data sets
CSEPs	Full list of CSEPs	Pedersen et al., 2012
CSEPs	Full list of CSEPs with haustoria/ epiphytic hyphae expression ratio above 50	Pedersen et al., 2012
CELPs	Full list of CELPs-haustoria only proteins	Bindschedler et al., 2011
CSEPs + CELPs	Full list pf CSEPs + full list of CELPs	Pedersen et al., 2012; Bindschedler et al., 2011
HYPHAE	Hyphae-only proteins	Bindschedler et al., 2011

This promoter study confirmed the presence of a -GCCAAG- motif (IUPAC name - TGCCAAR) in 65 out of 97 genes in the 1000 bp promoter region of these haustoria specific CSEPs in comparison to only 9 out of 71 promoters from the negative gene control set (Table 6-3). The same motif was enriched in 40 out of 83 CSEPs with haustoria/hyphae expression ratio above 50 (Table 6-3). Another motif with IUPAC alphabets CWTGGCA, which was a reverse complement of -GCCAAG- motif, was shown to be enriched in 58 out of 127 candidates included in the combined list of CSEPs and CELPs (Table 6-3). Interestingly, no motifs were identified in the CELPs only list (Table 6-3). Based on the higher enrichment of the -GCCAAG- motif in multiple data sets of haustoria-specific effector candidates, this motif was submitted to the motif comparing TOMTOM tool within the MEME suite to identify any transcription factor that may bind to this motif.

The motif comparing TOMTOM tool, which compares the submitted domains to the yeast transcription factor binding domains included in the JASPAR CORE 2014 database (Gupta et al., 2007), identified this -GCCAAG- motif as the pH responsive element (PRE) binding the pH sensitive transcription factor RIM101 from *S. cerivisae* (Table 6-4), RIM101 being the equivalent of PacC.

Next the promoter regions of a few validated effectors acting as virulence factors, i.e. the *Blumeria* metallo-protease-like effector BEC1019 and the effector candidates belonging to the family 21 like CSEP0264(BEC1011), CSEP0064 (BEC1054), CSEP0065 and CSEP0066 were manually queried for the presence of this PRE sequence. This led to the confirmation of the presence of one or more PRE motifs in the promoter regions of these *B. graminis* effectors (Figure 6-2). These results match the outcome of the study in chapter 3 querying the regulation of *Bgh BEC1019* by the pH sensitive

transcription factor PacC, where PREs were independently identified in the promoter region of *Bgh BEC1019*, a known haustoria-specific *Blumeria* virulence factor (Figure 5-9). These findings suggest that *Blumeria* effector expression might be regulated by the pH sensitive transcription factor PacC/RIM101.

Table 6-3: Motifs enriched during promoter analysis of B. graminis effectors.

Table describing the frequency and nature of motifs enriched in the promoter region of different data sets of haustoria specific effector candidate (CSEPs) as compared to to hyphae specific proteins (negative control). The data was generated by ab initio discovery of motifs using MEME suite.

Dataset	Motifs discovered (IUPAC alphabet)	Motifs discovered (Logo)	Motif discovered (Reverse complement logo)	E-value	Frequency of motif occurrence in queried dataset	Frequency of motif occurrence in negative control
Full CSEP list	TGCCAAR			7.1e-003	65/97	8/71
CSEPs with ratio above 50	TGCCAAR			1.7e-002	40/83	8/71
Full CELPs list	No motifs found					
CSEPs + CELPs	CWTGGCA			7.0e-008	58/127	7/71

Table 6-4: Description of the haustoria-specific effector enriched -GCCAAG- motif

Motif identified by the TOMTOM software used to query the JASPER database of transcription factor binding promoter elements in yeast.

NAME:	RIM101
MATRIX ID:	MA0368.1
CLASS:	C2H2 zinc finger factors
FAMILY:	Factors with multiple dispersed zinc fingers
COLLECTION:	CORE
TAXON:	Fungi
SPECIES:	*Saccharomyces cerevisiae*
DATA TYPE:	PBM, CSA and/or DIP-chip
VALIDATION:	19111667
UNIPROT ID:	P33400

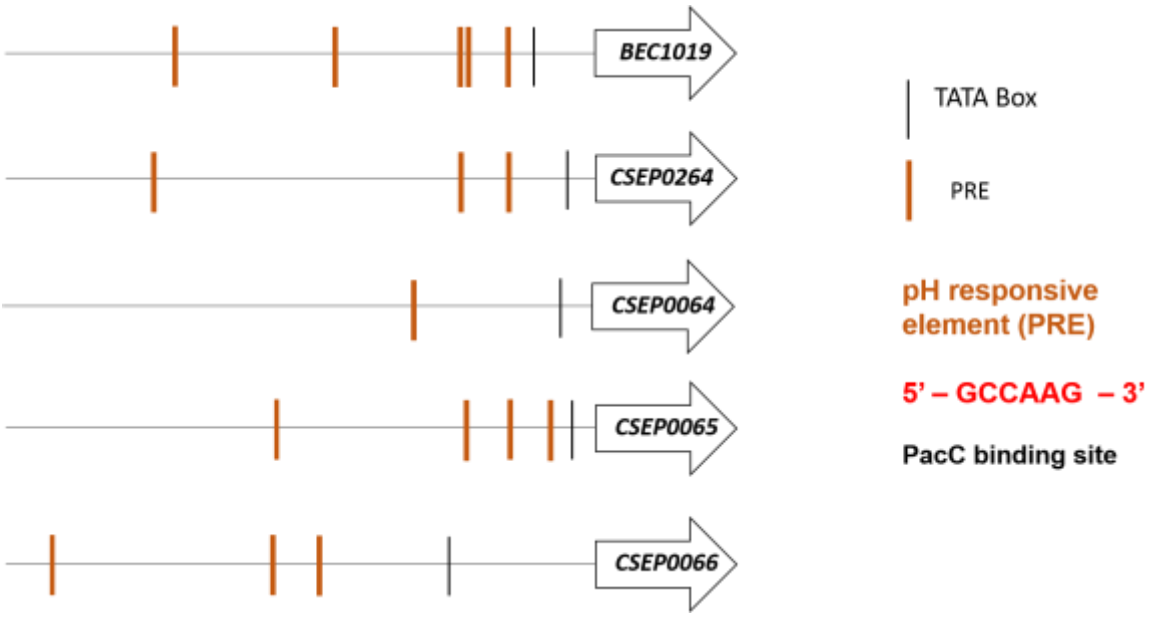


Figure 6-2: pH responsive element (PRE).

Prediction of putative pH responsive elements (PRE) (5'-GCCAAG-3') in the promoter regions of B. graminis candidate secreted effector candidates (CSEPs) belonging to family 21 and BEC1019.

6.3.2 *Bgh PacC* impact on virulence by controlling the expression of *Bgh* effectors

Since PacC influences *C. albicans* and *A. fumigatus* virulence by regulating the expression of *Bgh BEC1019* homologues *PRA1* and *AspF2* as described in detail in chapter 5, the role of *Bgh PacC* in powdery mildew infection development and regulation of *Bgh BEC1019* expression was queried. On discovery of the PREs in the promoter regions of *Blumeria* effector candidates, this study was further extended to investigate the role of *Bgh PacC* in regulation of the effector *Bgh BEC1011*.

This was tested by studying the impact of *Bgh PacC* silencing on powdery mildew infection development and transcript levels of *Bgh BEC1019* and *BEC1011*. AsPTO based *PacC* silencing reduced its transcript levels by 63% (Figure 6-3B), confirming *Bgh PacC* silencing. This was accompanied by a reduction in barley mildew infection development by 48% (Figure 6-3A), confirming a role of PacC in *Blumeria* virulence. Interestingly, this reduction in *PacC* transcript level was also accompanied by a corresponding reduction in transcripts of *BEC1019* and *BEC1011* by 58% and 51% respectively (Figure 6-3B). The expression of *BEC1011* and *BEC1019* were relative to *Bgh GAPDH*, confirming that the reduction in effector transcript levels was not due to the reduced fungal biomass post *PacC* silencing. These findings confirm that PacC may be one of the factors regulating the expression of *B. graminis* effectors/virulence factors BEC1011 and BEC1019 during barley powdery mildew infection.

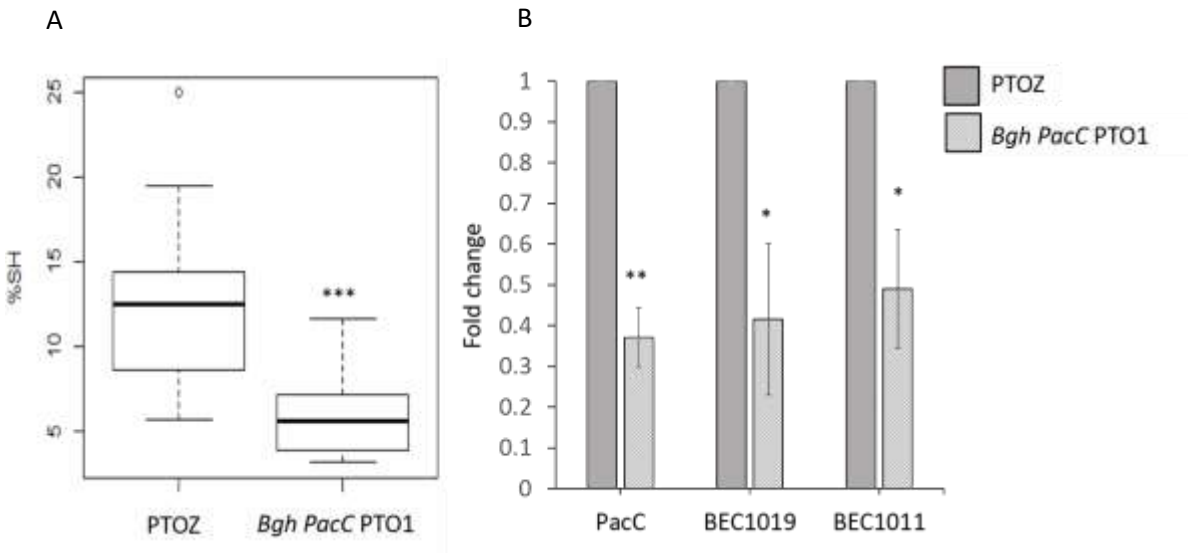


Figure 6-3: Effect of Bgh PacC on barley powdery mildew infection and effector expression.

Impact of silencing Bgh PacC on A. barley powdery mildew infection, as measured via the proportion of secondary hyphae (%SH) formed per germinated conidia, B. PacC and BEC1019 transcript levels. PTOZ targeting a seed protein Z was used as a control. Silencing was carried out on 7 days old barley (c.v. Golden Promise) excised leaves. For infection assay, at least 250 appressoria forming conidia were counted and the accompanying number of non-germinated conidia and hyphae were noted. Bgh GAPDH was used as a reference gene for qRT-PCR. Data derived from 3 biological replicates for infection and 4

*biological replicates for qRT-PCR, where each repeat consisted of 6 leaves. Significance was calculated in R using the general linear model for (A) and 1 sample t-test for (B) * P<0.05, ** P<0.01, *** P<0.001.*

-

6.4 Discussion

Effectors and virulence factors are central to successful host colonisation and infection development by various fungal pathogens (reviewed by Lo Presti et al., 2015). Effectors have been shown to be instrumental in subverting host metabolism by detoxifying defence compounds (reviewed by Shalaby & Horwitz, 2015) , by dampening the host immune response (Blümke et al., 2014; Marshall et al., 2011) and for nutrient acquisition during infection (Citiulo et al., 2012; reviewed by Divon & Fluhr, 2007). Even though many studies in the recent times have focussed on understanding the functions and targets of these effectors, our knowledge of effector transcriptional regulation is still limited.

Some recent functional studies have unearthed transcription factors important for pathogenicity of different fungal phytopathogens (Cao et al., 2016; Cho et al., 2012; Park et al., 2013; Son et al., 2011). Thus far, 12 transcriptional factors belonging to 4 families have been identified with a role in regulating the expression of candidate effectors (reviewed by Tan & Oliver, 2017). These transcription factor families include the zinc-finger containing transcription factors, the APSAS (Asm1, Phd1, Sok2, Efg1, and StuA), the WOPRs and the fork-head domain containing transcription factors (reviewed by Tan and Oliver, 2017). The APSAS family consists of basic helix-loop-helix containing transcription factors that are specific to the wheat pathogen *Parastagnospora nodorum*. *SnStuA1*, a member of this APSAS family was shown to regulate the fungal growth and virulence by regulating the expression of the genes involved in carbon metabolism, synthesis of the mycotoxin alternariol and the *P. nodorum* effector *SnTox3* (IpCho et al., 2010). Similarly, The WOPR family of transcription factors are characterised by the presence of two dissimilar but highly conserved globular N-terminal DNA binding domains (Lohse et.al., 2010), and have been discovered in many pathogenic fungi (reviewed by Tan & Oliver, 2017). The *Fusarium oxysporum* transcription factor SGE1 (SIX Gene Expression 1), a member of the WORP family, was shown to impact on tomato wilt development by regulating the expression of the *SIX* genes encoding fungal effectors (Michielse et al., 2009). In the third transcription factor family, the fork head is a "conserved helix-turn-helix DNA binding domain with a winged helix structure" (reviewed by Tan & Oliver, 2017). *Fox1*, a member of this fork-dead domain family, is believed to affect *U. maydis* virulence by positively regulating six putative effector candidates (Zahiri et. al., 2010).

Amongst these families, the zinc-finger containing transcription factors are the biggest family of

transcription factors found in fungi (reviewed by Tan and Oliver, 2017). Members of this family harbour at least one zing finger, where zinc binds to either cysteine (C) or histidine (H) residues (MacPherson et.al., 2006). These zinc-fingers are also the sites of zinc dependent binding of DNA by these transcription factors (Frey et.al., 2011). AbPf2 from *Alternaria brassicicola,* a Zn2Cys6 binuclear cluster domain transcription factor, is an important member of this zinc-finger containing family of transcription factors. The deletion of *AbPf2* led to reduced fungal virulence accompanied by a reduction in expression of 8 cysteine-rich small secreted candidate effector proteins (Cho et. al., 2013), confirming its indirect role in *Alternaria* virulence through regulation of putative effector expression. Similarly, the AbPf2 ortholog in *Parastagnospora nodurom,* PnPf2, was shown to impact on fungal virulence by positively regulating the expression of the necrotrophic effectors *SnToxA* and *SnTox3* (Rybak et al., 2017). This zinc-finger family also includes the zinc sensitive transcription factor ZAP1 and the pH sensitive transcription factor PacC. ZAP1 (extensively described in chapter 3) has been shown to be required for fungal growth under zinc limiting conditions and to regulate virulence factors of human fungal pathogens such as *C. albicans* and *A. fumigatus* (Amich et al., 2010; Böttcher et.al., 2015; Citiulo et al., 2012; Moreno et al., 2007). PacC, on the other hand, modulates fungal response to ambient pH and is therefore required for virulence in many, but not all fungal pathogens (Alkan et al., 2013; Bignell et al., 2005; Calcagno-Pizarelli et al., 2007; Landraud et al., 2013). PacC was also shown to both positively and negatively regulate fungal virulence factors (Amich et al., 2010; Citiulo et al., 2012; Merhej et. al., 2011).

In this study, the role of the *Bgh* PacC transcription factor in virulence of the obligate biotrophic pathogen *B. graminis* f. sp. *hordei* was demonstrated. PacC, along with the transcription factor ZAP1 (described in chapter 5) are the first transcription factors shown to be important during barley powdery mildew infection development. This study indicates that PacC may influence *B. graminis* virulence by regulating the haustoria-specific expression of virulence factors like *BEC1011* and *BEC1019* (Section 6.3.2). However, the expression of other *Blumeria* effector candidates may also be regulated by PacC, as indicated by the presence of PacC binding pH responsive elements (PREs) (Espeso et al., 1997) in their promoter regions (Table 6-3; Table 6-4; Figure 6-2). The *Bgh PacC* sequence alignment with other fungi like *Aspergillus* sp. showed the presence of three zinc-fingers, amongst which the zinc fingers 2 and 3 were conserved (described in chapter 5). These two zinc fingers were shown to be the point of contact between PacC and the PREs (Espeso et al., 1997). However, this interaction is yet to be tested in *B. graminis*. This could be attempted by yeast-one-hybrid studies, and if proven will further strengthen the hypothesis of *Blumeria* effector regulation by *Bgh PacC*.

Even though not much is known about the manipulation of host apoplastic pH by *Blumeria,* the

change in barley apoplastic and intracellular H^+ levels post barley powdery mildew infection was studied for short and long term changes (Felle et. al., 2004; Felle et. al., 2008). Interestingly, between 2 to 4 hpi after *B. graminis* inoculation, a transient increase in apoplastic pH accompanied by a reduction in intra-cellular pH was observed in the infected barley leaves. This pH change was believed to be in response to fungal pathogen perception by the host PAMP receptors. Similarly, at 24 hpi, the intracellular pH increased again, this time by two units. This was believed to be as a result of mounting of host immune responses rather than fungal manipulation of the host to increase compatibility (Felle et al., 2008). PacC was shown to be activated at pH 6.5 by proteasome cleavage in *A. nidulans* (Peñas et al., 2007). So maybe a short spike in alkalinity during host penetration is enough to activate PacC which in turn regulates effector expression in haustoria, as supported by the *PacC* expression localisation in *Blumeria* haustoria along with *Bgh BEC1019* (as described in section 5.3.11). Moreover, effectors like *Bgh BEC1011* and *Bgh BEC1019* have been shown to be induced at 24hpi (Pliego et al., 2013; Dasgupta S., unpublished data), when the barley intracellular pH is likely to be high as mentioned in the above described study (Peñas et al., 2007). This indicates that the expression of *Blumeria* effectors in the haustoria may be induced in response to extracellular neutral/alkaline pH. But this hypothesis is yet to be experimentally tested. However, this is a difficult experiment to perform for a biotrophic pathogen, as it cannot be done *in vitro*. Interestingly, another study demonstrated the dependency of *Blumeria* host penetration on host cytoplasmic pH. Increase in cytoplasmic pH in barley coleoptile cells increased powdery mildew susceptibility by increasing *Blumeria* host penetration (Yamaoka et al., 2000). These observations further support our hypothesis that the pH sensitive transcription factor PacC may impact on *B. graminis* virulence by globally regulating the expression of effectors/virulence factors required for barley powdery mildew infection development.

This study has provided another novel target for barley powdery mildew disease control. *Bgh PacC*, a transcription factor which potentially regulates the expression of multiple *Blumeria* effectors/ virulence factors, can be a good target for silencing or fungicide-based inhibition for successful disease control. *PacC*, with its conserved role in controlling fungal growth under alkaline conditions, is less like to mutate and hence its targeting may provide durable resistance against powdery mildew. Moreover, PacC which is conserved amongst fungi does not show very high sequence identity amongst its homologues but possess two conserved zinc-fingers which mediate DNA binding for gene regulation (section 5.3.3). Therefore pathogen-specific fungicides/silencing molecules can be designed which would not adversely impact useful fungi.

Chapter 7 Adaptation of the PTO based gene silencing method for *in planta* and *in vitro* gene silencing of *Fusarium graminearum* and *Zymoseptoria tritici* genes

7.1 Introduction

7.1.1 Gene silencing in fungal pathogens

In the recent years, gene silencing has become an emerging approach for functional genomics studies and disease control in numerous fungal phytopathogens (Panwar et.al., 2013; Gu et al., 2019; Nowara et al., 2010; Park et al., 2012; Pliego et al., 2013; Wang et.al., 2016). These gene silencing approaches were all mediated by RNA interference (RNAi) depending on gene specific dsRNA (Gu et al., 2019; Koch et. al., 2016) and hairpin RNA (hpRNA) (Nowara et al., 2010; Panwar et al., 2013; Park et al., 2012; Pliego et al., 2013). The silencing molecules where initially delivered by virus induced gene silencing, using hpRNA cloned into viral vectors and delivered into hosts by viral infection for virus induced gene silencing (VIGS) (Panwar et al., 2013). However, the use of viral vectors such as BSMV have been shown to trigger the plant immune system, rendering this system unsuitable for functional genomics studies (Tufan et.al., 2011). Host plants stably transformed for expressing a fungal specific gene hpRNA were also used for fungal disease control. For instance, FHB resistance was enhanced in leaves and spikes of stable transgenic wheat lines expressing a gene specific hpRNA targeting the fungal β-1, 3-glucan synthase gene (Chen et al., 2016). In the obligate biotrophic pathogen *Blumeria graminis*, host induced gene silencing (HIGS) was also achieved by biolistic delivery into barley leaves of a plasmid carrying an expression cassette allowing the expression of a hpRNA targeting fungal effector genes in epidermal barley cells (Pliego et al., 2013b). Although, these approaches are not suitable for their application in crop protection due to complications involving host transformation. Consequently, new approaches involving direct delivery of dsRNA have been explored in numerous studies for both *in vitro* (Koch et al., 2013) and *in planta* (Gu et al., 2019; Koch et al., 2016; Song et. al., 2018; Wang et. al., 2016) silencing of fungal genes. The *in vitro* gene silencing approach was achieved by uptake of dsRNA targeting *F. graminearum* CYP genes directly from the fungal medium, resulting in reduced fungal growth (Koch et al., 2013). *In planta* gene silencing, on the other hand, exploited direct delivery of gene specific short dsRNA sprayed on plant tissues such as leaves, inflorescence, fruits and vegetables, resulting in reduced disease symptoms (Gu et al., 2019; Koch et al., 2016; Song et.al., 2018; Wang et al., 2016). Nevertheless, a recent study for silencing *Fusarium asiaticum* showed that the spraying of dsRNA targeting fungal genes resulted in a silencing effect only for up to 9 hours (Song et al., 2018),

requiring multiple application of dsRNA for a long term silencing effect. However, spraying with dsRNA targeting plant genes resulted in a longer silencing effect, suggesting that this system was limited by the fungal siRNA secondary amplification machinery (Song et.al., 2018). Therefore, new silencing molecules that can silence fungal and plant genes by direct delivery into plant hosts need to be explored.

In the Bindschedler laboratory, the use of phosphorothioate modified antisense short oligodeoxynucleotides (PTO modified ASO) for *in planta* gene silencing has been developed as a methodology to silence fungal and plant genes in the barley-powdery mildew pathosystem (Orman, Das Gupta, manuscript in prep). In PTO-modified ASO, one of the non-bridging oxygens of the DNA phosphodiester bond is replaced by a sulphur atom (Figure 1.17), resulting in higher stability of the silencing molecule due to increased resistance to host nucleases (Stein et.al., 1988). Silencing of a specific barley or *Blumeria* gene was achieved by direct uptake of a gene specific PTO modified ASO into excised barley leaves by transpiration. These PTO modified ASO treated leaves were then inoculated with *B. graminis* spores and the effect of gene silencing on powdery mildew disease development was monitored by scoring the disease development microscopically at 48 hpi , measuring the number of spores successful in producing hyphae (Orman, Das Gupta et.al., manuscipt in prep) (Section 2.3). So far, this methodology has been validated by silencing multiple *B. graminis* effector genes, previously known to be involved in virulence and barley genes known to be involved in host susceptibility. The PTO mediated ASO targeting of these genes led to reduced powdery mildew infection (Orman et.al., manuscipt in prep). Earlier in this project, this PTO modified ASO based gene silencing was successfully utilised to silence *B. graminis* housekeeping genes and barley susceptibility genes, suggesting that this strategy is suitable for disease control in multiple barley cultivars (chapter 3) as well as for discovery of new players involved in *B. graminis* virulence or plant susceptibilty (chapters 3,5,6). Therefore, this PTO modified ASO approach will be investigated for its potential and adaptation for *in vitro* and *in planta* gene silencing in other fungal pathogens such as *F. graminearum* and *Z. tritici.*

7.1.2 Fungicide and fungicide resistance in *Fusarium graminearum* and *Zymoseptoria tritici*

Fungicides have been traditionally used in agriculture for controlling fungal diseases. These include fungicides targeting different defence mechanisms and metabolic processes like respiration (Miles et.al., 2012; Xiong et al., 2015), sterol synthesis (Ma et.al., 2006), cell division (Qiu et al., 2011), amongst others (explained in detail in section 1.3). For example, fungal respiration is targeted by succinate dehydrogenase inhibitors (SDHI) (Xiong et al., 2015) and Quinone outside inhibitors (QoI) (Miles et al., 2012). Fungal cytoskeleton and cell division are affected by methyl benzimidazole carbamates (MBC) that target the fungal β-tubulin gene (Qiu et al., 2011). Similarly, the

demethylation inhibitors (DMI) such as the triazoles target the fungal sterol biosynthesis enzyme C14-demethylase (Ma et al., 2006).

In the wheat pathogen *F. graminearum,* the number of effective fungicides for FHB control is limited. Till date, FHB control only relied on MBC and a few azole fungicides (Lucas et al., 2015). Worldwide *F. graminearum* strains are naturally resistant to QoI fungicides (Dubos et.al., 2011). The European and North American *F. graminearum* strains are also resistant to SDHI fungicides (Dubos et al., 2013). The extent and type of fungicide resistance also varies between wheat cultivars. There are contrasting reports about fungicide resistance incidence, likely dependant on the *F. graminearum* strains associated with certain wheat cultivars. For instance, a study conducted between 1992-1993 reported about FHB insensitivity to several fungicides such as benomyl, chlorothalonil, fenbuconazole, flusilazole, myclobutanil, potassium bicarbonate, propiconazole, tebuconazole, thiabendazole and a combination of triadimefon and mancozeb (Milus and Parsons, 1994). These fungicides had little effect on development and DON production by *F. graminearum* during infection of the American soft red wheat cultivar Florida 302 . In contrast, another study conducted between 1994 -1997 showed reduced FHB incidence and severity in hard red spring wheat cultivars Norm and 2375 in response to the MBC fungicide benomyl and the triazole fungicide tebuconazole (R. K. Jones, 2000).

Fungicide pressure has triggered evolution of *F. graminearum* isolates with increased fungicide resistance. In many cases, fungicide resistance is due to point mutations introduced in the target gene of single site fungicides (Qiu et al., 2011; Scalliet et al., 2012). Therefore, the use of traditional multisite fungicides is being recommended, as pathogens are less likely to develop resistance against such group (AHDB). However, other molecular mechanisms explaining increased fungicide resistance have also been observed. These include the upregulation of the fungicide target gene (Cools et.al., 2012,) as well as increased efflux of the fungicide by alteration in ABC transporters (Sanglard et al., 1995). Therefore, improved management of fungicide administration and other agricultural practises, the study of mechanism of fungicide resistance, resistance monitoring and resistance prediction models are being used to control the development of fungicide resistance (Hawkins & Fraaije, 2016). For example, some *F. graminearum* isolates resistant to azole fungicides are characterised by upregulation of the *CYP51A* gene in response to azole application (Fan et al., 2013). In recent years, *F. graminearum* strains with acquired resistance to both MBC and azole fungicides have been identified across the world. First identified in China, *F. graminearum* isolates resistant against the MBC fungicide carbendazim are characterised by F167Y or E198K substitutions in the β2-tubulin gene (Chen et al., 2009; Zhang et al., 2009). Azole resistant *F. graminearum* strains have also been identified in China and US. But for these, the mechanism of azole resistance is not yet

understood. However, one of the studies suggests a role of the ABC transporter 3 as evidenced by increased fungicide susceptibility in *FgABC3* deletion mutants (Ammar et al., 2013).

The Septoria tritici blotch pathogen *Z. tritici* has also developed resistance to multiple fungicide groups. The MBC resistance has been shown to be conferred by E198A substitution in β-tubulin which might have occurred around 1984 (Griffon and Fisher, 1985). Moreover, a study of the *Z. tritici* stains isolated from stored grains from long term wheat trials at Rothamsted Research detected the presence of the original β-tubulin in samples harvested prior to 1984, which was subsequently replaced by the E198A resistant allele (Lucas and Fraaije, 2008). But the situation is even worse for controlling the STB disease, since QoI fungicides are completely ineffective against *Z. tritici* in UK due to a G143A substitution in the cytochrome b, which is the target of QoI (McCartney et.al., 2007). The QoI resistance is also prevalent in *Z. tritici* isolates from other western European countries, as well as a few isolates from USA and New Zealand (Lucas et.al., 2015). Moreover, European *Z. tritici* strains have also developed multiple mutations in the DMI target gene *CYP51*, of which the substitutions V136A, I381V, S524T, a double deletion ΔY459/G460 and point mutations at positions 459 or 561 have been associated with DMI resistance (Cools et al., 2011; Fraaije et al., 2007; Leroux et.al., 2007).

Therefore, there is an immediate need for development of strategies for mitigating fungicide resistance by either molecular studies or alteration of agricultural practises. One approach considered is to alternate gene expression of the cognate gene of the fungicide targeted protein, using gene specific silencing approaches.

7.1.3 2-Glycosyl transferase (*GT2*)

Glycosyl transferases are enzymes that catalyse the transfer of a glycosyl group from an active sugar donor to a nucleophilic acceptor resulting in the formation of glyosidic bonds. In particular, some glycosyl transferase are involved in catalysing the addition of sugar moieties to non-sugar acceptors such as proteins and lipids, resulting in the formation of glycoproteins and glycolipids (Lairson et.al., 2008). Glycoproteins and glycolipids are an integral part of the fungal cell wall (Gow et.al., 2017) and therefore, glycosyl transferases are believed to be important for cell wall synthesis and capsule development in fungi (King et al., 2017; Klutts et.al., 2006).

GT2 belongs to the family 2 of glycosyl transferases that use UDP-glucose, UDP-N-acetyl-galactosamine, GDP-mannose or CDP-abequose (also called 3,6-Dideoxy-D-Xylo-Hexopyranose) as a sugar donor and transfer the glycosyl group to a variety of substrates such as cellulose, dolichol phosphate (amily of long-chain polyisoprenols, containing several units of isoprenes and a phosphate group) and teichoic acids (co-polymers of glycerol phosphate or ribitol phosphates)

(InterPro id: IPR001173). Although the enzyme activity of GT2 is not yet known, GT2 was shown to be required for fungal growth and virulence in both wheat pathogens *F. graminearum* and *Z. tritici* (King et al., 2017). Both *F. graminearum* and *Z. tritici GT2* deletion mutants were non-pathogenic, very likely due to the impairment in hyphae formation and host penetration, thus confirming the role in fungal virulence and fitness (King et al., 2017). *Z. tritici GT2* deletion mutants also showed cell wall deformities, untimely expression of the *Zt* effector *Mg3Lysm* on the leaf surface rather than *in planta* during asymptomatic phase, and constitutive overexpression of the *alpha-1,3-glucan synthase* during the *Z. tritici* growth on the wheat leaf surface. The *alpha-1,3-glucan synthase* gene was believed to be overexpressed to compensate for the cell wall deformities caused by *GT2* deletion. However, the untimely expression of *Mg3Lysm* suggested that Z. tritici GT2 may be modulating this effector gene expression in response to sensing the host leaf surface (King et al., 2017).

Therefore, *Fg GT2* and *Zt GT2* were chosen as suitable targets for gene silencing using gene specific PTO modified ASOs.

7.1.4 Trichodiene synthase (*TRI5*)

TRI5, the gene encoding the enzyme trichodiene synthase is part of a gene cluster responsible for the synthesis of a large family of trichothecenes toxins that act as protein inhibitory mycotoxins. The trichothecene family also includes the deoxynivalenol (DON) toxin in *F. graminearum* (Makoto Kimura et al., 2003). In *F. graminearum*, this gene cluster is located on chromosome 2, however four other genes needed for trichothecene synthesis are located on other chromosomes (Alexander et.al., 2004). The trichodiene synthase catalyses the first step of trichothecene synthesis involving the cyclization of farnesyl diphosphate to trichodiene (Rynkiewicz et.al., 2001).

The DON toxin has been shown in several instances as to be required for necrosis and virulence *F. graminearum* infection of wheat heads and has been shown to be produced at the growing tip of the hyphae during fungal colonisation of the host (Boenisch et al., 2011; N. A. Brown et al., 2011). Therefore, *F. graminearum* Δ*TRI5* that are unable to produce DON toxin have reduced virulence on wheat heads (Cuzick et.al., 2008).

Hence, *TRI5* can be used as a suitable target for gene silencing in *F. graminearum*.

7.1.5 β- Tubulin

Alpha and beta tubulins are 55 kD subunits of tubulin that polymerise to form microtubules in eukaryotes (Joshi & Cleveland, 1990). Within the microtubule lattice, α and β tubulins associate in a head to tail fashion which defines the polarity of the microtubule. Fluorescent microscopic studies

have confirmed that β tubulin is usually associated with the plus-ends of the microtubule (Mitchison, 1993). Microtubules assembly regulates important biological processes like cytoskeleton organisation, organelle trafficking and chromosome segregation during cell division (Brouhard & Rice, 2018). In fungi, microtubules and tubulin play a role in rapid and polarised growth of the fungal hypha, as evidenced by loss of fungal hyphal growth when microtubules were disrupted using a drug. In fungi, microtubules maintain a continuous tip growth throughout the cell cycle, which further enables the maintenance of an appropriate mass of cytoplasm for the multinucleate system (Horio, 2007).

Fungal β tubulins are also a target of the MBC fungicides such as benzimidazoles (Hollomon et.al., 1998). Fungal isolates which have developed resistance to the MBC fungicides have been characterised to have a point mutation in the β-tubulin gene that results in the replacement of Glu198 with either Ala, Val, or Gly, or the replacement of Phe200 with Tyr (Hollomon & Butters, 1994). Replacement of Glu198→Gly198, was shown to disrupt the binding of benzimidazoles to the β-tubulin fusion protein of *Aspergillus nidulans* (Hollomon et al., 1998).

Therefore, β tubulin is also a suitable target for gene silencing in fungi that can be tested by change in sensitivity of the fungus towards benzimidazole fungicide like carbendazim.

7.2 Objectives and experimental rationale

7.2.1 In planta silencing of Fusarium genes for reducing fungal virulence in barley leaves

With the aim of developing a PTO modified ASO based gene silencing method for *in planta* gene silencing of *F. graminearum* genes, an infection assay for *F. graminearum* infection of barley leaves was set up to allow for the silencing of *F. graminearum* genes previously described as required for *F. graminearum* infection. Then *Fg GT2* and *Fg TRI5* were targeted by gene specific PTO modified ASOs to estimate their effect on *F. graminearum* virulence.

7.2.2 *In vitro* silencing of fungicide targets to increase sensitivity of *Zymoseptoria* towards fungicides

Similarly, with the aim of silencing *Z. tritici* genes either for functional genomic studies or for disease control, the silencing approach using PTO-modified ASOs was employed in an attempt to silence genes of *Z. tritici in vitro*, choosing gene targets which are also known targets of fungicides. For this the MBC fungicide carbendazim, which targets *β-tubulin,* was chosen. Carbendazim concentration still giving a sigmoid growth curve for *Z. tritici* was chosen as condition to evaluate the impact of silencing the *Z. tritici β-tubulin* gene with *β-tubulin* specific PTO-modified ASOs. The silencing effect was compared to similar conditions where a “no gene target” PTO-modified ASO, was used as

negative control. The effect of *β-tubulin* silencing was assessed for its capacity at increasing *Z. tritici* sensitivity towards carbendazim.

7.3 Results

7.3.1 Establishment of a *Fusarium graminearum* spore droplet inoculation assay on barley leaves, excised or on seedlings

With the aim of silencing *F. graminearum* genes in planta, an infection assay was developed *for F. graminearum* infection on barley leaves, using a protocol modified that published by Koch et al., 2016. The methodology is described in detail in the material and method chapter, but the workflow is described in brief here. For this, 7 days primary leaves attached to hydroponically grown barley seedlings or 14 days old secondary leaves excised from plants grown on soil, were used. For the infection assay these were transferred to agar plates containing a low concentration of benzimidazole. A small piece of filter paper was placed on the leaf surface to help with applying a spore droplet in an aqueous solution, as the barley leaf is very hydrophobic. *F. graminearum* spore suspension (20 µl of $4x10^4$ spores/ml, corresponding to $0.8x10^6$ total spores) was placed directly above the filter paper. The lid of the petri dish was closed and sealed with parafilm to maintain high humidity. The petri dishes were then incubated in a growth chamber at 25°C, 70% RH and 16h-photperiod for 5 days. A 20 µl droplet of water was used as a mock inoculation control.

At 5 dpi, lesions were visible on the *F. graminearum* inoculated leaves, while no lesions were observed on the mock controls, confirming a successful *F. graminearum* infection (Figure 7-1).

Figure 7-1: F. graminearum infection assay on barley leaves.

Pictures showing barley primary leaf inoculated with $0.8x10^6$ spores F. graminearum (Fg) spores or water as a mock inoculation control.

7.3.2 Effect of targeting of *Fg GT2* by root and leaf delivery of PTO modified ASOs on *F. graminearum* virulence

With the aim of silencing *F. graminearum* genes *in planta*, *Fg GT2* was targeted in attempt to silence it using a gene specific PTO-modified ASO, subsequently monitoring the effect of such treatment on *F. graminearum* virulence. Since, *Fg GT2* deletion mutants has cell wall deformities and were non-pathogenic on wheat, it was assumed that if *Fg GT2* silencing is successful, a decrease infection should be observed.

In order to silence *GT2*, PTO-modified ASOs were delivered via the root system into 7 days old barley seedlings (c.v. Golden Promise) or by direct uptake into 14 days old barley secondary leaves under continuous light for 24 hours. The PTO modified ASO treated seedlings were then placed on agar and as described above. The effect of *Fg GT2 silencing* was assessed by measuring the lesion length at 5 dpi. The PTO-modified ASO "PTOZ" with no gene target in barley and *F. graminearum* was used as a control.

The lesion development was highly variable between leaves treated with the same PTO modified ASO. Cumulatively, average lesion length from three independent biological replicates (each comprising of 8-9 leaves/treatment) showed no discernible difference between the *Fg GT2* targeting and control treatments by either root delivery (Figure 7-2) or direct leaf uptake (Figure 7-3), indicating that no successful *Fg GT2* silencing may have been achieved using PTO mediated ASOs.

The slight reduction in lesion length in the *Fg GT2* targeting by root delivery of silencing molecule maybe from the first biological repeat, where a slight reduction (although, non-significant) in lesion length was observed. However, the infection development was highly variable, as no filter papers were used. Use of filter papers in the subsequent experiments reduced this variation, however, these subsequent experiments did not show any significant change in lesion length post *Fg GT2* targeting by root delivery of PTO modified ASOs.

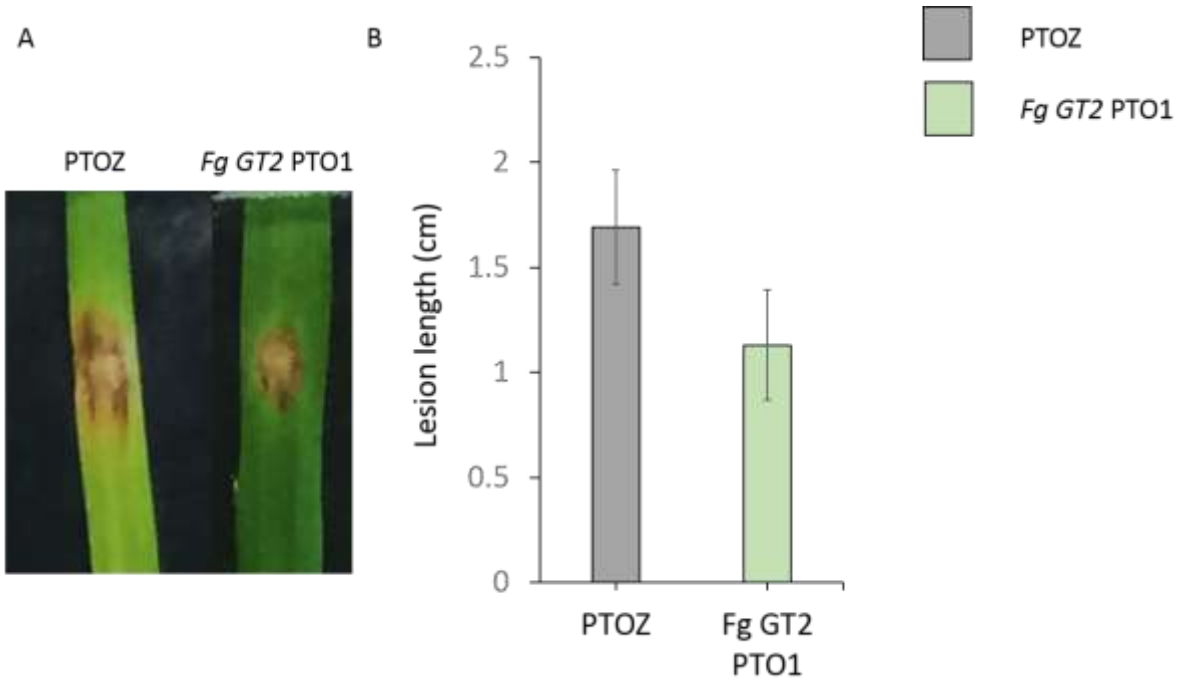


Figure 7-2: In planta silencing of FgGT2 by root delivery of PTO modified ASOs into barley seedlings.

*F. graminearum infection sites observed in primary leaves of barley from whole seedlings to which the PTO-modified ASOs were delivered through the root system: PTOZ for the negative control or Fg GT2 PTO1 to silence F. graminearum GT2. F. graminearum was subsequently inoculated with 0.8x10^6 spores in a 20 µl droplet. Observations were made at 5 dpi. A. Photograph of typical lesions observed. B. Histogram showing average lesion length measured at 5 dpi. Data were acquired from 3 independent biological replicates, each comprising of 6 to 9 leaves per treatment. Significance (One-sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

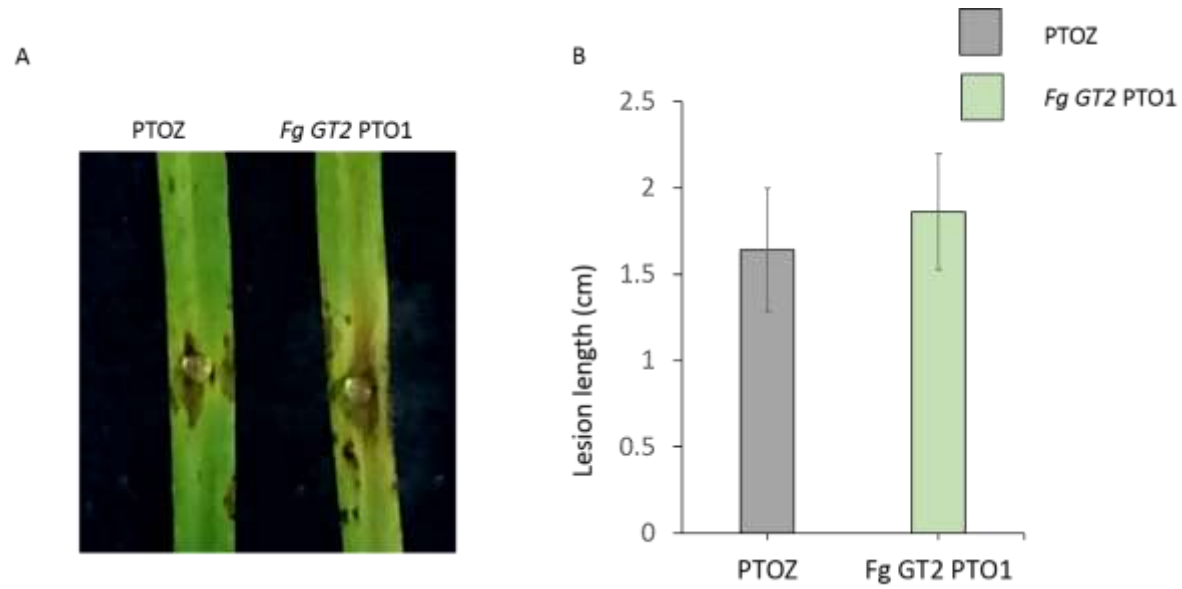


Figure 7-3: In planta silencing of Fg GT2 via direct uptake of PTO modified ASOs by excised barley leaves.

F. graminearum infection sites observed in excised secondary leaves of barley to which the PTO-modified ASOs were delivered by direct leaf-uptake by transpiration: PTOZ for the negative control or Fg GT2 PTO1 to silence F. graminearum GT2. F. graminearum was subsequently inoculated with 0.8x106 spores in a 20 µl droplet. A. Observations were made at 5

*dpi. A. Photograph of typical lesions observed. B. Histogram showing average lesion length measured at 5 dpi. Data were acquired from 3 independent biological replicates, each comprising of 6 to 9 leaves per treatment. Significance (One-sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

7.3.3 Effect of targeting of *Fg TRI5* by root and leaf delivery of PTO modified ASOs on *F. graminearum* virulence

With the same aim as in the previous section, of *in planta* silencing of *F. graminearum* genes, *TRI5*, the gene coding for trichothecene synthase, an enzyme involved in the synthesis of the fungal virulence factor DON toxin (Makoto Kimura et al., 2003) was also used as a suitable target. *TRI5* was targeted for silencing using gene specific PTO modified ASOs and the effect of gene silencing on FHB infection was assessed by measuring the lesion length five days after *F. graminearum* inoculation, assuming that *F.* graminearum lesions should be decrease, if the *Tri5* gene was successfully silenced.

The PTO modified ASO treatments were carried out with 7 days old barley seedlings by root delivery or direct delivery via the vascular tissue, into 14 days old excised secondary barley leaves as described above, prior infection with *F. graminearum*. The effect of *Fg TRI5* silencing was assessed at 5 dpi comparing the Tri5 silencing treatment to the negative control "PTOZ", which had no gene target in either barley or *F. graminearum*.

When observing lesions on barley primary leaves infected with *F. graminearum* at 5 dpi , no significant difference in lesion length was observed between the *TRI5* targeting and control treatments administrated to whole hydroponically grown seedlings with root delivery of PTO-modified ASO (Figure 7-4).

Similarly no differences *in F. graminearum* lesion size were observed when *Tri5* targeting PTO or PTOZ negative control were delivered via direct uptake into excised leaves (Figure 7-5), indicating that no successful *Fg TRI5* silencing may have been achieved using PTO mediated ASOs, or Tri5 is dispensible for barley infection of *F. graminearum* (Maier et al., 2006).

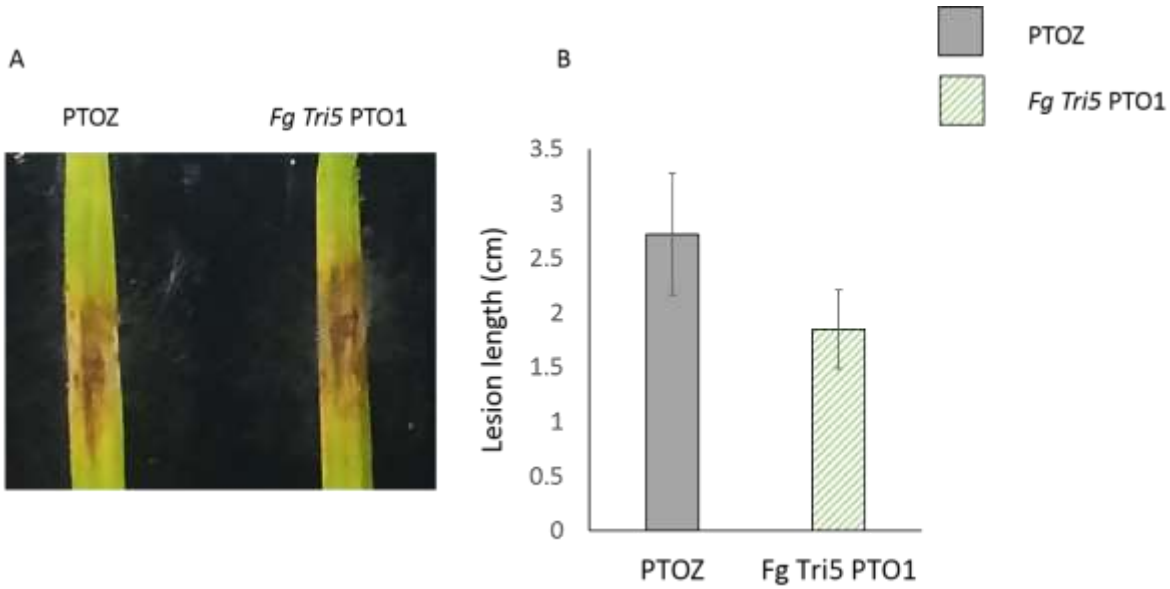


Figure 7-4**: **In planta silencing of Fg Tri5 by root delivery of PTO modified ASOs into barley seedlings

*F. graminearum infection sites observed in primary leaves of barley from whole seedlings to which the PTO-modified ASOs were delivered through the root system: PTOZ for the negative control or Fg Tri5 PTO1 to silence F. graminearum TRI5. F. graminearum was subsequently inoculated with 0.8x10^6 spores in a 20 µl droplet. Observations were made at 5 dpi. A. Photograph of typical lesions observed. B. Histogram showing average lesion length measured at 5 dpi. Data were acquired from 2 independent biological replicates, each comprising of 6 to 9 leaves per treatment. Significance (One-sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

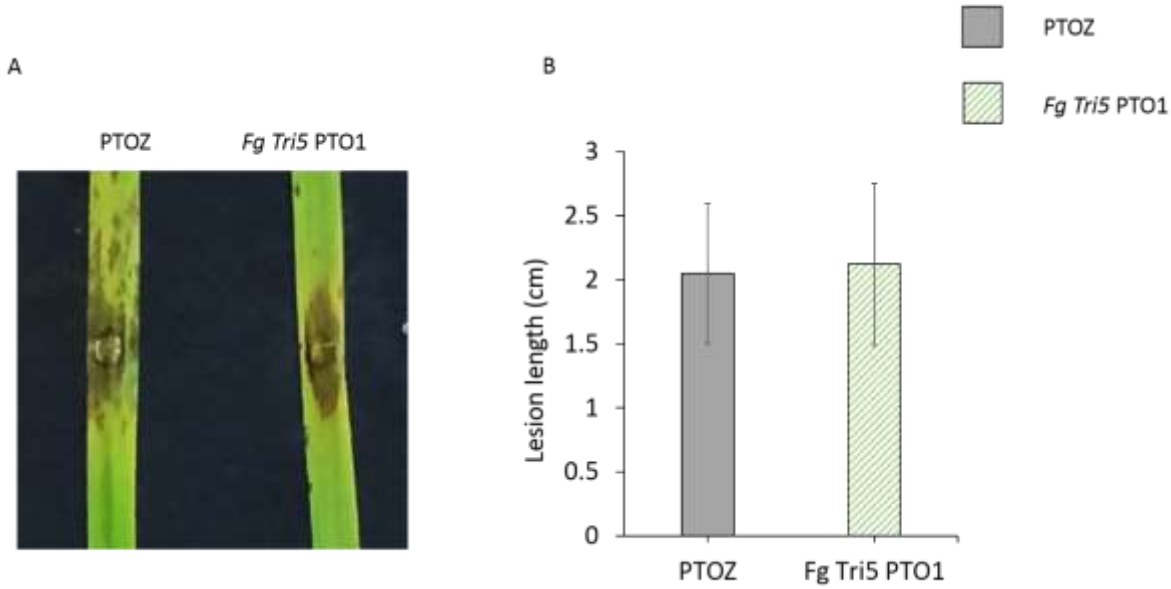


Figure 7-5: In planta silencing of Fg Tri5 via direct uptake of PTO modified ASOs by excised barley leaves

F. graminearum infection sites observed in excised secondary leaves of barley to which the PTO-modified ASOs were delivered by direct leaf-uptake by transpiration: PTOZ for the negative control or Fg GT2 PTO1 to silence F. graminearum GT2. F. graminearum was subsequently inoculated with 0.8x106 spores in a 20 µl droplet. A. Observations were made at 5 dpi. A. Photograph of typical lesions observed. B. Histogram showing average lesion length measured at 5 dpi. Data were

*acquired from 3 independent biological replicates, each comprising of 6 to 9 leaves per treatment. Significance (One-sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

7.3.4 Effect of targeting of *Fg GT2* by droplet inoculation of PTO modified ASOs on *F. graminearum* virulence

Since no significant could be obtained when delivering PTO-modified ASO, either through root delivery or through direct uptake by the vascular tissue of excises leaves, an alternative delivery method of the PTO-modified ASO was attempted. As previous work suggested that both GT2 and Tri5 are required for *F. graminearum* virulence, thus it would have been expected that a phenotype of infection could have been observed if the gene targeted were silenced efficiently. The negative outcome, more likely reflects absence of silencing, possibly through the absence of an efficient delivery or cross kingdom transfer of the PTO-modified ASO between the barley host *and Fusarium graminearum*. Thus, different delivery techniques for silencing of *F. graminearum* genes using PTO modified ASOs, were attempted. *Fg GT2* was targeted by droplet inoculation of *F. gramineraum* in presence of gene specific PTO modified ASOs, and its effect on lesion lengths caused by *F. graminearum* infection was assessed.

A combined inoculation of the silencing molecule and the fungal spores was carried out by inoculating a 20 µl droplet containing 10 µM of the *Fg GT2* targeting PTO modified ASO and $0.8x10^6$ spores of *F. graminearum* s onto the adaxial side of the primary leaf of 7 days old plantlets placed on agar. The effect of the attempt of silencing *Fg GT2* was assessed by measuring the lesion length at 5 dpi, comparing plant treating with Fg GT2 silencing PTO with the PTOZ negative control. Again, as for the previous treatments, at 5 dpi, no significant difference in the length of the lesions was observed ibetween the two treatments, indicating that *Fg GT2* gene silencing may not have been achieved by PTO modified ASO droplet inoculation.

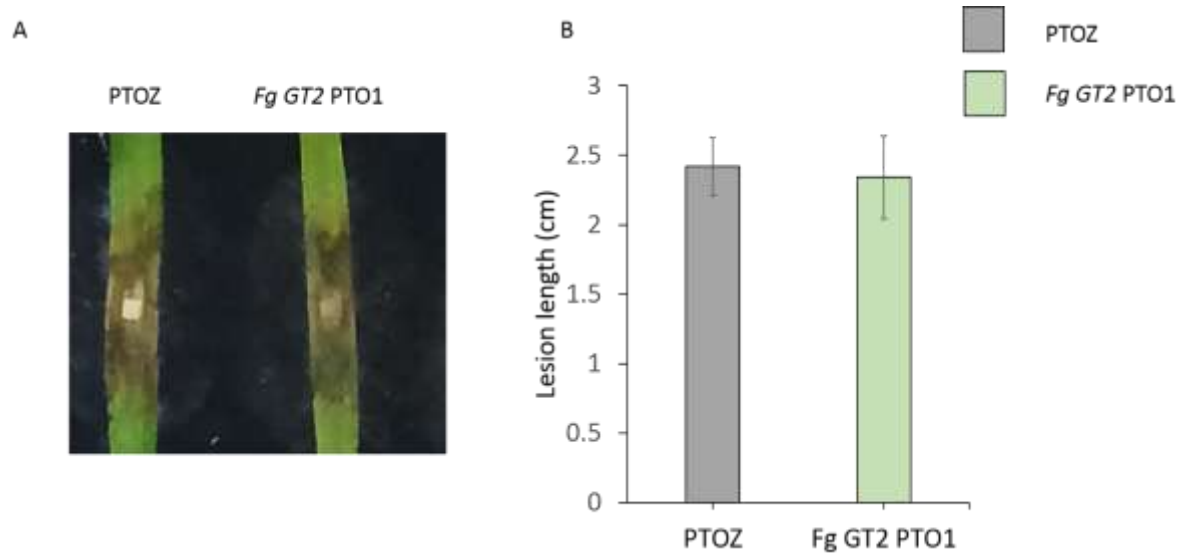


Figure 7-6: In planta silencing of Fg GT2 via droplet inoculation of PTO modified ASOs on attached barley leaves.

*A. Infection symptoms, B. lesion length developed post F. graminearum infection of 7 days old attached barley primary leaves treated with a PTO modified ASO targeting FgGT2 (Fg GT2 PTO1) and a target PTOZ, targeting wheat seed protein Z. 10µM solution of PTO modified ASO was inoculated as a part of a 20 µl droplet containing the silencing molecule and $4x10^4$ spores/ml of F. graminearum spores. Lesion length was measured at 5 dpi. Data acquired from one independent biological replicate comprising of 8 leaves per treatment. Significance (One-sample t-test) * P<0.05, ** P<0.01, *** P<0.001.*

7.4 Discussion

7.4.1 Do PTO- modified ASOs mediate *in planta* gene silencing of *F. graminearum* genes

In planta silencing of *F. graminearum* has recently been achieved in several studies using RNAi mediated silencing using hairpin and dsRNA silencing products either generated in transgenic lines or sprayed as ectopic application on cereal leaves and inflorescence (Cheng et al., 2015; Koch et al., 2016). For instance, transgenic wheat lines expressing three hpRNas targeting the *F. graminearum* chitin synthase gene *Chs3b* showed reduced host colonisation by *F. graminearum* as a result of *Chs3b* silencing (Cheng et al., 2015). Ectopic application of long dsRNA targeting the three *Fg CYP51* genes sprayed onto barley leaves also led to reduced *F. graminearum* virulence (Koch et al., 2016). For *Fusarium asiaticum,* silencing of *Fg Myo5* by ectopic application of dsRNA onto wheat coleoptiles before fungal inoculation led to reduced lesion size. Moreover, *F. asiaticum* strains with silenced *Fg Myo5* showed increased sensitivity to the phenamacril fungicide (Song et.al., 2018). However, some of these results have not been reproducible in other laboratories (Personal communication, Prof. K. Hammond-Kosack, Dr. G. Scalliet).

Even though, the mechanism by which dsRNA is taken up by the pathogen is still unclear, cross kingdom RNA exchange has been reported in many pathosystems. For example, the rot pathogen *B. cinerea* secretes sRNA into the plant host *Arabidopsis*. These sRNAs interact with an *Arabidopsis*

dicer protein and hijack the host RNAi machinery to silence host immune genes, facilitating fungal virulence (Weiberg et al., 2013). Similarly, human erythrocytes use micro RNA to counter malaria by targeting genes of the protozoan pathogen *Plasmodium falciparum* (LaMonte et al., 2012). This dsRNA mediated silencing is believed to be mediated by DICERs which cleave the target mRNA using the RNAi machinery in the target pathogen (Wang et. al., 2016; Weiberg et al., 2013). However, there are exceptions like *P. falcifarum,* which lacks an RNAi machinery, but still undergoes host miRNA mediated gene silencing by a yet unknown mechanism (LaMonte et al., 2012). The uptake of cross-kingdom dsRNA is believed to be mediated by exosomes ore extracellular vesicles (EVs), which are endogenous vesicles shown to naturally carry nucleic acids between two organisms and regulate gene expression in the recipient (Valadi et al., 2007).

Antisense oligodeoxynucleotides are alternative gene silencing molecules, that are widely used in animal systems for both functional genomics studies and for clinical applications (Gewirtz et.al., 2013). For instance, ASO mediated exon skipping has been used to treat the genetic disease Duchene muscular dystrophy (DMD) (Echevarría et.al., 2018). Recently, ASO based gene silencing therapies are also undergoing clinical trials for treating cancer (Moreno & Pêgo, 2014) as well as neurological disorders (Rinaldi & Wood, 2018). The applications of ASOs in plant sciences is still emerging. A limited number of studies have used ASOs for *in planta* silencing of plant genes (Dinc et al., 2011; Sun & Ho, 2005). In the Bindschedler lab, PTO modified ASOs have been successfully used for the first time for discovering genes that control barley and wheat powdery mildew disease by *in planta* silencing of fungal and plant genes (Orman, Das Gupta, manuscript in prep). However, this is the first attempt of using ASOs for silencing genes potentially relevant for the Fusarium head blight pathosystem. So far, all the attempts made have not been promising. This could be due to the absence of transfer between the host cells and *F. graminearum*, which first grow extracellularly in the apoplastic space (N. A. Brown et al., 2011). The success of the system in *B. graminis* may be due to the presence of a haustorium, a structure specific to obligate biotrophic pathogens, which acts as a direct interface between host and pathogen involved in facilitating the exchange of molecules between the two organisms (Both et al., 2005). Therefore, the transfer of ASOs into *B. graminis* is believed to occur through the host-fungal haustorial interface, resulting in successful gene silencing. In plants, Sun et al (2007) proposed that ASO uptake through the plant membrane barrier occurs via sugar translocators, where fluorescently labelled ASOs were visualised in barley leaves where ASOs were provided in the presence of 200 mM of sugars such as glucose, fructose, maltose and sucrose. According to this study, ASO uptake was absent in barley leaves incubated with sugar alcohols, osmotic agents and sucrose analogue glucose-1,6-fructose, which is neither taken up nor metabolised by the plant cell (Sun et.al., 2007). Moreover, ASO uptake was shown to occur through

active transport. Addition of the CCCP uncoupler, which dissipated the H^+ gradient across the cell membrane, prevented ASO uptake even in the presence of sugars, confirming the involvement H^+ coupled sugar symport systems for ASO uptake by plants (C. Sun et al., 2007). Interestingly, sugar transporters have been shown to be overexpressed on haustorial membrane (Voegele et.al., 2001), facilitating sugar uptake through the haustorium. This further explains why successful ASO mediated gene silencing was achieved in *B. graminis,* but it does not explain in first instance the successful uptake by the barley cells, including barley epidermal cells, unless there is sufficient sucrose transporters present. However, sugar transporters have also been discovered in *F. graminearum* (Gonçalves et.al., 2016). Therefore, there is a possibility that fungal gene specific ASOs could be delivered into *F. graminearum in vitro*. However, *in planta* delivery of PTO modified ASOs into *F. graminearum* cells is more complicated. Microscopic studies motoring the uptake of fluorescently labelled ASOs carried out in Bindschedler lab showed a strong fluorescence in the vasculatures which were loaded with the ASOs, this progressively reduced in the cytoplasm of the epithelial and mesophyll cells (Orman et. al., manuscript in prep). Therefore, PTO modified ASOs may still be inaccessible by the *F. graminearum* cells growing apoplastically. The uptake of ASOs by *F. graminearum* could be queried *in vitro* using ASOs labelled with fluorescent dye cyanine 3 which can be studied by fluorescent microscopy. The addition of sugar to the PTO modified ASOs might facilitate the upload of the PTO modified ASOs into plant cells, as suggested by Sun et. al. (2007). However, it is important to remember that plant uptake of dsRNA during SIGS does not need any additives.

The lack of significant reduced infection phenotype following silencing attempts of *F. graminearum* genes known to be required for virulence might also be due to the selection of the target gene. For instance, *TRI5* encodes for trichothecene synthase, an enzyme important for synthesis of the DON toxin, which is known to be an important virulence factor for FHB development (Proctor et.al., 1995). However, DON toxin was shown to act as a virulence factor during *F. graminearum* infection of wheat inflorescence but not *Arabidopsis* inflorescence, as evidenced by reduced virulence of *F. graminearum ΔTRI5* mutants in wheat heads and normal virulence in *Arabidopsis* florets (Cuzick et al., 2008). DON toxin usually facilitates wheat head colonisation by *F. graminearum* by countering host cell wall thickenings induced to limit infection spread (Jansen et al., 2005). However, fungal colonisation by *F. graminearum* wild type and *ΔTRI5* mutants in barley inflorescence was inhibited at the rachis node and rachilla, limiting infection of adjacent florets through the phloem and along the surface of the rachis due to heavy cell wall thickenings at infection sites (Jansen et al., 2005). Therefore, *Fg TRI5* may not act as a virulence factor for the barley-*F. graminearum* pathosystem during leaf infection. Alternatively, a wheat coleoptile infection assay could be used for future gene

silencing attempts involving targeting of *Fg TRI5.* Interestingly, *F. graminearum* susceptibility can vary between different organs of the same wheat cultivar. For instance, the wheat cultivar Sumai 3 is resistant to *F. graminearum* infection in the ears (Anderson et al., 2001) but it is susceptible to *F. graminearum* infection in the coleoptile (Shin et al., 2014).

Silencing a new target, *Fg MAP1,* gene encoding a MAP kinase, could also be attempted. *Fg ΔMAP1* mutants were asymptomatic during infection in wheat heads (Urban et.al., 2003), *Arabidopsis* (Cuzick et al., 2008) and soya bean (Sella et.al., 2014), increasing the chances of *MAP1* to act as a virulence factor during *F. graminearum*-barley interaction. Alternatively, *F. graminearum* gene *Chs3b* can also been targeted as, as chitin synthase is a housekeeping gene important for fungal cell wall synthesis and growth and is not dependent on the host (Cheng et al., 2015).

Furthermore, all the successful attempts of RNAi mediated gene silencing of *F. gramiearum* genes used 20-30 seedlings to assay the effect of fungal gene silencing on FHB development (Gu et al., 2019; Koch et al., 2016). In this study, only 8-9 leaves were assayed per treatment. As gene silencing usually has a weaker phenotype than gene deletion, due to the presence of a still functional gene, even if in smaller amount, a larger study group might help to acquire a significant change in infection phenotype post PTO mediated ASO silencing of *F. graminearum* genes in leaf assays. In *B. graminis,* end capped PTOs showed a stronger silencing effect that the fully modified PTOs. Silencing of *F. graminearum* genes may also be tested using gene specific end capped PTOs.

7.4.2 PTO modified ASO mediated *in vitro* gene silencing of *Z. tritici* genes

As mentioned earlier, cross-kingdom RNAi silencing is an emerging tool for functional genomics studies and disease control in many fungal pathosystems (Nowara et al., 2010; Panwar et al., 2013b; M. Wang, Weiberg, Lin, Thomma, & Huang, 2016). RNAi based gene silencing is mediated by dsRNA or hairpin RNA that bind the target mRNA and lead to its degradation by the dicer and RISC complex from the RNAi machinery of the fungus (Nowara et al., 2010; M. Wang, Weiberg, Lin, Thomma, & Huang, 2016) . Cross kingdom RNAi has been shown to affect both fungal virulence as well as host immunity against pathogens. The RNAi machinery affect fungal virulence by producing sRNAs that act as fungal effectors. For instance, dicer deletion mutants in *Botrytis cinerea* have reduced virulence due to inability to suppress host immunity genes through secretion of pathogen sRNA effectors (Weiberg et al., 2013). Similarly, sRNA produced by RNAi machinery also facilitate host defence as evidenced by the production of miRNA by that target *Plasmodium falciparum* genes to counter the spread of malaria (LaMonte et al., 2012). Interestingly, *P. falciparum* does not possess any RNAi machinery, therefore sRNA mediated gene silencing may be achieved by a yet unknown alternative mechanism (LaMonte et al., 2012).

However, RNAi based gene silencing has not been achieved in the wheat pathogen *Zymoseptoria tritici* (Kettles et al., 2019). Hairpin RNA mediated virus induced gene silencing of known fungicide targets in *Z. tritici* such as *Zt α-tubulin, Zt β-tubulin* (Hollomon et al., 1998) and *Zt CYP51* (Siegel, 1981) as well as the fungal housekeeping gene *Zt Alg1* (encoding α-1,2- mannosyl transferase), important for hyphae development and pathogenicity (Motteram et al., 2011), did not affect *Z. tritici* virulence, as evidenced by no change in STB lesion length in wheat plants expressing hpRNA targeting these genes as compared to control plants expressing just the viral vector (Kettles et al., 2019). Moreover, to test the presence of cross kingdom RNAi transfer in the Septoria tritici blotch pathosystem, computational analysis of fungal sRNA and host mRNA sequencing datasets was carried out which led to the identification of 389 *in planta*-induced *Z. tritici* sRNA loci, where products of these sRNA loci where hypothesised to target wheat genes. However, molecular studies proved otherwise. Deletion of *Z. tritici* single gene coding dicer like proteins (*Zt ΔDcl1)* and the two genes coding for Argonauts (*Zt ΔAGO1* and *ΔAGO2*) did not affect fungal pathogenicity, as evidenced by no change in leaf lesion area and pycnidia density in the deletion mutants of these genes as compared to wild type *Z. tritici* (Kettles et al., 2019). These results indicate at a lack of RNAi machinery in *Z. tritici.* Interestingly, certain sRNAs were shown to be expressed in *Z. tritici* in the absence of *Zt Dcl1*, suggesting the presence of an alternative Dcl1-independent sRNA generation machinery in *Z.tritici* (Kettles et al., 2019).

PTO modified ASOs are alternative silencing molecules already used successfully to silence both plant and fungal genes in the barley powdery mildew pathosystem (Orman, Das Gupta et.al., manuscript in prep). PTO modified ASO silencing is mediated by either post transcriptional degradation of target mRNA by RNase H or by stearic hinderance caused by binding of ASOs to ribosomes, leading to translational arrest (Shen & Corey, 2018). This study, for the first time, attempted *in vitro Z. tritici* gene silencing by direct uptake of gene specific PTO modified ASOs. *Z. tritici* has developed resistance to multiple fungicides such as methyl bezimidazole carbamates (MBCs), QoIs and demethylation inhibitors (DMIs) (reviewed by Lucas et al., 2015). RNAi mediated silencing of fungicide targets have led to increased fungicide sensitivity and reduced virulence in other fungal pathogens. For instance, spraying of dsRNA specific to MBC fungicide target *β- tubulin* from *F. asiaticum* onto barley, cucumber and soyabean reduced infection development of *M. oryzae, B. cinereal* and *C. truncatum* respectively due to silencing of the *β- tubulin* gene in these fungal pathogens (Gu et al., 2019). Similarly, silencing of MBC fungicide target *β- tubulin* in *F. asiaticum* increased fungal sensitivity to the MBC fungicide carbendazim (Gu et al., 2019). In the present study, *Zt β- tubulin* was selected as a suitable target for gene silencing in *Z. tritici,* where the silencing effect could be observed as the change in *Z. tritici* sensitivity to the MBC fungicide carbendazim. In this

study, *Z. tritici* was grown in the AE minimal medium supplemented with either 10^{-2} or 10^{-3} mg/L carbendazim, in the presence of 10 µM PTO modified ASO targeting *Zt β-tubulin* for silencing or with a no gene target PTO-modified ASO, as negative control. The preliminary data showed reduced *in vitro Z. tritici growth in the presence of Zt tubulin* targeting PTO modified ASO. However, these results were acquired from a single replicate, therefore, no conclusions could be drawn, and the data was not included in the results section. These results need to be repeated to get more robust data. Study of change in *Zt β-tubulin* mRNA amounts following treatment with PTO modified ASOs needs to be monitored to confirm that the observe phenotype is linked to gene silencing. Moreover, uptake assays studying the uptake of fluorescently labelled PTO modified ASOs by *Z. tritici* will further confirm whether *Z. tritici* is capable of taking up PTO modified ASOs required for gene silencing.

Chapter 8 : General Discussion

This project exploited gene silencing approach with short antisense oligonucleotides ASOs, in particular phosphorothioate modified ASOs, as a functional genomics tool, as well as a putative strategy to control crop diseases in different fungal pathosystems of wheat and barley. This successfully allowed for the discovery of new key players of fungal virulence in the biotrophic *Blumeria graminis*, causing wheat and barley and wheat powdery mildew. However, although promising, the approach still needs further development for its application to wheat-*Fusarium graminearum* and wheat-*Zymoseptoria tritici* pathosystems. Attempts were also made to adapt the technology as a fungicide alternative for the control of cereal powdery mildew in different barley cultivars and in wheat, developing an *in planta* gene silencing assay in whole plants, exploiting root delivery of ASO gene specific silencing molecules.

8.1 PTO modified ASO mediated gene silencing

A phosphorothioate (PTO) modified ASO based gene silencing assay first developed for targeting *B. graminis* f.sp *hordei* and barley genes in excised leaves (Orman, PhD thesis) was further exploited for gene specific silencing of housekeeping and host susceptibility genes in the barley powdery mildew pathosystems, as an approach to develop a disease control strategy efficient in different barley cultivars. This methodology was also adapted for *in planta* silencing of wheat powdery mildew genes, where other transient gene silencing assays, such as the host inducing gene silencing assay (HIGS) requiring biolistic delivery of a plasmid carrying a silencing construct, have failed (Patrick Schweizer, personal communication). Thus, this is the first known, convenient, ready and easy to use, host induced gene silencing methodology in the wheat-powdery mildew pathosystem. As earlier gene silencing technologies in this pathosystem involved the silencing of genes by producing transgenic wheat lines expressing gene specific hpRNA delivered by transformation via viral vectors (REF). These methodologies have limitations arising from use of viral vectors and complications involved in genetic manipulation. This technology was further tailored for root induced gene silencing or RIGS of fungal genes in whole wheat and barley seedlings in these cereal-powdery mildew pathosystems. *In vitro* targeting of *Zymosetoria tubulin* gene by direct uptake of PTO modified ASOs from the medium was also attempted reducing *in vitro* fungal growth, however no conclusive results were obtained as not enough repeats were done. Similarly attempts for PTO modified ASO based *in planta* gene silencing did not produce any significant changes in lesion lengths in barley leaves infected with *F. graminearum*.

Although PTO modified ASOs have been extensively and successfully used for functional genomics and for clinical applications in animal models, which include recent tests for therapies for genetic

disorders, cancer and neurological diseases (Echevarría et.al., 2018; Moreno & Pêgo, 2014; Rinaldi et.al., 2018), such an approach has been hardly exploited in plant systems. *In planta* gene silencing was also successfully attempted by direct uptake of ASOs into plant cell cultures (Tsutsumi et.al., 1992), excised leaves (Dinc et al., 2011; C. Sun & Ho, 2005) and pollen tubes (Mizuta & Higashiyama, 2014a). ASO based gene silencing occurs through either RNase H mediated degradation of target mRNA resulting in post transcriptional gene silencing (Eckstein, 2000; Wu et al., 2004), by DNA methylation (Wojtasik et.al., 2014) or by binding of ASO to ribosomes resulting in translational arrest (Baker et al., 1997) . ASO uptake into plant cells is believed to occur through sugar transporters, as evidenced by the ASO uptake into barley excised leaves in the presence of sugars like glucose, fructose, mannose and sucrose, but not in the presence of sugar alcohols, osmotic agents like sorbitol and sucrose analogue glucose-1,6-fructose, which is neither taken up nor metabolised by the plant cell (Sun et.al., 2007). Addition of a H^+ gradient disrupter like CCCP de-coupler, further disrupted the ASO uptake even in the presence of sugars, indicating at an involvement of active transport through H^+ sugar symport systems for ASO uptake into plants (Sun et al., 2007). The ASO uptake by fungal pathogens may also be mediated by active sugar transport. For instance, it is speculated that uptake of silencing molecules into the obligate biotrophic pathogen *B. graminis* occurs through the haustorium, the feeding structure and interface between the host and pathogen, responsible for nutrient uptake and effector secretion (Both et al., 2005). Haustoria are well characterised as sites of sugar uptake in obligate biotrophs (Staples, 2001). Moreover, sugar transporters are highly expressed on the haustorial membrane as compared to the hyphal membranes, as was reported for another obligate biotroph, *Puccinia graminis* (Voegele et.al., 2001), further supporting the hypothesis that ASO uptake through fungal haustoria is likely to be efficient. Perhaps, ASO mediated gene silencing was not successful in *F. graminearum* due to lack of haustoria through which nutrients and effector exchanges take place. However, sugar transporters have been identified on *F. graminearum* membranes (Gonçalves et.al., 2016), suggesting that there is potential for ASO uptake by this fungus. Similarly, LEN2 sugar transporters have been identified in the *Z. tritici* genome (Goodwin et al., 2011), which may be a putative mode of ASO uptake for silencing of *Z. tritici* gene *in vitro*. The ASO uptake by these fungi could be confirmed by fluorescent microscopy to visualise the uptake of fluorescently labelled PTO modified ASO (Dinc et. al., 2011). In the case of barley and wheat excised leaves, fluorescently labelled PTO modified ASO and non-modified ASO were shown to be loaded through the vascular tissue, and translocated to cells, in particular epidermal cells, in absence of any sugar addition in the ASO solution (Orman thesis, and this work). Therefore, the presence of sugar in the solution to facilitate ASO uptake is debatable, but it cannot be denied that sugar transporters might be the route of transport, unless the ASOs are also

transported through endocytic vesicles. Exosomes have been reported to carry nucleic acid cargo between cells in both plants and animal systems (Cai et al., 2018; Ridder et al., 2014). For instance, genetic information in the form of mRNA was shown to be transported between the haematopoietic cells and the brain via the exosomes, in response to inflammation (Ridder et al., 2014). Similarly, cross kingdom RNAi transport between *Arabidopsis* and *B. cinerea* was also shown to be mediated via the exosomes (Cai et al., 2018). Interestingly, exosomes were observed in the extracellular matrix of the oomycete *Golovinomyces orontii* in the electron microgram (Micali et.al., 2011). Therefore, there is a possibility that ASO uptake in biotrophic pathogens may also be mediated via the exosomes present in the fungal haustorium.

In the recent years, RNAi based gene silencing was successfully attempted for gene function studies and disease control in multiple fungal plant pathosystems (Koch et al., 2016; Nowara et al., 2010; Panwar et.al., 2013; Wang et.al., 2016). Initially, gene silencing was attempted via viral delivery of gene specific hairpin RNA (hpRNA) in host plants (Panwar et al., 2013b) or by host plants expressing hpRNA targeting specific fungal genes (Cheng et al., 2015). An alternative host induced gene silencing (HIGS) strategy mediated by biolistic bombardment to deliver a plasmid carrying a cassette to express the hpRNAs *in planta*, was developed for the delivery of hpRNA for silencing *B. graminis* effectors (Nowara et al., 2010) and further exploited for putative effector functional studies (Aguilar et. al., 2015; Ahmed et al., 2016; Pliego et al., 2013; Zhang et al., 2012). Transgenic host plants expressing gene specific dsRNA of the pathogen have also been successfully used for control of nematodes and insect pests (Gordon & Waterhouse, 2007). For instance, cotton bollworm feeding on plant leaves expressing hpRNA targeting fungicide target cytochrome P450 reductase regained their susceptibility to pyrethroid insecticide (Mao et al., 2007). Similarly, expression of dsRNA targeting nematode parasitism gene *16D10* rendered the plant resistant to root knot nematodes *Meloidogyne sp.* (Huang et.al., 2006). In humans, RNAi based gene silencing strategies have also been extensively developed for therapies for curing genetic diseases (Martínez et.al., 2013). In the case of gene silencing RNAi is mediated by the host RNAi machinery. In hpRNA expressed or delivered in the hosts are first cleaved by the host dicer proteins producing primary siRNA, which are then amplified by the host RNA dependent RNA polymerases (RdRP). The host siRNA is then transferred into the pathogen, where it amplifies via the pathogens RdRP and binds the target mRNA resulting in its degradation via the recruitment of the pathogen RISC complex. Interestingly, *Arabidopsis Dcl2, Dcl3* and *Dcl4* triple mutants produced intact hpRNA resulting in more efficient silencing of insect P450 gene in cotton bollworm, indicating that efficient gene silencing may be dependent on host hpRNA production at a rate faster than the rate at which they can be diced (Mao et al., 2007). Moreover, a small feed of hpRNA resulted in complete gene silencing in cotton

bollworm, suggesting that the ingested hpRNA is likely to be amplified by the insect's RNAi machinery (Mao et al., 2007). However, unlike plants and nematodes, insects lack RNA dependent RNA polymerase (RdRP). Therefore, it was speculated that as cotton bollworm cytochrome P450 is expressed in the insect midgut, the continuous availability of hpRNA in the insect gut due to feeding on transgenic plants might be responsible to gene silencing in the absence of the RdRP (Mao et al., 2007). In the case of fungal plant interactions, recent gene silencing studies have achieved fungal gene silencing and disease control by direct uptake of dsRNA by the plant host (Gu et al., 2019; Koch et al., 2016; Koch et al., 2013; M. Wang et. al., 2016). For example, *F. graminearum* CYP51 genes were targeted for silencing, ether by direct delivery and uptake of dsRNA to fungal spores in medium, or by spraying the dsRNA on excised barley leaves. These treatments led to reduced fungal growth and virulence (Koch et al., 2016; Koch et al., 2013). Similarly, the spraying of fruits, leaves or flowers with dsRNA to target the *B. cinerea Dcl1* gene also led to reduced fungal virulence as seen by decreased infection symptoms and decreased mRNA levels of the targeted gene (Wang et. al., 2016). This was also supported by the discovery of naturally occurring cross kingdom RNAi transfer between hosts and pathogens. For instance, the plant pathogen *B. cinerea* produces siRNA-based effectors that supress host immune response facilitating fungal virulence (Weiberg et al., 2013). Cotton plants were also showed to transfer miRNA in *Verticilium dahliae* to silence virulence genes (Zhang et al., 2016). Another study reported the preferential loading of 17 nt long "tiny RNAs" into the extracellular vesicles of *Arabidopsis.* These tiny RNAs were postulated to be small activating RNAs, capable to activating gene expression (Baldrich et al., 2019). Similarly, human hosts produce siRNA that control the spread of malaria by silencing *Plasmodium falciparum* genes (LaMonte et al., 2012). Interestingly, gene silencing in *Plasmodium falciparum* occurs in the absence of a RNAi machinery (LaMonte et al., 2012). The uptake of dsRNA is believed to be mediated by exosomes, which are endogenous secreted vesicles evidenced by the presence of extracellular vesicles or EVs shown to naturally carry nucleic acids between two interacting organisms and modulate or regulate gene expression of gene targets in the recipient (Baldrich et al., 2019; Cai et al., 2018; Valadi et al., 2007). However, a maximum of 9 days of silencing effect from a single dose of dsRNA spray, requiring multiple applications for a long-term effect. This was believed to be due to limitation of the fungal RNAi machinery, as the silencing effect for plant genes lasted longer (Song, Gu, Duan, Xiao, Hou, Duan, Wang, Yu, et al., 2018). Interestingly, application of dsRNA was coated on to nanoparticles sustained a silencing effect for 21 days when targeting viral genes (Mitter et al., 2017).

As mentioned earlier, in the initial studies the silencing molecule was delivered into the host by viral vectors (Panwar et al., 2013b) or by biolistic bombardment (Nowara et al 2010; Pliego et al., 2013).

These methods are limited due to host immune activation by the viral vector (Tufan et.al., 2011) as well as complications related to genetic manipulation, that make them unsuitable for agricultural use. Direct delivery of dsRNA by spraying leaves or watering growth medium (root uptake in whole plants) is more applicable to agriculture, however, as mentioned earlier, spray induced gene silencing effect was limited to 9 days in some studies (Song et al., 2018). However, delivery of dsRNA by coating them onto clay nanoparticles have been shown to prolong the gene silencing effect resulting in control of plant viral diseases for up to 30 days (Mitter et al., 2017).

The present work is the first description of a gene silencing method involving root delivery of the silencing molecule. Root uptake of ASOs very likely occurs through root hairs along with water, where they enter the xylem either symplastically through the plasmodesmata, apoplastically through intracellular space or by transmembrane transport through membrane water channels (Newman, 1976). Once delivered into the leaves by the vasculature using capillary movement of water triggered by transpiration, the ASOs might eventually enter the plant epithelial cells (Newman, 1976) and then the fungal haustoria through H^+-sugar symport pathways, where ASOs target fungal genes. Root delivery may counter the problems faced associated with instability of dsRNA sprayed onto leaves. However, for fungal pathogens like *F. graminearum* and *Z. tritici* which grow in the plant apoplastic space, PTO modified ASP delivery by infiltration might be more effective. Root treatments can potentially allow for the delivery of the silencing molecules to the whole plant rather than a single organ. However, the long-term effect of PTO modified ASO mediated gene silencing is yet to be assessed. As growth of obligate biotrophs is dependent on the longevity of the host, the root delivery assays using attached leaves are better suited for study of long-term silencing effects as compared to excised leaf assays showing faster leaf senescence. A comparison of gene silencing mediated by PTO modified ASOs and dsRNA may also be done. However, initial experiments involving direct uptake of 21 nt long 10 µM, 1 µM of sRNA led to shrivelling of the barley leaves. Moreover, no silencing effect was achieved when the sRNA concentration was reduced to 0.1 or 1 µM (Unpublished data, Snehi Gazal).

The unmodified ASOs are not easily taken up by the cells due the repulsion between the negative charge of DNA and the negative charge on the cell membrane (Stein et al., 1993). Therefore, ASO carriers such as positively charged liposomes were developed. These are positively charged vesicular colloid vesicles generally composed of bilayers of phospholipids and cholesterol which easily integrate into the cell membrane facilitating the ASO delivery into the cells (Ma & Wei, 1996). The stability of the silencing molecule and its uptake can be increased by introducing chemical modifications such as phosphorothioate (PTO) modifications, where one of the bridging oxygen atoms of the phosphodiester bond s replaced by a sulphur (Stein and Subasinghe, 1988). PTO

modification stabilises the nucleic acid against host nucleases (Stein and Subasinghe, 1988) and facilitates its uptake by the host increasing its bioavailability (Koller et al., 2011). Moreover, PTO modified ASO acts as a substrate for RNase H leading to target mRNA degradation and post transcriptional gene silencing (Eckstein, 2000; Wu et al., 2004) . Moreover, the present study has shown that end capped PTO modified ASOs containing PTO modifications in the last two phosphodiester bonds in each end of the ASO have higher silencing efficiency than fully PTO modified ASOs. This observation is akin to the results obtained in animal models, where end capped PTOs delivered higher silencing efficiency without the cytotoxic effects associated with a fully PTO modified ASO (Hebb & Robertson, 1997). Moreover, end capped PTOs are more economical and reduce the cost of the assay, making the root assays more financially viable. Other ASO chemistries have been successfully trialled for gene silencing in animal models. These include secondary modifications like 2'-O-Methyl (2'-OMe) and 2'-O-Methoxyethyl (2'-MOE) modification of the ribose sugar in the DNA backbone. These modifications reduce the RNase H affinity for the DNA-RNA heteroduplex resulting in suppression of RNAse H mediated gene silencing. Therefore, mixed backbone silencing molecules consisting of 10 PTO modifications flanked by 5 OMe or MOE modified ASOs have been designed. Then these modifications still allow RNase H binding at the chimeric junctions supporting RNase H degradation along with stabilising the ASO against nucleases (Chan et.al., 2006; Zhou & Agrawal, 1998). Secondary modified ASOs have been successfully used for exon skipping for gene therapy against Duchene Muscular Dystrophy (Cirak et al., 2011). Tertiary modifications have also been introduced into ASO backbone to improve ASO stability, target specificity and pharmacokinetics. These include furanose ring modifications resulting in peptide nucleic acid (PNA) (Rozners, 2012), locked nucleic acid (LNA) (Swayze et al., 2007) and phosphoroamidate morpholino oligomer (PMO) modified ASOs (Lebleu et al., 2008). Since these tertiary modified ASOs do not act as a substrate for RNase H, gene silencing occurs by translational arrest (Lebleu et al., 2008). These tertiary modifications have also been used as a part of a mixed backbone ASO. For instance, locked nucleic acids were successfully used for gene silencing by creating LNA−DNA mixmers and LNA−DNA−LNA gapmers, resulting in successful gene silencing without the cytotoxic effects associated with PTO modifications in animal cells (Wahlestedt et al., 2000).

PTO modified ASO based gene silencing method uses short gene specific ASOs that decreases non-specific binding. So far, this method has been used to silence fungal virulence/housekeeping genes and plant susceptibility genes in the barley and wheat powdery mildew pathosystems. However, a complete loss of pathogenicity has not yet been achieved by silencing of any of these genes. Therefore, complexing assays may need to be developed where a combination of genes can be

silenced for crop protection. This would firstly require an understanding of the lowest concentration of PTO modified ASO at which significant silencing effect can be achieved, and showing a dose effect, in order to minimise amount for cost reasons as well as toxicity. Based on this combination of multiple PTO modified ASOs might produce an additive or synergic silencing effect resulting in better fungal disease control. But, to date, only a couple of studies have shown successful silencing of a family of genes using a single silencing molecule, rather than silencing multiple genes (Koch et al., 2016; Koch et al., 2013). Therefore, this strategy of silencing multiple genes is yet to eb explored. Another strategy to improve the impact of ASO based silencing on reducing fungal virulence could be to silence global transcription factors regulating the expression of the prevalent virulence factors of the pathogens. This was attempted by silencing the pH sensitive transcription factor PacC, as it was shown to be involved in virulence, potentially by regulating the expression of many *B. graminis* effectors. But *PacC* silencing led to only 50% reduction of infection. However, *PacC* silencing could be combined with transcriptomic studies to validate or identify the downstream genes and pathway regulated by the PacC transcription factor. This will in turn help in enhancing the understanding of *B. graminis* growth and virulence as a response to ambient pH conditions.

For high throughput analysis, the ASO mediated gene silencing could potentially be a solution as it involves no cloning or the preparation of transgenic plants. However, one of the limitations of the silencing assay in the powdery mildew pathosystem is the time needed for scoring the infection success rate, measured as the % of conidia forming secondary hyphae, which is a very time-consuming process. Nevertheless, recent data from the Bindschedler laboratory showed reduced host ROS development following the silencing of the barley susceptibility gene *MLO1* (Lambertucci et.al., 2019). This phenotype was easy to visualise and score more rapidly than the infection success rate. It involved detecting ROS in infected barley epidermal cells by diaminobenzidine (DAB) staining. A brown precipitate forming in responding cells could be easily observed, even at low magnification, and thus, could be quickly scanned. The reduction in host ROS development was associated to an increase in host immunity as the result of the silencing of host susceptibility genes (Lambertucci et.al., 2019). An increase in ROS production was also associated with the silencing of several *B. graminis* effector genes (Orman et.al., manuscript in prep). Therefore, there may be a possibility of assessing the gene silencing effect via DAB staining in the future experiments, making the process more time efficient.

During the *in planta* gene silencing of *F. graminearum* genes, the selection of gene targets may need to be reconsidered, as *Fg TRI5* was shown to act as virulence factor during the wheat- *F. graminearum* pathosystem when considering infection of the wheat ears, but not during *F. graminearum* infection of barley and *Arabidopsis* (Cuzick et.al., 2008; Maier et al., 2006). Since assay

involving wheat ears is not convenient for high throughput assays, leaf infection assays were attempted, but no significant effect was seen, although a trend was observed when silencing the 2-glycosyl transferase. This was possibly the case because of variation in infection with such assay, probably requiring a larger experimental set. Alternatively, a previously published wheat coleoptile assay might be adapted for *in planta* silencing of *F. graminearum* genes using PTO modified ASOs (Jia et al., 2017). However, the difference in virulence role of *F. graminearum* genes between different hosts and host tissues diminishes the chances of developing a single assay targeting a single gene that can be used for both laboratory and field applications. Alternatively, host susceptibility genes could be targeted for *F. graminearum* disease control. In the initial part of the study, most of the potential susceptibility genes investigated were discarded because silencing them rendered the host susceptible to other fungal pathogens, particularly when comparing obligate biotrophs to pathogens with other lifestyles. For instance, deletion of *Arabidopsis ATG* genes involved in autophagy and thus important for host PCD, rendered the host resistant to biotrophs like *P. syringae* and susceptible to necrothrophs like *Alternaria brassicicola* (Lenz et al., 2011). Similarly, silencing of the *B. graminis* susceptibility gene MLO1, rendered barley resistance to obligate biotrophic *B. graminis* and susceptible to the hemibiotroph *F. graminearum* (McGrann et al., 2014). On the other hand, deletion of the *Arabidopsis DMR1* gene encoding a homoserine kinase, rendered the host resistant to both biotrophic and necrotrophic pathogens including the hemibiotroph *F. graminearum* (Brewer et.al., 2014). However, the *DMR1* deletion mutants also produced dwarf plants, which is not convenient for disease control applications. Nevertheless, gene knock down by silencing might not have a strong impact on growth as compared to complete loss of gene functionality as a consequence of its deletion (Brewer et.al., 2014). Therefore, barley *DMR1* homologue could be trialled as a suitable target for *in planta* gene silencing using PTO modified ASOs in the *F. graminearum* – barley or *B. graminis*-barley pathosystems.

Some preliminary data generated in the present study showed an increase in *Z. tritici* susceptibility to the MBC fungicide carbendazim as an effect of PTO modified ASO mediated targeting of the fungicide gene target *Zt β- tubulin*. These experiments need to be repeated to check their reliability. Moreover, an uptake assay confirming the uptake of fluorescently labelled PTO modified ASOs by *Z. tritici* will further strengthen the findings from this study. As *Z. tritici* has developed resistance to multiple fungicides limiting the options available for STB disease control, a combination of PTO modified ASO mediated gene silencing and fungicide can potentially facilitate STB disease control by mitigating fungicide resistance in *Z. tritici*.

8.2 Role of metals in plant – pathogen interactions

Zinc is an essential element for plant growth that drives multiple cellular functions (Broadley et.al., 2007). Zinc has a role in structural, catalytic and regulatory role in plants. For instance, zinc contributes to the catalytic role of superoxide dismutase which is responsible for mitigating ROS related damage in organisms (Kang et al., 2002). One of the largest groups of zinc requiring proteins are the zinc-finger containing transcription factors that regulate different growth processes (MacPherson et.al., 2006). Therefore, it is not surprising that zinc is associated with approximately 6% of the prokaryotic proteome and about 9% of the eukaryotic proteome (Andreini et.al., 2005). Interestingly, zinc hyperaccumulating plants are more resistant to certain bacterial pathogens (Fones et al., 2010) as well as herbivores (Pollard & Baker, 1997). Moreover, application of exogenous zinc has been shown to alleviate symptoms generated by abiotic stress such as drought and salt (Disante et.al., 2011; Tavallali et.al., 2010). For instance, exogenous zinc application on drought stressed *Quercus suber L.* seedlings reduced the intensity of drought symptoms. High zinc availability also increased the water content of seedlings to 75% as compared to 50% in seedlings with low zinc availability. Moreover, zinc availability also reduced stomatal conductance and photosynthetic rate, resulting in increased stress tolerance (Disante et al., 2011). Similarly, in pistachio seedlings, exogenous zinc application alleviated salt stress by production of antioxidants like ascorbic acid, increasing cell membrane stability by inhibiting lipoxegenase (LOX) activity and also increasing the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Tavallali et al., 2010). Plants acquire zinc primarily from soil but 60% of arable soil is deficient in zinc (Noulas et.al., 2018). Therefore, a tightly regulated zinc homeostasis involving import, trafficking, sequestration and export is of paramount importance to ensure plant health, yield and indirectly the health of the human population who acquire their zinc from their food sources.

The plant roots acquire zinc from the soil which then enters the symplast, a network of live tissues. Uptake of zinc into the symplast is through zinc transporters belonging to the ZIP family. These transporters are also involved in root to shoot translocation of zinc. For instance, in rice, the zinc transporters OsZIP1 and OsZIP3 are involved in root zinc uptake by roots whereas OsZIP4, OsZIP5, and OsZIP8 are involved in root to shoot translocation (Ishimaru et.al., 2011). In living cells, the absorbed zinc faces alkaline conditions which renders it insoluble, necessitating the presence of zinc chelators to ensure zinc translocation. For instance, in the zinc hyperaccumulating plant *Arabidopsis helleri,* zinc is translocated from the roots to the shoots by binding to nicotianamine. Suppression of *Ah-NAS2* gene encoding for the nicotianamine synthase, strongly reduced root nicotianamine accumulation and an associated reduction in root-to-shoot translocation of Zn (Deinlein et al., 2012).

Nicotianamine is also important for intercellular mobility of zinc and mobility of zinc in the phloem enhancing the zinc content of the seeds. Interestingly, nicotianamine-bound zinc is more bioavailable as evidenced by rice lines overexpressing the nicotianamine synthase *NAS* gene having twice the amount of zinc in the seed endosperm (S. Lee et al., 2011). Nicotianimine also binds other metals such as iron and copper (Clemens et.al., 2013). Similarly, museneic acid (MA) family of phytosiderophores are also involved in zinc uptake and transport in rice plants (Bashir et.al., 2010).

In fungi, zinc is also required for growth and development. Fungal pathogens acquire zinc from their host, as evidenced by the presence of zinc sequesters such as AspF2 in *Aspergillus fumigatus* and PRA1 in *Candida albicans,* both of which contribute to fungal growth and virulence. Since, alkaline conditions render the zinc insoluble, these fungal sequesters are activated under alkaline conditions to ensure zinc supply (Amich et.al., 2010; Citiulo et al., 2012). Indeed, the expression of these zinc sequestering virulence factors are regulated by homologues of zinc sensitive (ZAP1) and a pH sensitive transcription factors (PacC) (Amich et.al., 2010; Citiulo et al., 2012). Interestingly, animal hosts express calprotectin, a zinc chelator that sequesters the zinc away from the fungal pathogen calprotectin (Mambula et.al., 2000; McCormick et al., 2010). This phenomenon of limiting pathogen growth and infection by limiting the availability of nutrients is termed as nutritional immunity (Hood & Skaar, 2012). In the present study, it was shown that the expression of the *B. graminis* virulence factor *BEC1019* is also regulated by a zinc sensitive (ZAP1) and a pH sensitive transcription factor (PacC). Therefore, *Bgh BEC1019* metalloprotease-like protein might also be involved in fungal zinc sequestration under alkaline conditions. However, so far not much is known about nutritional immunity in plants against fungi, although some studied reported this in plant immunity against bacteria (Fones & Preston, 2013). However, as mentioned earlier, metal hyperaccumulating plants do possess zinc chelators produced by enzymes such as nicotinamine synthase (Deinlein et al., 2012). Moreover, some zinc hyperaccumulators are highly resistant to bacterial pathogens such as *Pseudomonas syringae* (Fones et al., 2010). Similarly, zinc-binding phytochelatins have been shown to be associated with plant basal defences where phytochelatins induce callose deposition in *Arabidopsis* during non-host resistance against bacterial pathogens (Clemens & Peršoh, 2009). Zinc and manganese binding metallothionines are also induced during *Colletotrichum* infection of *Arabidopsis*, tobacco and velvet leaf (Dauch & Jabaji-Hare, 2006). Therefore, there is a possibility that plant zinc sequesters play a role in plant nutritional immunity.

Other metals are also believed to play a role in plant responses to biotic stress. Plants hyperaccumulating metals like Ni, Cd, AS and Se are shown to be resistant to pathogens, insects and herbivores. For instance, Ni hyperaccumulation renders the plant *Streptanthus polygaloids* resistant to tissue chewing herbivore insect pests and depiteran rhizovores cabbage maggots (Jhee et.al.,

2005). Similarly, arsenic hyperaccumulation in the Chinese brake fern *Pteris vittata* deters herbivorous grasshoppers like *Schistocerca americana* (Rathinasabapathi et al., 2007). Selenium hyperaccumulation by plants also reduced arthropod load in the fields (Galeas et al., 2008). These plants have developed mechanisms for metal homeostasis that protects them from the toxic effects of metal hyperaccumulation, as evidenced by the high constitutive expression of metal homeostasis genes in the metal hyperaccumulator *Arabidopsis halleri* (Becher et.al., 2004). Moreover, in arsenic hyperaccumulating plants *Ptesseri vittata*, hyper-tolerance is mediated by phytochellatin mediated sequestration of arsenic (Zhao et al., 2003). Similarly, tolerance to copper hyperaccumulation in *Silena vulgaris* is mediated by overexpression metallothionine-like gene *SvMT2b* (van Hoof et al., 2001). Sometimes, metal accumulation can result in plant responses alike immune response to abiotic or biotic stress but may uncouple the stress response pathways. For instance, Cd hyperaccumulation induced SA synthesis in *Thymus praecox* which renders the plant resistant to the biotrophic fungal pathogen *Erysiphe cruciferarum*. However, Cd accumulation diminished the host's capacity to produce SA in response to fungal attack (Llugany et.al., 2013). In another study, zinc hyper accumulation in the aerial tissues of *Noccaea caerulescence* renders them toxic to many pathogens due to ROS species accumulation. In these plants, superoxide dismutase is highly expressed to prevent ROS related damage in host tissues. Interestingly, *Noccaea* challenged with *P. syringae* did not elicit a ROS burst typical of defence against *P. syringae*, however the plants were resistant to the bacterial pathogen as a result of ROS accumulation triggered by metal hyperaccumulation. The plant also lacked ROS signalling mediated plant defence responses like callose deposition and PR gene expression, however SA production was intact (Fones et.al., 2013).

Metal homeostasis in the pathogens also plays a role for their virulence. Therefore, controlling metal availability in the plant during invasion might be a strategy to control disease. For instance, bacterial Fur transcription factors which regulate iron uptake and homeostasis in an iron dependent manner, play a critical role in defence against oxidative stress, acid stress and expression of virulence factors (Kitphati et.al., 2007). Moreover, Fur deletion mutants in *P. syringae* have reduced virulence in tobacco, which may be linked to reduced iron uptake and siderophore synthesis but may also be linked to reduced production of tabtoxin in the Fur mutants (Ratledge & Dover, 2000). Siderophores have been shown to be important for pathogenicity of other bacterial pathogens such as *Klebsiella* and *Yersinia pestis* during infection in mouse models (Bearden et.al., 1997; Lawlor et.al., 2007). Competition for iron between hosts and bacteria also plays an important role in bacterial pathogenicity, as demonstrated by loss of bacterial pathogenicity and increase in iron deficiency in host plant *Saintpaulia ionantha* as a direct result of production of the siderophore chrysobactin in the bacterial pathogen *Erwinia chrysanthemi* (Neema et.al., 1993). Similarly, the plant bacterial

pathogen *Xanthomonas campestris* possess Zur zinc sensitive transcription factors , that play a part in zinc homeostasis and bacterial virulence by regulating the expression of bacterial effectors (Huang et al., 2008; Huang et al., 2009; Tang et al., 2005).

Therefore, there is a possibility that plants have a nutritional immunity strategy just like animals, and *Bgh BEC1019* might facilitate fungal zinc sequestration to overcome limitations in zinc bioavailability caused by the plant host nutritional immunity strategies.

8.3 Role of ambient pH in plant pathogen interactions

Intracellular pH modulates biological processes in plants. For instance, cellular pH regulates protein modifications and sorting via the secretory pathway (Wu et al., 2001). Moreover, the cellular compartments have a distinct pH that mediates their biological function. For example, the plant cytoplasm is alkaline whereas the vacuole tonoplast is acidic (Pittman, 2012). pH regulation also helps the cells to cope with potentially toxic alkaline/acidic conditions. Therefore, for homeostasis, the cellular pH is tightly regulated in plant cells by the H+/ ATPase proton pumps that maintain the H+ gradient across the cell membrane, as well as between cellular compartments (Gout et.al., 1992; Zhang et al., 2017). The proton motive force generated across the cell membrane H^+ gradient is also responsible for transport of water and nutrients across the cell membrane (Gaxiola et.al., 2007; Zhang et al., 2017). Interestingly in a study, the plant cytoplasmic pH is independent of the external pH in the range of 4.5-7.5. In contrast, the intracellular pH drops rapidly below pH 4.5 and increased progressively over pH 7.5 in response to the extracellular pH (Gout et al., 1992). In the same study, the drop in the extracellular pH below 7.5 was accompanied by acceleration in oxygen uptake and reduction in cellular ATP levels. It was believed that the ATP was being rapidly utilised by the H^+/ATPase pumps to counter the rapid influx of H+ ions. As the extracellular pH reached 4.5, the activity of H^+/ATPase pumps was insufficient to prevent the rapid H+ influx, resulting in the intracellular pH drop. Similarly, as the extracellular pH increased from 4.5, the pH increase was countered by a simultaneous synthesis of organic acids like maleic and citric acids in the cytoplasm. This increase in organic acid production may be due to a marked activation of the cytoplasmic phosphoenolpyruvate carboxylase induced by an increase in cytoplasmic bicarbonate concentration. As the extracellular pH reached 7.5, a progressive passive influx of OH^- occurred in the cytoplasm resulting in a corresponding increase in cytoplasmic pH (Gout et al., 1992).

Alike plants, the cytoplasmic and organellar pH is highly regulated in fungal cells, as it has a direct impact on protein activity and cellular processes. For instance, the pH gradient across the inner mitochondrial membrane is responsible for ATP synthesis and ion transport (Reviewed by Kane, 2016). In *S. cerevisiae,* the plasma membrane H^+-pump Pma1 and the V-ATPase play a role in cytosol

and organellar pH regulation (Serrano et. al. 1986). Pma1 is a single-subunit P-type H^+-ATPase that belongs to the same family as Na^+, Ca^{2+} and Na^+/K^+-ATPases of mammalian cells and have homologues in both plants and fungi (Serrano et al., 1986). Homology modelling of Pma1 homologue is *Neurospora crasa* showed them to be a H+/ATPase pump (Kühlbrandt et.al., 2002), which was later confirmed when the X-ray crystal of Pma1 homologue in *Arabidopsis* was obtained (Pedersen et.al., 2007). Alike P-type proton pumps, the fungal cell membrane ATPase (Pma1) consists of 3 cytosolic domains for nucleotide-binding (N), phosphorylation (P) and actuator (A) (Figure 8-1A) (B. P. Pedersen et al., 2007). ATP hydrolysis results in conformational changes that are communicated to the transmembrane domains that drive the transmembrane ion exchange (Moller et.al., 2005). The pH regulating plasma membrane localised H^+/ATPases are also essential for fungal growth, as evidenced by *S. cerevisiae Pma1* mutants showing defect in pH regulation, cell cycle progression, increased sensitivity to osmotic pressure and cold stress (McCusker et.al., 1987) In yeast, the plasma membrane ATPases are regulated by glucose levels, intracellular and extracellular pH (Carmelo et.al., 1997; Pilar et.al., 1987; Serrano, 1983). For instance, glucose addition to the medium resulted in 10-fold increase in Pma1 ATPase activity, which was reversed in the absence of glucose. Glucose induced ATPase activity was also accompanied by reduction in K_m for ATP and shift towards neutral pH optimum for enzyme activity (Serrano, 1983). Glucose is believed to prevent the autoinhibition of Pma1 mediated by an interaction between the Pma1 c-terminal tail and the catalytic site, resulting in its activation (Eraso & Portillo, 1994). Glucose mediated activation of Pma1 also requires the fungal protease Ptk2, which causes S899-dependent phosphorylation of a Pma1 C-terminal peptide in vivo (Eraso et.al., 2006). However, phosphoproteomic analysis of the Pma1 C-terminal tail has confirmed that phosphorylation of S911 and T912 are the primary mediators of glucose activation of Pma1 (Lecchi et al., 2007). As mentioned earlier intra and extra cellular pH also regulated Pma1 activation which helps yeast to survive under highly acidic conditions (pH 2-3). ATPase pump in activated in the growing yeast cells as the extracellular pH falls, maintaining a constant intracellular pH. However, this acid mediated activation is growth dependent and only logarithmic and stationary phase cells can activate the ATPase under acidic extracellular conditions, indicating at a role of the ATPase in promoting fungal growth under acidic conditions (Pilar et. al., 1987). The ATPase activation is also dependent on availability of glucose along with acidic pH in the growing cells, which results in maintenance of an intracellular neutral pH (Holyoak et al., 1996). However, when glucose is depleted in the stationary phase, intracellular acid production occurs and the intracellular pH falls to 6.0 (Orij et al., 2012). As an electrogenic pump, Pma1 activity is also sensitive to plasma membrane potential. For instance, K^+ addition promotes H^+ efflux from yeast cells treated with glucose to activate Pma1 (Martínez-Muñoz & Kane, 2008).

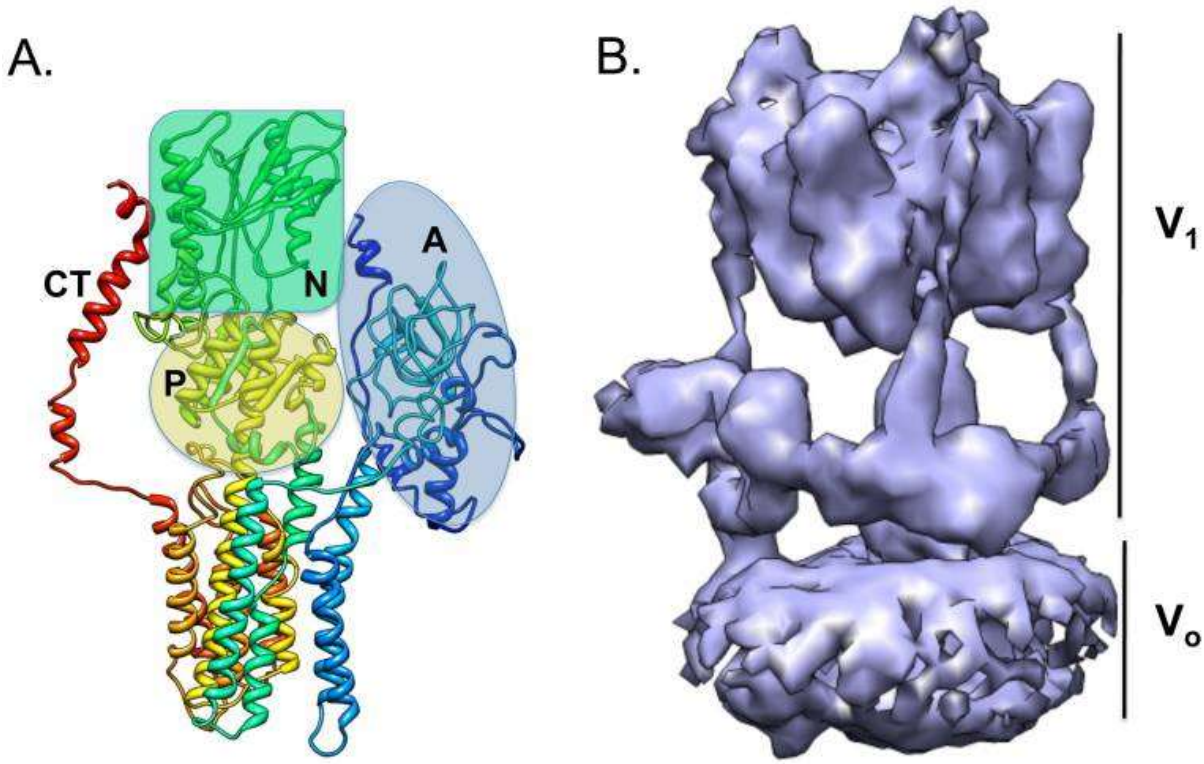


Figure 8-1: P-type and V-type ATPase structures.s

A. Structure of P-type (Pma1) ATPase associated with fungal cell membrane consisting of the cytosolic nucleotide binding (N), phosphorylation (P) and arbitrator (A) domains. B. V-ATPases associated with fungal organellar membrane: They contain a peripheral membrane associated V1 structure consisting of hexamer of catalytic and regulatory subunits and the Vo subunit a. Ion exchange occurs at the interface between the V1 and Vo subunits. Diagram extracted from Kane, 2016.

Fungal organellar pH regulation is mediated by V-ATPases or Vacuolar proton-translocating ATPases that are multi-subunit proton pumps responsible for acidification of intra organellar space of vacuole/lysosomes, endosomes and Golgi apparatus in eukaryotes (Reviewed by Kane, 2006). Evolutionarily, the V-ATPases are closely linked to F-type ATPases as well as archaeal ATP synthases and ATPases (Grüber et.al., 2014; Kibak et.al., 1992). The peripheral membrane structure (V_1) consists of the regulatory and catalytic subunits. The V-ATPases exhibit a rotary binding catalysis mechanism, where ATP binding and hydrolysis results in conformational changes in the catalytic units that is transmitted to central rotor stalk in the centre of the catalytic complex. The rotor stalk then rotates the membrane rotor complex, which consists of several protonatable proteolipid subunits. Proton transport then occurs at the interface between the proteolipid subunits and a single larger membrane subunit (V_0 subunit a). Rotor catalysis also requires 3 peripheral stators that hold together the hexamer of catalytic and regulatory subunits (V_1) and the V_0 subunit a. Two bridging subunits, V_1 subunits H and C, provide the primary contacts between the V_1 and V_o sectors (Reviewed by Kane, 2016) (Figure 8-1B). Like the p-type ATPase Pma1, V-ATPases are also regulated by glucose and pH (Diakov & Kane, 2010; Kane, 1995). For instance, glucose deprivation led to

disassembly of V_0-V_1 complex, resulting in V-ATPase deactivation and the V-ATPase complex was intact in glucose-replete cells (Kane, 1995). The V-ATPase activity is also regulated by the extracellular pH as evidenced by the increased activity of V-ATPases when yeast cells were grown in a medium buffered at pH 7.0 as compared to a medium at pH 5.0 (Diakov & Kane, 2010). The plasma membrane ATPase pump and the vacuolar ATPase pump are believed to work in coordination to maintain the intracellular pH in fungi being activated under different pH conditions (Carmelo et al., 1997).

Ambient pH plays an important role in fungal virulence (Alkan et.al., 2013). For instance, in *M. oryzae,* alkaline pH induces spore germination whereas mild acidic conditions facilitate host colonisation (Landraud et.al., 2013). Therefore, the fungal pathogens have developed ambient pH sensory mechanisms that sense the change in pH and mount a response for fungal survival under changing pH conditions (Reviewed by Vylkova, 2017). Fungal pH sensing occurs through Pal pathway in *Aspergillus sp.* (RIM pathway in *S. cerevisiae)*, where membrane associated PalH receptors perceive change in ambient pH through sensing the alkaline pH induced alteration in the lipid symmetry of the cell membrane (Nishino et.al. 2015). This in turn activates the Pal pathway resulting in the proteolytic cleavage-based activation of the pH sensitive transcription factor PacC (RIM101 in *S. cerevisiae)* (Denison et.al., 1995; Xu & Mitchell, 2001). Once active, PacC mediates fungal growth under alkaline pH by activating alkaline-expressed genes and by suppressing acid-expressed genes (Tilburn et al., 1995). PacC mediated gene regulation involves binding of PacC to conserved pH sensitive regulatory elements in the promoters of the target genes (Espeso et al., 1997). PacC homologues have been shown to regulate fungal virulence by regulating the expression of virulence factors like *AspF2* in *A. fumigatus* (Amich et al., 2010)*, PRA1* in *C. albicans* (Citiulo et al., 2012), secreted lytic enzymes in *M. oryzae* (Landraud et al., 2013) and DON toxin in *F. graminrearum* (Merhej et.al., 2011). In this study we have shown that PacC homologue in *B. graminis* also influences barley powdery mildew disease development by regulating the expression of *Bgh* effectors *BEC1019* and *BEC1011*. Interestingly, the PacC binding PREs were also enriched in the promoters of haustoria specific candidate effectors in *B. graminis*, indicating a role of PacC in regulation of the expression of multiple effector genes. PacC is the first *B. graminis* transcription factors shown to regulate effector expression in barley powdery mildew. This can be further validated by confirming PacC binding to the promoter region of *Blumeria* effector candidates using yeast-one-hybrid assays.

Fungal pathogens are also capable of manipulating the host pH for facilitating host colonisation. Fungi acidify the host by producing organic acids like oxalic acid, citric acid and maleic acid amongst others (Vylkova, 2017). For instance, acidification of the host environment by the necrotrophic

pathogen *B. cinerea* induces the secretion of the fungal virulence factors such as aspartate proteases in order to promote host invasion and necrotrophy (Manteau et.al., 2003). A similar activation of pathogen aspartyl proteases by pathogen mediated acidification of the host environment also promotes host colonisation in other fungal pathogens such as *C. albicans* (Naglik et.al., 2003). Host acidification also inhibit host ROS production during *Colletrotichum* infection (Chen & Dickman, 2005). In contrast, *Colletrichum* alkalinise the host environment to induce appressorium formation and secretion of pectin lyases (Shnaiderman et.al., 2013). Similarly, the human pathogen *C. albicans* neutralises the normally acidic phagosome by raising the pH by several units (Vylkova et al., 2011). Alkalisation of the host environment by fungal pathogens primarily involves ammonia production. Ammonia is produced either extracellularly or as a by-product of amino acid synthesis pathway (Vylkova et al., 2011). For instance, in *C. albicans* ammonia production requires amino acid uptake by lyases and its mobilisation to the fungus vacuoles where they are catabolised releasing ammonia (Vylkova et al., 2011). Ammonia accumulation is also favoured by carbon limitation, as lack of carbon prevents the utilisation of ammonia for amino acid biosynthesis, resulting in its accumulation (Barad et.al., 2016; Vylkova et al., 2011). Some fungi such as *F. oxysporum* alkalinises the host apoplast by secreting alkalising peptides called Rapid Alkalising Factors or RALFS (Masachis et al., 2016; Prusky et.al., 2001). Little is known about how host ambient pH is manipulated by the obligate biotrophic pathogen *B. graminis.* Nevertheless, during barley powdery mildew infection, host pH changes were witnessed in infected barley cells (Felle et.al., 2004; Felle et.al., 2008). A transient increase in barley apoplastic pH was observed between 2-4 hpi after *B. graminis* inoculation of barley leaves, which was accompanied by a simultaneous reduction of the cytoplasmic pH (Felle et al., 2008). This rise in host apoplastic pH was believed to be the result of *Blumeria* PAMP recognition by the host receptors. Later at 24 hpi, the intracellular pH increased and peaked again, this time by two units during the incompatible interaction. However, this change in the host apoplastic pH is not attributed to the pathogen pH manipulation to increase host compatibility, but was rather believed to be a response attributed to the activation of the host immune response, as it was more pronounce in incompatible interactions where the host was resistant to the inoculated *Blumeria* isolate (Felle et.al., 2008). It can therefore be speculated that PacC, which is activated at pH 6.5 or above, might be activated during either the initial transient alkalisation or the later increased pH observed at the level of the host apoplast and leaf surface on which *Blumeria* spores germinates prior to forming a penetration peg and the first haustorium (Felle et al, 2008). However, at the 2-4 h stage, *B. graminis* secondary germ tube is slowing down, dwelling and transforming into the appressorium structure prior host penetration (Both et.al., 2005). So, at this stage, it is likely that the fungus can perceive the pH at the leaf surface rather than in the leaf apoplast, despite there being a correlation between

the pH at the surface of the leaf and in the apoplast (Feller et al, 2008). Interestingly, leaf surface pH in many plants varies from slightly acidic to alkaline (Oertli et.al., 1977). Moreover, germ tubes of other obligate biotrophs such as *P. graminis* have been shown to sense pH gradient on host leaf surface (Edwards & Bowling, 1986). Therefore, there is mounting evidence that the PacC protein might be activated as a response to pH rise from slightly acidic to alkaline pH on the barley leaf surface, at early stages following inoculation with *B. graminis*. Alternatively, PacC activation maybe as a response to intercellular alkalisation of barley epithelial cells at 24 hpi (Felle et al., 2008), when *B. graminis* has already formed the primary haustorium. pH sensing receptors PalH homologues maybe present on the extra-haustorial membrane which may sense the alkalisation of host intercellular space, resulting in activation of PacC. From the work presented in this thesis, *Bgh PacC* was shown to be dominantly expressed in *B. graminis* haustoria at 24 hpi. Even though expression of a gene does not confirm the activation of the protein, it certainly increases the possibility of increased PacC activity in *B. graminis* haustoria in response to alkalisation of barley intercellular space. Moreover, PacC binding pH responsive elements (PREs) in promoter regions of haustoria-specific *Blumeria* effector candidates, reinforce the hypothesis that PacC is placed upstream of multiple *Blumeria* effector candidates to activate their expression. Moreover, PacC was shown to influence *B. graminis* virulence by regulating the expression of the effector *Bgh BEC1011* and the virulence factor *Bgh BEC1019*, since silencing of PacC led to a relative decrease of the two cognate transcripts. Interestingly, the present study also showed the localisation of *Bgh BEC1019* and *Bgh BEC1011* transcripts to be dominant in *B. graminis* haustoria at 24 hpi. This further supports the hypothesis that *B. graminis* may respond to the intracellular alkalisation of barley plants inoculated with *B. graminis* by activating *Bgh* PacC in haustoria, which in turn induces the expression of effectors that are required for virulence and promoting barley powdery mildew infection and development. However, experimental proof of this hypothesis is difficult to acquire as it is impossible to work with *B. graminis in vitro*. Interestingly, another study demonstrated the dependency of *Blumeria* host penetration on host cytoplasmic pH. Increase in cytoplasmic pH in barley coleoptile cells increased powdery mildew susceptibility by increasing *Blumeria* host penetration (Yamaoka et al., 2000). These observations further support the hypothesis that the pH sensitive transcription factor PacC may impact on *B. graminis* virulence by globally regulating the expression of effectors/virulence factors required for barley powdery mildew infection.

Moreover, PacC may also be developed as a suitable target for fungal disease control as silencing of a single transcription factor may supress the expression of multiple fungal effectors, resulting in disease control, at least for powdery mildews, possibly other biotrophs such as Puccinia sp. and very likely other fungi which requires neutral to basic conditions for growth and virulence. This not the

case of every phytopathogenic fungi. For instance, *F. graminearum* PacC homologue was shown to negatively regulate DON synthesis, a virulence factor for FHB development, which needs acidic pH (Merhej, Richard-forget, et al., 2011). In addition, Fusarium head blight virulence was unchanged in the Fusarium knockout mutants for *Fg BEC1019,* which is also regulated by *Fg Pac1,* the PacC homologue in *F. graminearum.* Therefore, PacC is possibly a negative regulator of virulence in *F. graminearum*.

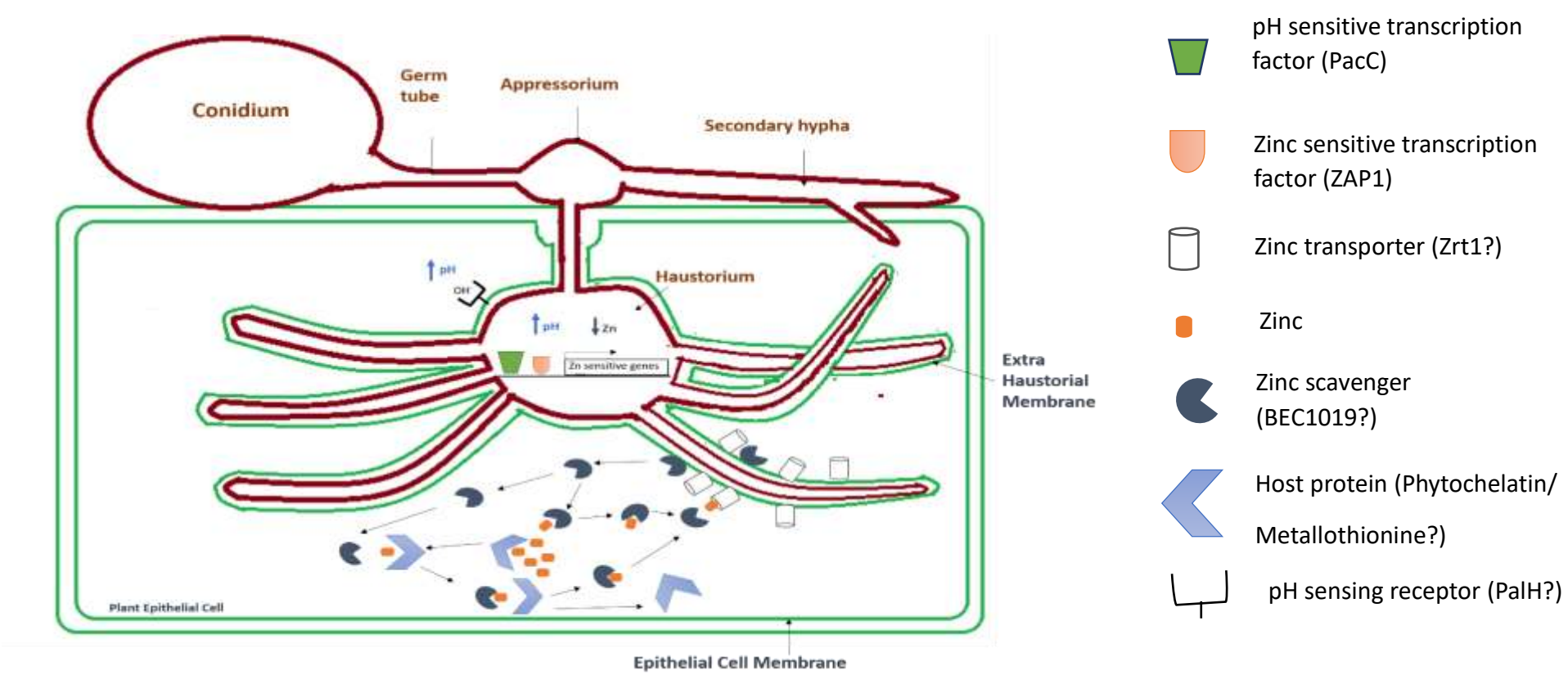


Figure 8-2: Proposed model for role of Bgh BEC1019 in B. graminis virulence.

A potential model was proposed for the role of BEC1019 in B. graminis. During B. graminis infection of barley epithelial infection, B. graminis may perceive alkalisation of host epithelial cell, maybe via receptors like PalH present on the Blumeria extra haustorial membrane, which may trigger the activation of pH sensitive transcription factor PacC within the Blumeria haustorium. Under alkaline pH and zinc deprivation, zinc sensitive transcription factor Bgh ZAP1 maybe activated. PacC and ZAP1 together trigger the expression of zinc sensitive genes like Bgh BEC1019 (potential zinc scavenger) and Bgh Zrt1 (potential zinc transporter) in the B. graminis haustorium. BEC1019 may then be secreted into the host cells where it may compete for the available zinc with host zinc-sequestering proteins combating plant nutritional immunity. The uptake of zinc may then support fungal growth and host colonisation.

8.4 Conclusions

This PhD work successfully exploited a previously developed PTO modified ASO based gene silencing assay using excised barley leaves as a suitable functional genomics tool for the barley powdery mildew pathosystem. This assay is very gene specific as the ASO being only 18-19 mer long allows for the targeting of single genes, and unlikely off targets. This assay allowed for the discovery or validation of *Blumeria* genes required for fungal virulence/housekeeping or barley genes required for host susceptibility. The assay was also successful in various barley cultivars. Moreover, the technology was further adapted for the first time for *in planta* gene silencing in whole plants by root delivery of PTO modified ASOs, suggesting its potential translation for crop protection. Although there was always a substantial residual powdery mildew infection, for any of the target silenced so far. Therefore, further improvements are still required before it could be used in agriculture. Several venue of improvement can be considered, by either altering the ASO chemistry, silencing more than one gene at a time, or by using a combination of silencing and fungicides targeting the same gene and its cognate gene product, hopefully reducing the amount of fungicide required by increasing the fungus sensitivity to a given fungicide or attenuating fungicide resistance. However, the length of the silencing effect is not known to this day and needs investigated before ringing too many bells.

Also, the PTO modified ASO based gene silencing was the first known functional genomics tool suitable for the wheat powdery mildew pathosystem, where other transient assays such as HIGS/ VIGS with biolistics have failed (Nowara 2010 or Pliego 2013, P. Schwiezer personal communication) and possibly for *in vitro* gene silencing in another wheat pathogen *Zymoseptoria tritrici.* Attempts were also made for *in planta* gene silencing of *Fusarium graminearum* using leaf assays, but although encouraging trends, no significant effect has been produced yet. Therefore, improving the leaf assay and the ASO delivery as well as selecting more potent virulence factors or vital genes is required.

Finally, in the barley powdery mildew pathosystem, in the work presented here alone, new fungal virulence factors, vital genes and their transcription factors *(BEC1019, CYP51, actin, GADPDH, GT2, PacC, ZAP1, Zrt1)* and plant susceptibility genes (*MLO, PR5 isoform TLP5)* were discovered or validated using the ASO technology. Previously other members of Bindschedler lab have used the technology to validated new CSEPs as *Blumeria* effectors (BEC1011 and the whole CSEP family 21 constituted of *CSEP0064 (BEC1054) CSEP0065, CSEP0066, CSEP0264 (BEC1011)* and the susceptibility genes, *Bluefensin 1, MLO* and *PR5* isoform *TLP5.* As such this contributes to understanding powdery mildew disease development and the interaction between *Blumeria* and its barley host. Potentially this could be transformed into a high throughput assay with simple fast and cheap image analysis, by checking the production of ROS or other hallmark of plant immunity. Alternatively, image analysis

automation systems might speed up the process of evaluating the disease scoring, or if the silencing effect can be shown to be long term, then one can also score the disease macroscopically 6-7 days post inoculation when the powdery mildew pustules are visible.

Supplementary figures and tables

S1: Culture media and solutions used

Fusarium graminearum media

Synthetic Nutrient Deficient Agar (SNA Agar) (Poor medium)

0.1% KH_2PO_4, 0.1% KNO_3, 0.1% $MgSO_4$ x 7 H_2O, 0.05% KCl, 0.02% glucose, 0.02% saccharose, 2% bacto agar, pH 5.0

Potato Dextrose Broth (Rich medium)

Obtained as a pre-made medium from Sigma Aldrich (Product code: P6685-250G)

Dissolved 24 g/l in distilled water and autoclaved at 121°C for 15 minutes.

Sucrose medium overlay and Sucrose medium bottom agar: Make up 1.6 M Sucrose medium separate from 2X Regeneration medium. Autoclave and mix with an equal volume 2X Regeneration medium.

Driselase: from Basidiomycetes, Protein content – 15%, crude powder containing laminarinase, xylanase and cellulase.

Preparation of enzyme mix: 100 mg Sigma Lysing Enzyme, 500 mg Driselase, 1 mg Chitinase (Sigma, high concentration);
dissolve in 20 ml 1 M Sorbitol
Mix stirred for 30 min at RT to allow Driselase to dissolve;
Centrifuge to get rid of unsolved particles

YEPD-2G : 3 g/L Yeast extract, 10 g/L Bacto-peptone, 20 g/L D-Glucose (Dextrose), pH 5.0.

Zymoseptoria tritici media

YG Broth: 30 g/l Glucose and 10 g/l Yeast Extract

YPD Agar (Rich medium): 10 g/l Yeast Extract, 20 g/l Peptone, 20 g/l Glucose, 20 g/l Agar (2%).

Czapek Dox Broth (Minimal medium): Obtained as a pre-made medium from Sigma Aldrich (Product code: C1551-250G)

Suspended 35.0 g/L in water, boiled to dissolve the medium completely. Sterilized by autoclaving at 121°C for 15 minutes.

Composition: 30 g/L Sucrose, 2 g/L Sodium nitrate, 1 g/L Dipotassium phosphate, 0.5% Magnesium sulphate, 0.5 g/L Potassium chloride, 0.01 g/L Ferrous sulphate, pH 7.5.

V8- Agar : 200mL V8 juice, $CaCO_3$ 3 g/L, Agar 2%

Protocol:

- Mix V8 juice, Agar and ca. 300mL distilled water and warm in the microwave until the agar dissolves.
- Add $CaCO_3$ in 200mL distilled water and gently warm up on an warming electric plate until $CaCO_3$ solubilizes.
- Pour solubilized $CaCO_3$ into the 300ml Medium
- Adjust pH to 6.3 and make up the final volume to 1000 ml using distilled water.
- Autoclave 30 Minutes at 121°C.

Liquid AE Medium: Yeast extract 10 g/L, $MgSO_4 * 7\ H_2O$ 0.5 g/L, $NaNO_3$ 6 g/L, KCl 0.5 g/L, KH_2PO_4 1.5 g/L, Glycerol 20 ml/L

Sterilised by autoclaving at 121°C for 15 minutes.

Agrobacterium Transformation of *Zymoseptoria*

LB + Kanamycin plates (Kan 50µg/ml)

***Agrobacterium tumefaciens* Induction Medium (IM)** and **Induction Medium plates**

Prepare stock solutions of:

Stock A (1000ml): $MgSO_4$ 10g, KH_2PO_4 29g, NaCl 3g.

Stock B (1000ml): K_2HPO_4 40.5g, $(NH_4)_2SO_4$ 10g

Stock C (1000ml): $CaCl_2$2g

These stock solutions do not need to be autoclaved

9mM $FeSO_4$ (Ferrous sulphate mw 278.02): 2.5mg/ml

1M MES (pH5.3) (mw 195) (MES hydrate Sigma M8250)

Filter sterilise – DO NOT autoclave

Heat to re-dissolve if precipitates out of solution during storage

39g in 200ml

Adjust to pH 5.3 with (~3ml) 5M KOH

(5M KOH is 11.2g in 40ml water)

Store Δ 4°C

Complete Stock (1000ml) **Stores for 1 week only**

(10mM K_2HPO_4,10mM KH_2PO_4, 2.5mM NaCl, 2mM $MgSO_4$, 0.7mM $CaCl_2$, 4mM $(NH_4)SO_4$, 9µM $FeSO_4$)

Stock A: 50ml

Stock B: 50ml

Stock C: 50ml

9mM $FeSO_4$: 1ml

DO NOT autoclave as salts precipitate out of solution

50% glycerol

20% glucose

Induction Medium (IM) (100ml per strain to be transformed)

(10mM Glucose, 40mM MES, 0.5% glycerol)

20% Glucose0.9ml

1M MES4ml

50% glycerol1ml

Make up to 100ml with Complete Stock and autoclave

Prepare the day before required

IM plates (500ml)

20% glucose: 4.5ml

1M MES: 20ml

50% glycerol: 5ml

1.3% agar: 6.5g

Make up to 500ml with Complete Stock and autoclave.

Add 500µl acetosyringone to the media prior to pouring.

Require 5 plates per strain transformed.

Make up fresh on the day required. Discard after use.

LB Mannitol (low salt) (40ml in 250ml flask)

Bacto tryptone10g

Yeast extract 5g

NaCl2.5g

Mannitol10g

Make up to 1l with dH_2O and autoclave.

***Aspergillus nidulans* Minimal Medium**

Prepare the salts and the trace elements in advance.

20x Salts

$NaNO_3$120g

KCl10.4g

$MgSO_4.7H_2O$10.4g

$KH_2PO_4$30.4g

Add to 1l water, autoclave, store at 4ºC (stored on bench)

Trace Elements

$ZnSO_4.7H2O$2.2g

$H_3BO_3$1.1g

$MnCl_2.4H2O$0.5g

$FeSO_4.7H2O$0.5g

$CoCl_2.5H2O$ 0.16g

$CuSO_4.5H2O$0.16g

$(NH_4)_6Mo_7O_{24}.4H2O$0.11g

Na_4EDTA5g

Add in order to 80ml water. Allow one to dissolve before adding the next. Heat to boiling, cool to 60º, adjust pH to 6.5 – 6.8 with 5M KOH. Cool and adjust volume to 100ml. Autoclave, then store in the dark at 4º. (Stored on the bench - $FeSO_4$ may ppt out of solution but still works).

Aspergillus MM agar plates (1000ml)

20 x Salts50ml

Trace elements1ml

Glucose10g

Add to 800ml water and adjust pH to 6.5 with 5M KOH (~1ml), then adjust volume to 1l.

For solid medium add 10g Agar No. 1 and autoclave.

Prepare fresh on the day of use.

Kanamycin (50mg/ml)

Use at a final concentration of 50µg/ml (1:1000 dilution)

Filter sterilise and store in aliquots Δ -20°C

Hygromycin (50mg/ml)

Use at a 1:500 dilution of the stock solution.

i.e. Final conc of 100µg/ml (1ml in 500 ml medium)

Timentin (50mg/ml)

Use at a 1:500 dilution of the stock solution.

Filter sterilise and store in aliquots Δ -20°C

Acetosyringone (screw-top tube)

200mM in 100% DMSO, use at a 1:1000 dilution

200mM is 40 mg/ml

No need to filter sterilise. Store in aliquots Δ -20°C. Acetosyringone is light sensitive.

Equipment to autoclave:

250ml flasks (two per agrobacterium culture)

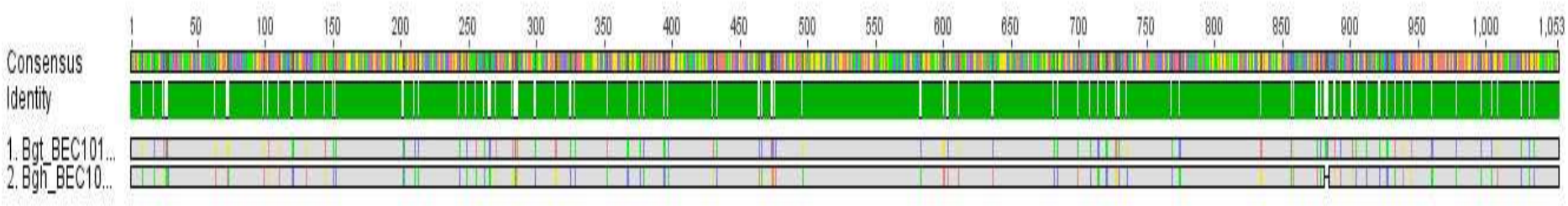
Cocktail sticks

dH_2O

Cellulose disks – intercalate cellulose disks with filter paper disks and autoclave in a foil-wrapped glass petri dish.

S2: Nucleotide sequence alignment of BEC1019 homologues

CDS Alignment: *Bgh* BEC1019 and its *Bgt* homolog

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Identities = 962/1053 (91%),
Positives = 962/1053 (91%), Gaps = 3/1053 (0%)

Bgt_BEC1019 homolog_CDS      1 ATGCAGTCGGTATTGCTCTTGACCATCGTTACACAATCGTTTATAGCTACAGCTTCTCCT   60

                                 ATGCAGTC GTATTGCT TTGACC T  TTACACAATCGTTTATAGCTACAGCTTCTCCT

Bgh_BEC1019 CDS             1 ATGCAGTCTGTATTGCTTTTGACCGTTCTTACACAATCGTTTATAGCTACAGCTTCTCCT   60


Bgt_BEC1019 homolog_CDS     61 CTGGTAGAAAGGGCTACACCACTCTCTTTTGCAGAGAAGAGACCACAAAGAGTCTCCTAT  120

                                 CT GTAGAAAG  CTACACCACTCTCTTTTGCAGAGAA AG CCACAAA AGTCTCCTA

Bgh_BEC1019 CDS             61 CTAGTAGAAAGATCTACACCACTCTCTTTTGCAGAGAAAAGGCCACAAAAAGTCTCCTAC  120
```

*Bgt*_BEC1019 homolog_CDS 121 GACTGGACAGCACCTTATGTCAAAGATTTTATAATTCACGGGTCATGTAATGCTACACAG 180

GACTGGACA CACCTTATGTCAA GATTT A AATTCACGGGTCATGTAATGCTACACAG

*Bgh*_BEC1019 CDS 121 GACTGGACAACACCTTATGTCAAGGATTTCACAATTCACGGGTCATGTAATGCTACACAG 180

*Bgt*_BEC1019 homolog_CDS 181 ACAAATGTACTCCGCCGTGGTCTAGAAGACGCCGTAACCCTTGCTCAGCATGCCAAAGAG 240

ACAAATGTACTCCGCCGTGG TAGAAGA GC GTAACCCTTGCTCAGCATGCCAAAGAG

*Bgh*_BEC1019 CDS 181 ACAAATGTACTCCGCCGTGGCTTAGAAGATGCTGTAACCCTTGCTCAGCATGCCAAAGAG 240

*Bgt*_BEC1019 homolog_CDS 241 CATAGTACGTATTTAATCTCTTTTCCTCCAAGCAATACTGAAACGATCCGGCGAAGTTTT 300

CA AGTA GTATTT ATCTC TTT TCC AGCAATACTGA A TCCGGCGAAGTT T

*Bgh*_BEC1019 CDS 241 CACAGTATGTATTTTATCTCCTTTTTTCCGAGCAATACTGAGATAGTCCGGCGAAGTTCT 300

*Bgt*_BEC1019 homolog_CDS 301 GGTTCACGGAAAGAAATCACCAATTTATCAAAAGTATTATGGGGCGCTGCCAACAGGGGC 360

GGTTCACGGAAAG AATCACCAAT TA CAAAAGTATTATGGGGCGCTGCC ACAGGGGC

*Bgh*_BEC1019 CDS 301 GGTTCACGGAAAGGAATCACCAATCTACCAAAAGTATTATGGGGCGCTGCCTACAGGGGC 360

*Bgt*_BEC1019 homolog_CDS 361 AGTGATTGGATGGTTTGACACGATAGCGACAGCTAACCGGGCCGGTGTTACCTTCCGTTG 420

AGTGAT GGATGGTT GA ACGATAGCGACAGC AA CGGGCCGGTGTTACCTTCCGTTG

*Bgh*_BEC1019 CDS 361 AGTGATCGGATGGTTCGATACGATAGCGACAGCCAATCGGGCCGGTGTTACCTTCCGTTG 420

*Bgt*_BEC1019 homolog_CDS 421 TGATGACCCAGATAAAAAATGTGCAACCGAGAACCGTACGTATCACCCGGGTACACACCA 480

TGATGACCC GA AAAAAATGTGCAACCGAGAACCGTACGTAT A CCGGGT A ACCA

*Bgh*_BEC1019 CDS 421 TGATGACCCGGACAAAAAATGTGCAACCGAGAACCGTACGTATAATCCGGGTGAAAACCA 480

*Bgt*_BEC1019 homolog_CDS 481 CGGCTAGTTGACAATGGAATAGGATGGGCTGGTCACTGGCGCGGGAAAGATGCTCCTTCA 540

CGGCTAGTTGACAAT GAATAGGATGGGCTGGTCACTGGCGCGGGAAAGATGCTCCTTCA

*Bgh*_BEC1019 CDS 481 CGGCTAGTTGACAATTGAATAGGATGGGCTGGTCACTGGCGCGGGAAAGATGCTCCTTCA 540

*Bgt*_BEC1019 homolog_CDS 541 GAGACGGTCATCTGTGATGTTTCCTTCTTTGAACGGCTTCCTCTGGAGGATTTGTGCTCG 600

GAGACGGTCATCTGTGATGTTTCCTTCTTTGAACGGCTTCCT TGGAGGATTTGTGCTC

*Bgh*_BEC1019 CDS 541 GAGACGGTCATCTGTGATGTTTCCTTCTTTGAACGGCTTCCTTTGGAGGATTTGTGCTCA 600

*Bgt*_BEC1019 homolog_CDS 601 CGCGGCTACAGAATTGCAACCGGTAAAGTATACTCCTACTGGGGGGCTGATCTAATCCAT 660

CG GGCTACA AATTGCAACCGGTAAAGTATACTC TACTGGGGGGCTGATCTAATCCAT

*Bgh*_BEC1019 CDS 601 CGAGGCTACAAAATTGCAACCGGTAAAGTATACTCATACTGGGGGGCTGATCTAATCCAT 660

*Bgt*_BEC1019 homolog_CDS 661 CGCATGTTTCATGTGGACATTGTTGGTCAGAACGCCATTACTCATGCTTCACACGGTTAT 720

CGCATGTTTCATGTGGACAT GT GGTCAGAACGCCAT ACTCATGC TCACA GGTTA

*Bgh*_BEC1019 CDS 661 CGCATGTTTCATGTGGACATCGTCGGTCAGAACGCCATAACTCATGCCTCACATGGTTAC 720

*Bgt*_BEC1019 homolog_CDS 721 CAAGATACTCTCAAGCTTGCCGCGGGCCAAAATTACACCCAGACTGCTACAAACACCGAC 780

CAAGAT C CTCAA CTTGCCGCGGGCCAAAATTACACCCAGACTGC ACAAA ACCGAC

*Bgh*_BEC1019 CDS 721 CAAGATGCCCTCAACCTTGCCGCGGGCCAAAATTACACCCAGACTGCCACAAATACCGAC 780

*Bgt*_BEC1019 homolog_CDS 781 TCTCTCATTTACTTTGCGGTTGAGGCGTATGCGTTTGACATCAGCGTGCCCGGAGAGGGC 840

TCTCTCATTTACTTTGCGGTTGAGGCGTATGCGTTTGACATCAGCGTGCCCGG GAGGGC

*Bgh*_BEC1019 CDS 781 TCTCTCATTTACTTTGCGGTTGAGGCGTATGCGTTTGACATCAGCGTGCCCGGGGAGGGC 840

*Bgt*_BEC1019 homolog_CDS 841 TGTGCTGGCCAAGCCCCGTCTATTCCCGATACGATAGCCATTCCTCCAACTGCTCCAAAC 900

TGTGCTGGCCAAGCC C TCTATTCCCGATACGA AG CA CTCC ACTG TCCAAAC

*Bgh*_BEC1019 CDS 841 TGTGCTGGCCAAGCCTCATCTATTCCCGATACGAAAGTCA---
CTCCGACTGATCCAAAC 897

*Bgt*_BEC1019 homolog_CDS 901 ACGTTGCCAGATTCAATCGTTATTCCTTCCCCAAAAGAGCCCGAAAAGAAAGACGAGCCC 960

CG TGCCAGA TCAATCGT ATTCC TCCCC AAAGA CCCGA AAGAAAGACGAGCC

*Bgh*_BEC1019 CDS 898 GCGCTGCCAGACTCAATCGTCATTCCCTCCCCTAAAGAACCCGAGAAGAAAGACGAGCCT 957

*Bgt*_BEC1019 homolog_CDS 961 GAGAAAAAAGCCGAGCCCGAAAAGAAGGCCGAGCCCGAAGCACCAGGGAAGAATTGCCAT 1020

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                                    GAGAAAAAAGCCGAGCC GAAAAGAAGGCCGAGCC GAAGCAC AGG AAGAATTGCCAT

Bgh_BEC1019 CDS             958 GAGAAAAAAGCCGAGCCTGAAAAGAAGGCCGAGCCTGAAGCACTAGGAAAGAATTGCCAT 1017


Bgt_BEC1019 homolog_CDS   1021 ACCCATGACGACGGTGAGGTTCATTGTGTCTAG 1053
                                 ACCCA GACGA GG GAGGTTCATTGTGTCTAG
Bgh_BEC1019 CDS           1018 ACCCACGACGATGGCGAGGTTCATTGTGTCTAG 1050
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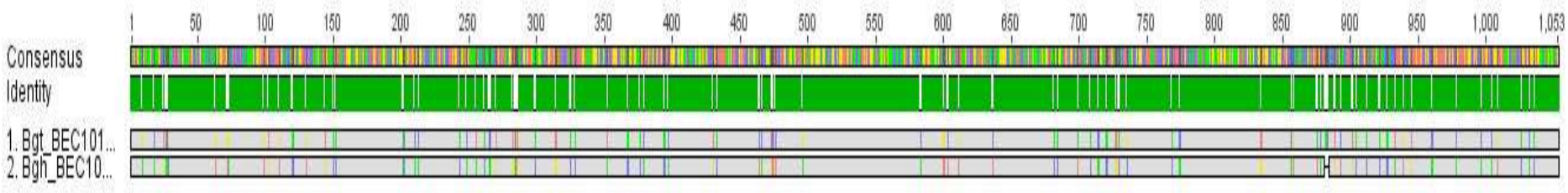
CDS Alignment: *Bgh* BEC1019 and its *Fg* homolog

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Score = 426.0, Identities = 544/1080 (50%),
Positives = 544/1080 (50%), Gaps = 219/1080 (20%)

Fg_BEC1019 homolog_CDS      1 ATGATGT-TCAAGTCC--ACCACCGCGGCTATGCTCCTCTTCGGAGCCGCCACTGCCACT   57
                              ATG  GT T  A T C     ACCG    TA  C     TT   AGC    AC GC   T
Bgh_BEC1019 CDS             1 ATGCAGTCTGTATTGCTTTTGACCGTTCTTACACAATCGTTTATAGC---TACAGC--TT   55

Fg_BEC1019 homolog_CDS     58 CCCCTCTTTGGCCGTGCCGAGGCCAGTCAGACCAAGTCTGCTTCTCAGTCTTCCA-AGAC  116
                                C CCTCT      GT         AG  AGA C A  C  CT CTC  T TT CA AGA
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Bgh_BEC1019 CDS            56 CTCCTCT-----AGT---------AGAAAGATCTACACCACT-CTC--TTTTGCAGAGAA   98

Fg_BEC1019 homolog_CDS     117 AAG---TGAGAGCAGCTCCTACAACTGGTCAGAGGGTTGGACCAAGGATTACCCCATCCA  173
                               AAG     A A    CTCCTAC ACTGG CA     TT    CAAGGATT C C AT CA
Bgh_BEC1019 CDS             99 AAGGCCACAAAAAGTCTCCTACGACTGGACAACACCTTATGTCAAGGATTTCACAATTCA  158

Fg_BEC1019 homolog_CDS     174 CCAGTCTTGCAACGCGACACTCCGTCATCAGTTGTCCAGCG---CTCTGGATGAGACTGT  230
                               C  GTC TG AA GC ACA  C G CA   GT  TCC  CG   CT T GA GA  CTGT
Bgh_BEC1019 CDS            159 CGGGTCATGTAATGCTACA--CAGACAAATGTACTCCGCCGTGGCT-TAGAAGATGCTGT  215

Fg_BEC1019 homolog_CDS     231 CCAGCTTGCTCAGCATGCCAAAGACCACATTCTG--------CGACAT-----GGACACA  277
                                   CTTGCTCAGCATGCCAAAGA CACA T TG        C  C T      GA  CA
Bgh_BEC1019 CDS            216 AACCCTTGCTCAGCATGCCAAAGAGCACAGTATGTATTTTATCTCCTTTTTTCCGAG-CA  274

Fg_BEC1019 homolog_CDS     278 AGTCGGAGTTTTTC--ACCAAGTACT--TT---GGAAATG-----CC--TCCAC------  317
                               A  C GAG T  TC   C AAGT CT  TT   GGAAA G     CC  TC AC
Bgh_BEC1019 CDS            275 ATACTGAGATAGTCCGGCGAAGTTCTGGTTCACGGAAAGGAATCACCAATCTACCAAAAG  334

Fg_BEC1019 homolog_CDS     318 --TT------CTCAGCCTA------------TTGGATGGTACGACCGTGTTGTCAACGCT  357
                                 TT      C C GCCTA            T GGATGGT CGA     T G  A  GC
Bgh_BEC1019 CDS            335 TATTATGGGGCGCTGCCTACAGGGGCAGTGATCGGATGGTTCGATACGATAGCGACAGCC  394

Fg_BEC1019 homolog_CDS     358 GACAAGACTGGTGTTCTCTTCCGTTGCGATGACCCTGACAAGAACTGTGCTA--------  409
                                A   G C GGTGTT  CTTCCGTTG GATGACCC GACAA AA TGTGC A
Bgh_BEC1019 CDS            395 AATCGGGCCGGTGTTACCTTCCGTTGTGATGACCCGGACAAAAAATGTGCAACCGAGAAC  454

Fg_BEC1019 homolog_CDS     410 ------------------------CAC----AG--GAC---------GCATGGGCTGGAC  430
                                                       CAC    AG  GAC         G ATGGGCTGG C
Bgh_BEC1019 CDS            455 CGTACGTATAATCCGGGTGAAAACCACGGCTAGTTGACAATTGAATAGGATGGGCTGGTC  514

Fg_BEC1019 homolog_CDS     431 ACTGGCGAGGCGACAATGCCACCTCCGAGACAGTCATCTGCCCCCTCTCTTTCGA-GATC  489
                               ACTGGCG GG  A  ATGC  C TC GAGAC GTCATCTG     T TC TTC   GA C
Bgh_BEC1019 CDS            515 ACTGGCGCGGGAAAGATGCTCCTTCAGAGACGGTCATCTGTGATGTTTCCTTCTTTGAAC  574
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Fg_BEC1019 homolog_CDS    490 CGCCGCAACCTCGACTCTGTCTGCAATCTGGGCTACACTGTCGCCAATTCCAAGTTGAAC  549
                              GC  C    T GA   T T TGC   C  GGCTACA   T GC A     AA  T  AC
Bgh_BEC1019 CDS           575 GGCTTC-CTTTGGAGGATTTGTGCTCACGAGGCTACAAAATTGCAACCGGTAAAGTATAC  633

Fg_BEC1019 homolog_CDS    550 ACTTTCTGGGCCACCGACCTTCTCCACCGGGTCTTGCACGTCCCCATCATCAGCGAGAAG  609
                               C T CTGGG   C GA CT  TCCA CG  T TT CA GT   CATC TC G  AGAA
Bgh_BEC1019 CDS           634 TCATACTGGGGGGCTGATCTAATCCATCGCATGTTTCATGTGGACATCGTCGGTCAGAAC  693

Fg_BEC1019 homolog_CDS    610 ACTGTCGATCACTTCGCTGAGAACTACACTGATGCTATTGCTCTTGCC-AAGTCC--GAC  666
                               C  T   TCA   C C  A    TAC   GATGC  T    CTTGCC   G CC   A
Bgh_BEC1019 CDS           694 GCCATAACTCATGCCTCACATGGTTACCAAGATGCCCTCAACCTTGCCGCGGGCCAAAAT  753

Fg_BEC1019 homolog_CDS    667 CCCTCCAAGAGCGTCATCGACAGCGACGCACTGCAGTACTTTGCCATTGATGTTTGGGCT  726
                                 C CC AGA  G CA   A A CGAC C CT    TACTTTGC  TTGA G  T  GC
Bgh_BEC1019 CDS           754 TACACCCAGACTGCCACAAATACCGACTCTCTCATTTACTTTGCGGTTGAGGCGTATGCG  813

Fg_BEC1019 homolog_CDS    727 TATGACATCGCTGCTCCTGGTGAGGGATGCACTGGTGAGGTTGAGGATGAGACCGAAGAG  786
                              T TGACATC   G  CC GG GAGGG TG  CTGG  A G       T A  CC  A A
Bgh_BEC1019 CDS           814 TTTGACATCAGCGTGCCCGGGGAGGGCTGTGCTGGCCAAGCCTCATCT-ATTCCCGATAC  872

Fg_BEC1019 homolog_CDS    787 GAGAAG-----CCTACTGCTACCAA------GTCTGACTC--------TTCC--------  819
                              GA AAG     CC ACTG T C AA      G C GACTC        TTCC
Bgh_BEC1019 CDS           873 GA-AAGTCACTCCGACTGATCCAAACGCGCTGCCAGACTCAATCGTCATTCCCTCCCCTA  931

Fg_BEC1019 homolog_CDS    820 ----------------------------------AAGCCTAGCGCTACCAAGGAGGCC---  843
                                                                AAGCC AGC CT   AAG AGGCC
Bgh_BEC1019 CDS           932 AAGAACCCGAGAAGAAAGACGAGCCTGAGAAAAAAGCCGAGC-CTGAAAAGAAGGCCGAG  990

Fg_BEC1019 homolog_CDS    844 CC------------CAAGGAGTGCCACACTCACGACGACGGTGTTGTTCACTGCTCTTAA  891
                              CC             AAG A TGCCA AC CACGACGA GG G  GTTCA TG    TA
Bgh_BEC1019 CDS           991 CCTGAAGCACTAGGAAAGAATTGCCATACCCACGACGATGGCGAGGTTCATTGTGTCTAG 1050
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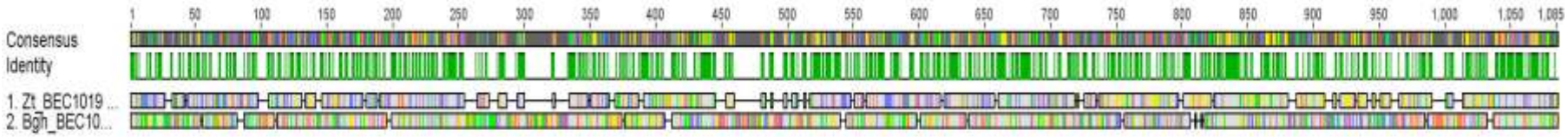
CDS Alignment: *Bgh* BEC1019 and its *Zt* homolog

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Score = 167.0, Identities = 532/1085 (49%),
Positives = 532/1085 (49%), Gaps = 214/1085 (19%)
Zt_BEC1019 homolog_CDS      1 ATGCTGACCTCCCTCCTCCTAACCGC-----CATCGGCGCCT-CAGCCGCCGCCCTCCCA   54
                              ATGC G C     T CT  T ACCG      CA    CG  T  AGC  C GC  TC C
Bgh_BEC1019 CDS             1 ATGCAGTCTGTATTGCTTTTGACCGTTCTTACACAATCGTTTATAGCTACAGC-TTCTCC   59

Zt_BEC1019 homolog_CDS     55 TCCGTCCCATCCCTCGAAACCCGCCAAGCCCCCTTCG-------AAGTCCTCCAAGCCGC  107
                              TC      A    TC A ACC     A  C C TT G       AAG CC  CAA   G
Bgh_BEC1019 CDS            60 TCTAGTAGAAAGATCTACACC-----ACTCTCTTTTGCAGAGAAAAGGCC-ACAAAAAGT  113

Zt_BEC1019 homolog_CDS    108 CCCCTGGGAC--GCCGGCGCC----GTGACCGAATGGCAAATCCACCCCTCCTGCAA--C  159
                              C CCT  GAC  G C  C CC    GT A  GA T    AAT CAC   TC TG AA  C
Bgh_BEC1019 CDS           114 CTCCTACGACTGGACAACACCTTATGTCAAGGATTTCACAATTCACGGGTCATGTAATGC  173

Zt_BEC1019 homolog_CDS    160 TCCTCCGA-ACGTCTCCAACTCCAACAGGGCCTCGACGAAGCCGTCACTCTCGCCACCCA  218
                              T C C GA A  T T   ACTCC  C  GGC T GA GA GC GT AC CT GC    CA
Bgh_BEC1019 CDS           174 TACACAGACAAATGT---ACTCCGCCGTGGCTTAGAAGATGCTGTAACCCTTGCTCAGCA  230

Zt_BEC1019 homolog_CDS    219 CGCCAAAGCCCACA---------TCAACCGCTG------GGGCAAC-------TCGTCCG  256
                               GCCAAAG  CACA         T A C  CT       G GCAA        T GTCCG
Bgh_BEC1019 CDS           231 TGCCAAAGAGCACAGTATGTATTTTATCTCCTTTTTTCCGAGCAATACTGAGATAGTCCG  290
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Zt_BEC1019 homolog_CDS    257 --------------------AAG----------TCTACCGCAAATACT-TCGGGAACTCC  285
                                                  AAG          TCTACC  AA TA T T GGG  CT C
Bgh_BEC1019 CDS           291 GCGAAGTTCTGGTTCACGGAAAGGAATCACCAATCTACCAAAAGTATTATGGGGCGCTGC  350

Zt_BEC1019 homolog_CDS    286 CCTC----CCTTTGAAGCCGCGGGCGC-ATACGACATCATCATCAATGGAGATCGTGCGG  340
                                C  C     C  TG A C G  GG  C ATACGA A C  CA C A     ATCG GC G
Bgh_BEC1019 CDS           351 CTACAGGGGCAGTG-ATCGGATGGTTCGATACGATAGCGACAGCCA-----ATCGGGCCG  404

Zt_BEC1019 homolog_CDS    341 GAGCGTTGTTTAGATGTGATGATCC-------AGATGGG--------------------A  373
                                G G     TT  G TGTGATGA CC       A ATG G                    A
Bgh_BEC1019 CDS           405 GTGTTACCTTCCGTTGTGATGACCCGGACAAAAAATGTGCAACCGAGAACCGTACGTATA  464

Zt_BEC1019 homolog_CDS    374 ATT---GTG-------CGGC--GTTTCC---TGA--TGAATGGGCCGGCCACTACCGCGG  416
                                AT    GTG       CGGC  GTT  C   TGA   G ATGGGC GG CACT  CGCGG
Bgh_BEC1019 CDS           465 ATCCGGGTGAAAACCACGGCTAGTTGACAATTGAATAGGATGGGCTGGTCACTGGCGCGG  524

Zt_BEC1019 homolog_CDS    417 CCCCAACG--GCACCCAC-GAAACAGTAATCTGCCCCCTCTCCTACACCTCCCGGCAACA  473
                                  AA G  GC CC  C GA AC GT ATCTG     T TCCT C      CGGC
Bgh_BEC1019 CDS           525 ---GAAAGATGCTCCTTCAGAGACGGTCATCTGTGATGTTTCCTTCTTTGAACGGCTT--  579

Zt_BEC1019 homolog_CDS    474 CCTCTCGCAAATCTGT--TCCCAAGGCTACGACGTGCGCACCGGCTCCCGAACCCTC-TA  530
                                CCT T G   AT TGT  TC C AGGCTAC A  T  GCA C G T   G A  CTC TA
Bgh_BEC1019 CDS           580 CCTTTGGAGGATTTGTGCTCACGAGGCTACAAAATT-GCAACCGGTAAAGTATACTCATA  638

Zt_BEC1019 homolog_CDS    531 CTGGGGTTCCGACCTCATCCATCGTCTCTACCACATGCCGGCCTACGGCGCCAACGGC-T  589
                                CTGGGG  C GA CT ATCCATCG  T T  CA  TG     C  CGG    AACG C T
Bgh_BEC1019 CDS           639 CTGGGGGGCTGATCTAATCCATCGCATGTTTCATGTGGACATCGTCGGTCAGAACGCCAT  698

Zt_BEC1019 homolog_CDS    590 A---CATCGACCAC-TACGCAGAAGGATGGGCTGGCATCGTCACCGCCGCGAAAGAGGGG  645
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                                A   CAT    CAC T    A  A GATG  CT  CA C T  CCGC G   AA A
Bgh_BEC1019 CDS           699 AACTCATGCCTCACATGGTTACCAAGATGCCCT--CAACCTTGCCGCGGGCCAAAATTAC  756

Zt_BEC1019 homolog_CDS    646 AGTCCGAATTCGACCCA---CGACTCGGATGGACTGCAGTTT-TTCGCGCTGGAGGCGTA  701
                                A  C GA T C AC  A   CGACTC   T   CT CA TT  TT GCG T GAGGCGTA
Bgh_BEC1019 CDS           757 ACCCAGACTGCCACAAATACCGACTC---T---CT-CATTTACTTTGCGGTTGAGGCGTA  809

Zt_BEC1019 homolog_CDS    702 TGCTTATGATGTGATTTATCCGGGGGTTGGGTGTCCGGGT------TCGAATGCGCCGGT  755
                                TGC T TGA  T A     CC GGGG  GG TGT C GG       TC   T   CC G
Bgh_BEC1019 CDS           810 TGCGTTTGACATCAGCGTGCCCGGGGAGGGCTGTGCTGGCCAAGCCTCATCTATTCCCGA  869

Zt_BEC1019 homolog_CDS    756 TACGGAGG-----AGGA--GATGGAGGGGAG-TGGGGGAC---ATGG--AGCGGCTGC--  800
                                TACG A G       GA  GAT  A   G G TG   GAC   AT G  A    CT C
Bgh_BEC1019 CDS           870 TACGAAAGTCACTCCGACTGATCCAAACGCGCTGCCAGACTCAATCGTCATTCCCTCCCC  929

Zt_BEC1019 homolog_CDS    801 ----GGCGGCGACGACGACGTCGATGGCTG---------CTGCGCC------GGCGGCGG  841
                                    G    CGA  A  A G CGA G CTG         C G GCC      G  GGC G
Bgh_BEC1019 CDS           930 TAAAGAACCCGAGAAGAAAGACGA-GCCTGAGAAAAAAGCCGAGCCTGAAAAGAAGGCCG  988

Zt_BEC1019 homolog_CDS    842 TGACGGATGCGCCGGCTGGACAGAACTGCCACACCCACGCCAACGGCGAGCTGCACTGCA  901
                                 G C GA GC C     GGA AGAA TGCCA ACCCACG C A GGCGAG T CA TG
Bgh_BEC1019 CDS           989 AGCCTGAAGCAC---TAGGAAAGAATTGCCATACCCACGACGATGGCGAGGTTCATTGTG 1045
Zt_BEC1019 homolog_CDS    902 CTTGA  906
                                T
Bgh_BEC1019 CDS          1046 TCTAG 1050
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S3: List of candidate secreted effector proteins (CSEPs) of *B. graminis* obtained from Pliego et. al. 2013

CSEP ID	GENE ID	SYNONYMS	FAMILY	PROTEOME AND GENOME DATA	HAUSTORIA/EPYPHYTIC EXPRESSION RATIO
CSEP00133	bgh03692 polypeptide		24	-	940
CSEP0371	bghG002857000001001		34	-	717
CSEP0182	bgh04105 polypeptide		NF	Haustoria only	566
CSEP0443	bghG005948000001001		23	Haustoria only	483
CSEP0119	bgh03594 polypeptide	**BEC1062**	NF	Seen in Hyphae or (Haustoria + Hyphae)	467
CSEP0005	bgh00020 polypeptide	**BEC1002**	25	Seen in Hyphae or (Haustoria + Hyphae)	446
CSEP0001	bgh00012 polypeptide	**BEC1007**	24	Seen in Hyphae or (Haustoria + Hyphae)	438
CSEP0091	bgh03275 polypeptide		12	Seen in Hyphae or (Haustoria + Hyphae)	434
CSEP0101	bgh03441 polypeptide		21	Seen in Hyphae or (Haustoria + Hyphae)	429
CSEP0075	bgh02934 polypeptide		44	Haustoria only	415
CSEP0210	bgh04352 polypeptide	**BEC1035**	NF	-	412
CSEP0073	bgh02925 polypeptide	**BEC1059**	44	Haustoria only	381

CSEP0142	bgh03731 polypeptide		NF	Haustoria only	372
CSEP0485	bghG008560000001001		66	-	370
CSEP0081	bgh03006 polypeptide		12	-	364
CSEP0262	bgh06494 polypeptide		66	-	360
CSEP0380	bghG003355000001001		35	Haustoria only	336
CSEP0285	bghG000389000001001		45	Haustoria only	317
CSEP0273	bghG000012000002001		NF	Haustoria only	317
CSEP0106	bgh03462 polypeptide		32	Haustoria only	308
CSEP0055	bgh02653 polypeptide		43	Seen in Hyphae or (Haustoria + Hyphae)	286
CSEP0102	bgh03443 polypeptide		20	Seen in Hyphae or (Haustoria + Hyphae)	278
CSEP0027	bgh01362 polypeptide	**BEC1032**	41	Haustoria only	277
CSEP0372	bghG002861000001001		NF	Haustoria only	267
CSEP0099	bgh03377 polypeptide		5	Seen in Hyphae or (Haustoria + Hyphae)	247
CSEP0145	bgh03736 polypeptide		5	Haustoria only	243
CSEP0097	bgh03375 polypeptide	**BEC1061**	45	Haustoria only	230
CSEP0105	bgh03459 polypeptide		31	Haustoria only	230
CSEP0122	bgh03625 polypeptide		43	Haustoria only	229

CSEP0008	bgh00029 polypeptide		NF	Seen in Hyphae or (Haustoria + Hyphae)	226
CSEP0066	bgh02877 polypeptide		21	-	220
CSEP0095	bgh03312 polypeptide	**BEC1041**	NF	Haustoria only	206
CSEP0135	bgh03694 polypeptide	**BEC1018**	63	Haustoria only	204
CSEP0086	bgh03058 polypeptide		25	Haustoria only	203
CSEP0096	bgh03316 polypeptide	**BEC1017**	47	Haustoria only	196
CSEP0123	bgh03629 polypeptide		61	Haustoria only	193
CSEP0104	bgh03457 polypeptide	**BEC1027**	24	Haustoria only	193
CSEP0257	bgh0580 polypeptide		31	-	184
CSEP0069	bgh02918 polypeptide	**BEC1055**	30		183
CSEP0486	bghG008575000001001		21	Seen in Hyphae or (Haustoria + Hyphae)	177
CSEP0264	bgh0653 polypeptide	**BEC1011**	21	Seen in Hyphae or (Haustoria + Hyphae)	175
CSEP0229	bgh05069 polypeptide		18	Haustoria only	169
CSEP0112	bgh03568 polypeptide		5	-	157
CSEP0082	bgh03015 polypeptide		NF	Seen in Hyphae or (Haustoria + Hyphae)	150
CSEP0017	bgh00458 polypeptide		NF	Seen in Hyphae or (Haustoria + Hyphae)	149

CSEP0196	bgh04255 polypeptide		NF	Haustoria only	141
CSEP0065	bgh02875 polypeptide		21	-	141
CSEP0074	bgh02928 polypeptide		44	Haustoria only	139
CSEP0491	bgh06518 polypeptide	**BEC1016**	5	Haustoria only	136
CSEP0232	bgh05102 polypeptide		NF	Haustoria only	124
CSEP0094	bgh03293 polypeptide		12	Seen in Hyphae or (Haustoria + Hyphae)	120
CSEP0141	bgh03730 polypeptide		64	Haustoria only	116
CSEP0070	bgh02922 polypeptide		30	Haustoria only	116
CSEP0080	bgh02998 polypeptide	**BEC1014**	45	Haustoria only	116
CSEP0059	bgh02778 polypeptide		29	Seen in Hyphae or (Haustoria + Hyphae)	112
CSEP0036	bgh01628 polypeptide		4	Seen in Hyphae or (Haustoria + Hyphae)	111
CSEP0168	bgh04018 polypeptide		13	Haustoria only	108
CSEP0004	bgh00016 polypeptide	**BEC1003**	36	Seen in Hyphae or (Haustoria + Hyphae)	105
CSEP0228	bgh05066 polypeptide	**BEC1033**	1	Haustoria only	102
CSEP0048	bgh02376 polypeptide		NF	-	100
CSEP0152	bgh03782 polypeptide		NF	Haustoria only	99
CSEP0202	bgh04268 polypeptide		4	-	98

CSEP0355	bghG002593000001001		18	Haustoria only	96
CSEP0002	bgh00013 polypeptide		10	Haustoria only	93
CSEP0071	bgh02923 polypeptide	**BEC1057**	30	Haustoria only	92
CSEP0153	bgh03786 polypeptide		4	-	83
CSEP0128	bgh03686 polypeptide	**BEC1063**	16	-	82
CSEP0231	bgh05096 polypeptide	**BEC1024**	18	Haustoria only	75
CSEP0130	bgh03689 polypeptide		16	-	73
CSEP0489	bghG009555000001001		35		72
CSEP0384	bghG003525000001001		54	Haustoria only	71
CSEP0064	bgh02874 polypeptide	**BEC1054**	21	Haustoria only	65
CSEP0032	bgh01406 polypeptide		10	Haustoria only	64
CSEP0134	bgh03693 polypeptide		60	Haustoria only	63
CSEP0266	bgh06578 polypeptide		64	Haustoria only	64
CSEP0137	bgh03703 polypeptide		22	Haustoria only	59
CSEP0146	bgh03739 polypeptide		22	-	57
CSEP0198	bgh04262 polypeptide		4	-	56
CSEP0271	bgh06899 polypeptide		4	-	56
CSEP0138	bgh03709 polypeptide	**BEC1028**	4	Haustoria only	55
CSEP0162	bgh03874 polypeptide		4	Haustoria only	55
CSEP0089	bgh03138 polypeptide		4	Haustoria only	53

CSEP0045	bgh02262 polypeptide		NF	Haustoria only	53
CSEP0346	bghG002234000001001		1	Haustoria only	46
CSEP0042	bgh02080 polypeptide		NF	Haustoria only	45
CSEP0342	bghG002084000001001		NF	Haustoria only	43
CSEP0111	bgh03481 polypeptide		24	Haustoria only	41
CSEP0121	bgh03613 polypeptide		47	Haustoria only	40
CSEP0230	bgh05086 polypeptide		6	Haustoria only	37
CSEP0192	bgh04225 polypeptide		6	Haustoria only	35
CSEP0191	bgh04220 polypeptide	**BEC1038**	10	-	34
CSEP0269	bgh06709 polypeptide		10	Haustoria only	29
CSEP0061	bgh02835 polypeptide		60	Haustoria only	24
CSEP0275	bghG000026000001001		67	Haustoria only	16
CSEP0352	bghG002403000001001		10	Haustoria only	16
CSEP0176	bgh04077 polypeptide	**BEC1066**	6	Haustoria only	15
CSEP0072	bgh02924 polypeptide		30	Haustoria only	14

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