Investigating the interactors of the Blumeria Effector BEC1054

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This thesis is a presentation of the author's own work and all other work is appropriately referenced.

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Abbreviations

Abbreviation	Description
3AT	3-Amino-1,2,4-triazole
3.3.12/3.3.14	Numbered designation of a transgenic wheat line, with the first number being the T_0 line, each successive number representing a plant from the next generation
5FOA	5-Fluoroorotic Acid
18S	The structural ribonucleic acid of the small subunit of eukaryotic cytoplasmic ribosomes
28S	The structural ribonucleic acid of the large subunit of eukaryotic cytoplasmic ribosomes
40S	The structural ribonucleic acid and proteins of the small subunit of eukaryotic cytoplasmic ribosomes
60S	The structural ribonucleic acid and proteins of the large subunit of eukaryotic cytoplasmic ribosomes
+/+	Transgenic wheat homozygous for *Blumeria* Effector Candidate 1054
-/-	Transgenic wheat azygous for *Blumeria* Effector Candidate 1054
ACTB	Actin
ANOVA	Analysis of Variance
APS	Ammonium Persulfate
Ara	*Arabidopsis thaliana* Rab GTPases
AVR	Avirulence
BAC	Bacterial Artificial Chromosome
BEC(s)	*Blumeria* effector candidate(s)
β-gal	β-galactosidase
BiFC	Bimolecular Fluorescence Complementation
BLAST	Basic Local Alignment Search Tool
BLASTp	Protein BLAST
CcdB	Control of Cell Death B (part of the CcdA/CcdB Type II Toxin-antitoxin system)
cDNA	complementary DNA
CDS	Coding sequence
CELP	Candidate Effector Like Protein
CI	Confidence Interval
Co-IP	Co-Immunoprecipitation
CPRG	chlorophenolred-β-D-galactopyranoside
CSEPs	Candidate Secreted Effector Proteins
C_T	The number of thermal cycles required for a fluorescent signal to cross the threshold (*i.e.* to become greater than the background

	level)
cv.	cultivar
ddH_2O	double-distilled water
DEPC	Diethylpyrocarbonate
DNApol	DNA polymerase
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
dpi	Days post inoculation
DSF	Differential Scanning Fluorimetry
dsRED	*Discosoma* species red fluorescent protein
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
eEF	Eukaryotic Elongation Factor
eIF	Eukaryotic Initiation Factor
EST	Expressed Sequence Tag
Fluorescence Resonance Energy Transfer	FRET
f.sp.	*forma specialis*; an informal taxonomic group given to a pathogen adapted to a specific host
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
geNorm	Gene Normalisation Experiments
GFP	Green Fluorescent Protein
GLM	Generalized Linear Model Model
GLMM	Generalized Linear Model Mixed Model
GST	Glutathione-S-Transferase
His	Histidine
his3	A yeast gene, encoding the enzyme Imidazoleglycerol-phosphate dehydratase which catalyses the sixth step of histidine biosynthesis
hpi	Hours post inoculation
HSP82	82 kDa Heat Shock Protein
H3	histone 3
IBSC	International Barley Sequencing Consortium
JIP60	Jasmonate Induced Protein 60
JIP60ml	JIP60, with a peptide in the N-terminal domain removed and replaced with a methionine-leucine linker
kDa	kilodalton
lacZ	The gene encoding β-galactosidase enzyme
LB	Luria Broth
LCMS	Liquid chromatography–mass spectrometry
Leu	Leucine
LIC	Ligation Independent Cloning
logEC50	The inflection point of a sigmoidal curve which indicates the “melting point” of BEC1054
LSU	Large Subunit

M	The internal stability of a control gene, calculated as the average pairwise variation of a particular gene with all other control genes
MDH	Malate Dehydrogenase
MGLL	Monoglyceride lipase
MeJA	Methyl Jasmonate
MMA buffer	10 µM MES (2-[N-morpholino]ethanesulfonic acid) and 10 µM MgCl2, pH 5.7
mRNA	Messenger RNA
mYFP	Monomeric yellow fluorescent protein
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide plus Hydrogen
NDPK	Nucleoside Diphosphate Kinases
NIAB	National Institute of Agricultural Botany
Ni-NTA	nitrilotriacetic acid
NIP1	Necrosis inducing protein-1
OD_{595}	Optical density of a sample measured at a wavelength of 595 nm
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PR	Pathogenesis Related
propH	Proportion of conidia which germinated to form at least one haustorium
PVX	Potato Virus X
qPCR	Quantitative PCR (Real-time PCR)
R	Resistance genes or proteins
RALPHs	RNase Like Proteins associated with Haustoria
RFP	Red Fluorescent protein
RIN	RNA Integrity Number
RIPs	Ribosome Inactivating Proteins
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SC	Synthetic Complete Media
SRL	Sarcin-Ricin Loop
SSU	Small Subunit
T_0	The first generation grown from transformed seeds
$T_{1....}T_n$	T_1 is the first generation descended from transformed seeds/seedlings which have been selfed, with subsequent generations being T_{n+1}
TBE	Buffer solution containing Tris base, boric acid and EDTA
TEMED	Tetramethylethylenediamine
T_m	The midpoint (melting temperature) of the

	protein unfolding
Trp	Tryptophan
TUBA	α-tubulin
TUBB	Barley β-tubulin
TUBBw	Wheat β-tubulin
U36	Unique Identifier sequence for the database HarvEST
UniProt	Universal Protein Resource database
UniRef	Unique reference for the database UniProt
UniRef90	a UniProt database identifier
Ura	Uracil
ura3	A yeast research marker gene, encoding Orotidine 5'-phosphate decarboxylase, which catalyzes the decarboxylation of orotidine 5-phosphate to uridine monophosphate
wBEC	The wobble BECs are synthetic genes which do not possess a signal peptide, and which have silent "wobble" mutations which minimize the nucleotide sequence identity with the wild-type barley genes
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
V_i	Initial reaction velocity (gradient) of an enzyme reaction curve
Y2H	Yeast two hybrid
Y3H	Yeast three hybrid
YFP	Yellow Fluorescent Protein
zGSTs	Class *zeta* Glutathione-S-Transferases

1. Abstract

Obligate biotrophic pathogens, for example the powdery mildew *Blumeria graminis* f.sp. *hordei*, must counteract the host's defenses if infection is to be successful and maintained. Effectors are secreted by fungi in order to regulate host immunity. One such effector, BEC1054 possesses a structure similar to that of an RNase, but the key catalytic site for RNase activity is not conserved. A total of 247 putative protein interactors were identified solely with BEC1054 through *in vitro* affinity chromatography. Comparison of these sequences with an unrelated BEC and empty Ni-NTA columns demonstrated that a significantly higher percentage of ribosomal large subunit and elongation factor related proteins were found with BEC1054. In the literature, many of these proteins have also been found to occur with Ribosome Inactivating Proteins (RIP)s. I hypothesise that BEC1054 competes with ribosome inactivating proteins, for example JIP60, preventing them from cleaving host cell's ribosomes. This would prevent the host cell's death, and therefore rejection of the powdery mildew.

In this investigation, I found that BEC1054 interacted with five proteins in yeast and *in planta*: a Pathogenesis Related protein 5 (PR5), PR10, Glutathione-S-Transferase (GST), eukaryotic Elongation Factor 1 Alpha (eEF1A) (1), eukaryotic Elongation Factor 1 Gamma (eEF1G), the latter two of which are associated with ribosomes. In addition, I identified an interaction between GST and PR10 in yeast and *in planta*, and a novel interacting protein was found for Jasmonate Induced Protein 60 (JIP60) in planta: eEF1a(3).

Control genes were assayed, to determine the best combination for normalisation of barley and *B. graminis* genes during quantitative real-time PCR. The optimal *B. graminis* housekeeping genes were found to be *act, gapdh* and H3; and for barley *gapdh*, *ubi* and *tuba2b*. The housekeeping genes investigated were shown to vary significantly between species (*B. graminis* and barley) and tissues, demonstrating the necessity for appropriate controls for each qPCR assay. The control genes were used to normalise four members of Candidate Secreted Effector Protein (CSEP) family 21 across a *B. graminis* infection time course in both epidermal and epiphytic material. The four CSEPs showed an early peak in transcript abundance in epiphytic material, which had not previously been identified in the literature. In addition, the four CSEPs demonstrated peaks in abundance at 24-48h in epiphytic material. In epidermal material, the CSEPs showed more diverse patterns of expression, with transcript abundance peaks occurring at ca. 24 or 48 hpi.

In vitro BEC1054 was found to interact with oligo(poly)nucleotides in a concentration-dependent manner. In addition, BEC1054 and JIP60 interacted with RNA in yeast; and the selective media assay indicated that this interaction may be specific for the region of the ribosome containing the ribosomal SRL.

Expression of BEC1054 in *N. benthamiana* and wheat affected the resistance of the host plants to the biotrophic pathogens *Peronospora tabacina* and *B. graminis* f.sp. *tritici* respectively. Finally, we found that *B. graminis* f.sp. *tritici* prevented degradation of the ribosome by ribosome inactivating proteins; and that BEC1054 appeared to partially prevent ribosome degradation.

2. Introduction

2.1. Barley and Wheat

Wheat (*Triticum* spp.) and barley (*Hordeum vulgare*) are economically important crops belonging to the Poaceae, which are mainly grown in the Northern Hemisphere (Hejgaard *et al.,* 1991, Gale and Devos 1998, Oerke and Dehne 2004). Their uses include food, biofuels, animal feed (the main use of barley), and alcohol production (Gale and Devos 1998, Cooper 2009).

2.2. Crop protection

Traditional crop protection strategies, for example crop rotation, are often unsuccessful for biotrophic pathogens such as *Blumeria graminis*, as the pathogen spreads efficiently and with great rapidity (Sanchez-Martin *et al.,* 2011). Resistant crop cultivars reduce the need for chemical crop protection and therefore reduce environmental damage, but new pathogen races can occur which overcome resistance (Marris *et al.,* 2008, Sanchez-Martin *et al.,* 2011).

In *Arabidopsis* C24*,* broad-spectrum resistance to downy mildew has been observed, involving multiple resistance loci (Lapin *et al.,* 2012). Future pathogen resistant crop plants may involve utilizing a polygenic base, with host responses selected to act at multiple stages of fungal infection; or the use of multiple forms of resistance (for example loss of susceptibility) (Prats *et al.,* 2007, Pavan *et al.,* 2010).

2.3. Compatible and incompatible interactions

Compatible interactions are those that lead to disease. During a compatible interaction, *B. graminis* successfully penetrates the plant cell, forms a haustorium that assimilates

nutrients from the host, and secondary hyphae are developed. Incompatible interactions occur when the resistance of the host plant to the pathogen is effective. In an incompatible interaction, attempted cell penetration is prevented through papillae formation, or through the hypersensitive response, which is characterized by rapid cell death in the sites surrounding infection. The hypersensitive response thus restricts the pathogen's growth, and its spread to other parts of the plant (Morel and Dangl, 1997). For haustorial biotrophs, preventing cell entry and haustorial formation precludes parasitism (Morel and Dangl 1997, Huckelhoven *et al.,* 1999, Heath 2000, van der Hoorn and Kamoun 2008).

The vast majority of plant hosts are immune to most microbial pathogens. Non-host, or race non-specific resistance, is a polygenic broad-spectrum resistance that occurs in all members of a plant species to all isolates of a microorganism that are pathogenic to other plant species (Clifford *et al.,* , Jorgensen 1994, Hammond-Kosack and Jones 1996). Isolates of *B. graminis,* in the UK, which infect cereals have been shown to be specialized to their hosts for example, isolates of f.sp. *avenae*, *hordei*, *secalis* and *tritici* from the UK are only able to infect the species from which they had been collected (Wyand and Brown 2003).

2.4. AVR and R genes

In the plant-pathogen gene-for-gene interaction model, an individual gene for plant resistance (R), and an individual, complementary, pathogen avirulence gene (AVR), account for AVR-R mediated resistance and pathogen recognition (Flor 1971, Jones and Dangl 2006). In "effectoromics", AVR effectors from pathogens have been utilised to help identify natural resistance R genes from plant germplasm. This method was first

used for *Phytophthora infestans* and potato, and has allowed catalogues of R and avirulence (Avr) genes to be developed (for a review, see (Vleeshouwers and Oliver 2014)). Functional assays have been used to accelerate the cloning of R genes, through transient complementation tests, allowing the laborious/time consuming process of creating stable transformants to be circumvented, with methods such as Agroinfiltration and Potato virus X (PVX) agroinfection being used to deliver effectors (Rietman *et al.,* 2012, Du *et al.,* 2014). Effectors have been used to identify homologs of resistance genes in other species, for example PVX agroinfection of the *P. infestans* effector AVRblb1 in *Solanum stoloniferum* (which can be crossed with cultivated potato) allowed the identification of *Rpi-stol1*, a functional homologue of *Rpi-blb1* (which occurs in *Solanum bulbocastanum*, which cannot be crossed with cultivated potato) (Vleeshouwers *et al.,* 2008).

The necrotrophic pathogen *Pyrenophora tritici-repentis* is the causal agent of wheat tan spot disease. One of the proteinaceous effectors produced by this pathogen is ToxA, which is recognized by the product of the *tsn1* gene (Faris *et al.,* 2010). Susceptibility to *P. tritici-repentis* was found to be correlated to the sensitivity of cultivars to the ToxA effector (Adhikari *et al.,* 2009, Faris *et al.,* 2013). Semipurified ToxA, produced using *E. coli* expression systems, has been used by Australian wheat breeders to test the sensitivity of wheat cultivars to this effector. Effector assays can be carried out on seedlings up to ca. six weeks old, and allows several thousand plants a day to be tested by a single person, producing scorable results within one week. These assays have helped efforts to eliminate *tsn1*, allowing breeders to get rid of sensitive plants immediately, saving time and resources (Vleeshouwers and Oliver 2014). Many more

examples of effector use in translational biology are available (for a review, see (Vleeshouwers and Oliver 2014)).

2.5. Powdery mildews

Powdery mildews (Erysiphales, Ascomycota) are biotrophic, economically important fungal plant pathogens (Huckelhoven 2005). They are amongst the most frequently encountered plant pathogenic fungi worldwide, infecting nearly ten thousand angiosperms. (Braun *et al.,* 2006, Glawe 2008). Their spores spread over great distances through high-altitude air currents; or locally through wind dispersal (Limpert *et al.,* 1999, Vogel and Somerville 2002). They infect the stems, leaves and fruits of their hosts, producing white, powdery clumps of spores, from which they derive their name (Jarvis *et al.,* 2002).

2.6. Blumeria graminis

The disease powdery mildew is caused by fungi belonging to the genus *Blumeria*. *Blumeria graminis* (DC) Speer infects grasses belonging to the Poaceae. It possesses a very high degree of host specificity, with eight *formae speciales* (f.sp.), each infecting one host genus; for example *Blumeria graminis* f.sp. *hordei* (barley powdery mildew) infects barley and *B. graminis* f.sp. *tritici* (wheat powdery mildew) infects wheat (Wyand and Brown 2003, Braun *et al.,* 2006, Dean *et al.,* 2012). *Blumeria graminis* f.sp. *hordei* is economically important; and of the powdery mildews it is the best studied (Both and Spanu 2004, Bindschedler *et al.,* 2009). It will be referred to as *B. graminis* hereafter unless stated otherwise.

2.6.1. Infection and life cycle

2.6.2. Asexual reproduction

Epidemic spread of *B. graminis* is caused by asexual conidia; the infection cycle is initiated by a conidium landing on a susceptible host plant (Figure 1) (Glawe 2008). The conidia produce an extracellular matrix (ECM) 20 s to 1 min after landing on the leaf surface (Kunoh *et al.,* 1988, Carver *et al.,* 1999, Kunoh 2002).

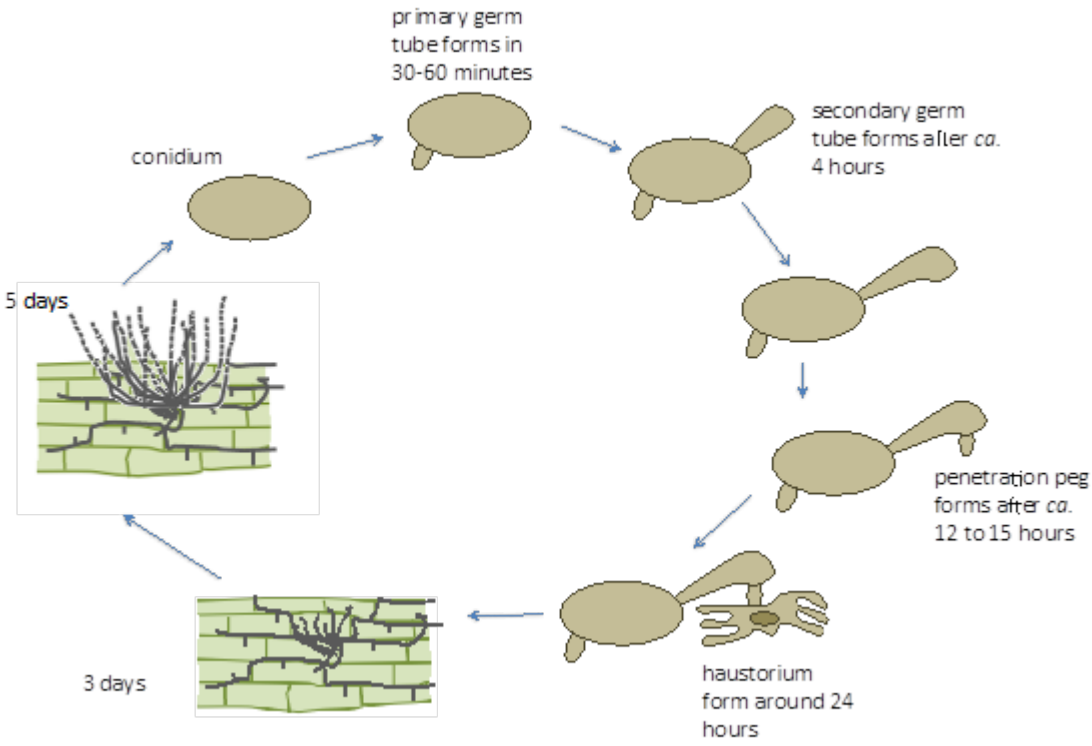


Figure 1: **Asexual lifecycle of *Blumeria graminis*.** Once a conidium has landed on a susceptible host, initiation of the germ-tube occurs within 60mins to one hour. Following the detection of the host, the primary germ-tube initiates secondary germ-tube formation (Kunoh 2002, Both *et al.,* 2005). Swelling at the apex of the secondary germ tube produces an appressoria by around 8 h of infection. By 12 h post infection (hpi), the appressoria has formed a penetration peg. If the host cell wall is successfully penetrated by the penetration peg, it invaginates the host cell's plasma membrane, continuing into the cell. Once inside, it forms a digitate haustorium; these are fully functional by 24 hpi. As the infection progresses, epiphytic mycelia are produced on the surfaces of the leaf; and conidia are formed within 3 to 5 days, which upon release begin the cycle again (Both *et al.,* 2005). Adapted from (Both *et al.,* 2005).

2.6.3. Sexual reproduction and variation of virulence

The sexual reproductive stage for *B. graminis* f.sp. *hordei* increases genetic diversity. Sexual reproduction occurs via structures named chasmothecium (formerly cleistothecium), a type of ascocarp that can survive harsh conditions. The chasmothecium contains multiple asci, each of which can release ascospores (Both and Spanu 2004). Towards the end of the growing season, as temperatures rise, random intercrossing occurs between compatible mating types (Braun *et al.,* 2002). Combined with the ease with which the asexual conidia are dispersed by the wind (Wolfe and McDermot, 1994), this allows frequent gene exchange amongst different populations. Sexual reproduction can lead to variation in pathogen virulence, with different effectors, for example AVR_{A1} being present amongst different *B. graminis* f.sp. *hordei* populations (Marris *et al.,* 2008, Sanchez-Martin *et al.,* 2011, Hacquard *et al.,* 2013, Zhu *et al.,* 2015).

2.6.4. Divergence and expansion of effector complements

The genome sizes of the powdery mildews are significantly larger than other ascomycetes (Spanu et al., *2010).* This expansion is believed to be due to reterotransposons, which can be observed as repetitive elements within the genome. In contrast, the number of curated identified by Spanu *et al.,* (2010) was 5854, which is towards the smaller end of fungal genome sizes. The low number of protein coding genes has occurred through the loss of some metabolic pathways, a decrease in the number of paralogous genes, and an overall reduction in gene family size.

In contrast to the reduction in gene family size and gene number, a massive expansion has occurred in the Candidate CSEPs, which represent greater than 7% of the barley powdery mildew protein coding genes. In contrast with other powdery mildew genes,

many of the CSEPs have been found to have paralogs that can be grouped into clearly identifiable families (Spanu *et al.,* 2010). Evidence of strong, positive diversifying selection is shown by the CSEPs, and this is associated with amino acids predicted to occur on the surface of proteins (Pedersen *et al.,* 2012).

The CSEP genes are closely linked to retro-transposable elements, indicating that the expansion of the CSEPs may have occurred through retro-transposon driven illegitimate recombination (Pedersen *et al.,* 2012). This gene duplication would allow increased expression, through the increased copy number of the CSEP, and would allow fivergense through the selection of mutated CSEP genes (Spanu, 2013). This retro-transposon based proliferation of CSEPs may confer an advantage to the pathogen, by providing an efficient means for the generation of effector genes. The majority of the CSEPs have been identified solely with the *Blumeria*, as poopsed to with the pea or *Arabidopsis* powdery mildews, demonstrating that the powdery mildew genomes have species specific innovations. These may have evolved through cospeciaton of the pathogens with their plant hosts (Sacristan *et al.,* 2009, Spanu *et al.,* 2010).

2.6.5. *Blumeria formae speciales*

Classically, eight *formae speciales* (*f.sp.*) are described, each infecting a single host genus (Troch *et al.,* 2014) (*Agropyron*, *Bromus*, *Dactylis* and *Poa* for the wild grasses, and *Avena*, *Hordeum*, *Secale* and *Triticum* for cultivated grasses) (Marchal 1902, Oku *et al.,* 1985) cit. in (Troch *et al.,* 2014). The corresponding *formae speciales* are named *agropyri*, *bromi*, *dactylidis*, *poae*, *avenae*, *hordei* and *tritici* respectively. Although this taxonomy for *B. graminis* has increasingly been brought into question, for example f.sp. *avenae, hordei* and *tritici* have been shown to be able to infect wild grasses from

multiple genera in Israel (Eshed and Wahl 1970), the f.sp. for *B. graminis* strains that infect cultivated cereals are generally supported (Troch *et al.,* 2014). The cereal clades of *B. graminis* have been shown to be specialized to these hosts, for example isolates of f.sp. *avenae*, *hordei*, *secalis* and *tritici* from the UK were only able to infect the species from which they had been collected (Wyand and Brown 2003).

2.6.6. *Blumeria*-pathogen host interactions

During *B. graminis* infection, the sole part of the fungus within the host cell is the haustorium. Host response and nutrient transfer both play key roles in determining whether infection is successful and maintained. The fungal pathogen absorbs nutrients and photoassimilates through the haustorium, allowing secondary epiphytic hyphae to grow (Green *et al.,* 2002, Zhang *et al.,* 2005, O'Connell and Panstruga 2006). Small proteins, including fungal effectors, are delivered at the haustorial complex, with both host and pathogen involved in "secretory warfare". The extrahaustorial matrix separates the perihaustorial membrane from the haustorial wall matrix (O'Connell and Panstruga 2006, Panstruga and Dodds 2009).

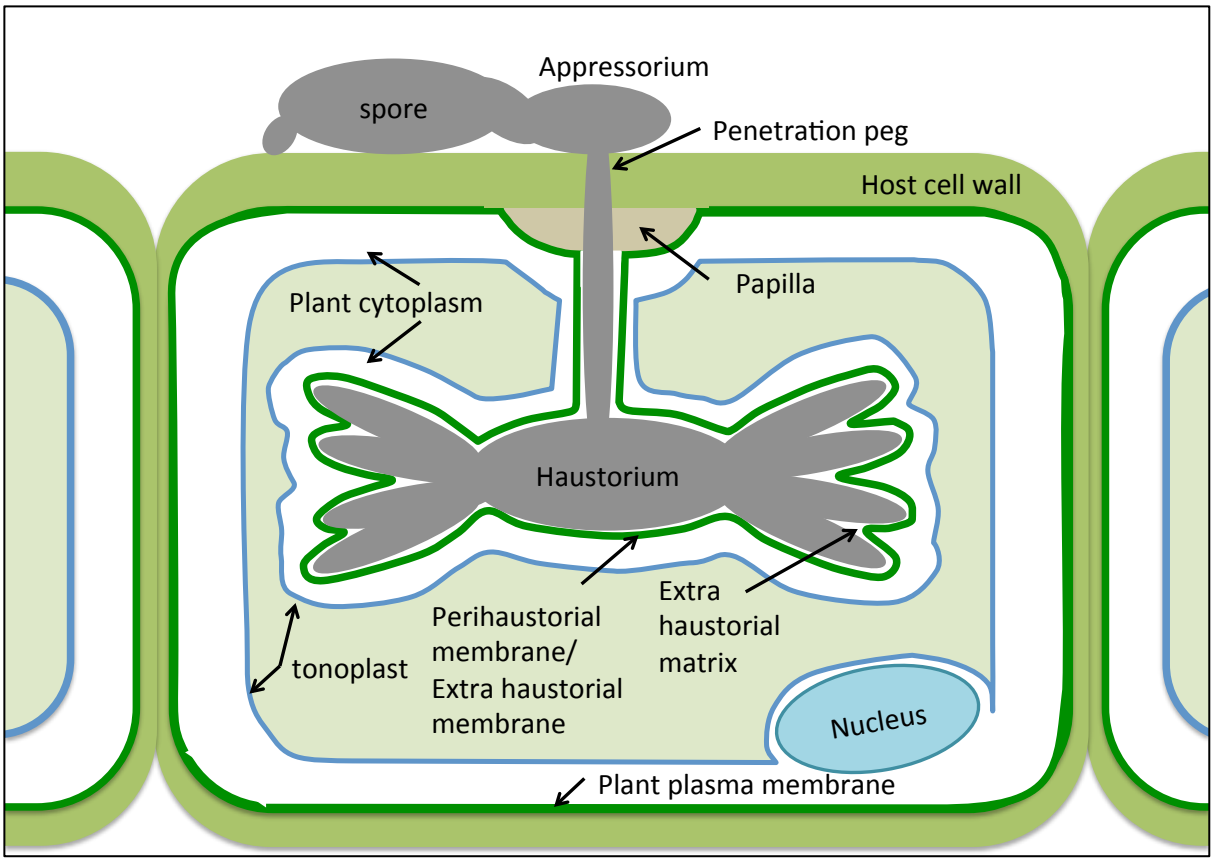


Figure 2: **The haustorial complex is formed from the perihaustorial membrane, haustorium and extrahaustorial matrix**. Infection of barley by the pathogen *Blumeria graminis* f.sp. *hordei* causes powdery mildew disease. The pathogen penetrates the cell wall, and forms digitate haustoria within the host cell. The haustorial complex is formed from the perihaustorial membrane, haustorium and extrahaustorial matrix. The perihaustorial membrane (or extrahaustorial membrane) is a plant plasma membrane, induced by the fungus.

2.7. Why study effectors?

In this study, the term "effector" has been utilised to indicate molecules, produced by pathogens, which have an effect on one or more genotypes of plants, which are either host or non-host (Vleeshouwers and Oliver 2014). This definition does not vary for cytoplasmic or apoplastic effectors, or between effectors that confer an advantage or disadvantage in the establishment of infection.

Cytoplasmic effector proteins are secreted into host cells, and apoplastic effectors into extracellular spaces, by microbial pathogens, including nematodes, oomycetes and fungi, to facilitate infection (Oliva *et al.,* 2010). Insect pathogens of plants, such as aphids, have also been shown to utilise effectors (Bos *et al.,* 2010b, Jaouannet *et al.,* 2014), as have pathogens which infect animals and humans, for example commensal bacteria utilise effectors to interact with human hosts (Cohen *et al.,* 2015), and the mosquito bacterial symbiont, *Pantoea agglomerans,* secretes effector proteins in the mosquito midgut (Bisi and Lampe 2011, Wang *et al.,* 2012). Various crucial roles are played by these effectors, including targeting of proteins or systems involved in host immunity. This has been shown to be the case for bacterial pathogens, for example *Pseudomonas syringae,* which injects type III effector (T3E) proteins into host cells, and uses them to alter organelle function, block RNA pathways, interfere with immune receptor signaling and block vesicle traficking (Block and Alfano 2011, Deslandes and Rivas 2012, Feng and Zhou 2012). Filamentous pathogens, including fungi and oomycetes, also subvert the host immune system using effectors (for reviews, see (de Jonge *et al.,* 2011, Bozkurt *et al.,* 2012, Dou and Zhou 2012, Rafiqi *et al.,* 2012).

2.7.1. *Blumeria* effector candidates

The identification of *B. graminis* effector candidates (BECs) occurred through identification of genes up-regulated specifically during infection (Thomas *et al.,* 2001, Both *et al.,* 2005); the identification of RNA associated specifically with haustoria (Godfrey *et al.,* 2010, Spanu *et al.,* 2010); identification of proteins expressed only in haustoria (Bindschedler *et al.,* 2009, Bindschedler *et al.,* 2011); through the use of transcriptomic, genomic, proteomic, and structural prediction methods/analyses to characterize effectors in the *B. graminis* genome (Pedersen *et al.,* 2012); and through

identification/characterisation of avirulence (AVR) genes, the products of which are recognized by R proteins in a gene-for-gene manner (Ridout *et al.,* 2006, Sacristan *et al.,* 2009).

2.7.2. BEC1054

Fifty *Blumeria* Effector Candidate (BEC) genes were chosen from the 71 identified by Bindschedler *et al.,* 2011 and screened by Host-Induced Gene Silencing (HIGS) to identify whether they played a role in pathogen virulence (Pliego *et al.,* 2013). Of the 50, 8 were shown by Pliego *et al.,* (2013) to contribute significantly to infection (BEC1005, BEC1016, BEC1018, BEC1019, BEC1038, BEC1040, BEC1054 and its paralog BEC1011), as silencing of these genes decreased the relative frequency of full haustorial development. The remainder could not be shown to be significant, but their silencing still decreased the haustorial index. The most significant effects on virulence were identified for BEC1011 and BEC1054. Furthermore, BEC1011 suppresses host programmed cell death, which occurs following pathogen recognition by the host (Lamb and Dixon 1997, Wang and Huang 2011, Pliego *et al.,* 2013). Most, but not all, BECs are also Candidate Secreted Effector Proteins (CSEPs), for example BEC1011 and BEC1054. The CSEPs are defined as proteins which are predicted to be secreted, which lack transmembrane domains, and which lack BLAST hits outside of the powdery mildews (Pedersen *et al.,* 2012). Both BEC1054 and BEC1011 are RNase like proteins, which are expressed in haustoria; whereas BEC1005 (used as a control in protein-protein interactions throughout this study) is predicted to be a β1,3-endoglycosidase, due to its similarity to fungal glucosyl transferases, with homologous proteins in yeast being involved in cell wall remodeling. BEC1019 is predicted to be a protease, and the functions of the others are not known (Pliego *et al.,* 2013).

2.8. BEC1054 is an RNase like effector

Pedersen *et al.,* (2012) found that many of the *B. graminis* CSEPs were similar to RNases. BEC1054 is a CSEP (equivalent to CSEP0064) with a structure similar to microbial RNases (Figure 3), but the catalytic active site residues required for RNase activity are absent (Pedersen *et al.,* 2012). This structure of BEC1054 has recently been confirmed experimentally (R. Jones, *pers. comm.*).

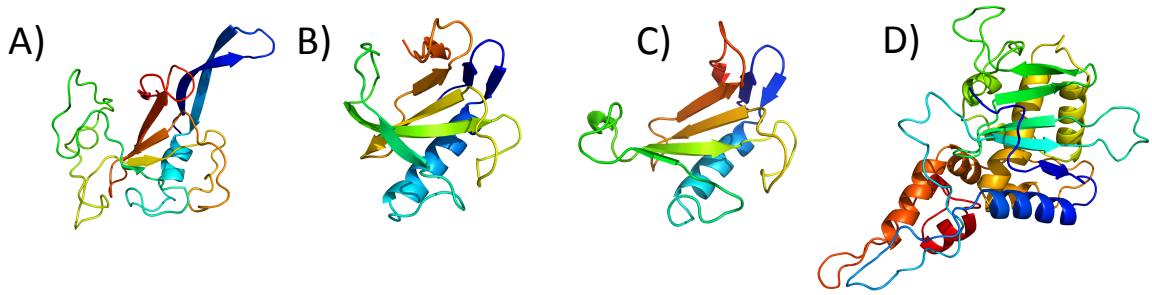


Figure 3: **RNase Like Proteins Expressed in Haustoria and RNases possess an RNA binding fold.** The PDB files of the three experimentally derived protein structures were uploaded in PyMol Version 1.5.0.4 and used to visualise the structures as "cartoons". The colour spectrum indicates the N terminus (blue) to C terminus (red), where A) is α-sarcin from *Aspergillus giganteus;* B) is Ribonuclease f1 from *Fusarium moniliforme* and C) is BEC1054 from *Blumeria graminis* and D) Is the N-terminall domain of Jasmonate Induced Protein 60 from barley (where the C-terminal domain is cleaved off post-translationally) (Jones et al, in preparation).

Almost all plant species have been found to possess a subclass of RNases, called Ribosome Inactivating Proteins (RIPs). They have been found to occur in maize (Roberts and Selitrennikoff 1990), *Sorghum* (Hey *et al.,* 1995), *Arabidopsis* (De-la-Pena *et al.,* 2008), castor oil plant (Sperti *et al.,* 1973), jequirity pea (Olsnes *et al.,* 1975), *Nicotiana tabacum* (N. Sharma *et al.,* 2004) and many more.

The large subunit of ribosomal RNA is depurinated by RIPs through the cleavage of an

N-glycosidic bond of an exposed ribosomal large subunit RNA loop, the Sarcin-Ricin Loop (SRL). This causes a sugar-phosphate backbone phosphodiester bond to become exposed to chemical hydrolysis within the cell (Endo *et al.,* 1988a, Endo and Tsurugi 1988, Endo *et al.,* 1988b, Barbieri *et al.,* 1993). If the depurinated rRNA is treated with aniline *in vitro*, aniline cleaves the sugar-phosphate backbone at site of the modified nucleotide(s), causing two rRNA fragments to be formed (Peattie 1979). Preservation of ribosomal integrity would help to maintain a living plant cell as a food source for the fungus. Binding of the eEF2/GTP complex to the ribosome is prevented by the cleavage of the sarcin-ricin loop. This inhibits protein synthesis at the elongation step (Wool *et al.,* 1992); and the expression of the barley RIP Jasmonate Induced Protein 60 (*jip60ml)* in *N. tabacum* has previously been shown to impair elongation, through a shift in the monosome/polysome ratio towards polysomes (Gorschen *et al.,* 1997). The RIPs, such as JIP60, have been shown to play a role in host induced cell death following pathogen detection (Reinbothe *et al.,* 1994).

2.9. Prior knowledge

Prior to the start of this study, the genome of *B. graminis* had been sequenced (Spanu *et al.,* 2010). The genomes of wheat (Mayer *et al.,* 2014) and barley (Mayer *et al.,* 2012) had also been sequenced, but their annotation and presentation were not yet complete. Before the *B. graminis* genome had been sequenced, a list of *Blumeria* Effector Candidates (BECs) was created through genomic, transcriptomic and proteomic methods. Genes up-regulated during haustorial formation were identified through the use of a microarray (Thomas *et al.,* 2001, Both *et al.,* 2005). The abundance of transcribed RNA associated specifically with haustoria (compared with conidia or

mycelia) were quantified using RNAseq, and included as BECs (Godfrey *et al.,* 2010, Spanu *et al.,* 2010). In addition, proteogenomic approach was used to identify *Blumeria* Effector Candidates (BECs) were identified which were associated specifically with host cells colonized by haustoria (Bindschedler *et al.,* 2009, Bindschedler *et al.,* 2011).

In *B. graminis*, a superfamily of candidate secreted effector proteins (CSEPs) containing 491 genes have been identified which do not possess a transmembrane domain, which have no significant BLAST matches outside of the Erysiphales, and which possess a predicted signal peptide. The exclusions of proteins with BLAST matches outside the Erysiphales allowed fungal proteins such as commonly secreted enzymes to be omitted (Panstruga and Dodds 2009, Spanu *et al.,* 2010). The initial characterisation of the Candidate Secreted Effector Proteins (CSEPs), specific to powdery mildews, was driven by bioinformatics predictions (Pedersen *et al.,* 2012). The CSEPs possessed features typically associated with effectors, but differ from the previously characterized avirulence proteins AVRK1 and AVRK10, which have no signal peptide (Ridout *et al.,* 2006, Sacristan *et al.,* 2009, Pedersen *et al.,* 2012).

In cereals, 17 functional "powdery mildew resistance 3" (*pm3*) alleles have been found to confer race-specific resistance to *B. graminis*. Much less is known about the corresponding powdery mildew *avr* genes. Recently, Bourras *et al.,* (2015) demonstrated that *pm3* race-specific resistance was multiallelic, and controlled by three genetically interacting fungal loci "*locus_1*", "*locus_2*" and *"locus_3*", with *locus_1* having been shown to be involved in all interactions of AVRPM3 effectors (which are canonical CSEPs) with PM3. The establishment (or failure) of a compatible infection for *B.*

graminis f.sp. *tritici* has been shown to depend upon a combination of avirulence genes, and suppressors of those avirulence genes. The *B. graminis* f.sp. *tritici* AVRPM3A effector was found to confer recognition specificity in a manner which was allele dependent. The gene *avrpm3*$^{a2/f2}$, cloned from *locus_2*, was found to be recognized by the R alleles *pm3a* and *pm3f*, thus demonstrating that AVRPM3A is an AVR factor. The authors linked these results to prior investigations of wheat *pm3* allelic specificity, and demonstrated that distinct Avr loci in *B. graminis* f.sp. *tritici* genetically controlled resistance specificities. In addition, distinct Avr factors were found to be recognized by different *pm3* alleles. Investigation of the expression of the gene *bcg1,* encoded by *locus*_1, and *avrpm3*$^{a2/f2}$, encoded by *locus_2*, demonstrated that differing levels of gene expression occurred between differing isolates, indicating a role for expression levels in avirulence. Together, these results showed that the *B. graminis* f.sp. *tritici* pathosystem is highly complex (Bourras *et al.,* 2015).

3. Materials and methods

All chemicals were obtained from VWR (VWR, Chicago, USA). Primers were obtained from Invitrogen (Invitrogen, Carlsbad, CA). Statistics were analysed using R v3.0.1 (http://www.r-project.org/), unless stated otherwise.

3.1. Protein interactors of BEC1054

3.1.1. Barley and *Blumeria graminis* growth conditions

Barley, *Hordeum vulgare* L. cv. Golden Promise, was grown in 13cm diameter pots filled with Levington® Seed and Modular Compost Plus Sand F2S (Everris, Ipswich, UK), with *ca.*30 seeds per pot. Wheat and barley plants were watered every two days, and grown with 16 h light, 8 h darkness, and 33% humidity at 21°C. Light intensity was set at approximately 130 μmol/m/s^2 with two alternating types of lighting tube: Philips Master 49W / 830 / TL5 HO (Philips, Guildford, UK) and Sylvania Lux Line Plus FHO 49W/ 840 / T5 (Havells Sylvania, NewHaven, UK).

Uninfected plants and infected plants were maintained under the same lighting and growth conditions. Seven days post germination, wheat or barley seedlings were transferred to 60cm^3 Perspex boxes, and used as the host for *Blumeria graminis* f.sp. *hordei* var. DH14 or *Blumeria graminis* f.sp. *tritici* respectively. Inoculation was performed, and the *B. graminis* lines maintained by shaking plants infected with *B. graminis* f.sp. *hordei* or *B. graminis* f.sp. *tritici* over seven day old barley or sheat seedlings respectively. This strain of barley was utilised due to its susceptibility to the *Blumeria* strain used.

3.1.2. Statistical analyses of *in vitro* liquid chromatography pull-downs

In vitro chromatography was performed by Dana Gheorghe and Laurence Bindschedler; I performed statistical analyses on the results.

3.1.1.2. Criteria used to count proteins from lists generated from Liquid Chromatography Mass Spectrometry data

In vitro chromatography was performed within the host laboratory to identify protein putative interactors for BEC1054. The bait was recombinant BEC1054 with an N-terminal His-tag, which had been expressed in *E. coli* and purified (Gheorghe, *pers. comm.*). The negative control was BEC1005 prepared in the same manner; and for experiment iii (listed below) a magnetic Ni-NTA column with no proteins attached was used as an additional control (Bindschedler, *pers. comm*). Three separate experiments were performed (Table 1).

Table 1: **Methodology summary describing the affinity pull-down experiments**. The pull-downs were performed as three biological replicates and included two technical replicates for each biological replicates of set A. i) Pull-down bait details; ii) experimental sample and affinity column/bead details.

i)

Purpose	**Control details**
Pull-down bait	*E. coli* expressed BEC1054 with N-terminal His tag BEC1054 (A, B, C)
Negative control 1	*E. coli* expressed BEC1005 with N-terminal His tag BEC1005 (A,C)
Negative control 2	Absence of bait (A, B,C)

ii)

Sample code	**Substrate**	**Affinity column/bead details**
A	48h Infected leaf epidermis	magnetic NTA agarose beads
B	Non-infected leaves	1ml NTA chromatography columns
C	7 day Infected leaves	magnetic NTA agarose beads

Protein extractions were performed on the harvested plant material as per Bindschedler *et al.,* (2011). Reverse phase liquid chromatography and nanoESI tandem mass spectrometry was performed in the collaborating laboratory as described by (Pennington *et al.,* 2016). Proteins were identified using Mascot (MatrixScience, London, UK), and BLASTed against the HarvEST (http://harvest.ucr.edu/) and International Barley Sequencing Consortium (IBSC) databases (http://www.public.iastate.edu/~imagefpc/IBSC%20Webpage/IBSC%20Template-home.html). Binomial proportion tests (Crawley 2005) were used to determine whether significantly more RNA-associated protein peptide sequences were identified with BEC1054 through *in vitro* chromatography than with negative controls. Search terms (Table 2) were used to determine the number of RNA-related sequences present; with each sequence being counted once per experiment to prevent pseudoreplication through the recognition of different peptides within the same protein. Upper and lower 95% confidence interval (CI) were used to calculate the standard error (Equation 1) (Higgins and Green 2011).

Equation 1: **Calculating standard error**. Standard error can be calculated from the 95% confidence interval and the t-value for a given dataset.

$$standard\ error = \frac{95\%\ confidence\ interval}{t - value}$$

Table 2: **Search terms used to identify RNA-related sequences from the protein descriptions of proteins identified through Liquid Chromatography Mass Spectrometry.** The following search terms were used in Excel to identify RNA-related sequences from the gene comments for genes from the International Barley Sequencing Consortium database, and from genes identified during interactions with BEC1054. * is a wildcard used as a substitute for any set of characters within the search. The left hand column contains the categories that the search terms divide into; for example 28S, 23S, 5.8S and 50S are all terms used to refer to the ribosomal large subunit, and were treated as one set of results.

Category	Search terms
large ribosomal subunit	28S
	23S
	5.8S
	50S
small ribosomal subunit	40S
	18S
	30S
	16S
ribosome	60S
	70S
	80S
	ibosom
	elongat*
	translation
	initiation
	RNA

3.1.3. Gene annotation and entry vector construction

3.1.1.3. Complementary DNA synthesis

RNA was extracted from infected barley leaf material using the QIAGEN RNeasy Mini Kit (QIAGEN, Crawley, UK). The A260/A280 ratio was measured to determine the resulting RNA concentration using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The cDNA was synthesized using the SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen, cat. No. 11917-010) using 3 µg RNA as a template.

3.1.1.3. PCR

Home-made Taq polymerase was used to perform PCR in a GS1-thermalcycler (G-Storm, Somerton, UK). PCR was performed in a 25 µl reaction, with 5 µl 10xPCR buffer (Applied Biosystems), 200 µM dNTPs (Applied Biosystems), 1.25U Taq and 10 pmol of the primers (Supplementary Table 13). Amplification was 3 min at 95°C, 40 cycles of 30 s at 94°C, 1 min annealing, 1 min at 72°C and 3 min at 72°C. Presence of PCR products was confirmed by gel-electrophoresis, with SYBR® Safe - DNA Gel Stain (Thermo Fisher Scientific) used for band visualization. PCR products were purified using the QIAprep Spin Miniprep Kit (QIAGEN). Gels were imaged/analysed under UV light using the InGenius Bio-imager and GeneSnap software (Syngene, Cambridge, UK). If multiple bands were obtained, the desired band was purified with a QIAquick Gel Extraction Kit (QIAGEN).

3.2.1.3. Protein interactor identification and amplification

Coding sequences for putative interactors were identified and annotated using the work flow described in Figure 4.

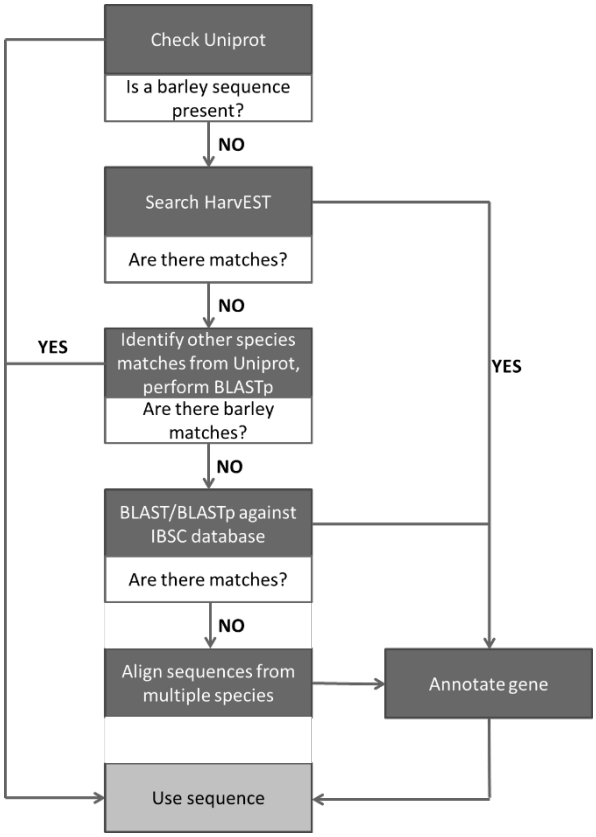


Figure 4: **Flowchart used for the identification and annotation of barley putative interactors.** Barley protein putative interactors, identified with BEC1054, were identified where possible through their "Unique Reference" code on the database UniProt (http://www.uniprot.org/). If they were not present on UniProt, the "Unique Identifier" was used to search the HarvEST U36 database (http://harvest.ucr.edu/). If the gene was not identified on either database, matches to other species from UniProt were translated to the protein sequence, and used on GenBank (http://blast.ncbi.nlm.nih.gov) to perform a protein-BLAST (BLASTp). Where barley sequences were available on GenBank, they were used to perform a BLAST or BLASTp against the IBSC database (http://www.public.iastate.edu/~imagefpc/IBSC%20Webpage/IBSC%20Template-home.html). If a match could not be identified, sequences from multiple plant species were aligned and used to select plausible START and STOP codons and open reading frames.

Genes of interest, including barley genes, BEC1054 and BEC1005, were amplified from cDNA made from 3 dpi barley epidermis using the primers listed in Supplementary Table 13.

3.3.1.3. Entry vector construction

The PCR products were inserted into pCR8 (Invitrogen), and transformed into competent DH5a or TOP10 *E. coli* (Invitrogen). Colonies were grown overnight at 37°C on LB media (5% yeast extract, 1 l ddH_2O, 1% NaCl, 1% tryptone, ± 1.5% agar, pH7.5) containing 100µg/ml spectinomycin. Plasmid DNA was analysed by directional PCR using M13 forward primer 5´-GTAAAACGACGGCCAGT-3' (a sequence of the vector pCR8) and the reverse primer from the gene (Supplementary Table 13). Where a product was detected, clones were selected, grown overnight at 37°C in liquid LB with100 µM spectinomycin, plasmids extracted using the QIAGEN Plasmid Midi Kit (QIAGEN), and sequenced (GATC, Cologne, Germany).

Genes of interest were re-amplified from entry vectors using primers that added either a START or a STOP codon, specified in Supplementary Table 14). The PCR products created using primers with the starting sequence "GGGGACAAGTTTGTACAAAAAAGCAGGCTTC" were amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Schwerte, Germany), as per the manufacturer's instructions. The PCR products were then cleaned using the DNA Clean & Concentrator™-5 kit (Zymo Research, Freiburg Germany); and inserted into pDONR201 (Invitrogen) using Gateway® BP Clonase® Enzyme Mix (Invitrogen), as per the manufacturer's instructions. The pDONR entry constructs were miniprepped using the NucleoSpin® Plasmid kit (Machery-Nagel, Eupen, Belgium). Products created using

the remaining primers were inserted into pCR8, as specified above. Following PCR, the reaction mixture containing the product from pCR8 was digested using Dpn1 (New England Biolabs) as per the manufacturer's instructions. The selective antibiotics were spectinomycin for pCR8 (final concentration 100 μM), and kanamycin (final concentration 50 μM) for pDONR201. Colony selection and culturing were performed as specified above. Plasmids were sequenced to confirm that the genes had inserted in frame/sense. Entry vectors created are summarised in Table 3.

Table 3: **Genes of interest and their entry vectors**. Genes were inserted into either pCR8, or into pDONR201. The entry vectors marked "*" was from (Morgan 2014) and ** from (Pliego *et al.,* 2013).

Gene name	Genes with neither START nor STOP codon	Gene with an added START codon (for N-terminal fusion)	Gene with an added STOP codon(for C-terminal fusion)
Glutathione-S-Transferase	pCR8	pCR8	pDONR201
Malate Dehydrogenase	pCR8	pDONR201	pDONR201
Pathogenesis Related protein 5	pCR8	pDONR201	pCR8
Pathogenesis Related protein 10	pCR8	pCR8	pDONR201
Eukaryotic Elongation Factor 1 Gamma	pCR8	pDONR201	pDONR201
Eukaryotic Elongation Factor 1 Alpha (1)	pCR8	pCR8	pDONR201
Eukaryotic Elongation Factor 1 Alpha (3)	pCR8	pDONR201	pDONR201
Ribosomal 40S protein 16	pCR8	pCR8	pDONR201
Nucleoside Diphosphate Kinase	pCR8	pDONR201	pDONR201
Blumeria Effector Candidate 1054	-	pCR8*	pCR8**
N-terminal Jasmonate Induced Protein 60 ml	pCR8	pDONR201	pDONR201

3.4.1.3. Jasmonate Inducted Protein 60 identification and amplification

The N-terminal domain of Jasmonate Induced Protein 60 (*jip60*) was amplified from 3 dpi cDNA, prepared as per section 3.1.1.3., using the primers specified in Supplementary Table 15. The PCR product was inserted into pCR8 (Invitrogen) according to the manufacturer's instructions. A peptide in the N-terminal domain; the removal of which is required for RIP activity (Chaudhry *et al.,* 1994); was excised by our collaborator Rhian Jones. A methionine-leucine linker was used to replace the peptide via the Q5® Site-Directed Mutagenesis Kit, with the primers listed in Supplementary Table 15. The resulting product is referred hereafter as "JIP60ml."

3.1.4. Yeast two hybrid

Cloning for the Y2H assay was performed with the help of Annabelle Damerum (Damerum 2013). She amplified the genes *eef1g* and *pr10*, and performed an LR recombination to transfer them to the pDEST plasmids.

3.1.1.4. Expression vector construction

Expression vectors were created using the entry vectors specified in section 4.3.1.3 using Gateway® LR Clonase® enzyme mix (Invitrogen) as per the manufacturer's instructions. Entry vectors were recombined with the yeast expression plasmids pDEST22 (which contained a C-terminal transcription factor activation domain) and pDEST32 (which contained a C-terminal transcription factor activation domain), to give the expression vectors pEXP22 and pEXP32 respectively (Table 7).

Table 4: **Combinations of plasmids used to investigate protein-protein interactions in yeast-two-hybrid.** The following plasmid pairs were used in a yeast-two-hybrid assay, to investigate the interactions of various proteins with BEC1054 and BEC1005. The following plasmids were provided with the commercial yeast-two-hybrid kit: pDEST22, pDEST32, pEXP32/Krev1, pEXP22/RalGDS-wt pEXP22/RalGDS-m1, and pEXP22/RalGDS-m2.

Bait plasmid (LEU2)	**Prey plasmid (TRP1)**	**Purpose**
none	none	Negative transformation control
pEXP32/Krev1	pEXP22/RalGDS-wt	Strong positive interaction control
pEXP32/Krev1	pEXP22/RalGDS-m1	Weak positive interaction control
pEXP32/Krev1	pEXP22/RalGDS-m2	Negative interaction control
pEXP32/BEC1054	pDEST22	negative activation control
pEXP32/BEC1005	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/MDH	interaction under investigation
pEXP32/MDH	pEXP22/BEC1054	interaction under investigation
pEXP32/MDH	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/GST	interaction under investigation
pEXP32/GST	pEXP22/BEC1054	interaction under investigation
pEXP32/GST	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/PR5	interaction under investigation
pEXP32/PR5	pEXP22/BEC1054	interaction under investigation
pEXP32/PR5	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/eEF1G	interaction under investigation
pEXP32/eEF1G	pEXP22/BEC1054	interaction under investigation
pEXP32/eEF1G	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/40S 16	interaction under investigation
pEXP32/40S 16	pEXP22/BEC1054	interaction under investigation
pEXP32/40S 16	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/eEF1A_1	interaction under investigation
pEXP32/eEF1A_1	pEXP22/B54	interaction under investigation
pEXP32/eEF1A_1	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/eEF1A_3	interaction under investigation
pEXP32/eEF1A_3	pEXP22/B54	interaction under investigation
pEXP32/eEF1A_3	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/PR10	interaction under investigation
pEXP32/PR10	pEXP22/B54	interaction under investigation
pEXP32/PR10	pDEST22	negative activation control
pEXP32/BEC1054	pEXP22/NDPK	interaction under investigation
pEXP32/NDPK	pEXP22/B54	interaction under investigation
pEXP32/NDPK	pDEST22	negative activation control

3.2.1.4. Yeast transformation

For Y2H a commercial kit was used (Invitrogen). Yeast strain MaV203 was made competent and transformed with plasmid pairs specified in Table 7. Transformed yeast was plated onto SC-Leu-Trp (20% glucose, 6.7g yeast nitrogen base without amino acids, 1.35g purine and amino acid powder mix missing Histidine, Leucine, Tryptophan, and Uracil (Sigma Aldrich, St Louis, USA), 0.32 mM uracil and 0.32 mM histidine, ±1% agar) and incubated at 30^{o}C for two days.

3.3.1.4. Quantitative β-galactosidase assay

Expression of the *lacZ* reporter gene can be quantified through its β-galactosidase activity. The maximum conversion rate, V_i (Figure 5) at the start of the hydrolysis of the yellow substrate CPRG to the red product chloramphenicol red (and D-galactose), was used as a quantitative indicator of the interaction between bait and prey (Figure 12).

Lines under investigation were streaked out onto SC-Leu-Trp, incubated (48 h, 32oC), aspirated and suspended in 1 ml phosphate-buffered saline. Samples were centrifuged, supernatant removed, and cells re-suspended in 200 µl phosphate-buffered saline. Cell density was measured for each sample by measuring the OD_{595}. A CPRG assay was performed as described by (Simon and Lis 1987) and the Y2H manufacturer (Invitrogen); but with 50 µl of the supernatant added to 100 µl of CPRG buffer2 (2.23 mM CPRG in Buffer1) in a 96-well microtitre plate (instead of 100 µl supernatant and 900 µl Buffer2 in a 1 ml cuvette). The OD_{595} was measured and used to calculate β-galactosidase activity for each time point, (Equation 2). The β-galactosidase activity was used to calculate the relative β-galactosidase activity (Equation 3).

An asymptotic exponential model (Equation 4) was fitted using the nls function in R v3.0.2, and used to calculate the β-gal activity within the first minute (for an example, see Figure 5)(Miller 1972, Miller 1992). The asymptotic exponential model was differentiated (Equation 5) to calculate the maximum gradient, i.e. the gradient at time zero (Equation 6).

Equation 2: **β-galactosidase activity equation.** The $OD_{595\ cell\ density}$ is the OD_{595} reading taken at the beginning of the assay as an approximation of cell density; $OD_{595\ CPRG}$ is the OD_{595} absorbance by chloramphenicol red (and light scattering by cell debris).

$$\beta - \text{galactosidase activity} = 1000 \times \frac{OD_{595\ CPRG}}{OD_{595\ cell\ density} \times volume}$$

Equation 3: **Relative β-galactosidase (β-gal) activity,** where "sample β-gal activity$_{t=n}$" is the current β-galactosidase activity and "sample β-gal activity$_{t=0}$" is the β-galactosidase activity at time zero (or the first reading).

$$relative\ \beta - gal\ activity = \frac{[sample\ \beta - gal\ activity_{t=n}] - [sample\ \beta - gal\ activity_{t=0}]}{[negative\ control\ \beta - gal\ activity_{t=n}]}$$

Equation 4**: β-galactosidase activity model.** An asymptotic exponential model fitted to the relative β-galactosidase activity, where "y" is the observed value, "a" is the asymptotic value of y, and "c" is the inflection point of the curve.

$$y = a - e^{-c \times x}$$

Equation 5: **Calculating β-galactosidase activity.** Differentiation of the equation $y=a-e^{-cx}$ can be used to find the gradient "m", where, "a" is the asymptotic value of y, and "c" is the inflection point of the curve.

$$m = a \times c(e^{-c \times x})$$

Equation 6: **Maximum β-galactosidase activity.** The gradient at time zero was calculated for a curve with the equation using the following equation, where "m" is the gradient, "a" is the asymptotic value of "y", and "c" is the inflection point of the curve (note that when x=0, $e^{-c \times x}=1$).

$$m = a \times c$$

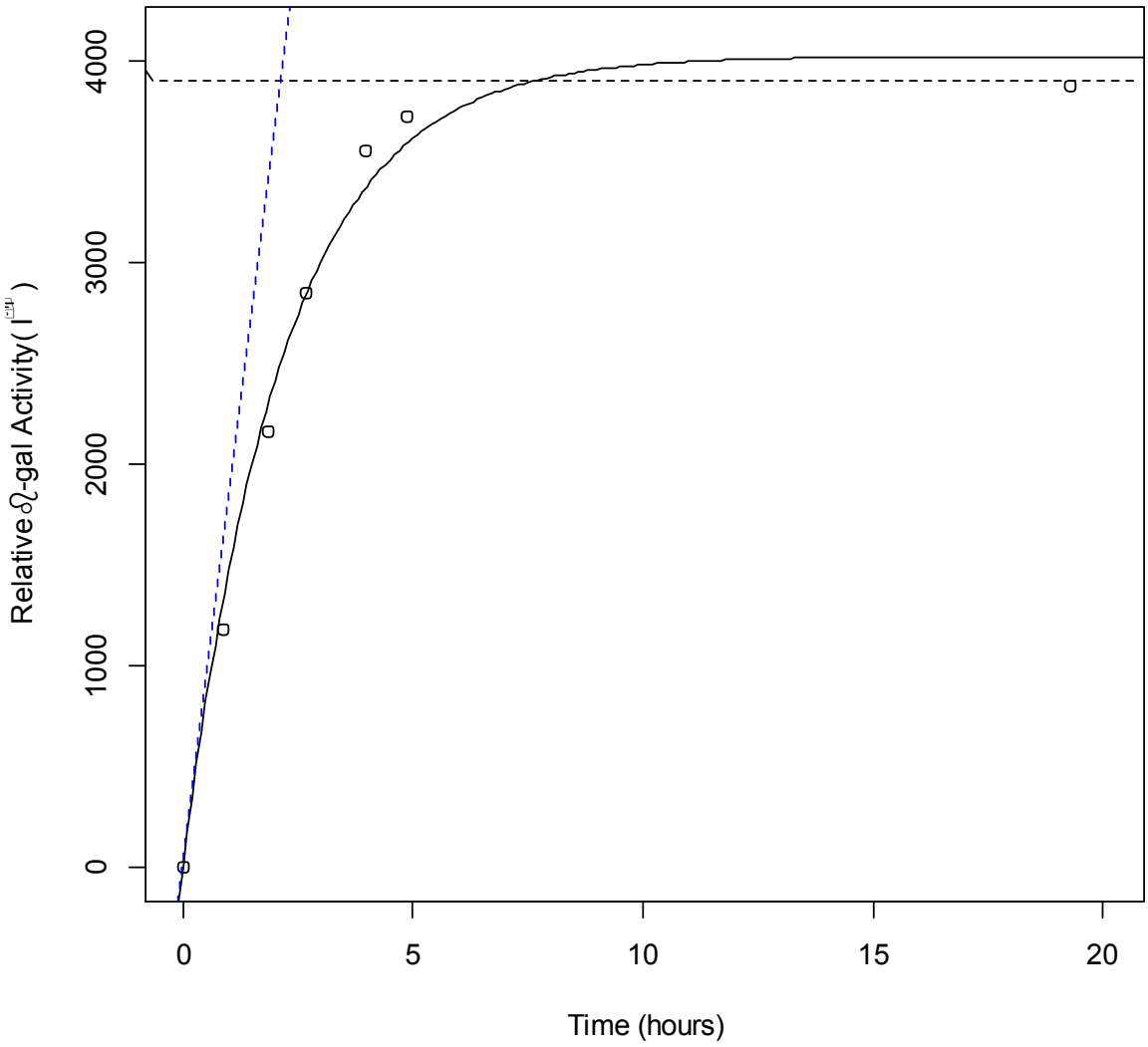


Figure 5: **Calculating the relative β-galactosidase gradient**. The black curve indicates the asymptotic exponential fitted to the data. The diagonal dotted line indicates the maximum reaction rate, V_i, (i.e. the gradient at time zero) for the relative β-galactosidase activity curve (in this case, V_i=1841.90). The horizontal dashed line indicates the asymptote.

3.4.1.4. Selective media plate assays

I transformed yeast with bait-prey pairs specified in Table 4. Single yeast colonies were picked using a 20 µl pipette tip, and resuspended in 1 ml of PBS to create a 1X dilution. This stock was then used to create 10X, 100X, 1000X and 10,000X dilutions in phosphate buffered saline (PBS). From these, 10 µl was plated onto selective media containing SC-Leu-Trp; SC-Leu-Trp-Ura; SC-Leu-Trp-His+10 mM, 25 mM or 50 mM 3AT; and SC-Leu-Trp + 2% 5FOA.

The yeast lines were analysed through plating onto selective media containing 5-fluoroorotic acid (5FOA), or onto media containing 3AT, but lacking histidine (Figure 12 and Table 10). Yeast line MaV203 shows a baseline level of histidine synthesis, which I was able to select against using 10 mM 3-Amino-1, 2, 4-triazole (3AT). The addition of 3AT suppressed basal HIS3 expression, allowing colonies to grow in the presence of 3AT. The URA3 gene allows both positive and negative selection, acting as both an auxotrophic marker, and resulting in the conversion of 5FOA to the toxic product 5-fluorouracil, (Boeke *et al.,* 1987).

3.1.5. Bimolecular fluorescence complementation (BiFC)

3.1.1.5. Expression vector construction

Gateway entry vectors were recombined with Gateway expression plasmids, all of which contained a 35S promoter, using the Gateway® LR Clonase® enzyme mix (Invitrogen), to produce the plasmids in Table 5. Plasmids were grown overnight on LB with 100 µM ampicillin, and a single colony picked and used to prepare a 200 µl culture for midiprep. Midipreps were performed using the NucleoBond® Xtra Midi kit

(Machery-Nagel) as per the manufacturer's instructions. Vectors were sequenced using the plasmids listed in

Supplementary table 16 to confirm the presence of the insert.

Table 5: **Combinations of plasmids used for protein expression and protein-protein interactions in barley.** The following plasmid pairs were used in for bimolecular fluorescence complementation, to investigate the expression patterns of *Blumeria* Effector Candidate 1054 (*bec1054*), Jasmonate Induced Protein 60 (*jip*60), Nucleoside Diphosphate Kinase (*ndpk*), Glutathione-S-Transferase (*gst*), Malate Dehydrogenase (*mdh*), eukaryotic Elongation Factor One Alpha (*eef1a*), eukaryotic Elongation Factor One Gamma (*eef1g*), ribosomal 40S protein 16 (*40s 16*), Pathogenesis Related protein 5 (*pr5*), PR5 without signal peptide *Δpr5*, and *pr10*. A plasmid expressing *dsred* was used to determine which cells had been transformed; pESPYCE was used to produce N-terminal fusions of the C-terminal fragment of YFP; pESPYNE was for N-terminal fusions of the N-terminal fragment of YFP; pUCSPYNE was for C-terminal fusions of the N-terminal fragment of YFP. Expression controls were performed using plasmids encoding C-terminal monomeric YFP (35S-GWY-mYFP) or N-terminal monomeric YFP (35S-mYFP-GWY) respectively.

Plasmid 1	**Plasmid 2**	**Plasmid 3**	**Purpose**
dsRED	NA	NA	autofluorescence control
dsRED	pESPYCE	pESPYNE	autofluorescence control
dsRED	pESPYCE	pUCSPYNE	autofluorescence control
dsRED	pESPYCE/BEC1054	pUCSPYNE	negative interaction control
dsRED	pESPYCE/BEC1054	pESPYNE	negative interaction control
dsRED	35S-mYFP-BEC1054	NA	positive expression control
dsRED	35S-BEC1054-mYFP	NA	positive expression control
dsRED	35S-mYFP-JIP60	NA	positive expression control
dsRED	35S-JIP60-mYFP	NA	positive expression control
dsRED	pESPYCE/BEC1054	pUCSPYNE/GST	interaction
dsRED	pESPYCE/BEC1054	pUCSPYNE/PR10	interaction
dsRED	pESPYCE/BEC1054	pUCSPYNE/NDPK	interaction
dsRED	pESPYCE/BEC1054	pUCSPYNE/eEF1a(1)	interaction
dsRED	pESPYCE/BEC1054	pUCSPYNE/eEF1a(3)	interaction
dsRED	pESPYCE/BEC1054	pUCSPYNE/eEF1G	interaction
dsRED	pESPYCE/BEC1054	pUCSPYNE/40S 16	interaction
dsRED	pESPYCE/BEC1054	pUCSPYNE/MDH	interaction
dsRED	pESPYCE/JIP60ml	pUCSPYNE/GST	negative interaction control
dsRED	pESPYCE/JIP60ml	pUCSPYNE/PR10	negative interaction control
dsRED	pESPYCE/JIP60ml	pUCSPYNE/NDPK	negative interaction control
dsRED	pESPYCE/JIP60ml	pUCSPYNE/eEF1a(1)	negative interaction control
dsRED	pESPYCE/JIP60ml	pUCSPYNE/eEF1a(3)	negative interaction control
dsRED	pESPYCE/JIP60ml	pUCSPYNE/eEF1G	negative interaction control
dsRED	pESPYCE/JIP60ml	pUCSPYNE/40S 16	negative interaction control
dsRED	pESPYCE/JIP60ml	pUCSPYNE/MDH	negative interaction control
dsRED	35S-GST-mYFP	NA	expression control
dsRED	35S-MDH-mYFP	NA	expression control
dsRED	35S-PR5-mYFP	NA	expression control
dsRED	35S-ΔPR5-mYFP	NA	expression control
dsRED	35S-eEF1G-mYFP	NA	expression control
dsRED	35S-40S 16-mYFP	NA	expression control
dsRED	35S-eEF1a(1)-mYFP	NA	expression control
dsRED	35S-eEF1a(3)-mYFP	NA	expression control
dsRED	35S-PR10-mYFP	NA	expression control
dsRED	35S-NDPK-mYFP	NA	expression control
dsRED	pESPYCE/BEC1054	pESPYNE/PR5	interaction

3.2.1.5. Preparation of micro-carriers

Micro-carriers (Gold powder, spherical, APS 0.8-1.5 micron, 99.96+% (metals basis, Alfa Aesar) were prepared as previously described (Elliott *et al.,* 2005; Schweizer *et al.,* 1999). In summary, micro-carriers were weighed out in 30 mg aliquots. They were then coated whilst being vortexed with 5 µl of DNA (1 µl of dsRED, 2 µl of bait plasmid and 2 µl of prey plasmid; or for the positive expression controls, 1 µl of dsRED and 2 µl of expression control plasmid; see Table 3 for details); followed by 50 µl of 2.5 M $CaCl_2$ and 20 µl of spermidine (Sigma-Aldrich, Munich, Germany). Micro-carriers were stored in 60 µl 100% ethanol and kept on ice until bombardment.

3.3.1.5. Bombardment

Primary leaves were harvested from eight day old *H. vulgare* c.v. Golden Promise, which had been grown under long day conditions (16 h light, 8 h darkness, 25^{o}C) in square 10cm pots filled with soil (Einheitserde, Frondenberg, Germany). Leaves were bombarded with the DNA coated micro-carriers. Bombardments were performed using a PDS-1000 / He™ System with a with Hepta™ Adaptor (Bio-Rad, Munich, Germany). For each micro-carrier, 7 µl aliquots were pipetted from a continuously vortexed eppendorf onto a macro-carrier disc (Bio-Rad) and the ethanol allowed to evaporate. The macro-carrier holder was inverted, intercalated with the stopping screen holder, and a clean stopping screen inserted. Rupture disks used were 900psi (Bio-Rad); with a vacuum of 27 inches of mercury.

3.4.1.5. Fluorescence microscopy

Barley leaves, three days post bombardment, were treated with perfluorodecalin, (Sigma-Aldrich) for at least 30 min before imaging, to enhance *in vivo* microscopy

resolution by filling the air spaces in the mesophyll. Perfluorodecalin has a refractive index closer to the cytosol than water, is non-toxic and non-fluorescent (Littlejohn *et al.,* 2010). A Leica TCS SP8-X laser scanning microscope, (Leica, Wetzlar, Germany) mounted with a 20x 0.75 numerical aperture water-immersion objective was used to analyze the leaf samples. For fluorescent protein detection and localisation, dsRED was excited at 561 nm using the 560 diode laser and the fluorescence emission was detected between 600 and 640 nm using a photomultiplier tube detector. The YFP fluorescence was excited at 514 nm with an argon laser and fluorescence emission was detected between 520 and 560 nm using a hybrid detector (Leica). Images and data captures were analyzed with Leica SP8 software.

3.2. Expression profiling of Candidate Secreted Effector Protein family 21

Quantitative real-time PCR (qPCR) work, and control optimization, was performed with the help of Linhan Li (Li 2014). Linhan collected and extracted the RNA for the majority of the experiments, performed repeats of the control optimization assay, and performed the qPCR reactions for the CSEPs, conidia-specific gene, and barley genes.

3.2.1. Sample collection

A vacuum pump was used for conidia collection. Epiphytic material was collected through immersion of barley leaves in 5% cellulose acetate dissolved in anhydrous acetone; evaporation of the acetone; and then collection of the cellulose acetate (which contained epiphytic material). Epidermal peels were then performed to obtain barley epidermal material (and the *B. graminis* material within it). Samples were flash-frozen in liquid nitrogen, and stored at -80^{o}C until further use.

3.2.2. Selected time-points

The samples for the gene normalisation (geNorm) experiments were collected at the following time-points, which represent the beginning, middle and end of *B. graminis*" asexual life cycle: 0 h post inoculation (hpi; ungerminated conidia); 16 hpi (penetration peg formation); two days post inoculation (dpi; new colony formation); and five dpi (colonies become abundant on the leaf surface). The members of CSEP family 21, and a conidia-specific gene, were also measured at four additional time points: four hpi (germinated conidia with primary and secondary appressoria); six hpi (appressorium formation); 24 hpi (haustorial formation) and 3 dpi (colonies become visible to the naked eye) (Both and Spanu 2004). Two biological replicates were used for the geNorm assay, as recommended by PrimerDesign, and three for the assays involving the CSEPs and conidia-specific gene.

3.2.3. Extraction, and analysis of RNA

Quartz sand (50-70 mesh, Sigma Aldrich, St Louis, USA**)** was added to the RNA samples (prepared as per 3.2.1), which were then ground in liquid nitrogen with a mortar and pestle. The RNA was extracted using the QIAGEN RNeasy Mini Kit (QIAGEN) as per the manufacturer's instructions, but with the following modifications, which were found to provide a higher RNA yield: ground samples were incubated in buffer RLT for 20 min; centrifuged (4^{o}C, 8000g, 20 min); and then transferred to the QIA shredder spin column. Two washes were performed using buffer RW1 (QIAGEN).

3.2.4. RNA quality control

A NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to determine the quantity of RNA post-extraction. Only RNA with an OD 260/230 greater than 1.5, and an OD260/280 greater than 1.8 were used for further work

(Manchester 1996, Sambrook and Russell 2001). Further analysis was performed using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, USA), with an Agilent 2100 Bioanalyzer (Agilent). Samples with an RNA Integrity Number (RIN; a measure of RNA integrity between one and 10) greater than 6.5 were used for further work (Fleige and Pfaffl 2006) (Supplementary Table 41).

3.2.5. Reverse transcription

A Precision DNase Kit (PrimerDesign, Southampton, United Kingdom) was used to remove genomic DNA (gDNA) from the RNA samples. Complementary DNA (cDNA) was synthesized, with a 1:1 mixture of Oligo-dT and random nonamer primers, using the NanoScript 2 Reverse Transcription Kit (PrimerDesign). Samples of cDNA were stored at -20^{o}C until further use.

A 7500-Fast Thermocycler (ThermoScientific, Loughborough, UK) was used to perform qPCR, with the primers described in

Supplementary Table 17, Supplementary Table 18, Suplplementary Table 19 and Supplementary Table 20. The PrecisionFAST Mastermix SYBR green detection kit (PrimerDesign) was used with 1 µl primer/probe mix; and 25ng of cDNA template in a 20 µl reaction. The reaction mix and primers were manually added to BrightWhite Real-Time PCR plates (PrimerDesign). The thermocycle program used was: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, and 59°C for 60s. The working concentration utilised for the CSEP primers (Suplplementary Table 19) was 300 nM; with 3 pmol of probe. The CSEP and barley gene primers were ordered, with desalting purification, from Invitrogen (Invitrogen, Carlsbad, CA). Validation of the control primers was performed by PrimerDesign. The optimal annealing temperature for the CSEP primers and barley gene primers was determined through gradient PCR; with an average temperature of 59°C being used. All primers utilised gave a single clean band of the expected size, which corresponded with the predicted length of the amplicon. The primer pairs were also found to give a single melt-curve peak with no shoulder.

3.3. Ribonucleic Acid interactors of BEC1054

Optimisation and troubleshooting of DSF was initially performed by Giulia Bonciani (Bonciani 2014).

3.3.1. Creating a model for barley rRNA

A model for the barley 28S rRNA could not be identified online or in publications. A wheat model was identified on GenBank (Sequence ID: PDB: 3IZ9_A). This model was used to perform a BLAST search of the 454BacContigs available from the International Barley Sequencing Consortium (IBSC)(http://webblast.ipk-gatersleben.de/barley/). An alignment was created using ClustalΩ (http://www.ebi.ac.uk/Tools/msa/clustalo/)

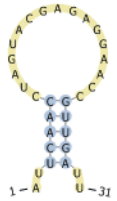
with sequences from wheat (*T. aestivum*; GenBank accession: AY049041), rye (*Secale cereale*; accession: JF489233), maize (*Zea mays*; accession: NR_028022), and a fragment of 28S barley rRNA which had incorrectly been labelled as mRNA (accession: AK248318; data not shown). The alignment was used to predict plausible beginning and end points for the 28S rRNA. The 28S RNA was located within the top BacContig match (HVVMRXALLeA0264I01_c1; IBSC).

The program RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to predict secondary structures of the 28S rRNA. Models created were viewed using PseudoViewer v3.0 (http://pseudoviewer.inha.ac.kr/PVWebService/WSPV_input.asp).

3.1.3.1. Defining the SRL loop

The eukaryotic model for the 28S sarcin-ricin loop (Endo *et al.,* 1988a) from rat (*Rattus rattus*) was aligned with barley 28S rRNA using ClustalW to identify the barley SRL, and used to design RNA fragments (Table 6). The MEGAshortscript T7 Kit (Applied Biosystems, Paisley, UK) was used to synthesise the RNA fragments *in vitro* from primer templates (Table 6).

Table 6: **RNA sequences and their secondary structures.** The RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to predict the thermodynamic ensemble secondary structures of the 28S rRNA. Models created were viewed using PseudoViewer version 3.0 (http://pseudoviewer.inha.ac.kr/PVWebService/WSPV_input.asp). The conserved "loop" part of the ribosomal Sarcin-Ricin Loop is highlighted in yellow for the rat and barley sequences.

Name	**Sequence**	**Secondary structure prediction**
Rat SRL +T7	ACCUGCUCAGUACGAGAGGAACCGCAGGU	
Barley SRL 3014-3044 +T7	AUUCAACCUAGUACGAGAGGAACCGUUGAUU	
Barley 76-114 +T7	UCCCCUAGUAACGGCGAGCGAACCGGGAACAGCCCAGC	

RNA synthesis reactions were terminated after 24 h, and phenol-chloroform extractions performed (Applied Biosystems), with the addition of one extra chloroform extraction step. Samples were precipitated (-80°C ≥30mins), centrifuged (≥10,000g, 45mins), ethanol removed, and RNA re-suspended in DEPC water. Synthesized RNA fragments were run on 17% polyacrylamide gels (6.4ml 40% acrylamide: bisacrylamide (37.5:1), 0.75ml 10xTBE, 4.2ml DEPC water and 8 M urea; with 7.5 µl TEMED and 30 µl 25% APS).

3.3.2. Yeast-three-hybrid assay

3.3.3. Construction of RNA expression plasmids

For the RNA expression plasmids, synthesis of cDNA was performed from uninfected barley rRNA as in chapter 3.1.1.3, using primer pairs specified in (

Supplementary Table 22). Phusion® High-Fidelity DNA Polymerase (NEB) was used to perform PCR of the RNA products. Primers were designed using the online NEBuilder assembly tool (http://nebuilder.neb.com/ , NEB). The RNA expression plasmid, pIIIA/MS2-2, was linearized using the restriction enzymes XmaI and SphI (NEB), and cleaned using the QIAquick PCR Purification Kit (QIAGEN), before insertion of the product through Ligation Independent Cloning (LIC) (New England Biolabs, MA, US) as per the manufacturer's instructions. For Y3H, strain YBZ1 was transformed as described in the Invitrogen protocol using the plasmids in Table 7; and plated onto SC-Leu-Ura. Protein plasmid construction was described in section 3.1.1.3.

3.3.4. Testing reporter genes

Colonies were assayed, and statistical analyses performed, as described in the Quantitative β-galactosidase assay (6.1.3.6.). Selective media assays were performed as per section 3.4.1.4, but with plates containing SC-Trp-Ura (tests transformation) and SC-Trp-Ura-His+10 mM 3AT (tests histidine synthesis).

Table 7: **Combinations of plasmids used to investigate protein-RNA interactions in yeast-three-hybrid.** The following plasmid pairs were used in a yeast-three-hybrid assay, to investigate the interactions of various proteins with BEC1054 and JIP60.

Bait plasmid	Prey plasmid	Purpose
pEXP32/BEC1054	pIIIA/rRNA2989_3139-MS2	interaction under investigation
pEXP32/BEC1054	pIIIA/rRNA13_363-MS2	interaction under investigation
pEXP32/BEC1054	pIIIA/IRE-MS2	negative interaction control
pEXP32/JIP60	pIIIA/rRNA2989_3139-MS2	interaction under investigation
pEXP32/JIP60	pIIIA/rRNA13_363-MS2	interaction under investigation
pEXP32/JIP60	pIIIA/IRE-MS2	negative interaction control
pAD-IRP	pIIIA/IRE-MS2	positive control
pEXP32/BEC1054	pIIIA/IRE	negative activation control

3.3.5. Differential Scanning Fluorimetry (DSF)

Expression and purification of BEC1054 was performed by R. Jones using the pET28a-LIC expression vector (Structural Genomics Consortium, Toronto, CA) in *Escherichia coli* strain Shuffle (New England Biolabs, MA, US). DSF was performed with 10 µM BEC1054, 1 µl SYPRO Orange (Sigma)(1:250 dilution in 20 mM Sodium Acetate (NaAc) pH 4.6, 150 mM NaCl), one to 20 µM template and buffer (20 mM NaAc, pH 4.6, 150 mM NaCl), reaction volume 40 µl; in a 7500-Fast Thermocycler (ThermoScientific, Loughborough, UK); temperature gradient from 25-99^{o}C, 1% ramp rate (i.e. where the temperature is increased by 1% of the total temperature gradient for each percentage of the allotted time). Templates were either a) synthesized RNA, b) DNA primers used in RNA synthesis or c) an 82 kDa Heat Shock Protein primer (HSP82): 5'-GCGTCGGACGTGCAGATGGGC-3'.

SigmaPlot v10.1 (Windows) was used to fit a "sigmoidal dose-response (variable slope)" (Equation 9) and derive the inflection point of the sigmoidal (logEC50) which indicates the "melting point" of BEC1054 (Figure 31).

Equation 7: **Calculating the inflection point of a sigmoidal curve**. Where "y" is the observed value, "max" is the maximum observed value, "min" is the minimum observed value, and "Hillslope" gives the largest absolute value of the slope of the curve.

$$y = \min + \frac{(\max - \min)}{1 + 10^{(logEC_{50} - \chi)^{Hillslope}}}$$

3.3.6. Expression of BEC1054 in wheat

Wheat, *Triticum aestivum* L. cv. Cerco, was grown as per section 3.1.1. It was used as the host for an unnamed Fielder wheat-compatible *Blumeria graminis* f.sp. *tritici* strain, and was provided by the NIAB (UK).

3.4. Stable expression of the *Blumeria* effector BEC1054 as a transgene in wheat

Genotyping and wheat non-host infection work was conducted with Peggy Luong (Luong 2014). Chlorophyll senescence assays and RNA extraction/analysis were conducted with Thomas Chandler (Chandler 2015) and Hannah Thieron. *Nicotiana benthamiana* non-host infection assays were performed with Hannah Thieron.

3.4.1. Genotyping

Triticum aestivum cv. Fielder had previously been transformed with wobble BEC1054 (*wbec1054*) or wobble BEC1011 (*wbec1011*) through *Agrobacterium*-mediated transformation at the National Institute of Agricultural Botany (NIAB, Cambridge). The wobble BECs do not possess a signal peptide, so that they remain within the plant cytosol. The wobble BECs are synthetic genes which do not possess a signal peptide, and

which have silent "wobble" mutations which minimize the nucleotide sequence identity with the wild-type *B. graminis* genes (Pliego *et al.,* 2013). All transgenic wheat seeds were sown in two by 2 cm chambered propagation trays in Levington® Seed and Modular Compost Plus Sand F2S (Everris) and 2 g/l Osmocote Patterned Release Fertiliser (Everris). Once they had begun to develop the second leaf, they were transferred to 9x9 cm square plastic pots containing the same mixture.

Work done at NIAB determined the copy number of the transgene in the T_0 generation. The T_1 lines PS1.16, PS2.4, PS3.3 and PS4.4 were selected for further investigation by Giulia Bonciani because they were one-copy lines with the highest expression (as determined through qPCR relative to wheat β-tubulin, TUBBw). This was further repeated for the T_2 generation. The lines 3.3.7 and 3.3.14 were homozygous (+/+) for wBEC1054 and had the highest amount of transcript relative to tubulin. The lines 3.3.11 and 3.3.12 were found to be homozygous null plants, and will be henceforth referred to as azygous (-/-), and were used as negative controls for further work (Bonciani 2014).

Seeds from the T_3 and T_4 generations of lines 3.3.14 and 3.3.12 were planted in work conducted with undergraduate student Peggy Luong. Seed dormancy was broken through five days of incubation at 32°C during the day and 4°C at night. Leaf disks were removed from the primary leaves using a flat-ended blunt 1mm diameter sterile needle. Leaf disks were used as the substrate for direct PCR, using the KAPA3G Plant Direct PCR kit (KAPA Biosystems, London, UK). Following experimental optimization, the following conditions were used: KAPA PCR mix contained 25 µl 2xKAPA Plant PCR buffer, 1.5 mM $MgCl_2$, 0.3 µM FWD primer, 0.3 µM REV primer, 0.5 µl KAPA Plant PCR enhancer and 1U

KAPA3G Plant DNApol. Amplification was performed in a GS1-thermalcycler (G-Storm), with 2 min at 95°C, 30 cycles of 40 s at 95°C, 20 s annealing at 50°C, 30 s at 72°C and 1 min at 72°C. Transgene expression was confirmed in the T_4 plants via endpoint reverse transcription. The total RNA was extracted, and cDNA made as per section (3.1.1.3). This cDNA was then used as the template for PCR, as per section (3.2.1.3), using the primers specified above for *wbec1054* and for *tubbw* (Luong 2014).

3.4.2. Phenotyping

Transgenic wheat from the T4 generation of lines homozygous for wBEC1054; or azygous (3.3.14 and 3.3.12 respectively); was assayed to determine whether wBEC1054 had an effect on the phenotypic characteristics of adult wheat (Figure 6). The eleven characteristics assayed were the total number of leaves (for all tillers); maximum height; subcrown length; ear length (including whiskers); peduncle (internode 1) length; the length of the three remaining internodes; the number of fertile tillers; the mass of the tillers; and the number of grains.

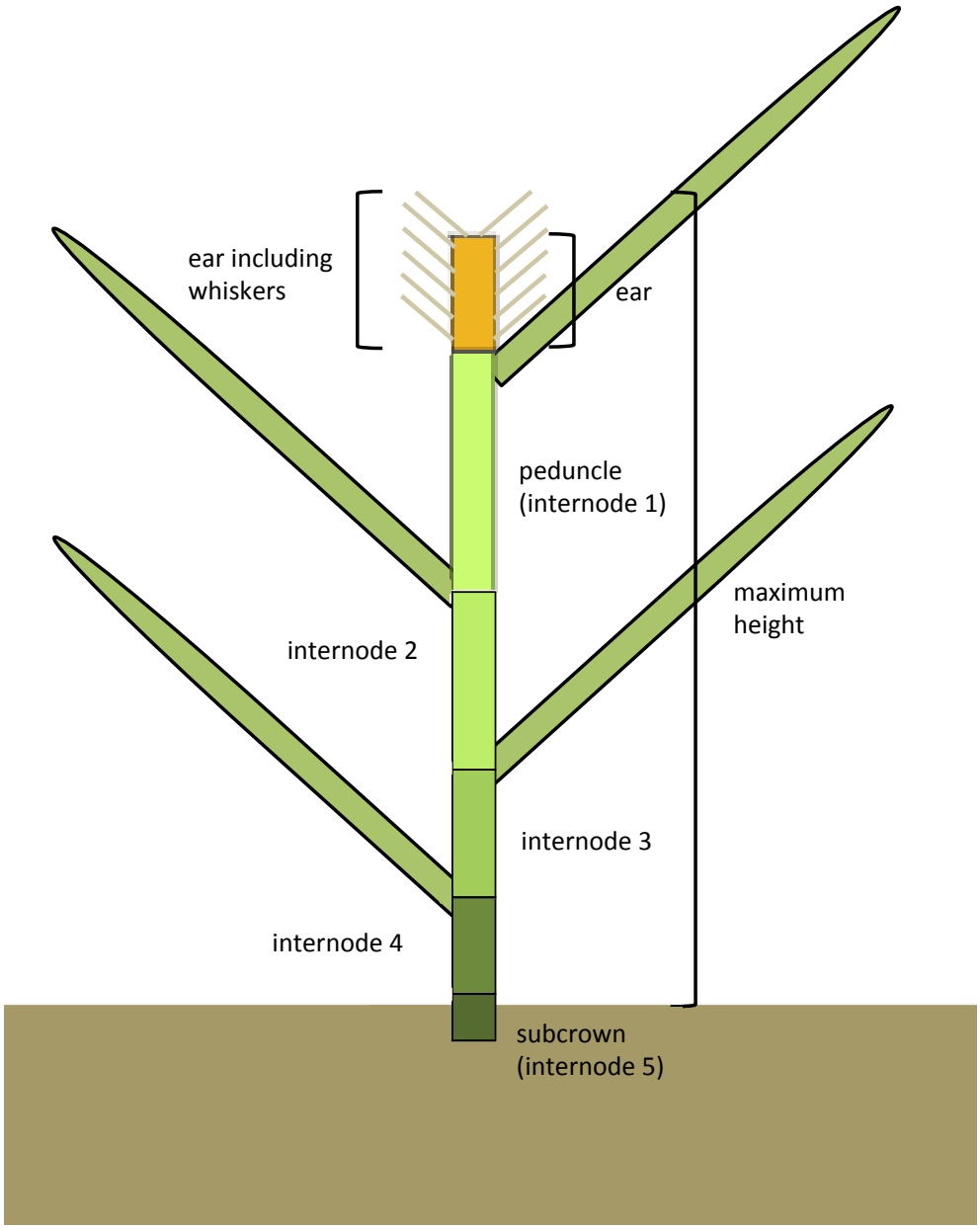


Figure 6: **Characteristics of wheat investigated in a phenotyping assay.** The stylized wheat diagram indicates the main characteristics investigated for wheat plants during a phenotyping assay.

3.4.3. Non-host *Blumeria* infection assay

Blumeria graminis f.sp. *tritici* was used to infect either T_4 plants grown for three weeks, or T_3 plants grown for 11 weeks, under the growth conditions listed in 3.1.1. One leaf was removed from each plant, and 3 2 cm-long segments taken from the tip, middle and base of the leaf using a flat blade. Segments were placed onto wet paper, and inoculated

with *Blumeria graminis* f.sp. *tritici* on the adaxial leaf surface. Even dispersal of conidia was achieved using a Hair Design 1000 hairdryer (Philips, Amsterdam, Holland). One hour following inoculation, leaf segments were transferred to water agar plates (0.5% agar supplemented with 16mg/L benzimidazole) with the adaxial side up. Plates were stored for three days within the same conditions as for *Blumeria graminis* f.sp. *hordei* (3.3.6).

The infected leaf segments were stained in 0.1% trypan blue stain in ethanolic lactophenol (1:3.35) (RAL Diagnostics, Martillac, France), and incubated for 2 h at 80^{o}C; followed by destaining in 2 mg per ml of chloral hydrate for 2 h. The fungal structures were viewed using a Carl Zeiss Axioskop 2 plus microscope (Zeiss, Cambridge, UK). The number of colonies, which formed epiphytic hyphae, was measured as a proxy for the presence of at least one functional haustorium. The proportion of germinated conidia that formed at least one haustorium (propH) was calculated using Equation 8.

Equation 8: **Calculating the proportion of conidia which formed at least one haustorium.** Where the abbreviation "propH" refer to the proportion of germinated conidia that formed at least one haustorium; "H" to the number of haustoria; and "NH" to the number which did not form haustoria.

$$propH = H/(H + NH)$$

3.4.4. Chlorophyll senescence assay

Transgenic wheat from the T_5 generations of wheat lines 3.3.7 (+/+), 3.3.14 (+/+), 3.3.11 (-/-) and 3.3.12 (-/-) was grown in the conditions described in section 3.1.1 for seven days. The wheat plants were then infected on three consecutive days via shaking *B. graminis* f.sp. *tritici* infected plants over them, to ensure a heavy infection, or they were left uninfected as a control.

Senescence was induced in the 10 day old seedlings through floating the detached primary leaves in 100ml of 45 µM MeJA (Chou and Kao 1992, Hung *et al.,* 2006), or in water, for a further five days in a 14cm diameter petri-dish. Petri-dishes were kept within the same controlled growth chambers as the wheat lines.

Leaf protein concentration was determined through the homogenization of a 1 cm leaf segment in 50 mM sodium phosphate buffer, pH 6.8. Samples were centrifuged (14,000g, 15 min, 4°C), and the supernatant used for protein quantification in a Bradford assay (Bradford 1976), but with 5 µl of supernatant being added to 250 µl in a 96-well microtitre plate. Absorbance was measured at 595nm in a Tecan 3000 (Tecan Group Ltd, Männedorf, Switzerland). The pellets produced through centrifugation were re-suspended in 96% ethanol to extract the chlorophyll; and re-centrifuged (14,000g, 15 min, 4°C). Absorbance was measured at 649, 665 and 750nm; and used to calculate the concentration of chlorophyll a and b (Equation 9 and Equation 10; from (Wintermans and de Mots 1965, Nakanishi *et al.,* 2001) also using a Tecan 3000. The chlorophyll concentrations were normalised using the results from the Bradford assay.

Equation 9: **Calculating chlorophyll a concentration.**

$$Chlorophyll\ a\ (\mu g/mL)\ =\ 13.70(A665 - A750) - 5.76(A649 - A750)$$

Equation 10: **Calculating chlorophyll b concentration.**

$$Chlorophyll\ b\ (\mu g/mL)\ =\ 25.80(A649 - A750) - 7.60(A665 - A750).$$

A GLM was performed in R (Equation 16). Following model simplification, a minimal model was produced (Equation 17).

Equation 11: **A maximal Generalised Linear Mixed Model for chlorophyll a data before simplification.** The model contains all possible interactions for the explanatory factors "Water_MeJA" (referring to treatment with water or methyl Jasmonate), "infection" referring to infection with *Blumeria graminis* f.sp. *tritici*, and "line" referring to which seed line was used.

$$glm(chlorophyll\_a \sim Water\_MeJA * Infection * Line)$$

Equation 12: **A minimal Generalised Linear Mixed Model for chlorophyll b data after simplification.** The model contains all possible interactions for the explanatory factors "Water_MeJA" (referring to treatment with water or methyl Jasmonate).

$$glm(chlorophyll\_b \sim Water\_MeJA)$$

3.4.5. RNA extraction and analysis

Total RNA was extracted from primary wheat leaf material. Extractions were performed using the Qiagen RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Up to 1 µg of extracted RNA was treated with 10 µl of 1 M aniline (≥99.5%, Sigma) at 60°C for 3 min. Following this, 2 µl of five molar ammonium acetate stop solution with 100 mM EDTA (Life Technologies, 2012) was added. Samples were mixed with 1 ml of 99.9% ethanol; frozen at -80°C for at least 20 min; and precipitated through centrifugation at 4°C for 45 min. The concentration of RNA post-aniline treatment was determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific); and the total RNA analysed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), with the Bioanalyzer RNA Nano 6000 kit. Peak area assignment was performed manually, as per Figure 7, using Agilent Technologies 2100 Expert Software (2100 Expert, 2009).

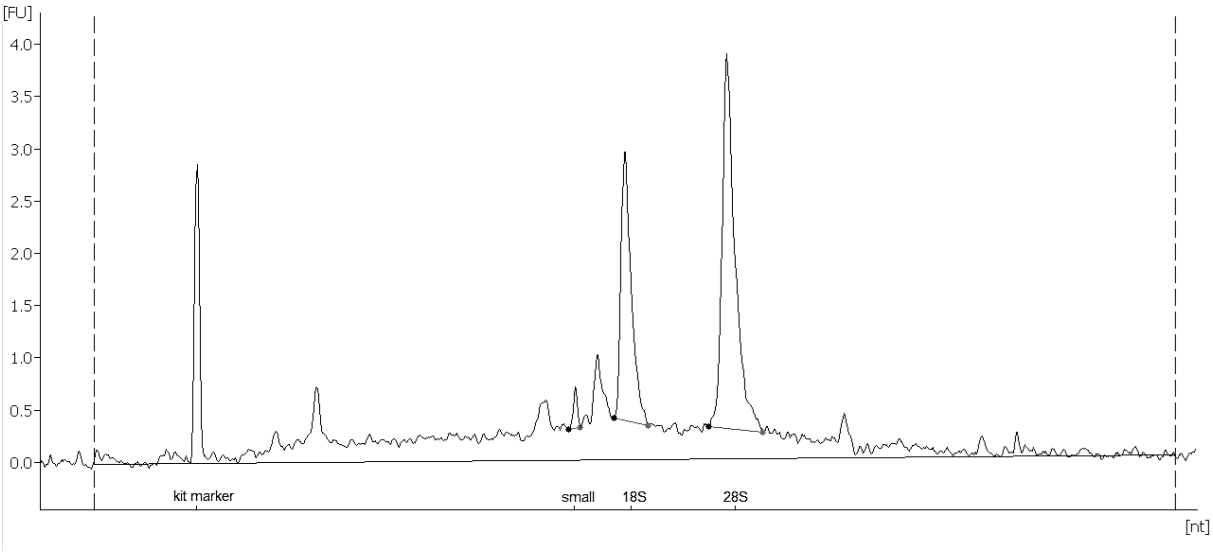


Figure 7: **Measuring Bioanalyzer RNA peaks**. Extracted total RNA was run on a Bioanalyzer RNA Nano 6000 chip, and analysed using Agilent Bioanalyzer 2100. Peak areas were assigned manually. The abbreviations "FU" stands for fluorescence units, "nt" for nucleotides", "kit marker" for a 25nt marker provided with the kit. The three peaks of interest are labelled "small" for the small diagnostic peak, "18S" for the 18S ribosomal subunit, and "28S" for the large ribosomal subunit.

3.5. Transient expression of BEC1054 in *Nicotiana benthamiana*

Nicotiana expression assays were performed with the help of Hannah Thieron and Michal Przydacz. Cloning was done with the help of Sian Morgan (Morgan 2014) and Joe Yu.

3.5.1. *Nicotiana benthamiana* growing conditions

Levingtons F2+S seed compost was mixed 2:1 with 2-5 mm Vermiculite (Sinclair, Lincolnshire, UK), and used to grow *Nicotiana benthamiana* in 9 cm square pots, with one plant per pot. Plants were watered once every four days, and grown with 16 h light, 8 h darkness, 33% humidity at 25^{o}C.

3.5.2. *Nicotiana* expression vector construction

Expression vectors were created using the entry vectors specified in section 3.3.1.3 using the Gateway® LR Clonase® enzyme mix (Invitrogen). Entry vectors were recombined with the *Agrobacterium* expression plasmids pK7FWG2 (which contains C-terminal GFP), pK7WGF2 (which contains N-terminal GFP), or pB7RWG2 (which contains N-terminal RFP) to give the expression vectors listed in Table 8.

Table 8: ***Agrobacterium* fluorescent expression plasmids**. The *Agrobacterium* expression plasmids pK7FWG2 contains C-terminal GFP, pK7WGF2 contains N-terminal GFP, and pB7RWG2 contains N-terminal RFP. Vectors marked "*" were already available at the beginning of this project. The plasmid pK7FWG2 strongly expresses GFP.

Gene	Expression vector
Blumeria Effector Candidate 1054	pB7RWG2/BEC1054
Blumeria Effector Candidate 1054	pK7FWG2/BEC1054*
Blumeria Effector Candidate 1054	pK7WGF2/BEC1054*
Ribosomal 40S protein 16	pK7FWG2/40S 16
Jasmonate induced protein 60 with methionine-leucine linker	pK7FWG2/JIP60ml
-	pK7FWG2*

3.5.3. Transformation of Agrobacterium

Agrobacterium tumifecans strain GV3101 was transformed with the constructs of interest (Table 8); and colonies were grown for two to four days at 28°C on LB-agar media containing 100µg/ml spectinomycin for the plasmids pB7RWG2, pK7FWG2, or pK7WGF2; or 50µg/ml ampicillin for the colocalisation vectors listed in section 3.5.3. *Agrobacterium* was aspirated from the surface of the plate, and suspended in MMA buffer (10 µM MES (2-[N-morpholino] ethanesulfonic acid) and 10 µM $MgCl_2$, pH 5.7). Bacteria were centrifuged (8000g, 5 min), and re-suspended in 10 mM MMA buffer. The OD_{600} was measured, and used to create 2 ml of bacterial suspension, with a final OD_{600} of 0.2 for GFP constructs, or 0.5 for RFP constructs.

3.5.4. Agroinfiltration

Healthy-looking, *Nicotiana benthamiana* plants, with suitable-sized leaves were infiltrated with *Agrobacterium*, for transient gene expression, at around three weeks old. A 1 ml syringe was used to infiltrate the suspension into the underside of leaves three and four; as these provided the best expression for transient *Agrobacterium* mediated gene expression (T. Bozkurt, *pers. comm*). Two to 4 dpi, leaves were harvested. Harvested leaves were maintained for agroinfiltration in clear plastic boxes on damp paper towel, with 16 h light, 8 h darkness, at 18^{o}C. Transformed areas of leaves were mounted in water, and analysed using a Leica SP5 resonant inverted confocal microscope with 63x objective. Excitation and emission wavelengths were 488 nm and 495 nm respectively for GFP, 488 nm and 680 nm for plastid autofluorescence, and 543 nm and 588 nm for RFP. The GFP and autofluorescence were excited using a helium-neon laser, and RFP with an argon laser. Image analysis was performed using Leica LAS X (Leica Microsystems, Milton Keynes, UK) and Fiji (Schindelin *et al.,* 2012).

3.5.5. BEC1054 colocalisation assay

Colocalisation was performed for the fluorescence signal from BEC1054-GFP with the fluorescence signal of RFP tagged subcellular markers for Golgi, mitochondria, ARA6 and ARA7 (Geldner *et al.,* 2009); and the mitochondrial marker with 40S 16-GFP. Colocalisation experiments were also performed for the RFP mitochondrial marker and 40S 16-GFP.

3.5.6. *Peronospora tabacina* non-host pathogenicity assay

The barley proteins JIP60 and BEC1054 were Agroinfiltrated into one half of an *N. benthamiana* leaf; and a GFP only construct into the other (Figure 8), using the plasmids in Table 8. Agrobacterium was used at an OD_{600} of 0.5. The plants were infected within 2

h of infiltration using *Peronospora tabacina*. For the infection, an infected leaf was shaken in 5 ml of H_2O; and the number of sporangia in 0.004 mm^3 counted. Sporangia were diluted to 25,000 in 10 µl, and the sporangia dilution was then pipetted onto six spots on each leaf (Figure 8). Nine dpi, three punches were taken from each leaf and shaken gently in 1 ml of H_2O. Sporangia were counted via microscopy, with five biological replicates (separate leaves from separate plants) being used for each pair of infiltrations.

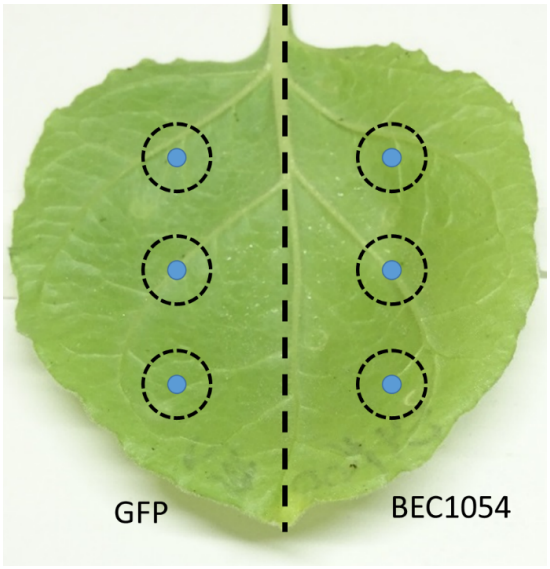


Figure 8: **Infiltration of *Nicotiana benthamiana* with *Agrobacterium* and infection with *Peronospora tabacina.*** Half of each *N. benthamiana* leaf was infiltrated with *Agrobacterium* transformed with the protein of interest, i.e. BEC1054, in the plasmid pK7FWG2. The other half of the leaf was infiltrated with *Agrobacterium* transformed with the plasmid pK7FWG2 containing GFP only. The black central line denotes the divide between the two halves. Within 2 h of infiltration, leaves would be infected with sporangia of P. tabacina suspended in H_2O. Nine days post infection, 1 cm punches were taken from the leaves (black circles), the spores suspended in H_2O, and counted.

4. Protein interactors of BEC1054

4.1. Introduction

Fungi (Panstruga and Dodds 2009, Spanu *et al.,* 2010), oomycetes (Ellis *et al.,* 2009, Tyler 2009), bacteria (Block *et al.,* 2008) and other pathogens secrete effector proteins into host plant cells in order to aid parasitism. The vast majority of the targets and activities of these effectors are unknown (Alfano 2009).

The discovery of effector targets, and their confirmation requires multiple, complementary approaches (Alfano 2009). Protein-protein interaction assays are the main method used for the identification of the host plant targets of effectors; for example the *Arabidopsis* RIN4 protein was identified as a target of *Pseudomonas syringae* AvrB through a yeast-two-hybrid (Y2H) assay (Mackey *et al.,* 2002). The *P. syringae* effectors AvrPto and AvrPtoB have been shown through Y2H to interact with *Solanum lycopersicum* Pto kinase (Tang *et al.,* 1996, Kim *et al.,* 2002); and protein co-immunoprecipitation or affinity purification (also known as pull-down) assays have been used to show that these effectors interact with pathogen-associated molecular pattern receptors (Zhang *et al.,* 2007).

In vitro approaches, such as pull-down assays, can be used to screen for putative interactors of effectors. The bait protein is expressed with a tag, for example a polyhistidine-tags (Hochuli *et al.,* 1987, Hochuli *et al.,* 1988); purified; and bound with an affinity resin containing a chelator such as nickel nitrilotriacetic acid (Ni-NTA) (Bornhorst and Falke 2000). Following this, the effector bound beads are incubated with plant lysate, washed, and the bound proteins eluted. Protein interactors can then

be identified through liquid chromatography mass spectrometry (LCMS). Affinity-LCMS techniques have a number of strengths: they can be used to isolate complexes of proteins from cell lysates; and they can be used to identify post-translational modifications which may be required for the interaction (Gingras *et al.,* 2007). Affinity purification is, however, associated with false positive results. Frequent contaminants include proteins which interact with the affinity matrix; proteins which bind unfolded peptides; and abundant proteins (for example ribosomal proteins, tubulin and actin) (Plocinski *et al.,* 2014). Ribosomal proteins, along with other abundant proteins, are common false positives identified in protein affinity-LCMS studies (Plocinski *et al.,* 2014). This is a problem when it is believed that the true interactors for the bait could be ribosomal proteins. In this study, three *in vitro* LCMS experiments were performed to identify putative protein interactors of BEC1054, BEC1005 (a putative glycosidase) or empty Ni-NTA columns, and the results compared using proportion tests.

The Y2H assay provides a useful method for the binary identification/validation of proteins and their interacting partners (Brueckner *et al.,* 2009). Interaction of the two hybrid proteins leads to the reconstitution of a functional GAL4 transcription factor, and expression of reporter genes (Figure 9)(Fields and Song 1989). The three reporter genes (*his3*, *lacZ* and *ura3*) each possess an independent promoter (MacDonald 2001), which helps to reduce false positives: only fairly strong transcriptional up-regulation will lead to the expression of all three genes. This can, however, lead to the missed detection of weak interactions. False positives can occur through the bait and prey proteins being strongly expressed within the same yeast cell compartment; they can interact with the reporter proteins, or the DNA binding protein; or they can bind

proteins in a non-specific manner. False positives can occur due to a lack of interacting partners or post-translational modification, misfolding of the protein of interest due to the fused yeast protein, the reaction involving a membrane protein, or due to the interaction being too weak/transient to detect (for a review, see (Brueckner *et al.,* 2009)).

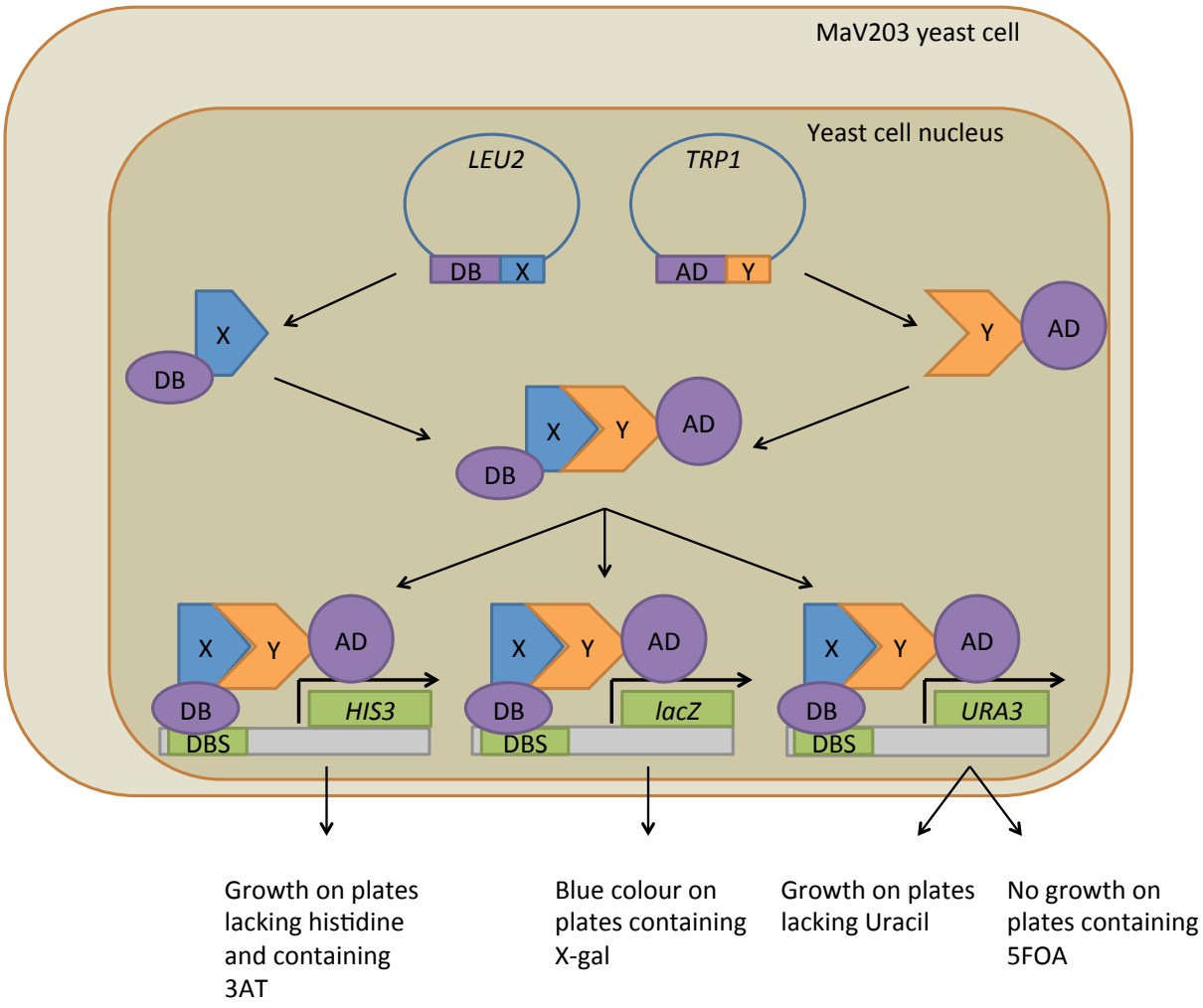


Figure 9: **Basis of the yeast-two-hybrid screen.** Yeast line MaV203 is transformed with plasmids carrying Leucine (LEU1) and Tryptophan (TRP1) selectable markers. The first plasmid encodes the first hybrid protein, the bait, which is composed of a DNA-binding domain (DB) fused to the protein of interest (X) to give DB-X. The second plasmid encodes the prey, which is composed of an activation domain (AD) fused to the putative interactor (Y) to give DB-Y. interaction of DB-X and AD-Y leads to the reconstitution of an active GAL4

transcription factor, leading to the transcription of reporter genes *his3, lacz* and *ura3* within the yeast chromosome (Fields and Song 1989).

Bimolecular fluorescence complementation (BiFC), also known as split-Yellow Fluorescent Protein (split YFP), is a powerful method used for the identification of protein-protein interactions in the host plant. The assay is based upon the reconstitution of a fluorescent protein, for example YFP, through the interaction of two proteins fused to the two halves of the fluorescent proteins (Figure 10) (Ghosh *et al.,* 2000). This method can be used to tell where proteins interact within the cell (Hu *et al.,* 2002), removing the requirement for exogenous staining . One of the main drawbacks of BiFC is the tendency for the two halves of the fluorescent protein to reassemble in the absence of interacting protein partners. This can be overcome through the use of appropriate controls (Horstman *et al.,* 2014).

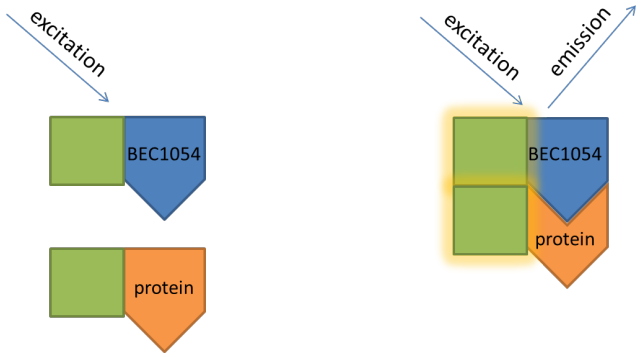


Figure 10: **Basis of Bimolecular Fluorescence Complementation (BiFC).** Two proteins of interest (i.e. BEC1054 and GST) are expressed fused to two fragments of a fluorescent reporter protein. The interaction of the two proteins of interest brings the two fluorescent fragments together. This allows the fluorescent reporter to reform, causing it to emit a fluorescence signal following excitation.

4.2. Aims and Objectives

AIM: To determine whether a greater proportion of ribosomal proteins were identified with BEC1054 *in vitro* than with an unrelated BEC, BEC1005.

OBJECTIVE: To produce count data for the frequency of occurrence of ribosomal proteins in a list of proteins identified through LCMS, and to determine the proportion of ribosomal proteins in comparison with the negative controls.

AIM: To investigate the interaction of BEC1054 with putative protein interactors through the use of a yeast-two-hybrid assay (Y2H).

OBJECTIVE: To utilise a Y2H assay to validate a series of protein interactors previously identified in the host laboratory through Liquid Chromatography Mass Spectrometry (LCMS).

AIM: To investigate the interaction of BEC1054 with protein putative interactors in barley through the use of Bimolecular Fluorescence Complementation (BiFC) analysis.

OBJECTIVE: To carry out experimental work in the laboratory of our collaborators, in Aachen, to determine whether BEC1054 interacts with the protein interactors in barley.

4.3. Individual Contributions

The leaf and epidermal material for the pull-down assays were harvested and processed by Dana Gheorghe. The LCMS, and analysis, were performed by Laurence Bindschedler.

I completed the Y2H work with the help of Masters Student Annabelle Damerum, whom I co-supervised. Annabelle cloned the barley genes *eef1g* and *pr10* into the entry vector pCR8, and recombined them into the expression vectors pDEST32 and pDEST22.

4.4. Results

4.4.1. Analysis of LCMS data

At the beginning of this study, pull-down assays and LCMS had already been performed for BEC1054, with an unrelated effector, BEC1005 as a negative control (Bindschedler, *pers. comm.*). I validated a number of these interactors through Y2H and BiFC, using the combination of the three different methods to confirm their interaction with BEC1054.

A list of putative interactors for BEC1054 (Supplementary Table 38) was generated, via a macro in Excel, comtaining proteins with at least two significant peptides. from the LCMS data. Proteins were included which occurred across two of the three datasets (A, B or C, see Table 1 for definitions) or which occurred at least twice in the replicates. Proteins highlighted in grey were selected for further validation via Y2H. Dark grey indicates that the protein was found in some negative controls, because groups of U36 HarvEST accessions were associated in the BEC1054 pull-downs with the same UniRef90 UniProt accessions (Supplementary Table 39). Interactors for further validation were initially selected from Supplementary Table 38. Included in this list were NDPK (accession Q9LKM0); eEF1A (accession Q9LN13) and 40S 16 (Q0IQF7). Re-analysis of the pull-down data, using the U36 accessions (Supplementary Table 40), identified these proteins as occurring in the negative controls. Therefore they may not represent specific interactors.

Many of the putative interactors identified through the UniRef90 database (Supplementary Table 38) were also identified as occurring solely with BEC1054 when

using the U36 accessions (Supplementary Table 38). We further investigated the interaction of BEC1054 with proteins from this list including the ribosomal elongation factors eEF1A (accession Q9ZSW2), and eEF1G (accession Q5Z627); a thaumatin-like PR5 protein (accession O23997); a PR10 protein (accession Q84QC7) and a zeta class glutathione-S-transferase (zGST IN2-1, accession Q8H8U5). A malate dehydrogenase (MDH, Q6YWL3) was also included as a possible interactor, despite being identified in a few negative controls, as it occurred in most BEC1054 pull-downs.

Further re-analysis of the data was performed, by our collaborators, using the IBSC barley database (Supplementary Table 40). This analysis was performed using more stringent criteria than those used for the U36 (HarvEST), with all proteins found in any negative control being excluded from the dataset. The putative interactors identified included a number of the targets under investigation (Supplementary Table 38 and/or 39), for example eEF1G, and the thaumatin-like PR5.

4.4.2. Statistical analysis of U36 HarvEST LCMS results

Statistical analyses were performed on the proportions of barley proteins identified via BLAST searching the identified peptides against the U36 HarvEST database. A total of 2508 sequences were identified through *in vitro* chromatography across the three experiments (Pennington *et al.,* 2016). Of these, 247 occurred only with BEC1054 and 1241 were found solely with BEC1005.

A greater percentage of ribosomal large subunit (LSU)-related sequences were identified with BEC1054 for non-infected leaf material (Table 9); BEC1054 with whole leaf infected material; and for BEC1054 as a whole. Similarly, a greater number of

ribosomal small subunit (SSU)-related sequences were identified with BEC1054 for infected epidermal material; non-infected whole leaf material; and infected whole leaf material. This increase was highly significant ($p<0.005$) for the total number of SSU-related sequences identified with BEC1054 when the empty agarose bead sequences were excluded. Furthermore, a greater number of elongation-related sequences was identified for non-infected whole-leaf material; infected whole-leaf material; and for the total number of sequences identified with BEC1054.

Greater numbers of RNA-related sequences were identified with BEC1005 for the search-terms "RNA", "initiation" and "ribosome" for nearly all experiments. The exceptions are for search term "RNA" with seven day infected leaves with magnetic Ni-NTA agarose beads and "ribosome" for non-infected leaf material with one ml Ni-NTA chromatography columns (no sequences were identified in this category for BEC1005 or BEC1054). Interestingly, there was no difference more significant than $p<0.1$ for any of the experiments for these three search terms.

Table 9: **Barley RNA-related sequences identified with *Blumeria* Effector Candidate 1054 (BEC1054) and BEC1005.** The proportions of *Blumeria* effector candidates identified with BEC1054 and 1005 were calculated; and used to perform a proportion test in Rv3.0.2. Three separate experiments were performed: "A': 48h infected leaf epidermis with magnetic Ni-NTA agarose beads; "B': non-infected leaf material with one ml Ni-NTA chromatography columns; and "C': seven day infected leaves with magnetic Ni-NTA agarose beads. The term "95%CI" refers to the 95% confidence interval; and "Chi sq" to the Chi squared value. Significantly different proportions are highlighted in blue. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005.

Search term	Experiment	BEC1054 percentage	BEC1005 percentage	Upper 95% CI	Lower 95% CI	Chi sq	Standard error	p-value	Significance
Large ribosomal subunit	A	3.21	3.41	-0.034	0.030	0.00	1.49	1.0000	
	B	7.41	3.77	-0.086	0.159	0.15	1.67	0.6994	
	C	10.94	2.94	-0.038	0.198	0.98	5.47	0.3229	
	total	5.67	3.46	-0.011	0.055	2.15	1.53	0.1427	
Small ribosomal subunit	A	3.85	3.41	-0.032	0.040	0.47	0.87	0.4927	
	B	14.81	4.18	-0.051	0.263	3.54	7.30	0.0598	.
	C	21.88	11.76	-0.070	0.272	0.91	7.94	0.3389	
	total	10.93	3.79	0.018	0.100	14.66	1.90	0.0001	***
elongation	A	0.64	1.76	-0.030	0.008	0.47	0.87	0.4927	
	B	11.11	1.26	-0.041	0.239	6.65	6.51	0.0099	**
	C	18.75	0.00	0.069	0.306	5.62	5.49	0.0177	*
	total	6.48	1.61	0.015	0.083	18.65	1.58	0.0000	***
RNA	A	4.49	4.75	-0.040	-0.001	1.60	0.92	0.2054	
	B	0.00	0.84	-0.050	0.016	0.00	1.54	1.0000	
	C	7.81	2.94	-0.061	0.158	0.27	5.09	0.6067	
	total	1.64	2.07	-0.024	-0.001	3.01	0.52	0.0829	.
initiation/	A	0.64	2.69	-0.040	0.035	0.00	1.76	1.0000	
translation	B	0.00	1.67	-0.028	0.012	0.00	0.93	1.0000	
	C	6.25	11.76	-0.201	0.091	0.32	6.79	0.5744	
	total	2.43	2.50	-0.028	0.033	0.00	1.42	0.9883	
ribosome	A	0.00	0.72	-0.016	0.002	0.27	0.42	0.6051	
	B	0.00	0.00	0.000	0.000	0.00	0.00	NA	
	C	0.00	2.94	-0.109	0.050	0.10	3.69	0.7465	
	total	3.24	0.00	-0.013	0.000	0.62	0.32	0.4302	

4.4.3. BEC1054 affects yeast growth

The maximum reaction rate (V_i) of many of the negative activation control lines was lower than the Invitrogen negative interaction control. In addition, during transformation of the yeast lines, it was observed that yeast transformed with constructs containing BEC1054 produced fewer, smaller colonies than constructs containing an unrelated BEC, BEC1005.

The growth of MaV203 yeast lines containing pEXP32/BEC1054 and pEXP32/BEC1005 were assayed in liquid culture, to find out whether BEC1054 had an effect on yeast growth (Figure 11). Yeast transformed with effector BEC1054 had a shorter lag time (λ) than yeast containing BEC1005, although this effect was not significant. The presence of BEC1054 was found to significantly decrease the maximum growth rate (μ) of the yeast; and to produce a significantly lower maximum cell density (A).

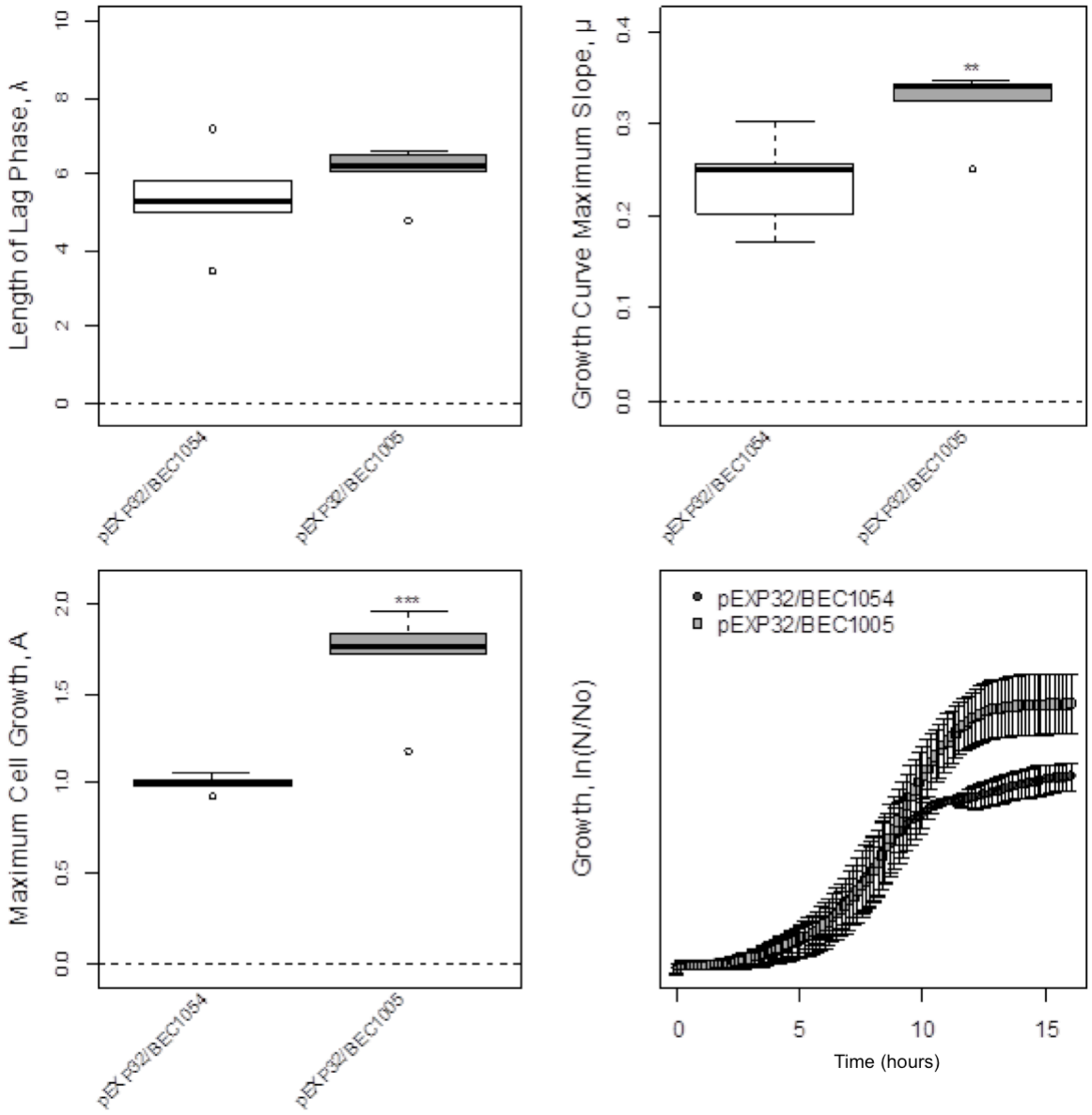


Figure 11: ***Blumeria* Effector Candidate 1054 (BEC1054) affects the growth of yeast line MaV203**. The fungal effector BEC1054 was expressed in yeast fused with a DNA-binding domain, on the plasmid pEXP32. The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. Generalized Linear Model Modelling was used to determine the significance, which is indicated by "**" for p≤0.01 and "***" for p≤0.005. Five independently transformed colonies were assayed for pEXP32/BEC1054, and six for pEXP32/BEC1005.

4.4.4. Yeast-two-hybrid

A yeast-two-hybrid (Y2H) approach was used to further validate a number of putative interactors identified through *in vitro* chromatography. The proteins investigated were NDPK, GST, PR5, MDH, PR10, eEF1A(1), eEF1A(3) and 40S 16.

Prior to the start of this PhD, the genome of barley had been sequenced (Mayer *et al.,* 2012), but the annotation and presentation was not yet complete. Gene models were not available for the genes of interest at the time. I therefore created models for the putative interactor proteins selected for Y2H analysis. Primers were designed to amplify the genes (Supplementary Table 13), and the PCR products inserted into the entry vector pCR8. The gene products were recombined into pEXP yeast expression plasmids. Following insertion into pCR8, three colonies containing the PCR product for eEF1A were sequenced. These were found to represent three different eEF proteins (Supplementary Figure 52; proteins identified by BLASTn and BLASTp), labeled eEF1A(1) to eEF1A(3) in all subsequent results. One (eEF1A(2)) was later found to be a *B. graminis* eEF, and so was excluded from the analysis.

Yeast lines containing the plasmids pDEST22 and pEXP32/BEC1054 were used as the negative control. The effector BEC1005 was initially used as a control, but I found that it showed β-galactosidase activity in all plasmid combinations, and it had previously been predicted to be a glycohydrolase (Pliego *et al.,* 2013). It was therefore excluded from further analysis. For each putative interactor, an additional negative control was also

used, consisting of yeast transformed with empty pDEST22, and the bait bound to pDEST32 (See Table 4 for the combinations of bait and prey proteins investigated).

4.4.5. β-galactosidase assay statistical analyses

Bartlett tests were performed to determine whether the variance of the data for the yeast lines was homogeneous (Crawley 2005). All but three datasets showed non-homogeneous variance (Supplementary Table 28). For the sake of consistency all datasets were treated as though non-homogeneous. Generalized Linear Model Modelling (GLM) allows the specification of error distributions, for example "gamma" distribution where the variance is assumed to increase faster than linearly with the mean (Crawley 2005). Response variables for datasets containing negative values were transformed to remove negative values through the addition of a constant to all data points (+4 for activity, and +205 for gradient). Datasets for the cell lines were each analysed separately with the controls, i.e. the strong, weak, negative, BEC1054 only, eEF1A(1) only, BEC1054+eEF1A(1) and eEF1A(1)+BEC1054 lines were analysed together, and are referred to hereafter as a dataset. Once GLMs had been created (Equation 7), and it had been determined that the yeast line had an effect, Games-Howell post-hoc tests (Crawley 2005) were performed to determine which pairs of means were significantly different.

Equation 13: **A Generalized Linear Model Model used to determine whether the response variable (gradient) changed with the yeast line under investigation.** A "gamma" error family was used to account for the overdispersion in the variance.

$$glm(gradient \sim yeast\_line, family = "Gamma")$$

The V_i for BEC1054-MDH, BEC1054-zGST and BEC1054-PR5 was higher than the negative controls in both bait-prey orientations (Figure 12 and Supplementary Table 29), indicating that BEC1054 interacts with these proteins in yeast. The V_i was also significantly elevated in one bait-prey orientation, compared with the negative controls, for BEC1054 with eEF1A(1), eEf1G and 40S 16 (pEXP32/BEC1054 with pEXP22/prey-protein). The V_i was not higher in the other bait-prey orientation (Figure 12 and Supplementary Table 29).

The proteins eEF1A(3) and PR10 did not show an increase in V_i in either bait-prey orientation (Supplementary Figure 51). In contrast, NDPK did show an elevation in Vi, compared with the BEC1054 negative control, but the NDPK negative control (pEXP32/NDPK with pDEST22) was also elevated.

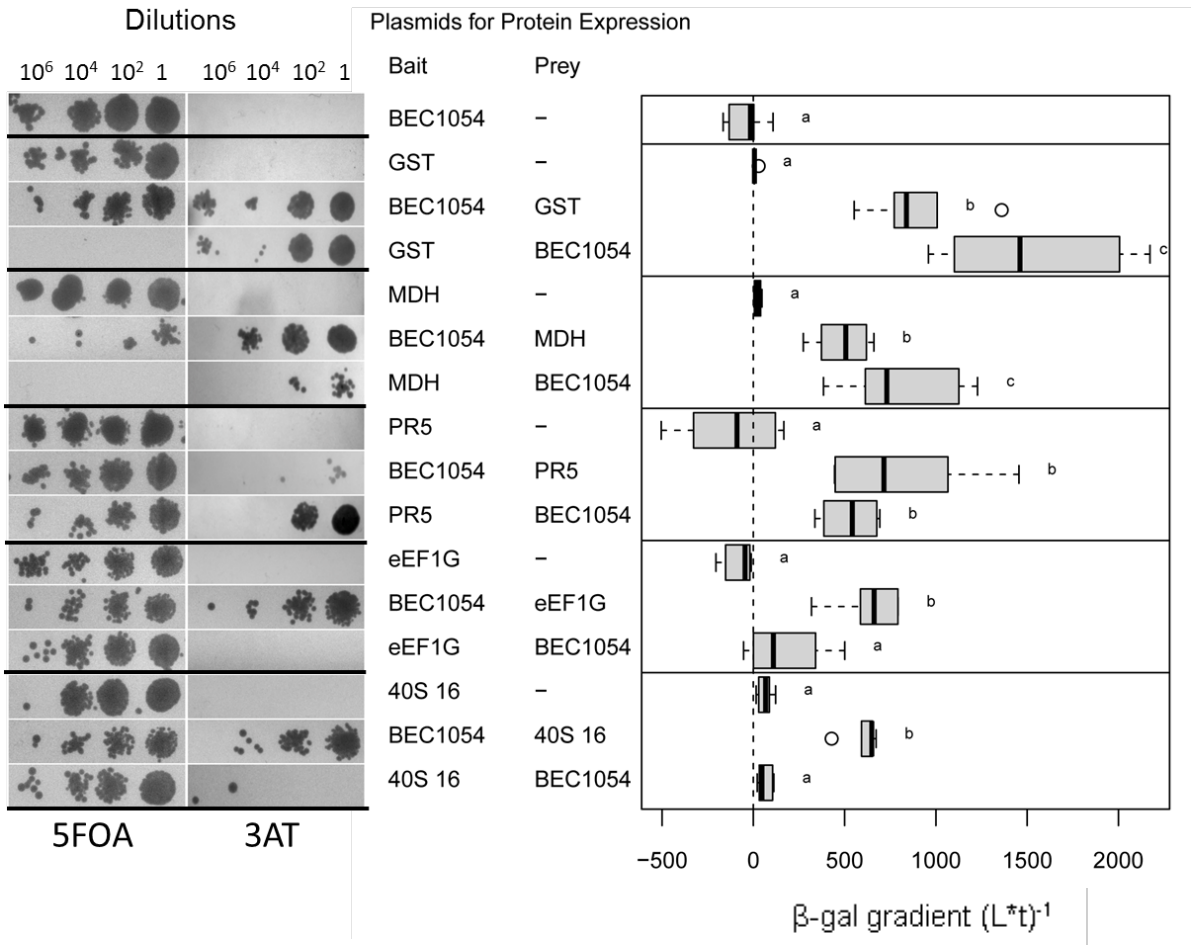


Figure 12: **Yeast-two-hybrid shows the interaction of fungal *Blumeria* Effector Candidate 1054 (BEC1054) with multiple plant proteins**. The protein interactors abbreviations are: GST, glutathione-S-transferase; MDH, malate dehydrogenase; PR5, pathogenesis-related protein 5; eEF1G, elongation factor 1 gamma; and 40S 16, 40S ribosomal subunit protein 16. **Left:** Inhibition in the presence of 5FOA (5-Fluoroorotic Acid) indicates an interaction; whereas growth promotion in the presence of 3AT (3-Amino-1,2,4-triazole) indicates an interaction. **Right:** A CPRG galactosidase assay was used to quantify the interaction between BEC1054 and putative interacting plant proteins in a yeast-two-hybrid assay. Yeast lines were lysed through freeze-thaw lysis, and the lysis supernatant added to buffer containing chlorophenolred-ß-D-galactopyranoside (CPRG). The names of the interactors are given first for the bait, and then for the prey, with a space referring to an empty plasmid, i.e. "BEC1054" is pEXP32/BEC1054 and pDEST22; whereas "BEC1054+eEF1G is pEXP32/BEC1054 and pEXP22/eEF1G. The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. Games-Howell posthoc tests were used to determine whether the mean V_i (the maximum rate of conversion of the yellow substrate CPRG to the red product chloramphenicol red (and D-galactose)) was significantly different for different yeast lines (line 1 and line 2). Significant difference is indicated by the letters "a", "b" and "c" ($p>0.05$), with bars labelled with different letters being significantly different. Six independently transformed colonies were used for each yeast line. Only yeast lines which showed evidence of an interaction are displayed here; the full set is presented in Supplementary Figure 51.

Growth on media containing 3AT, or inhibition of growth on media containing 5FOA indicated the interaction between the bait and prey proteins. Yeast lines, which co-expressed BEC1054 with MDH, PR5 or GST showed a decrease in growth on media containing 5FOA, and grew on media containing 3AT (Table 10). These results agree with those of the β-galactosidase assay (Figure 12), confirming that BEC1054 interacted weakly with these proteins in yeast. Yeast containing BEC1054 and 40S 16 in the bait-prey orientation pEXP32/BEC1054 pEXP22/40S 16 showed reduced growth on media containing 5FOA, and grew on media containing 3AT (Table 10). In contrast, yeast containing the opposite bait-prey pairing grew well on media containing 5FOA and did not grow on media containing 3AT. These results corroborate those seen in the β-galactosidase assay, demonstrating that BEC1054 interacts with 40S 16 in yeast in one bait-prey orientation. Yeast which expressed BEC1054 and eEF1G showed decreased growth on 5FOA in both bait-prey orientations (Table 10). Yeast lines containing BEC1054-eEF1G grew on 3AT, but the opposite bait-prey pairing did not Figure 12. Taken together with the β-galactosidase assay (Figure 12), these results indicated that eEF1G may interact weakly with BEC1054 in one bait-prey orientation. The four remaining proteins: eEF1A(1), eEF1A(3), NDPK and PR10 did not show evidence of interaction with BEC1054 in yeast, i.e. they grew well on media containing 5FOA and did not grow on media containing 3AT. Except for eEF1A(1), these results confirmed those seen in the β-galactosidase assay. None of the cell lines investigated grew on media lacking uracil, indicating only a weak interaction between the bait-prey pairs (Table 10).

Table 10: **Yeast-two-hybrid selective media assays indicate *Blumeria* Effector Candidate 1054 (BEC1054) interacts with multiple plant proteins in yeast.** Inhibition in the presence of 5FOA (5-Fluoroorotic Acid) indicates an interaction; whereas growth promotion with 3AT (3-Amino-1,2,4-triazole) indicates an interaction. Four independently transformed lines were plated out onto media at serial dilutions of 1:10, 1:100, 1:1000, and 1:10000, and compared with negative controls to determine whether growth was affected. The abbreviation "Y" stands for "yes, there was an interaction", "N" for "no, there was no interaction" and "W" for "there was weak evidence for an interaction", and "P" for "partial evidence of interaction". Assays were performed with BEC1054 as the bait, and the plant protein as the prey, and vice versa, which is why two results are shown for each cell.

Putative BEC1054 interactor	5FOA	3AT	-URA	Summarised Y2H evidence
GST	W/Y	Y/Y	N/N	Yes (weak)
MDH	Y/Y	Y/Y	N/N	Yes (weak)
PR5	W/Y	W/Y	N/N	Yes (weak)
eEF1G	W/W	Y/N	N/N	Yes (weak)
40S 16	W/N	Y/N	N/N	No
eEF1A(1)	N/N	N/N	N/N	No
eEF1A(3)	N/N	N/N	N/N	No
PR10	N/N	N/N	N/N	No
NDPK	N/N	N/N	N/N	No

Taken together, the Y2H results indicate that BEC1054 interacted directly with GST, PR5 and MDH in yeast in both bait-prey orientations (Figure 12 and Table 10). Furthermore, BEC1054 interacted with 40S 16 and eEF1G in one bait-prey orientation, demonstrating that BEC1054 was able to interact directly with a number of barley proteins. These results largely agreed with those seen in the LCMS pull-downs (Supplementary Table 25, Supplementary Table 26, Supplementary Table 27), where eEF1G, GST and PR5 were identified solely with BEC1054 (and not in the negative controls); MDH was found repeatedly in the pull-downs with BEC1054, and only rarely with the negative controls (Supplementary Table 25, Supplementary Table 26,

Supplementary Table 27). The greatest discrepancy was that 40S 16 was identified in numerous negative controls from the *in vitro* pull-downs, but showed a consistent weak interaction with BEC1054. Direct interactions were not confirmed for PR10, NDPK, eEF1A(1) or eEF1A(3), all of which were observed in some of the pull-down negative controls in the U36 HarvEST database (Supplementary Table 26).

An association was found in the literature between GST and PR10: PR10 undergoes post-translational S-glutathiolation in birch (Koistinen et al., 2002). I therefore decided to investigate the interaction of GST with PR10 (Table 25 and Figure 12). A CPRG assay was performed, as described above, to determine whether the interaction of GST and PR10 led to an increase in β-galactosidase activity. I performed a Bartlett test on the data, to determine whether the variance of the yeast Vi was homogeneous (Crawley 2005). It was not (Bartlett's K-squared=28.62, p-value<0.0001).

I found that the interaction of GST and PR10 elevated the Vi above that of the negative interaction controls (Figure 25). This elevation was significant in both bait-prey orientations. This increase in Vi was significant (Table 25).

Yeast lines, which co-expressed GST and PR10, showed no growth in the presence of 5FOA, but they did not grow on 3AT. The 5FOA results agree with the β-galactosidase assay, indicating that the two proteins interacted in yeast, whereas the 3AT results did not. The Y2H results for the interaction of GST and PR10 were therefore inconclusive.

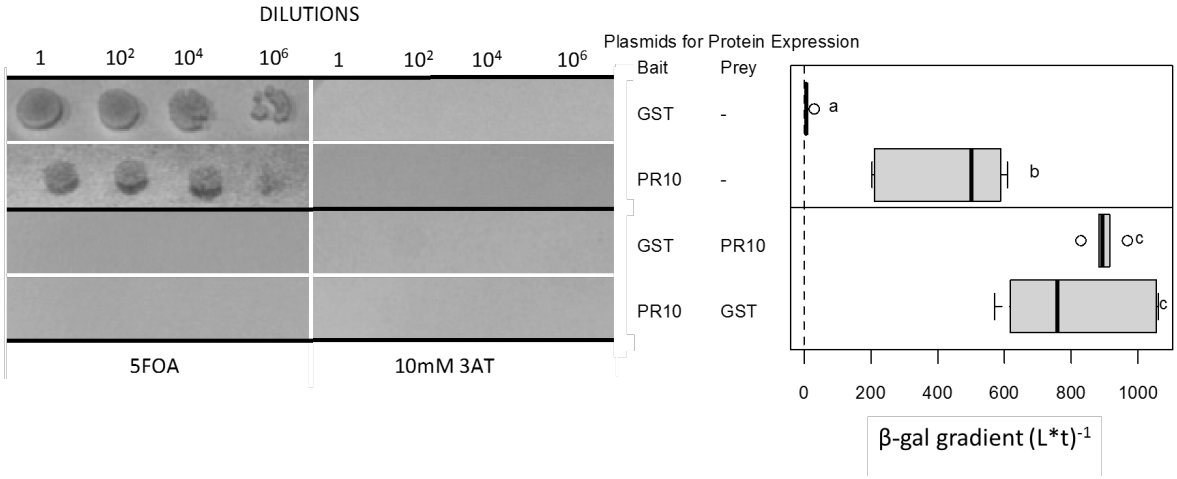


Figure 13: **Yeast-two-hybrid shows the interaction of barley Glutathione-S-Transferase (GST) and Pathogenesis Related protein 10 (PR10). Left:** Inhibition in the presence of 5FOA (5-Fluoroorotic Acid) indicates an interaction; whereas growth promotion in the presence of 3AT (3-Amino-1,2,4-triazole) indicates an interaction. **Right:** A CPRG galactosidase assay was used to quantify the interaction between BEC1054 and putative interacting plant proteins in a yeast-two-hybrid assay. Yeast lines were lysed through freeze-thaw lysis, and the lysis supernatant added to buffer containing chlorophenolred-ß-D-galactopyranoside (CPRG). The names of the interactors are given first for the bait, and then for the prey, with a space referring to an empty plasmid, i.e. "GST" is pEXP32/GST and pDEST22; whereas "GST+PR10 is pEXP32/GST and pEXP22/PR10. The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. Games-Howell posthoc tests were used to determine whether the mean V_i (the maximum rate of conversion of the yellow substrate CPRG to the red product chloramphenicol red (and D-galactose)) was significantly different for different yeast lines (line 1 and line 2). Significant difference is indicated by the letters "a", "b" and "c" ($p<0.05$), with bars labelled with different letters being significantly different. Six independently transformed colonies were used for each yeast line. Only yeast lines which showed evidence of an interaction are displayed here; the full set is presented in Supplementary Figure 51.

4.4.6. Bimolecular fluorescence complementation (BiFC)

I cloned the bait proteins, BEC1054 and JIP60ml (see footnote [1]) , into the pESPYCE plasmid, which encoded a C-terminal fusion of the C-terminal domain of the YFP protein (Table 5). I also cloned the plant prey proteins into pUCSPYNE which encoded an C-terminal fusion of the YFP N-terminus; and PR5 into pESPYNE, which has an N-terminal fusion of the YFP N-terminus. An interaction of the bait and prey proteins leads to the formation of a competent YFP protein (Ghosh *et al.,* 2000). In addition, BEC1054, JIP60ml and the putative plant interactors were cloned into a plasmid with either an N, or a C terminal fusion of a complete YFP protein, to determine their expression patterns *in planta.*

The fluorescent tags of the bait proteins, BEC1054 and JIP60ml, could be weakly seen in the nucleus and the cytoplasm (Figure 14). A Z-stack maximum projection view, demonstrated the presence of puncta (seen as faint yellow dots superimposing the yellow colour seen throughout the cytoplasm and nucleus). Only two epidermal cells were seen expressing JIP60ml, one of which is shown in Figure 14. The vast majority of cells in which the signal for JIP60's fluorescent tag was observed were guard cells.

The chloroplasts present in the mesophyll cells autofluoresce at the wavelengths used to detect YFP fluorescence (see Figure 14, Figure 16 and Figure 18 for examples). I performed a spectral scan to demonstrate that this signal was distinct from that of the YFP (Figure 15). Fluorescence of YFP can be seen represented in green, and chlorophyll autofluorescence in red. The round shape of the mesophyll cells can be observed

[1] Jasmonate Induced Protein 60 (JIP60) had an internal peptide removed, the removal of which is required for RIP activity, and replaced with a methionine-leucine (ml) linker (Chaudhry *et al.,* 1994). This protein was used as a negative control in the split-YFP experiments.

through the location of the chloroplasts, as they are effectively outlining the cells Figure 15. The maximum intensity wavelength shown in Figure 15 corresponds to the cell image in Figure 15, taken from within the Z-stack. The maxima for YFP and chloroplasts can be seen as the first and second peaks respectively. We measured YFP between 520 and 560 nm, and the maximum fluorescence falls within this range.

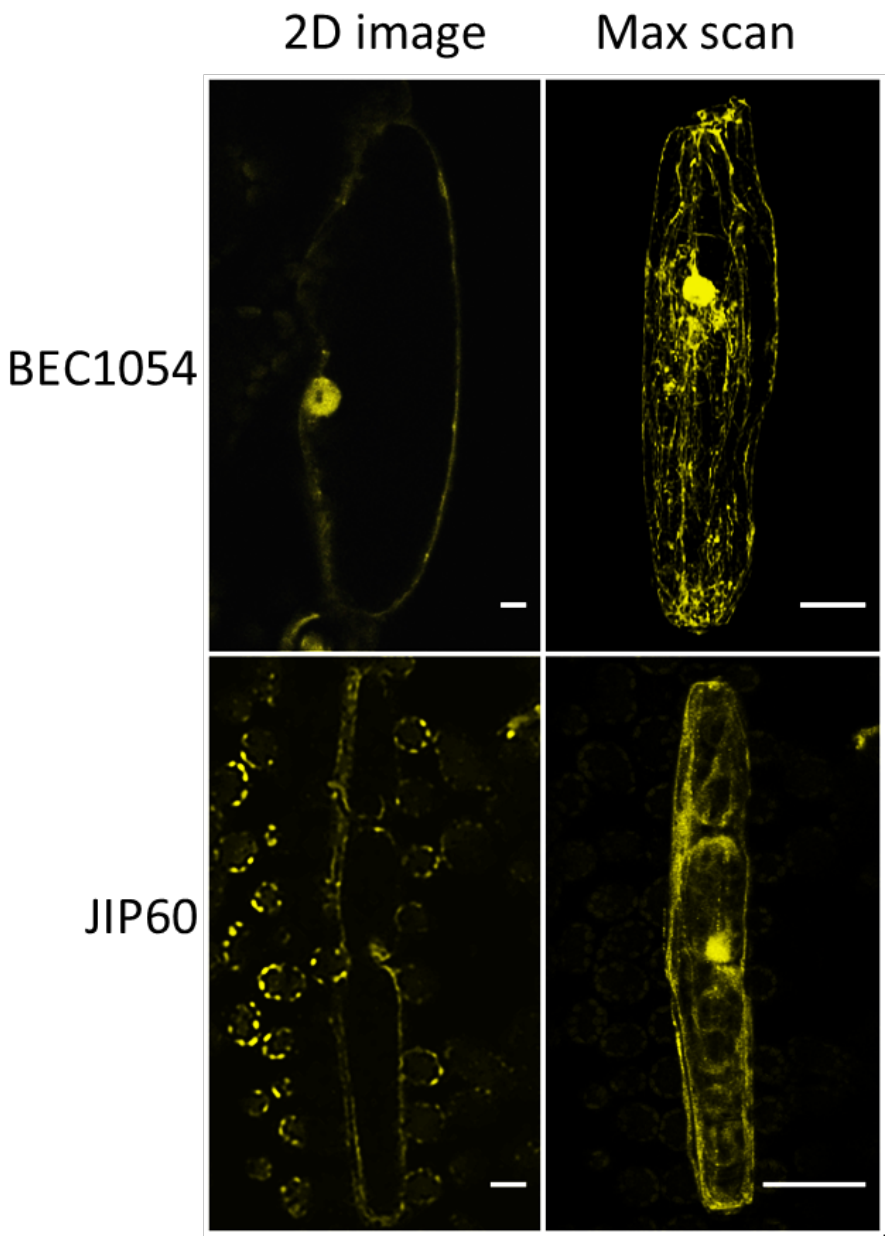


Figure 14: ***Blumeria* Effector Candidate BEC1054 (BEC1054) and Jasmonate Induced Protein 60 (JIP60) are expressed in the cytoplasm and the nucleus of the host plant, barley**. The 2D image is a single confocal image of the cell, whereas the "Max scan" shows the Z-stack maximum projection view. Scale bars are 20 µm. Both BEC1054 and JIP60 were cloned into the vector 35S-GWY-mYFP, and tagged at the C-terminus with monomeric yellow fluorescent protein.

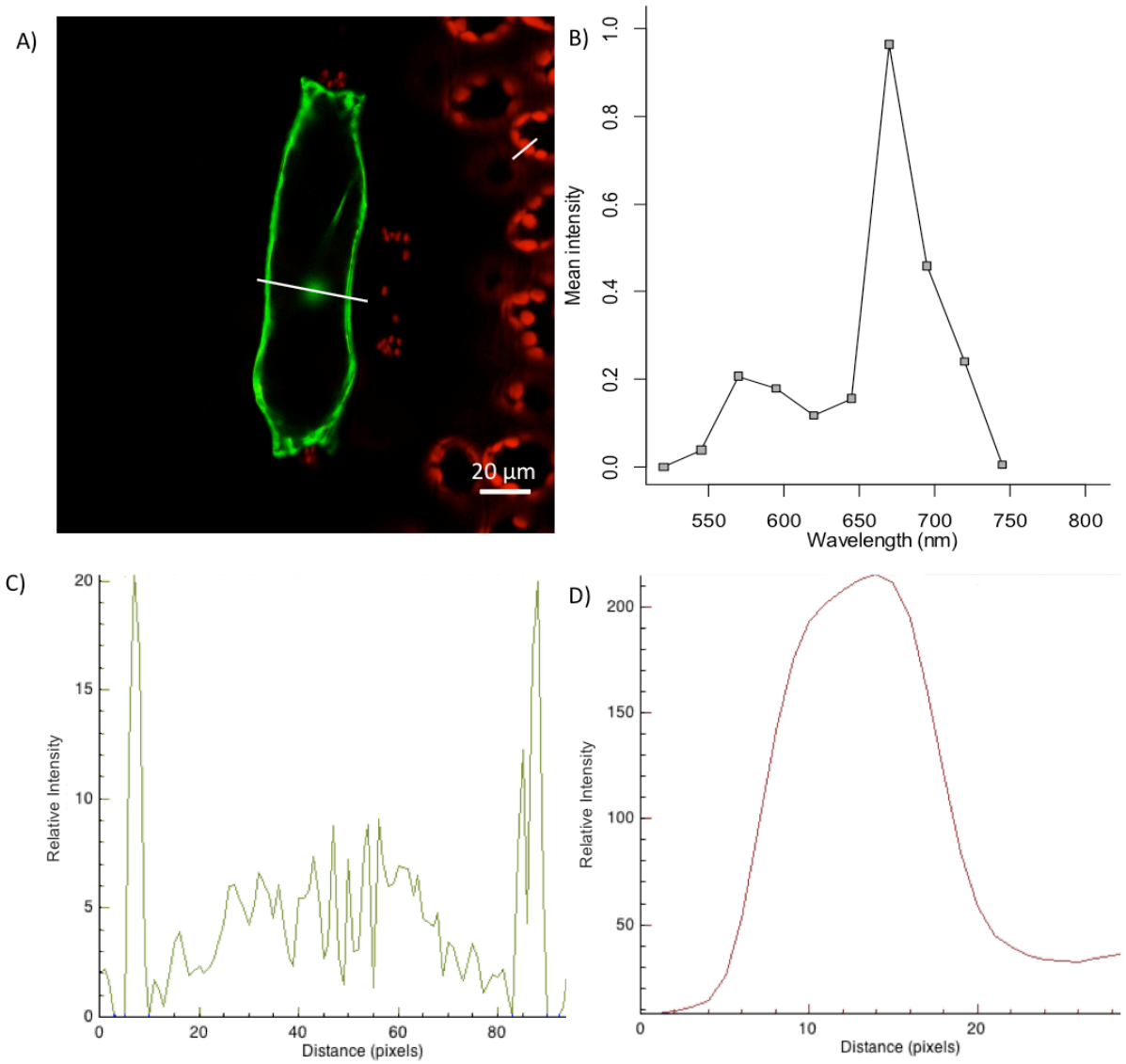


Figure 15: **The fluorescence of Yellow Fluorescent Protein (YFP) fused to eukaryotic elongation factor one alpha, and chloroplasts autofluorescence occur at differing wavelengths.** The plant protein eEF1A was cloned into pUCSPYNE which encoded an C-terminal fusion of the YFP N-terminus. A) YFP fluorescence is shown in green, and chloroplast autofluorescence in red. Two regions of interest, one across the transformed cell, and one across a chloroplast are marked by white lines. A 20 µm scale bar is also shown in white. B) Mean intensity at differing wavelengths. C) YFP relaive signal intensity across the cell wall and nucleus of the transformed cell. D) Chloroplast autofluorescence signal intensity actoss the marked chloroplast.

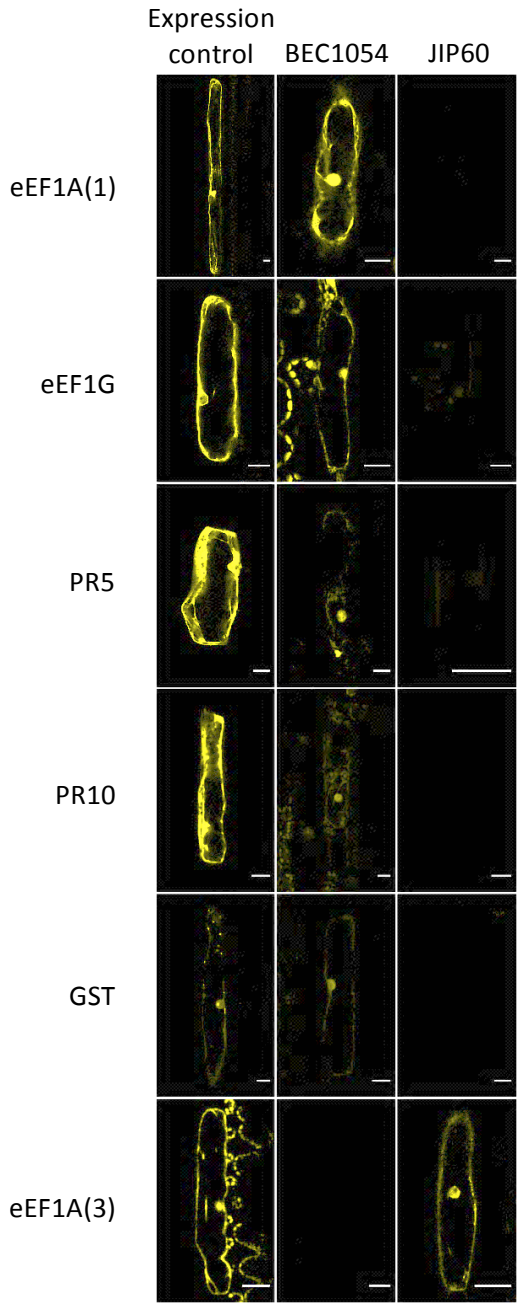


Figure 16: ***Blumeria* Effector Candidate 1054 (BEC1054) interacts with multiple proteins *in planta.*** A bimolecular fluorescence complementation assay was performed in the host-plant, barley. The left-hand column "Expression control" demonstrates the expression patterns of the five proteins of interest. The proteins listed down the left hand side were cloned with an N-terminal fusion of the complete monomeric yellow fluorescent protein (YFP). For the central column "BEC1054" indicates the interaction of proteins with BEC1054. For the right hand column, "JIP60" indicates the interaction with the negative control, Jasmonate Induced Protein 60. BEC1054 and JIP60ml were cloned into the pESPYCE plasmid, which encoded a C-terminal fusion of the C-terminal domain of the YFP protein. The plant prey proteins were cloned into pUCSPYNE which

encoded an C-terminal fusion of the YFP N-terminus; and PR5 into pESPYNE, which has an N-terminal fusion of the YFP N-terminus. The barley proteins were: eukaryotic Elongation Factor One Alpha (eEF1A) homologs one and three, eukaryotic Elongation Factor One Gamma (eEF1G) Pathogenesis Related protein 5 (PR5), PR10 and Glutathione-S-Transferase (GST). Scale bars are 20 μM.

Five of the prey proteins: eEF1A(1), eEF1A(3), PR5, PR10 and NDPK were expressed, and their fluorescent tag was visible in a pattern of cytoplasmic and nuclear expression (Figure 16 and Figure 17). One protein, eEF1G, showed strong expression in the cytoplasm, and weak expression in the nucleus. The expression of MDH was strong in the cytoplasm, weak in the nucleus and in filaments across the cell. The two proteins, GST and 40S 16, were expressed in a very weak and diffuse manner in the nucleus and cytoplasm, with the majority of the fluorescence occurring in the nucleolus, and in puncta throughout the cytoplasm.

There was evidence of interaction between BEC1054 and the prey proteins eEF1A(1), eEF1G, PR5, PR10 and GST (Figure 16). These proteins did not interact with the negative control protein, JIP60ml. In all cases, the interaction occurred mainly in the nucleus, and weakly in the cytoplasm, matching the expression patterns for the bait proteins. I found that the negative control protein, JIP60ml, interacted weakly with eEF1A(3) in the cytoplasm and the nucleus. The interactions observed in barley supported those seen during the Y2H assay. All five interactions confirmed through BiFC showed at least partial evidence for an interaction in yeast.

I investigated the interaction of GST and PR10, with PR10 expressed with a C-terminal fusion of the C-terminus of YFP, and GST with an N-terminal fusion of the N-terminus

(Figure 18). I found that the two proteins interacted in the host plant, barley. This result further supported that seen in the Y2H assay (Table 11).

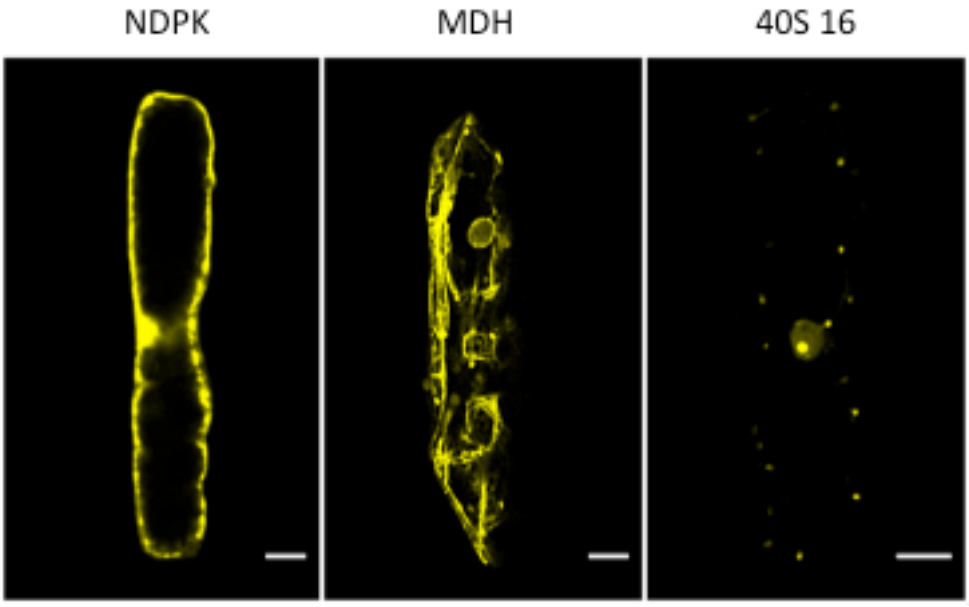


Figure 17: **Three plant proteins show diverse expression patterns**. The proteins Nucleoside Diphosphate Kinase (NDPK), Malate Dehydrogenase (MDH), and ribosomal Subunit 40S protein 16 (40S 16) were expressed with an N-terminal whole, monomeric YFP tag in the host plant, barley. Scale bars are 20 µm.

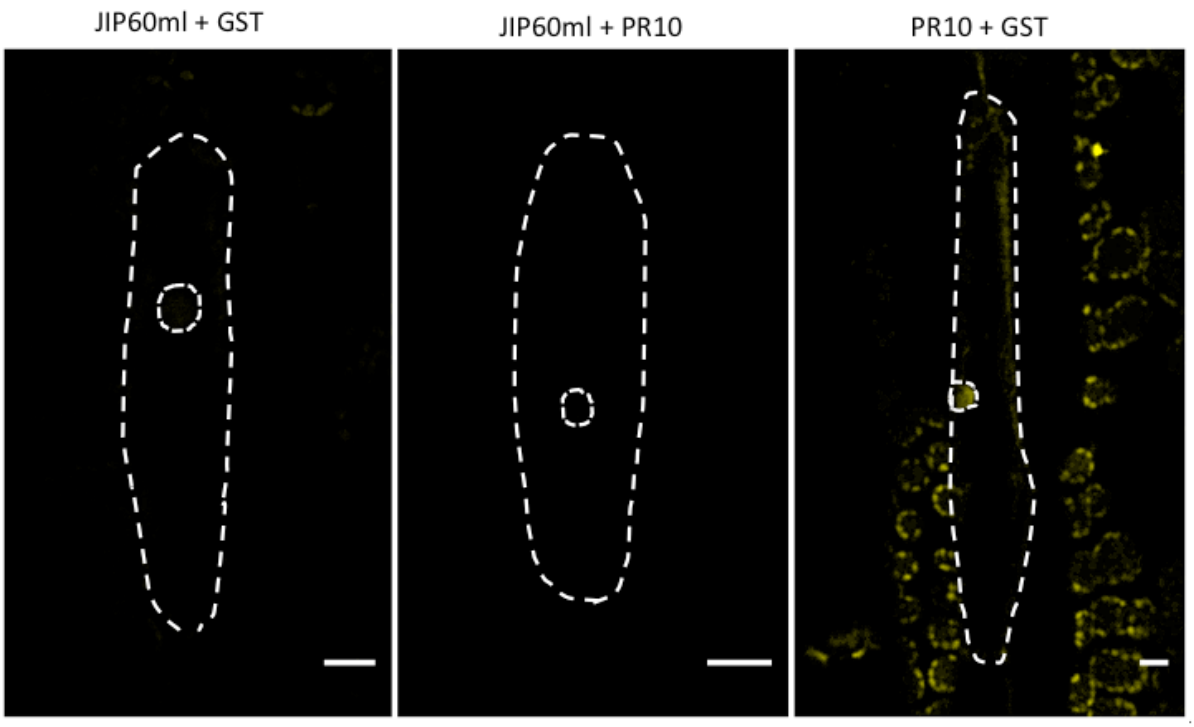


Figure 18: **The barley proteins Glutathione-S-Transferase (GST) and Pathogenesis Related protein 10 (PR10) interact weakly in the nucleus and cytoplasm.** Jasmonate Induced Protein 60 (JIP60) was used as a negative interaction control with both PR10 and GST. The white dashed lines indicate the nucleus and cytoplasm. The first protein stated in each pair was cloned into the pESPYCE plasmid, which encoded a C-terminal fusion of the C-terminal domain of the YFP protein. The second protein was cloned into pUCSPYNE, which encoded an C-terminal fusion of the YFP N-terminus. Scale bars are 20 μm.

4.4.7. Summary table for both BiFC and Y2H results

Table 11: ***Blumeria* Effector Candidate interacts with multiple plant proteins in yeast and *in planta*.** The abbreviation "Y" stands for "yes, an interaction was seen", and "P" for a "partial interaction was seen", and "I" for "Inconclusive".

Bait protein	**Prey protein**	**Y2H**	**Split YFP**
BEC1054	PR5	Y	Y
BEC1054	GST	Y	Y
BEC1054	eEF1G	Y	Y
BEC1054	PR10	I	Y
BEC1054	MDH	Y	-
BEC1054	40S 16	P	-
BEC1054	eEF1A(1)	P	Y
BEC1054	NDPK	-	-
PR10	GST	I	Y

The Y2H and BiFC assays are summarised in Table 11. For the Y2H assay, I found that five proteins interacted with BEC1054 in yeast, and four further proteins showed possible evidence for interaction. For the BiFC assay, five proteins showed evidence of interaction in the host plant, barley. All of interactions detected through BiFC were with proteins also detected through Y2H. In addition, PR10 and GST interacted weakly in both Y2H and BiFC.

4.5. Discussion

4.1.5.1. *The ribosome binding hypothesis* and ribosomal proteins identified through LCMS

A significantly greater total number of ribosomal small-subunit proteins were identified with BEC1054 (Table 9). Furthermore, a significantly greater proportion of elongation factor-related sequences were identified with BEC1054 for two of the three experiments (non-infected whole-leaf material "B" and infected whole-leaf material "C"); and a significantly greater number of elongation-related sequences identified overall. These results indicated that BEC1054 may indeed be binding to the ribosomal proteins.

A literature search was used to identify the roles of the different RNA-associated protein interactors. A number of them (for example S31) extend into the A-site; others (for example S6, S14 and the elongation factors) are located close to the SRL, or bind close to it (Klinge *et al.,* 2011, Rabl *et al.,* 2011). It may therefore be that the 40S, 60S and eEF proteins identified do indeed interact with BEC1054. Alternatively, if BEC1054 bound to the rRNA or other ribosomal proteins, the 40S and 60S proteins could be identified as interactors by also being bound to the ribosome.

4.2.5.1. *Validation of interactors through Y2H*

I used a targeted Y2H approach to further investigate the putative barley interactors identified, by our collaborators, through LCMS. The interactors investigated were PR5, PR10, MDH, GST, eEF1A(1), eEF1A(3), eEF1G, 40S 16 and NDPK. The ribosome associated proteins eEF1A and eEF1G were selected because they are involved in with

the translation of mRNA (a process which we believe the RNase Like Proteins associated with Haustoria (RALPH) effectors may target); and 40S 16 was selected because it is an intrinsic ribosomal protein. Although chromatography had given only marginal evidence for BEC1054 interacting with PR10 *in vitro*, PR10 was selected because it is an RNase (where BEC1054 is an RNase like protein). The protein MDH was selected as it occurred in most pull-downs, despite being identified in a few negative controls. The proteins PR5, GST and eEF1G were identified only with BEC1054, not with any of the negative controls.

Expression of the *lacZ* reporter gene can be quantified through its β-galactosidase activity. The maximum conversion rate, V_i (Figure 5), at the start of the hydrolysis of the yellow substrate CPRG, was used as a quantitative indicator of the interaction between bait and prey (Figure 12). Yeast containing the plasmids pEXP32/BEC1054 and pDEST22 were used as a negative control; as was the barley putative interaction protein bound to the bait protein (pEXP32/barley protein). In addition, lines expressing BEC1054 showed decreased growth when compared with the controls provided by Introgen. The decrease in growth may be due to BEC1054 binding ribosomes and altering their activity. Comparison with lines containing BEC1054 may therefore be more biologically meaningful than comparison with the negative control provided by Invitrogen.

It was not possible to use BEC1005 as a negative control for the CPRG assay, as it showed evidence of endogenous β-galactosidase activity. The negative control protein, BEC1005, is a putative glycosidase (Pliego *et al* 2013) and may therefore hydrolyze the CPRG substrate. No previous β-galactosidase activity had been detected for BEC1005, although this had been tested extensively within the host laboratory (Spanu, *pers. comm*.), which is why it was initially included. The CPRG assay is more sensitive than the traditionally used ONPG assay (Eustice *et al.,* 1991) which had previously been used. Moreover, for the ONPG assay previously conducted in the laboratory (Spanu, *pers. comm*), BEC1005 was expressed in *E. coli*. Its expression in a eukaryote (yeast) may have provided a more conducive environment for its enzyme activity. It may be that BEC1005 is post-translationally modified in eukaryotes, or other proteins present in yeast/plant cells but not in prokaryotes may be required for the interaction.

Yeast transformation with pEXP32/BEC1054 had a low success rate, and grew poorly (Figure 11). A low transformation rate for yeast containing a particular bait plasmid, and noticeable poor growth, are both phenotypic effects associated with toxic bait proteins (Serebriiskii 2010). The decrease in growth may be due to BEC1054 binding ribosomes and altering their activity. It is possible that in the host cell BEC1054 decreases ribosomal activity, but prevents it from being completely lost. This may explain why the V_i of many of the negative activation control lines was lower than the Invitrogen negative interaction control. The increase in V_i for lines containing the putative interactors and BEC1054 may therefore be more biologically meaningful than comparison with the negative control provided by Invitrogen.

The V_i for BEC1054-GST, BEC1054-MDH, and BEC1054-PR5 were elevated in both bait-prey orientations, when compared with the negative controls (Figure 12 and Supplementary Table 29). In addition, the V_i was higher in one bait-prey orientation (pEXP32/BEC1054 pEXP22/barley protein) for 40S 16, eEF1G and eEF1A(1) with BEC1054, when compared with the negative controls (Figure 12, Supplementary Table 29 and Figure 13). No significant increase in V_i was seen for BEC1054 with PR10, NDPK or eEF1A(3) (Figure 13 and Supplementary Table 29). Both PR10 and NDPK demonstrated an increased Vi, when expressed with the DNA binding domain (pEXP32/PR10 or pEXP32/NDPK), indicating that they may affect transcription.

The auxiliary domains of many RNA binding proteins undergo post-translational modification (see Glisovic *et al* 1996, for a review). The protein PR10 undergoes post-translational S-glutathiolation, catalyzed by GST in birch; but the glutathiolation does not affect its RNase activity (Koistinen *et al.,* 2002). Both PR10 and a GST were identified in the LCMS assay, and I investigated whether the two proteins interacted in yeast. The V_i for PR10 and GST in both bait-prey pairings was significantly elevated above that of the controls (Figure 13), indicating an interaction.

Transformed yeast lines were further investigated, through plating onto media containing 3AT or 5FOA. Growth on 3AT, or inhibition on 5FOA, when compared with the control lines, indicates an interaction between the bait and prey proteins (MacDonald 2001). Yeast expressing both PR10 and GST did not grow on media containing 5FOA, or on media containing 3AT. The lack of growth on 5FOA indicates that an interaction between the proteins may have occurred; whereas the lack of

growth on 3AT does not support this conclusion. As a result PR10 and GST could not be confirmed to interact through Y2H.

Yeast lines which co-expressed BEC1054 with MDH, PR5 or GST demonstrated decreased growth on media containing 5FOA, and showed increased growth on media containing 3AT (Table 10 and Figure 12). These results agree with the β-galactosidase assay, further demonstrating that these proteins interact with BEC1054 in yeast. Yeast expressing BEC1054 as the bait, and 40S 16 as the prey (pEXP32/BEC1054 and pEXP22/40S 16) showed reduced growth on media containing 5FOA, and grew on media containing 3AT (Table 10). The opposite bait-prey pair did not show altered growth. These results support those seen in the β-galactosidase assay, confirming that in yeast BEC1054 interacted with 40S 16 solely in one bait-prey orientation. Yeast co-expressing BEC1054 and eEF1G in either bait-prey orientation, did not grow reproducibly on 3AT media. The lines co-transformed with the plasmids pEXP32/BEC1054 and pEXP32/eEF1G demonstrated decreased growth on 5FOA media; but this was not observed for the opposite bait-prey pair (Table 10 and Figure 12). This result confirms the weak interaction seen in one orientation for the β-galactosidase assay.

The proteins PR10, NDPK, and both eEF1A homologues, did not show evidence of interaction within yeast in the selective media assays, i.e. they did not grow on media containing 3AT, and they grew well on media containing 5FOA (Table 10). The proteins eEF1A(1) and NDPK showed evidence of a weak interaction in the β-galactosidase assay in one bait-prey orientation (Supplementary Figure 51), but for NDPK, this result

cannot be relied upon without further evidence, due to its increased β-galactosidase activity; and for eEF1A(1), it could not be validated in yeast by any other means utilised. If BEC1054 interacts with ribosomes, it may be that ribosomal proteins (including eEFs, 40S and 60S proteins) do not interact directly with BEC1054, but instead were identified as components of an interaction complex in the *in vitro* chromatography through being bound to the ribosome.

Yeast lines were also plated onto selective media lacking uracil, but none of the lines tested grew (except for the strong and weak controls supplied by commercial kit (Invitrogen)), indicating that the interactions between BEC1054 and the plant proteins are weak, at best (Table 10). Whilst the use of a sensitive β-galactosidase assay may make it possible to detect weak interactions, it may also increase the rate of false-positive interactions identified. The use of other, complementary, assays such as the selective media growth assays is therefore essential to help determine whether an interaction has taken place.

The *his3* marker is reported to be more sensitive than the *ura3* marker. The promoter for *ura3* strongly represses transcription, as it contains the URS1 sequence (Gietz *et al.,* 1997, Vidal 1997, Pierce *et al.,* 1998). This helps to explain the lack of growth on media lacking uracil, seen for the weak positive control, and for any of our interactions (data not shown). These results demonstrate the importance of considering not only the quantitative CPRG data, but also the qualitative selective plate assay data (MacDonald 2001).

4.3.5.1. *Validation of interactions* in planta

One of the main limitations of BiFC is that the two halves of the fluorescent protein can reassemble in the absence of a true protein-protein interaction. The use of appropriate controls can help to overcome this problem. Ideally, the interacting proteins should be expressed with a mutated or truncated binding partner (Horstman *et al.,* 2014). This approach requires that the interacting amino acids/domains had been identified. This has not yet been achieved for BEC1054. Alternatively, a protein could be used that is related to the protein of interest, but which does not interact with the prey proteins. The protein JIP60ml was used as a control for the split YFP assay, as it is a RIP expressed in barley, whose function as a RIP has been validated (Chaudhry *et al.,* 1994, Reinbothe *et al.,* 1994). I used it as a control, as it belongs to the class of proteins that we predict BEC1054 may be outcompeting.

The protein JIP60 has previously been reported to be cytosolic (Hause *et al.,* 1994), and its N-terminal region shares sequence homology with type I and type II RIPs (Chaudhry *et al.,* 1994, Reinbothe *et al.,* 1994). The N-terminal region has been shown to act as a RIP, with the ability to cleave RNA in both animal and plant polysomes (Reinbothe *et al.,* 1994). It is believed to be synthesised as an inactive precursor, which requires the removal of a peptide from the N-terminus before activation (Chaudhry *et al.,* 1994). It also possesses a C-terminal domain similar to eukaryotic initiation factors of type 4E(Chaudhry *et al.,* 1994, Reinbothe *et al.,* 1994) and an S19 protein and GTP-binding elongation factors (Chaudhry *et al.,* 1994), indicating that the C-terminal region may associate with ribosomes (Chaudhry *et al.,* 1994, Reinbothe *et al.,* 1994). This tail also requires removal for activation of JIP60. Both N- and C-terminal processing steps are

needed for near complete inhibition of translation (Chaudhry *et al*, 1994). Our JIP60ml construct expressed only the N-terminal domain, with the N-terminal inhibitory peptide replaced by a methionine-leucine linker. Maize Ribosome-lnactivating Protein (b-32) also requires the removal of a peptide for activation, and its replacement with a methionine-leucine linker has been shown to produce an active product (Mak *et al.,* 2007). Both N-terminal and linker boundaries were selected based on Chaudhry *et al.,* (1994).

In barley, BEC1054 had a diffuse accumulation pattern throughout the cytoplasm and nucleus (Figure 14); as did JIP60ml. Both of these proteins were expressed weakly, with only a low level of visible YFP fluorescence. This may be due either to low expression, possibly caused by their inhibiting translation, or due to mis-folding of the YFP C-terminus when expressed fused to these proteins. Max scans performed for BEC1054 and JIP60ml, created through combining the maximum pixel intensity of the images obtained in a Z-stack, demonstrated the presence of weak puncta in the cytoplasm. These puncta may well represent sub cellular compartments, for example p-bodies, early or late endosomes, mitochondria, Golgi or vesicles.

Only two epidermal cells were identified expressing JIP60ml tagged with C-terminal YFP. The majority of the cells identified were guard cells (more of which are generally identified, as the nucleus occupies a much larger proportion of the cell, making the chances of hitting it with the gold bombardment particle rather higher); but there were still few of these observed, when compared with lines containing BEC1054 or other plant proteins. This result cannot be explained simply by JIP60ml's low fluorescence, as

all cells were cotransformed with an RFP marker, so that transformed cells could be identified even in the absence of an interaction. It may be that JIP60ml was toxic, killing the majority of cells that it was expressed in, or that it affected translation of both the transformation marker and the YFP vectors.

The barley proteins showed a range of accumulation localizations. Five were expressed in the cytoplasm and the nucleus (eEF1A(1), eEF1A(3), PR5, PR10 and NDPK), one accumulated in the cytoplasm, but very little in the nucleus (eEF1G); two were seen mainly in the nucleolus, and as puncta throughout the cytoplasm (GST and 40S 16) (Figure 16 and Figure 17).

Five proteins were found to interact, by BiFC, with BEC1054 *in planta*: PR5, PR10, eEF1A(1), eEF1G and GST (Figure 16). All of the interactions were seen weakly in the cytoplasm, and more strongly in the nucleus, matching the accumulation patterns of BEC1054 and JIP60ml. The GST, tagged with the YFP N-terminus, may well still be present in puncta throughout the cytoplasm, but unless BEC1054 were also within those compartments, then an interaction would not be seen there. The interaction of BEC1054 with these five proteins supported the results of both the *in vitro* chromatography, and the Y2H assay (Table 11).

The fluorescence from the complementation of tagged eEF1A(1) and BEC1054 was strongest when compared with the other interactions indicating the possibility of a direct interaction. In contrast, the fluorescence seen for the eEF1G- , PR5- , PR10- and GST- BEC1054 pairs were much weaker. This result is particularly surprising, as the

Y2H assay provided the weakest evidence for the interaction of BEC1054 with eEF1A(1), when compared with the other interactors identified through BiFC. It may be that other proteins, or post-translational modifications that occur in the plant, were required for the interaction of eEF1A(1) with BEC1054. Post-translational modification of eEF1A has been shown to occur in a wide range of species, including prokaryotes such as *Escherichia coli* (Litalien and Laursen 1979), protists for example *Euglena gracilis* (Toledo and Jerez 1990), fungi (*Mucor racemosus* (Hiatt *et al.,* 1982)), animals (rabbits, (Dever *et al.,* 1989), and plants (Ransom *et al.,* 1998). *In planta* post-translational modification of BEC1054 itself may have been required for the interaction to take place. Alternatively, the interaction with eEF1A(1) in BiFC may have been a false-positive (Huang and Bader 2009).

The interaction between GST and PR10 was investigated *in planta*, to further validate the Y2H results, and the aforementioned association between the proteins identified in the literature (Koistinen *et al.,* 2002). The two barley proteins were found to interact weakly *in planta* (Figure 18). These results indicated that PR10 and GST may be part of a complex pulled down during the LCMS assay. The lack of interaction of GST with JIP60ml indicated that it was not simply modifying proteins which possess RNA binding fold (which PR10, JIP60 and BEC1054 share).

In the Y2H assay, I found partial evidence for the interaction of BEC1054 with 40S 16. The interaction with BEC1054 in the LCMS was not entirely specific; and it could not be repeated in the BiFC assay, indicating that 40S 16's identification in the Y2H assay may have been a false positive. The interaction of MDH also could not be repeated using

BiFC. These, and the positive results above, highlight the need for multiple methods of analysis when investigating protein-protein interactions. If any single assay had been utilised, the conclusions drawn would have been different.

The barley RIP JIP60ml interacted with eEF1A(3) in barley. Cleavage of rRNA by ricin has been shown to be much slower for naked rRNA than for intact ribosomes (Endo and Tsurugi 1988). These results indicate that ribosomal proteins play a role in the binding and activity of RIPs, a concept which is supported by evidence of RIPs binding ribosomal proteins: the ricin A-chain has previously been chemically cross-linked to ribosomal proteins L9 and P0 (also known as L10e) (Vater *et al.,* 1995); pokeweed antiviral protein interacted with L3 in yeast (Hudak *et al.,* 1999, Rajamohan *et al.,* 2001); and trichosanthin interacted with P0, P1 and P2 in a Y2H and *in vitro* pull-down assay (Chan *et al.,* 2001, Chan *et al.,* 2007). The ribosomal stalk is composed of acidic P-proteins (including P0, P1 and P2), and plays a significant role in the binding of elongation factors to the ribosome. Anti-P-protein antibodies can be used to prevent binding of eEF1A and eEF2 to the ribosome (Uchiumi *et al.,* 1990). It is possible that JIP60, and BEC1054, interact with ribosomal, and ribosome associated, proteins *in vivo*.

4.4.5.1. Concluding remarks for protein interactions with BEC1054 *in vitro*, in yeast and *in planta*

Multiple proteins were identified interacting with BEC1054 *in vitro*, in yeast and *in planta* (Table 11; Figure 12 and Figure 16). Three approaches were used to validate these interactions: primary identification through affinity precipitation and mass spectrometry; Y2H; and BiFC. Three of these proteins were validated using all three approaches (PR5, eEF1G and GST). Only partial evidence was obtained through Y2H for

two further proteins: eEF1A(1) and PR10 interacting with BEC1054 (Table 11), although both were identified through LCMS and BiFC.

Fungal and oomycete effectors have been found to target more than one host protein; for example, the *Cladosporium fulvum* AVR2 targets both Rcr3 and PIP1 of tomato (Rooney *et al.,* 2005, Shabab *et al.,* 2008); the *Phytophthora infestans* AVR3a has 13 putative interactors of potato (Bos *et al.,* 2010a). Similarly, an investigation of three taxonomically unrelated pathogens identified effectors with multiple host protein targets; a number of which overlapped (Mukhtar *et al.,* 2011, Wessling *et al.,* 2014). Surprisingly for such a small protein with a simple domain structure, BEC1054 may have multiple targets amongst the identified protein putative interactors.

Proteins that bind RNA do so with differing affinities and specificities (Glisovic *et al.,* 2008). The activity of the RNA binding domains is altered by RNA binding scaffolds; and can be further changed by auxiliary domains. The auxiliary binding domains of RNA binding proteins are able to mediate their interaction with other proteins, affecting the generation of RNA binding protein complexes. The auxiliary domains of BEC1054 and JIP60 may mediate their interaction with their protein-binding partners.

The identification of multiple interactors for BEC1054 has produced a further challenge: identifying the mechanistic roles or biological significance of the interaction between these host proteins and BEC1054. The known activities and possibilities for the five validated proteins (PR5, PR10, eEF1A(1), eEF1G and GST) are discussed below.

4.5.5.1. Pathogenesis Related (PR) proteins

The PR proteins are inducible, defense related proteins expressed or up-regulated in response to pathogen invasion (Gregersen *et al.,* 1997, van Loon *et al.,* 2006, Gjetting *et al.,* 2007). They are up-regulated in response to viruses (Pierpoint *et al.,* 1981), bacteria (Pieterse *et al.,* 1996), oomycete (Alexander *et al.,* 1993), insect attack (Fidantsef *et al.,* 1999), viroids (Garcia Breijo *et al.,* 1990) and fungi (Cordero *et al.,* 1994).

The PR protein families are defined based on common biological (or biochemical) properties, and many are associated with limiting the spread, activity or growth of pathogens (Loon *et al.,* 1994, van Loon 1999). The functions of some PR proteins are understood, for example chitinases (Legrand *et al.,* 1987), endoglucanases (Ward *et al.,* 1991), and proteinase inhibitors (Fidantsef *et al.,* 1999), whereas others are unknown. The *B. graminis* effector protein CSEP0055 has been shown to interact with PR1 and PR17, indicating a role in the suppression of plant defense (Zhang *et al.,* 2012). It is possible that BEC1054 and CSEP0055 may moderate the antifungal activity of PR proteins.

4.6.5.1. Pathogenesis Related Protein 5 (PR5)

The PR5 protein, identified through *in vitro* chromatography, is a thaumatin-like PR protein. Little is known about the specific roles of PR5 protein(s), including barley PR5. They possess a range of functions including antifungal activity (Hejgaard *et al.,* 1991). Some are expressed in healthy developing barley seeds in a tissue-specific manner (Skadsen *et al.,* 2000), but they also accumulate in the leaves of barley infected with *B. graminis* (Bryngelsson and Green 1989). Some have been shown to bind 1,3-β-D-glucan, and to induce hyperpolarization of the *Fusarium graminearum* membrane (Osmond

2000), and this activity also occurs in other plant hosts (Hejgaard *et al.,* 1991, Anzlovar *et al.,* 1998).

The induction of PR5 requires salicylic acid signaling, which is associated with defense against biotrophs (as opposed to necrotrophs) (Thomma *et al.,* 1998, Kessler and Baldwin 2002, Glazebrook 2005).

4.7.5.1. Pathogenesis Related Protein 10 (PR10)

The PR10 proteins are cytosolic small highly conserved proteins (15-18 kDa)(Loon *et al.,* 1994, Markovic-Housley *et al.,* 2003) which possess antifungal and antibacterial activities (Flores *et al.,* 2002). Expression of PR10 genes is activated through infection and through the jasmonic acid and salicylic acid pathways (Fristensky *et al.,* 1988, Somssich *et al.,* 1988, Pinto and Ricardo 1995, McGee *et al.,* 2001); the induction can occur during developmental stages in a tissue- or organ- specific manner (Apold *et al.,* 1981, Crowell *et al.,* 1992, Warner *et al.,* 1994), (Markovic-Housley *et al.,* 2003). In barley, PR10 expression is epidermis specific, and occurs as a result of epidermal cell perception of a small phytotoxic protein (Steiner-Lange *et al.,* 2003) produced by barley pathogen *Rhynchosporium secalis* (Rohe *et al.,* 1995). The PR10 proteins possess a number of activities, including binding brassinosteroids (Markovic-Housley *et al.,* 2003), cytokinins (Fujimoto *et al.,* 1998), flavonoids and fatty acids (Mogensen *et al.,* 2002).

Protein PR10 is part of the Betv 1/PR10/MLP family. This family contains a number of toxic lectins, which are type II RIPs (Kourmanova *et al.,* 2004). Some PR10 proteins

have RNase activity (Bufe *et al.,* 1996, Bantignies *et al.,* 2000, Wu *et al.,* 2003). I propose that PR10 may possess RIP function.

4.8.5.1. Eukaryotic Elongation Factors 1 alpha and gamma

The majority of the eEFs identified were found to be part of the eEF1 complex (Figure 19); which occupies the A-site of the small ribosomal subunit, near the SRL; as does eEF2 (Figure 20)(Unbehaun *et al.,* 2007). Multiple copies of eEF1A were identified following insertion of PCR products into pCR8. Six eEFs and eIFs (eEF1A, eEF2, eEF3, eIF4A, eIF4G and eIF5A) are encoded by multiple genes; with the occurrence of multiple alleles for initiation and elongation factors being a feature conserved amongst eukaryotes (Firczuk *et al.,* 2013).

Ribosomal protein eEF1g is involved in formation of eukaryotic elongation factor-1 (eEF-1) complex (see above). It is associated with RIPs (Unbehaun *et al.,* 2007). Here, I have found that it interacts in one bait-prey orientation with BEC1054 (BEC1054+eEF1g) in yeast. Taken together, these results support the hypothesis that BEC1054 may interact directly with this RIP-associated ribosomal protein (Figure 18).

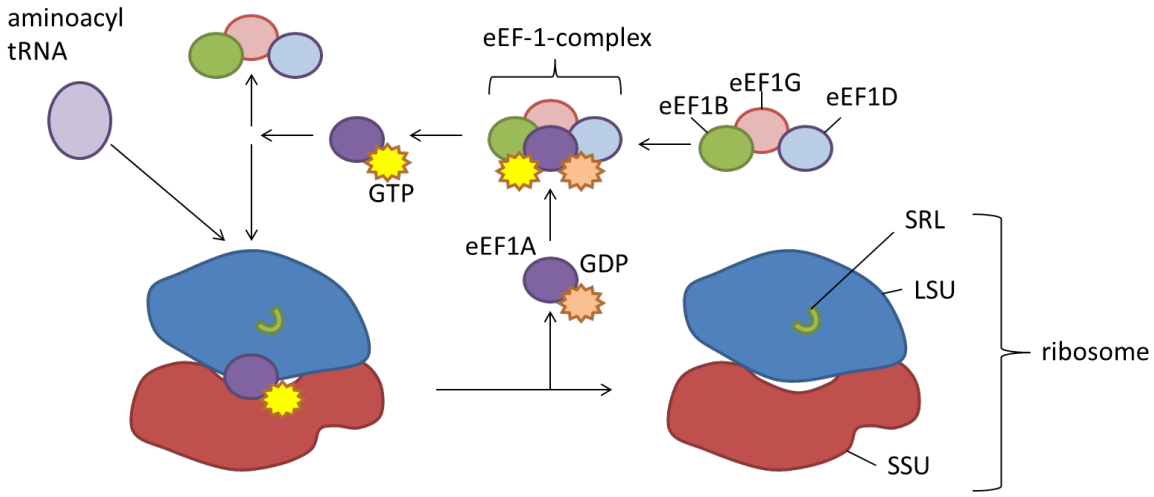


Figure 19: **Model of the formation of the eukaryotic elongation factor-1 (eEF-1) complex during translation elongation.** An aminoacylated transfer RNA is delivered to the ribosome "A" site by eukaryotic elongation factor 1 alpha (eEF1A) in complex with GTP. When codon-anticodon recognition occurs, the GTP is hydrolysed to GDP, and eEFA-GDP is released. The three subunits; eEF1B, eEF1G and eEF1D; together with eEF1A form the eEF-1 complex; where eEF1B, eEF1G and eEF1D act as the GTP exchange factor for eEF1A; allowing active eEF1A to be regenerated. "SRL" stands for the ribosomal sarcin-ricin loop, "LSU" for ribosomal large subunit, and "SSU" for ribosomal small subunit. Figure adapted from (Li *et al.,* 2013) and (Unbehaun *et al.,* 2007).

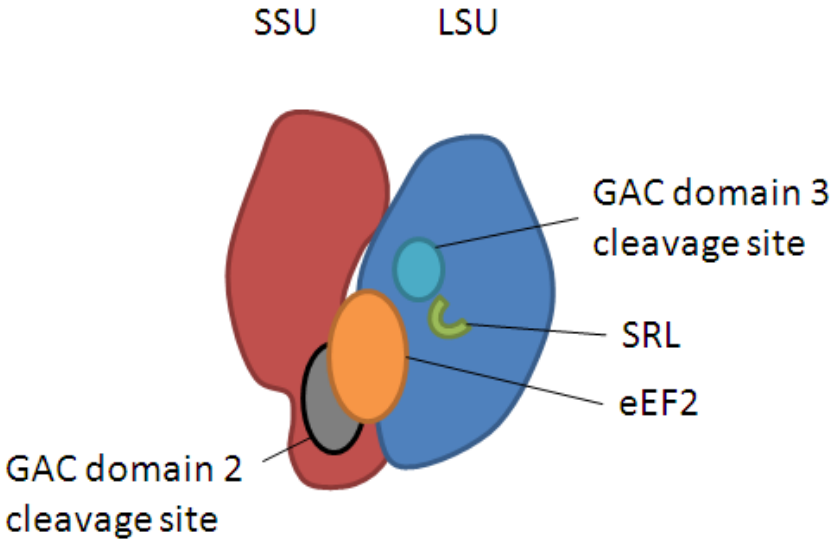


Figure 20: **Eukaryotic elongation factor 2 (eEF2) occupies the A-site of the small ribosomal subunit.** Where "SSU" stands for the ribosomal small subunit, "LSU" stands for the ribosomal large subunit, SRL for the ribosomal sarcin-ricin loop, GAC for "GTPase associated center."

4.9.5.1. Glutathione-S-transferase (GST)

The GSTs catalyse the conjugation of electrophilic molecules with glutathione (Dean *et al.,* 2005). They are involved in responses to pathogen invasion and stress through glutathione peroxidase activity, but the exact function of most GSTs is poorly understood (Edwards *et al.,* 2000, Dean *et al.,* 2005). In addition, they provide further protection through reduction of cytotoxic hydroperoxides, for example hydrogen peroxide or nucleic and fatty acid hyperoxides, to their respective alcohols (Li *et al.,* 1997, Dixon *et al.,* 2002, R. Sharma *et al.,* 2004).

The GST investigated in this study is a *zeta* class GST (zGST)(Edwards *et al.,* 2000). This class of GSTs possess glutathione peroxidase activity in a number of species including wheat (Cummins *et al.,* 1997), maize (Dixon *et al.,* 1997, Dixon *et al.,* 1998), soybean (Skipsey *et al.,* 1997) and *Arabidopsis* (Eshdat *et al.,* 1997). It is not clear why BEC1054 and GST interact, and this may be worth further investigation.

4.6. Conclusion

The fungal effector BEC1054 is an RNase like protein, which I propose may bind ribosomes, outcompeting RIPs. An *in vitro* pull-down demonstrated that BEC1054 was associated with significantly more ribosomal proteins than the negative controls used. I used three differing experimental methods to investigate the interaction of BEC1054 with proteins *in vitro*, in yeast and *in planta.* I found that five proteins interacted with BEC1054 in all experiments: PR5, PR10, GST, eEF1A(1) and eEF1G, the latter two of which are associated with ribosomes. In addition, GST and PR10, which is an RNase from a family containing RIPs, interacted with each other in yeast and *in planta*.

4.7. Further work

As in any investigation, there are always further controls, or further experiments that would have been completed if there were more time/resources available. This section, and the 'Further work' section at the end of each results chapter, indicates the ones that I would most like to have completed.

AIM: To validate further interactors from the LCMS analysis, focusing on other ribosomal proteins and the main targets identified in the latest LCMS re-analysis.

OBJECTIVE: To utilise Y2H and BiFC approaches to test further protein-protein interactions in a 1:1 manner.

REASONING: The latest re-analysis of the LCMS data was performed after I had finished the cloning for the Y2H assay, and just before I was due to move to Aachen. The re-analysis was performed against the IBSC database, using more stringent criteria than those used for the U36 (HarvEST), with all proteins found in any negative control being excluded from the dataset. Validation of these proteins as interactors for BEC1054 would help to validate the results seen in our LCMS assay, and to help determine whether more stringent analysis produced a more accurate representation of the host protein interactors of this effector.

AIM: To utilise further controls for the BiFC assay.

OBJECTIVE: To test the interactors previously identified against a series of BEC1054 mutants to identify the interacting sites.

REASONING: One of the major disadvantages of BiFC, is that the N and C terminal parts of the fluorescent protein can interact irreversibly without an interaction having

occurred between the two proteins of interest. Choosing one, or a selection, of negative controls, such as a version of the bait protein with a mutated binding site, is therefore critical when confirming a protein-protein interaction (Horstman *et al.,* 2014).

AIM: To perform further methods of protein-protein interaction validation

OBJECTIVE: To utilise fluorescence resonance energy transfer, to further validate the interactions identified through Y2H and BiFC.

REASONING: Methods which utilise FRET give information on both the protein-protein interaction, and also the localisation of the individual proteins (Horstman *et al.,* 2014). In addition, since FRET works through two fluorophores transferring excitation energy between them when they are in close proximity, not on the reassembly of a fluorescent molecule, the problem of the two halves of a fluorescent molecule reassembling is overcome.

AIM: To determine whether PR10 possesses RIP function

OBJECTIVE: To determine whether PR10 inhibits translation in wheat germ lysate, or rabbit reticulocyte lysate.

REASONING: The inhibition of translation is a technique used to identify RIP activity. The RIP proteins arrest protein synthesis at the translation step (Endo and Tsurugi, 1987, Sharma *et al.,* 2004). Protein PR10 is part of the Betv 1/PR10/MLP family. This family contains a number of toxic lectins, which are type II RIPs (Kourmanova *et al.,* 2004). Some PR10 proteins have RNase activity (Bufe *et al.,* 1996, Bantignies *et al.,* 2000, Wu *et al.,* 2003).

5. Expression profiling of Candidate Secreted Effector Protein family 21

5.1. Introduction

In *B. graminis*, the internal features (i.e. those that occur within the host plant) are comprised of haustoria formed within epidermal cells. The external features (those that occur on the surface of the leaf) include surface hyphae, spores, and primary and secondary germ tubes (Both and Spanu 2004). Significant changes in transcript abundance occur during infection (Both *et al.,* 2005). The prior alterations, i.e. promotion or inhibition of gene activity, required to cause these mean that the expression levels of housekeeping genes, and the abundance of gene transcripts, cannot be assumed to remain constant.

Transcript abundance can be assayed simultaneously across many different samples through quantitative real-time PCR (qPCR) (Higuchi *et al.,* 1993, Heid *et al.,* 1996, Fink *et al.,* 1998). The qPCR method is both more rapid and relatively high throughput, when contrasted with other methods of RNA quantification, for example competitive RT-PCR, northern blots or ribonuclease protection assays (Vandesompele *et al.,* 2002).

An alternative normalization control for qPCR is DNA. This can take the form of circularized or linear plasmid DNA, or genomic DNA (Yun *et al.,* 2006, Hou *et al.,* 2010). Supercoiled plasmid DNA has been shown to suppress PCR amplification, which can lead to elevated estimates of genomic DNA copy number, and presumably of RNA transcript abundance also. Genomic DNA or linear DNA (linearized plasmid or PCR

amplicons) can be used, but they are only subject to the amplification step, not to the reverse transcription step required for mRNA samples, meaning that the results may not be strictly comparable (Chen *et al.,* 2007, Hou *et al.,* 2010).

A number of variables need to be considered when investigating alterations in transcript abundance through qPCR, including template quality and quantity (Vandesompele *et al.,* 2002). Normalisation of transcript abundance can be done through traditional methods, including determining the amount of 18S/28S RNA (where the total amount can only be reduced, not removed), determining cell number (which is problematic in solid samples, i.e. barley leaves), or through determining the RNA mass quantity (which does not assess enzymatic efficiency or the quality of the substrate). Normalisation is carried out for qPCR through the use of internal control genes, frequently called "housekeeping" genes. These genes are used as a comparative reference for the genes of interest. Housekeeping genes should ideally be stably expressed, within the tissue under investigation, despite experimental treatment. These genes require validation of their stability, as expression can vary significantly (Thellin *et al.,* 1999, Bustin 2000, Suzuki *et al.,* 2000, Warrington *et al.,* 2000).

Vandesompele *et al.,* (2002) developed a method to validate control gene expression without a reliable measure with which to perform control normalisation. Their method utilises the idea that two ideal control genes would have identical expression ratios, regardless of experimental conditions or cell type. Any variation in the housekeeping genes" expression ratios would indicate that the genes were not constantly expressed; with increasing expression stability corresponding to a decreasing ratio.

As is the case for other haustorium-forming pathogens, *B. graminis* and its host perform "secretory warfare", with the haustorial complex acting as the delivery site for small effector proteins, such as the members of CSEP family 21 (O'Connell and Panstruga 2006, Panstruga and Dodds 2009). In this study, the levels of messenger RNA (mRNA) encoding members of CSEP family 21, which includes BEC1054 and its paralogs, were quantified along with a conidia-specific gene, and five proteins predicted to interact with BEC1054 (Pennington *et al.,* 2015).

5.2. Aims and Objectives

AIM: To identify and validate normalisation controls for use in qPCR across a *B. graminis* infection time course, for barley epidermal tissue, and for *B. graminis* epidermal and epiphytic material.

OBJECTIVE: To identify a selection of control genes from literature searches, and to utilise qPCR with geNorm control gene validation software, to identify the best controls from the selection screened.

AIM: To investigate the changes in transcript abundance for *bec1054* (*csep0065*), for the other members of CSEP family 21.

OBJECTIVE: To utilise qPCR, to determine changes in transcript abundance for members of CSEP family 21, and for a conidia-specific gene across an infection time course.

AIM: To investigate the changes in transcript abundance for putative barley interactors of BEC1054.

OBJECTIVE: To utilise qPCR, to determine changes in transcript abundance for protein interactors of BEC1054 identified through yeast-two-hybrid and bimolecular fluorescence complementation.

5.3. Individual Contributions

I applied for, and was awarded, a PrimerDesign Gold Studentship (2014-2015), which provided:

1. training, technical advice and support
2. access to geNorm software
3. a seminar series on qPCR given by Dr Alison Davis from PrimerDesign at Imperial
4. experimental chemicals/reagents required for this project.

Data was obtained with the assistance of the Masters student Linhan Li, who I supervised. Linhan completed, or took part in:

1. preparation of RNA from epidermal and epiphytic material
2. testing and validation of control genes using geNorm software
3. design and testing of qPCR primers for the four members of CSEP family 21 and the conidia specific gene
4. performance of qPCR reactions for the CSEPs and their controls, and collection of data necessary for the Pfaffl calculations

I completed, or took part in, a number of practical elements of this project, including:

1. identification of candidate control genes through literature searches
2. identification and obtaining of both gDNA and cDNA sequences for the candidate control genes, and the barley putative interacting protein sequences
3. submission of data/cDNA, required for primer design and optimization, to the sponsoring company PrimerDesign
4. troubleshooting of qPCR
5. testing and validation of control genes using geNorm software

5.4. Results

5.4.1. Selection of control genes

I selected six genes for investigation as *B. graminis* normalisation controls (

Supplementary Table 17): monoglyceride lipase (*mgll* (Both *et al.,* 2005, Nowara *et al.,* 2010)); β-tubulin (*tubb* (Zhang *et al.,* 2000, Hacquard *et al.,* 2013)); α-tubulin (*tuba* (Hacquard *et al.,* 2013)); histone 3 (*h3* (Both *et al.,* 2005)); actin (*actb* (Hacquard *et al.,* 2013)); and glyceraldehyde 3-phosphate dehydrogenase (*gapdh* (Hacquard *et al.,* 2013)). Three, *mgll, h3* and *tubb* had previously been characterized as being stable during the interaction of barley and *B. graminis* (Both *et al.,* 2005). Three of the selected genes have also been utilised for the normalisation of a number of fungal species, including *Fusarium graminearum* (Brown *et al.,* 2011); *Melampsora larici-populina* (Hacquard *et al.,* 2011); *Magnaporthe oryzae* (Kim *et al.,* 2009); *Aspergillus niger*, *Penicillium chrysogenum* and *Cladosporium cladosporioides* (Ettenauer *et al.,* 2014).

I selected five barley genes as normalisation controls for *H. vulgare* (Supplementary Table 18): *actb* (Jiang *et al.,* 2011, Ma *et al.,* 2013); *gapdh* (Besse *et al.,* 2011, Ma *et al.,* 2013) (a similar *gapdh* has also been used in the literature (Identities 813/987 (82%), Query cover 71%, Blastn-2-sequences) (Jarosova and Kundu 2010)); adenosine triphosphatase (*h+-atpase* (Besse *et al.,* 2011)); ubiquitin (*ubi* (Trujillo *et al.,* 2006, Besse *et al.,* 2011)); and *tuba* (*tuba2b* (Doblin *et al.,* 2009, Besse *et al.,* 2011)). An additional, similar, *tuba2b* was also tested as a normalisation control Accession: AK260165 (Identities 1600/1602 (99%), Query Cover 98%, Blastn-2-sequences).

The internal stability of a control gene (M), can be calculated as the pairwise variation of a control gene with all other control genes. For each control gene, I determined the pairwise variation from the standard deviation of the logarithmically transformed expression ratios of the transcript quantities. A high M indicates a large variation in the

abundance of transcripts, and a low M a small variation in abundance (i.e. stable expression). I determined the M value; excluded the least stable gene based on the average pairwise variation; and repeated the process in a stepwise manner to determine the two genes with the lowest M values (i.e. the most stable genes) (Table 12 and Figure 21). Originally, the algorithm used was not able to rank the two most stable genes (Table 12); as genes were excluded based on the average pairwise variation (Vandesompele *et al.,* 2002). Improvement of the geNorm software has allowed the identification of the most stable gene (qbase+ v3.0, Biogazelle, www.qbaseplus.com).

For *B. graminis,* I found the control genes *h3, gapdh* and *act* to be the most stable for both the *B. graminis* infected epidermis samples; and for the "combined analysis" sample (which was comprised of *B. graminis* epiphytic and epidermal material analysed together; Table 12). In epiphytic *B. graminis*, I found *gapdh, mgll* and *tuba* to be the most stable. In infected barley epidermis, I found that *tuba2b, ubi* and *gapdh* transcripts varied least.

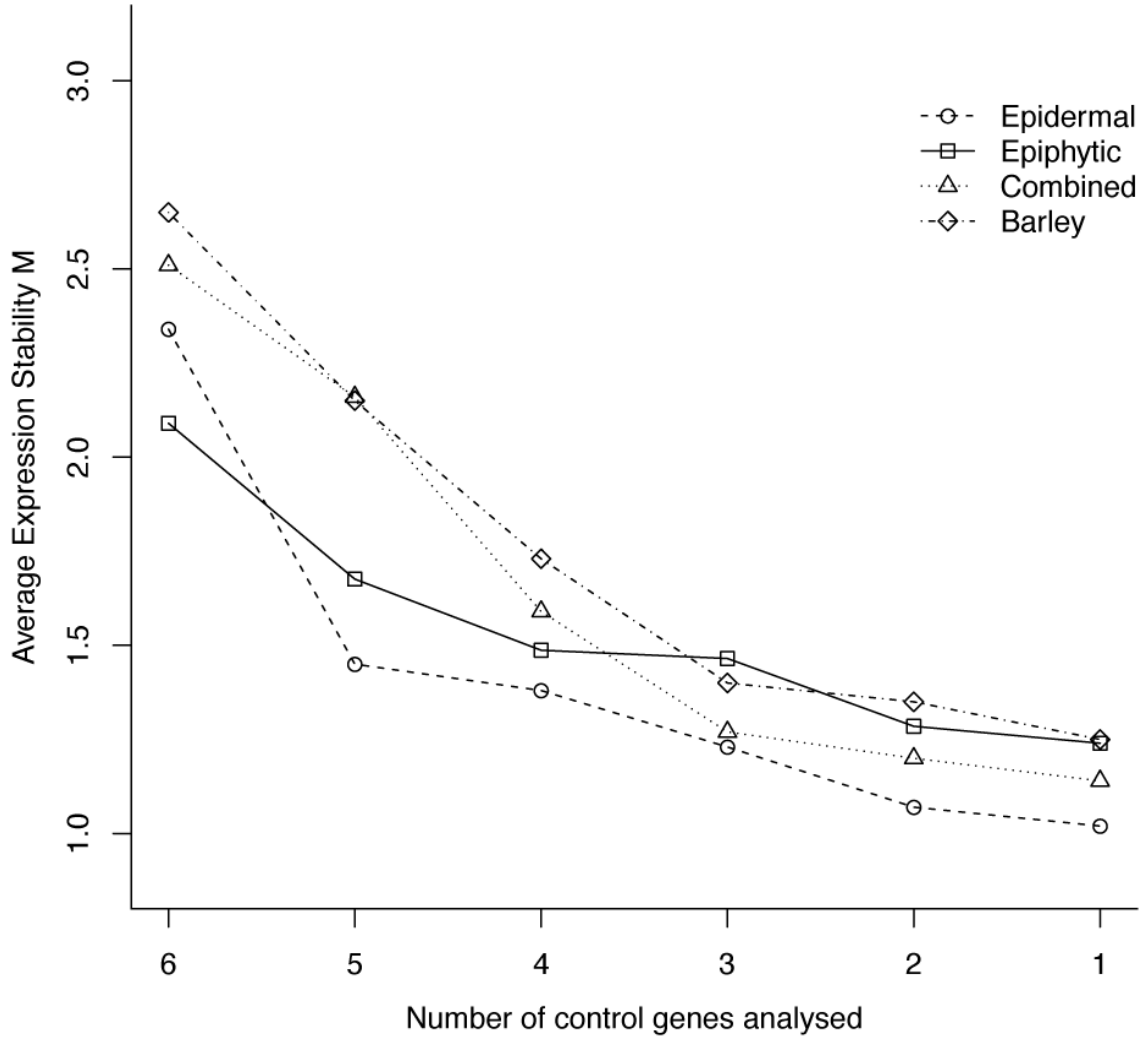


Figure 21: **Calculation of control gene stability (M).** The internal control gene stability can be calculated as the pairwise variation of a control gene with all other control genes. The M value was determined, the least stable gene excluded, based on the average pairwise variation; and the process repeated in a stepwise manner to determine the two genes with the lowest M values (i.e. the most stable genes). Improvement of the geNorm software has allowed the identification of the most stable gene. Control genes are ranked along the x-axis from left to right, in order of increasing stability. Six genes were investigated for each of the following substrates: *B. graminis* epiphytic material "Epiphytic"; *B. graminis* epidermal material "Epidermal"; combined *B. graminis* epidermal, and epiphytic material "Combined"; and barley epidermal material "Barley." Figure reproduced with permission from (Pennington *et al.,* 2015).

Table 12: **Control genes for B. graminis and barley ranked in order of expression stability**. The control genes are ranked from 1-6, where 1 is the most stable, and 6 is the least stable. Table reproduced with permission from (Pennington et al., 2015).

	Blumeria graminis **reference genes**			**Barley reference genes**
Rank	**Epidermal**	**Epiphytic**	**Combined**	**Epidermal**
1	*act*	*mgll*	*gapdh*	*gapdh*
2	*gapdh*	*tuba*	*act*	*ubi*
3	*h3*	*gapdh*	*h3*	*tuba2b*
4	*mgll*	*act*	*mgll*	*tuba*
5	*tub2b*	*h3*	*tub2b*	*atpase*
6	*tuba*	*tub2b*	*tuba*	*act*

5.4.2. Number of control genes

The "Pairwise Variation" (Vn/n+1) (where n is the number of genes, and $3 \leq n \leq 5$) was used to calculate the optimal number of reference genes (Vandesompele *et al.,* 2002). A large V indicates that the inclusion of the control gene has a significant effect; and that it should ideally be included for the calculation of the normalisation factor. I plotted the V values for the different substrates (Figure 22); and the results demonstrated that the optimal control gene numbers were three, four and three for *B. graminis* epiphytic, epidermal and combined qPCR substrates respectively. For barley epidermal material, I found the optimal number of control genes to be three. The minimum number of control genes recommended for normalisation was three (Vandesompele *et al.,* 2002). In our study, we used the best three controls for normalisation of barley and *B. graminis* transcript levels.

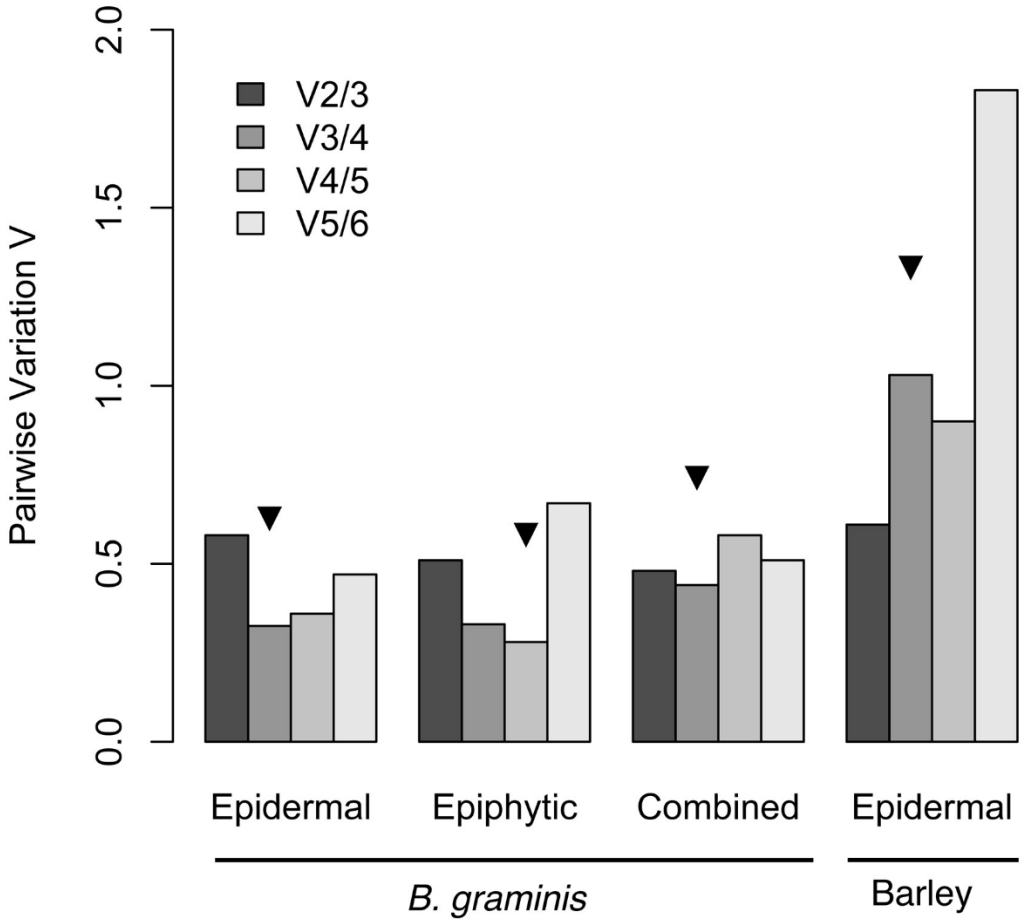


Figure 22: **Determining the optimal number of genes for gene normalisation.** Pairwise variation analysis was used to determine the number of control genes required for accurate normalisation. A high variation value (V) indicates a low correlation coefficient. The optimal number of control genes is marked by the symbol "▼'. The "Pairwise Variation" (Vn/n+1) (where n is the number of genes) was calculated between the normalisation factors NF, and NFn+1 to determine the optimal number of reference genes (see Vandesompele *et al.,* 2002 for details). The following substrates were used: *B. graminis* epiphytic material "Epiphytic"; *B. graminis* epidermal material "Epidermal"; combined *B. graminis* epidermal, and epiphytic material "Combined"; and barley epidermal material "Barley." Figure reproduced with permission from (Pennington *et al.,* 2015).

5.4.3. Control gene expression

We used the three optimal controls to normalise the remaining (non-optimal) control genes, and then plotted the Pfaffl values (Figure 23). For epiphytic material (Figure 23a), transcript abundance of the control gene *mgll* increased at 16hpi and decreased by 48hpi; but did not go back to pre-infection expression levels. The expression levels of *tuba* and *tub2b* fell, with minimum expression occurring at 48 hpi; the two controls then increased again by 120 hpi. We found that the pattern of transcript abundance for *tub2b* was the same in both combined and epidermal material (Figure 23b and c). For *tuba*, expression increased at 16 hpi; decreased; and then remained below the original level of *tuba* expression (Figure 23b and c). Variation was also seen in the expression levels of *mgll*, which increased at 16 hpi; fell at 48 hpi, and increased again at 120 hpi.

The transcript abundance of barley (Figure 23d) *act* decreased by 16 hpi, then increased by 48 hpi and again by 120 hpi. In contrast, *tuba2b* decreased by 16 hpi; following which it remained constant. The transcript abundance of *atpase* increased at 16 hpi and 48 hpi; and decreased by 120 hpi.

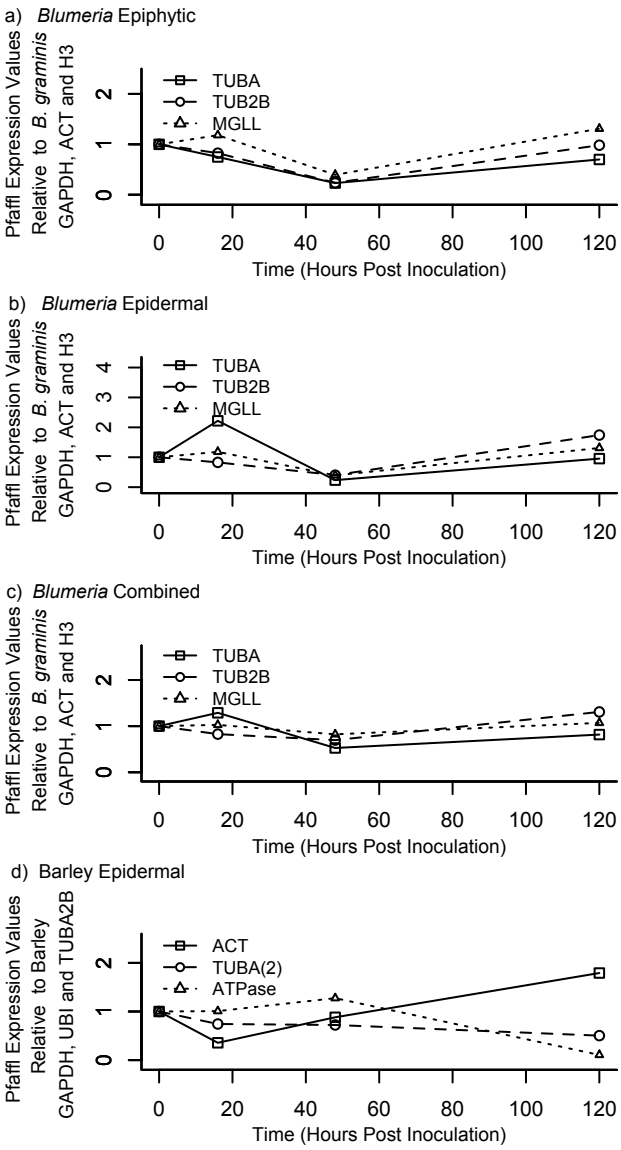


Figure 23: **Quantification of "control" gene RNA transcript levels.** Levels of RNA transcripts were calculated using the Pfaffl method, and are shown relative to 0 h post inoculation. The different substrates used were *B. graminis* a) epiphytic material; b) epidermal material; c) combined epiphytic and epidermal material; and d) barley infected epidermal material. Figure reproduced with permission from Pennington *et al.,* (2015).

5.4.4. CSEP expression

For the CSEPs, three biological replicates were used for each time point. Biological replicated were treated as independent samples for further analysis. Initial analyses, and calculation of the C_T values was done using 7500-Fast Software v1.0 (ThermoScientific). The "no-template" controls for the CSEPs and control primers did

not show amplification. Analysis of the control genes was performed using geNorm software (Vandesompele *et al.,* 2002). Further analysis of the controls, and between the controls and the CSEPs, was performed using the Pfaffl calculation method (Equation 14) (Pfaffl 2001).

Equation 14: **Relative quantification of target genes via the Pfaffl method.** Where "ratio" is the relative expression ratio, "E" is the real-time PCR efficiency, and "ΔCP" is the crossing point difference of a sample and a control.

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

We investigated the RNA transcript abundance of CSEP family 21 and a conidia-specific gene during the initial stages of *B. graminis* infection of barley (Figure 24). In general, the four CSEPs investigated showed the same trend in epiphytic material, with a primary peak of maximum expression at 6 hpi, and a broad secondary peak at 24 hpi (*csep0066*), or between 24 and 48 hpi (*csep0064, csep0065* and *csep0264*). The two CSEPs (0064 and 0066) demonstrated maximum transcript abundance in epidermal material at 48 hpi; which then decreased to near the initial level; whereas *csep0065* and *csep0264* increased in transcript abundance, with a maximum at 48 hpi, followed by a decrease in abundance by 120 hpi. The same overall trends were observed when using the two best controls (*act* and *gapdh*), as when using the three best controls (*act, gapdh* and *h3*). In contrast, use of the worst control (*tub2b*) obscured the second peak for all CSEP epiphytic material. This change in trend was the most pronounced for *csep0066* and *csep0264*; with *csep0066* even gaining an additional secondary peak.

Following infection, the conidia-specific gene showed a general decrease in expression for both epiphytic and epidermal material. We found this to be the case for normalisation against the best (*act, gapdh* and *h3*) and worst (*tub2b*) normalisation control genes.

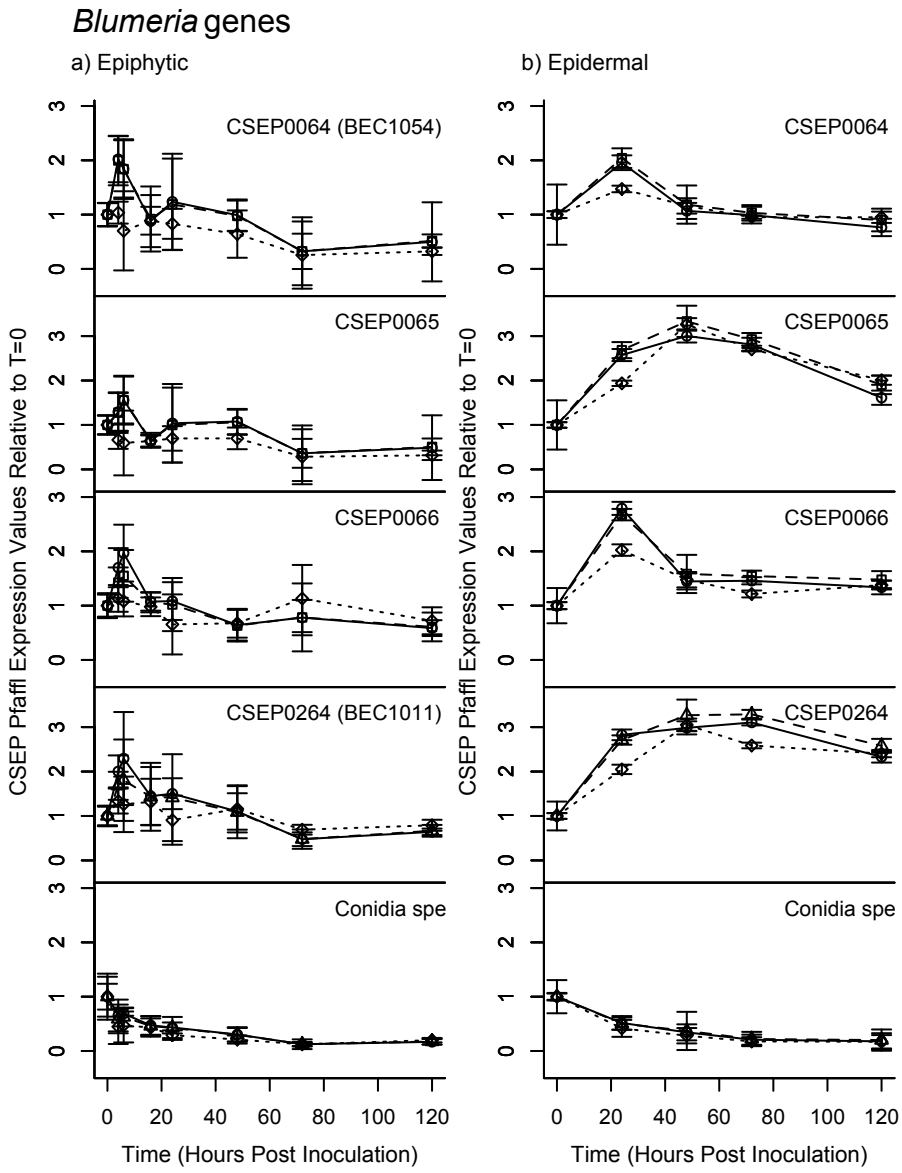


Figure 24: C**andidate Secreted Effector Protein (CSEP) family 21 expression during infection with *Blumeria graminis*.** Transcript levels were investigated using quantitative real-time polymerase chain reaction (qPCR). The RNA substrates were a) infected epiphytic material; b) infected epidermal material. Results are shown as Pfaffl values, relative to 0 h post inoculation (hpi) (=1) for tissue samples from zero to 120 hpi, with standard deviation error bars for three biological replicates. The normalisation control genes were actin (accession CCU76638), glyceraldehyde-3-phosphate dehydrogenase (accession CCU80715) and histone-3 (accession CCU82905). Figure reproduced with permission from Pennington *et al.,* (2015).

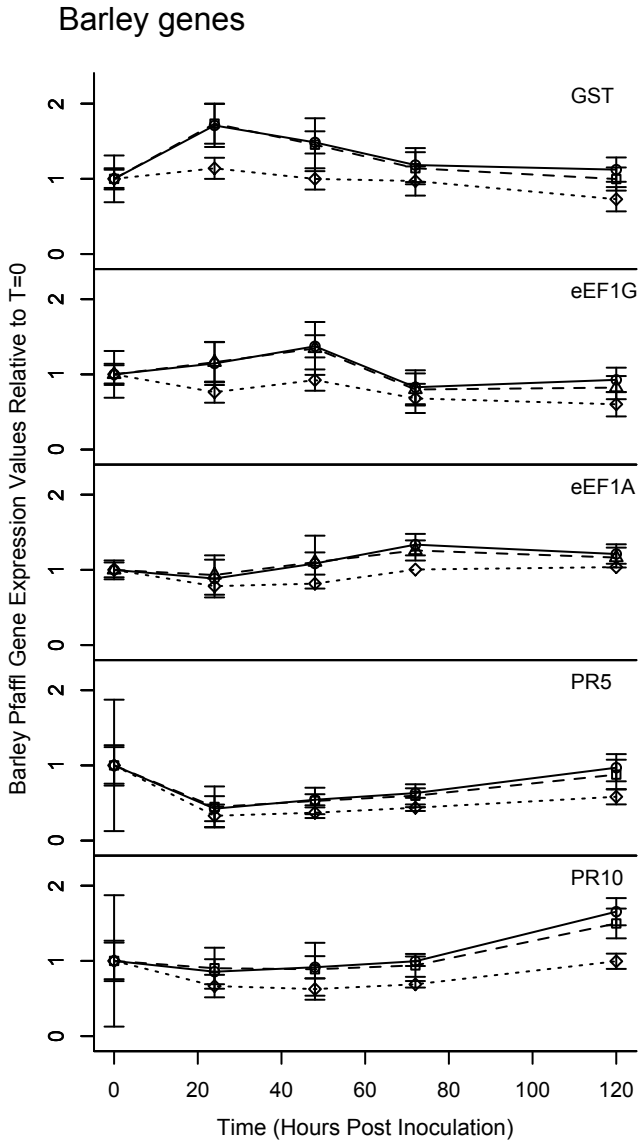


Figure 25: **Barley gene expression during infection with *Blumeria graminis*.** Transcript levels were investigated using quantitative real-time polymerase chain reaction (qPCR). The RNA substrates were a) infected epiphytic material; b) infected epidermal material. Results are shown as Pfaffl values, relative to 0 h post inoculation (hpi) (=1) for tissue samples from zero to 120 hpi, with standard deviation error bars for three biological replicates. The normalisation control genes were tubulin A (accession: U40042), ubiquitin (accession: X04133) and glyceraldehyde phosphate dehydrogenase (accession: X60343). Error bars represent the standard deviation of three biological replicates. The abbreviation GST stands for glutathione-S-transferase, eEF1A for eukaryotic elongation factor 1 alpha, eEF1G for eukaryotic elongation factor 1 gamma, and PR for pathogenesis response. Figure reproduced with permission from Pennington *et al.,* (2015).

5.5. Discussion

The issue of accurate and reliable quantification of gene expression has been increasingly highlighted in the literature, especially when the aim is to observe subtle changes, as changes in the abundance of housekeeping genes can obscure changes in the transcript abundance of the genes of interest (Pfaffl, 2004). The abundance of housekeeping genes has been shown in numerous studies to vary considerably (reviewed by Vandesompele *et al.,* (2002)).

The use of single control genes for qPCR normalisation is associated with error, caused by changes in the transcript abundance of the control gene used. The use of multiple control genes is therefore recommended (Vandesompele *et al.,* 2002). In this assay, during an infection time-course, six *B. graminis* and five barley genes were investigated as potential normalisation genes for *B. graminis* infected material, and for infected barley epidermal material. Optimal normalisation controls were selected (Table 12); and used for the normalisation of the remaining control gene Pfaffl values (Figure 23). These results demonstrated that control gene abundance varies between genes.

When compared with transcript abundance of the optimal *B. graminis* controls *act, h3* and *gapdh; tuba* transcript was found to accumulate at 16 hpi. At around this time, the fungus penetrates the cell of the host plant, forming complex multidigitate haustoria (Spanu and Kaemper 2010). The increase in *tuba* may represent the cytoskeletal changes taking place within the fungus.

Obligate biotrophs are challenging to investigate: the infection process and their life cycle are closely linked; they cannot be cultured outside of the host; and their structures differ inside and outside of the host (Spanu and Kaemper 2010). Dissecting the epidermal layer from barley leaves increases the percentage of the biomass represented by the fungus (although this still remains small). This method reduces the competing signal from the host tissue, without losing *B. graminis*, which grows only within epidermal cells. Our study found different normalisation optima for different tissue substrates (Figure 23 and Table 12). This result demonstrates the variation between cell types and tissue sources. The control genes *act, h3* and *gapdh* were used for *B. graminis* transcript abundance normalisation, as they were the least variable for the combined epidermal and epiphytic, and epiphytic qPCR substrates (Table 12).

Hacquard *et al.,* (2013), found *act* and *tubb* to be the two most stable controls for barley (compared with *gapdh*, eukaryotic elongation factor 1 alpha, ubiquitin conjugating enzyme *e2*, and *tuba*). In contrast, we found that the least stable normalisation control was *tubb* in epidermal material. These differences may be due to the differing experimental set-up, with different time-scales, control genes and sample materials (whole-leaf instead of epidermal tissue) used. The changes in the transcript abundance of some genes were found to be obscured by the use of the worst control (*tub2b*). For *csep0066* and *csep0264*, the use of the worst control produced an additional secondary peak, which was not observed when other controls were used (Figure 24). These differences in the observed results further emphasize the need for control genes appropriate to each experiment, and to the investigation in question.

The three most stable controls identified for barley, during infection, were *tuba2b, ubi* and *gapdh*. The least stable normalisation control was *act* (this contrasts with *B. graminis*, where *act* was one of the most stable). The two normalisation controls *atpase* and *act* were found to increase at 48 hpi. These results correlates with the formation of the haustorium, and extrahaustorial membrane formation (O'Connell and Panstruga 2006, Panstruga and Dodds 2009). The increase in transcript abundance of the cytoskeletal genes may reflect the epidermal cell response to the attempted penetration by the pathogen (Kobayashi *et al.,* 1992, Gross *et al.,* 1993, Both *et al.,* 2005).

A recent investigation (Ferdous *et al.,* 2015) also utilised the normalisation control genes *gapdh, tuba* and *act*, in addition to microRNAs and small nucleolar RNAs. They investigated their use in barley under a variety of experimental conditions, including the infection cycle of *Rhynchosporium commune*, a necrotrophic fungal pathogen. In our study, we found that of the three genes (*gapdh, tuba* and *act*), *tuba* was the most stably expressed, and *act* the least; whereas Ferdous *et al.,* (2015) found *act* to be the most stable, and *tuba* the least. The difference in results may be due to the different experimental conditions, or to the use of a different, necrotrophic, fungus. Their study found that different control genes were optimal under different experimental treatments, and that the order of stability varied. They did, however, demonstrate that an ADP-ribosylation factor-1 like protein and small nucleolar RNAs may perform well under a variety treatments/conditions.

Similar expression patterns were found for the four CSEP members of family 21, with peaks in transcript abundance occurring at 16 hpi, and 24 or 48 hpi. The former time

point represents the formation of the penetration peg, and the latter the stages between infection and the formation of fungal colonies visible to the naked eye on the surface of the leaf (Both *et al.,* 2005). The occurrence of multiple peaks indicates that these CSEPs may be involved in more than one part of the infection process. Overall, the results found for *csep0064* and *csep0066* agree with those previously published by Pliego *et al.,* (2013). The secondary peak detected occurred at the same time-point (ca. 24 hpi) (Pliego *et al.,* 2013). An expression maximum was also seen, in epidermal material, for *csep0064* and *csep0066* at 24 hpi. The patterns of transcript abundance were different for *csep0065* and *csep0246* in epidermal and epiphytic material. The two CSEPs showing general elevation and a maximum transcript abundance at 48 hpi, indicating that they take part in the later infection stages, possibly aiding the spread of epiphytic hyphae across the surface of the leaf and further preventing the recognition of the fungus.

There was no peak in transcript abundance seen for the conidia specific gene, indicating that the peak in transcript accumulation seen for the CSEPs in both epiphytic and epidermal substrate is biologically relevant, i.e. that it is not the product of normalisation bias.

The five barley genes identified through Y2H and BiFC as possible interactors for BEC1054 (Section 4) were also investigated; and their expression patterns were found to be very varied (Figure 25). The increase in expression for *eef1a, eef1g* and *gst* overlaps with the peak in abundance for *csep0064* seen in both epidermal and epiphytic material. In contrast, the two PR proteins, when compared with pre-infection expression levels, were found to be reduced at almost all time-points.

5.6. Conclusion

Six *B. graminis* housekeeping genes, and five barley genes, were investigated as potential qPCR normalisation controls for a *B. graminis* infection time course. The optimal *B. graminis* housekeeping genes were found to be *act, gapdh* and H3; and for barley *gapdh*, *ubi* and *tuba2b*. The housekeeping genes investigated were shown to vary significantly between species and tissues, demonstrating the necessity for appropriate controls for each qPCR assay. The four members of CSEP family 21 showed diverse expression patterns in epiphytic and epidermal material. In epiphytic material, all four CSEPs showed a primary peak of maximum expression at six hpi, and a broad secondary peak at 24 hpi (*csep0066*), or between 24 and 48 hpi (*csep0064, csep0065* and *csep0264*). In epidermal material, *csep0064* and *0066* demonstrated maximum transcript abundance at 24 hpi; which then decreased sharply to near the initial level by 48 hpi. In contrast, *csep0065* and *csep0264* increased in transcript abundance, with a maximum at 48 hpi, followed by a gradual decrease in abundance by 120 hpi.

A paper has been published on the work conducted in this chapter:

Pennington, H. G., Linhan., L. & Spanu, P. D. (2015). Identification and selection of normalisation controls for quantitative transcript analysis in *Blumeria graminis*. *Molecular Plant Pathology*. DOI: 10.1111/mpp.12300 [Epub ahead of print]

5.7. Further work

AIM: To investigate the relative transcript abundance of JIP60, and other barley RIPs, during a *B. graminis* time course.

OBJECTIVE: To determine, through qPCR, whether barley RIPs such as JIP60 are up-regulated following infection.

REASONING: This information would allow us to determine whether RIPs, including JIP60, are transcribed in a manner that changes following infection with *B. graminis*. Any changes in transcription may help to elucidate their role in the plant defense response.

AIM: To investigate the relative transcript abundance of the proteins identified through the re-analysis of the LCMS work.

OBJECTIVE: To utilise qPCR to determine whether the proteins identified from the more stringent analysis of the IBSC database were expressed within a time-frame in which they could interact with BEC1054.

REASONING: This information, in conjunction with further Y2H experiments conducted on these putative protein interactors (suggested in Further Work Section 4.7) would allow us to determine if they are expressed at the same time as BEC1054.

6. Ribonucleic acid (RNA) interactors of BEC1054

6.1. Introduction

The BECs were identified through proteomic analysis as proteins associated specifically with host cells colonized by haustoria. Structural prediction of the two functionally validated effectors, BEC1011 and BEC1054 determined that they possess an RNase like fold similar to a microbial RNase, but the catalytic active site residues required for RNase activity are absent (Bindschedler *et al.,* 2011, Pedersen *et al.,* 2012). This structure of BEC1054 has recently been confirmed experimentally (R. Jones, *pers. comm.*). This structure has led to these proteins being referred to as RNase-Like Proteins expressed in Haustoria (RALPH) effectors. The RALPHs represent the largest group of effectors within the *B. graminis* f.sp. *hordei* genome. Their abundance, and proliferation, within a genome, which is otherwise losing genes (Spanu *et al.,* 2010), indicates that they play a key role in the infection process.

Previous work performed within the host laboratory was unable to identify any RNase activity for recombinant *bec1054* expressed in *E. coli* (Kwon 2011). A general ribonuclease assay was performed, using methylene blue as an indicator of RNase activity. Methylene blue monomers intercalate into RNA, the degradation of which, through alkaline hydrolysis or enzymatic digestion, causes the absorbance at 688nm to decrease (Pritchard *et al.,* 1966, GreinerStoeffele *et al.,* 1996). Not only did induction of recombinant BEC1054 not show RNase activity, but its induction was associated with a relative decrease in RNase activity. This finding led to the proposal of a hypothesis in which BEC1054 competes with RIPs by binding to rRNA (Kwon 2011), preventing them

from cleaving a specific adenine base from the 28S rRNA sugar-phosphate backbone (Endo *et al.,* 1988b, May *et al.,* 1989, Funatsu *et al.,* 1991).

Ribonucleic acid binding proteins play roles in all parts of RNA biology, from pre-mRNA editing and splicing (Green 1991, Rueter *et al.,* 1999), transcription and polyadenylation (Wahle and Keller 1992), to translation (Shatkin 1985), transport and localisation (Kang and Cullen 1999), and degradation of RNA (Gherzi *et al.,* 2004). They can bind to RNA with varying degrees of sequence specificity and affinity; and the correct functioning of all of these networks and processes is essential as their disruption can lead to disease (Glisovic *et al.,* 2008). Determining RNA and protein binding partners can help to further elucidate these networks; with similar techniques being used to screen many different RNA-protein interactions. We decided to investigate the RNA binding activity of BEC1054, based on the aforementioned hypothesis that it may be binding to rRNA. To this end, a model of the 28S barley RNA was created, and used as a template for the synthesis of RNA fragments for further investigation.

Multiple *in vitro* and *in vivo* techniques are available for the study of protein-RNA interactions; their combined use provides a powerful method for determining interactions of proteins with RNA. For example, protein-RNA interactions can be investigated *in vitro* by differential scanning fluorimetry (DSF). This is a rapid method which, once a purified soluble protein has been obtained, allows rapid screening of ligands that bind to the purified protein (Senisterra and Finerty 2009). The method utilises a fluorescent dye which possesses an affinity for the protein's hydrophobic regions, and which is quenched in aqueous solution (Pantoliano *et al.,* 2001, Lo *et al.,*

2004). As the temperature of the solution is increased, the protein unfolds, leading to exposure of its hydrophobic core. This allows the dye to bind to the protein, and to unquench, leading to increased fluorescence. The T_m is defined as the midpoint of the protein unfolding (Niesen *et al.,* 2007). The comparison of the T_m with, and without, the presence of a ligand can be compared. The change in the T_m relates to the binding affinity of the protein and ligand; and can therefore be used as an indicator that binding occurred (Senisterra *et al.,* 2006, Vedadi *et al.,* 2006).

Alternatively, protein-RNA interactions can be investigated in yeast using a yeast-three-hybrid assay (Y3H; Figure 26)(SenGupta *et al.,* 1996). In this method, the RNA of interest is cloned into an RNA expression vector, which contains two RNA MS2 sequences, which bind to the bacteriophage MS2 protein. The bait-RNA is targeted to the DNA binding site by a hybrid protein, encoded in the yeast chromosome, composed of the LexA DNA binding domain and the MS2 coat protein. Interaction between the RNA of interest and the prey protein leads to the co-localisation of the activation domain to the reporter genes (Figure 26), which in turn leads to the activation of the reporter genes *his3* and *lacZ*. This method is rapid, as RNA sequences can be cloned into the pIIIA expression plasmid with relative ease. It has also been designed to be compatible with the prey-AD fusions, and the plasmids encoding them, used for most Y2H assays. We were therefore able to re-use the prey plasmids described in Section 4.4.4.

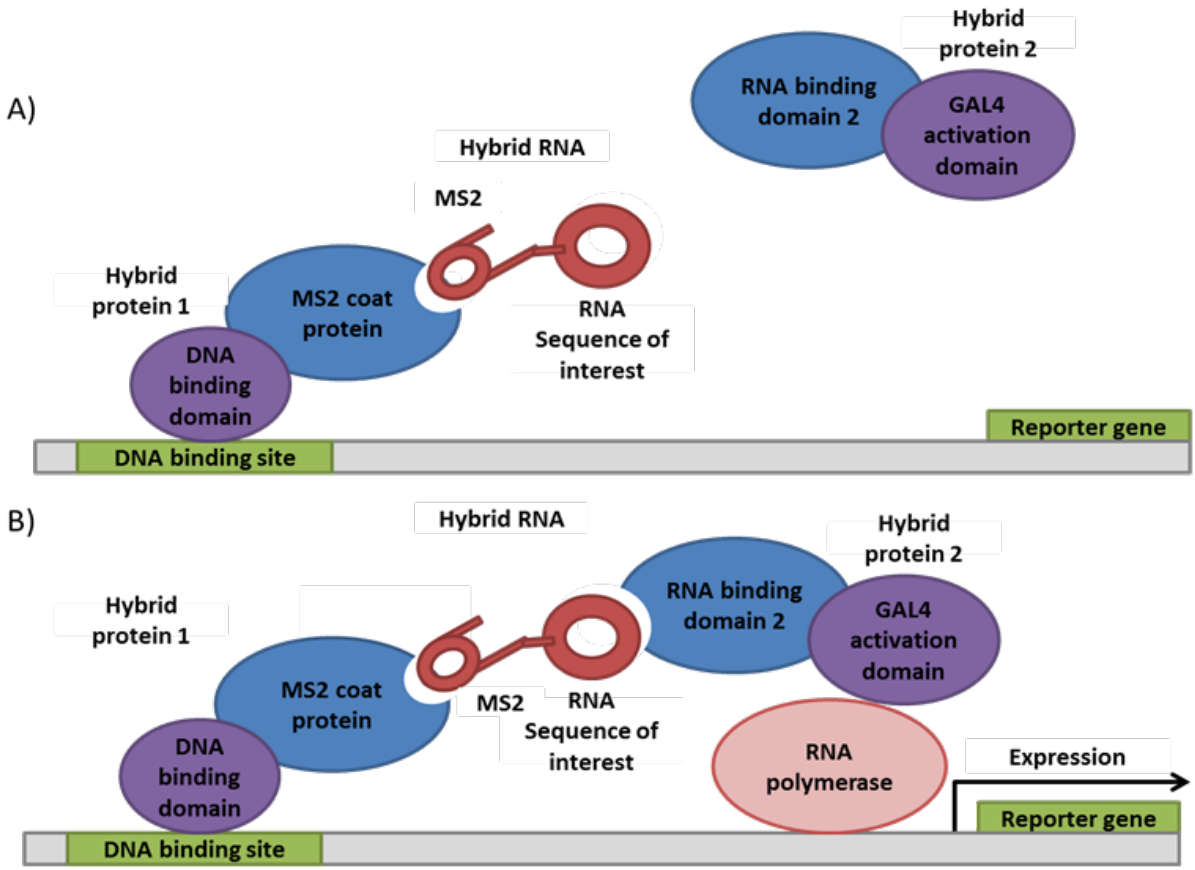


Figure 26: **Basis of the yeast-three-hybrid system.** A fusion RNA, composed of the RNA sequence under investigation and an MS2 binding site, is localised to the DNA binding site by a hybrid protein, encoded in the yeast chromosome, composed of or the LexA DNA binding domain and the MS2 coat protein. A) shows no interaction, where a second hybrid protein, composed of the GAL4 activation domain (AD) and the RNA protein of interest, does not interact with RNA. In B), The RNA binding domain of the second hybrid protein does interact with the RNA sequence of interest. The GAL4-AD is brought into close proximity with the regulatory region upstream of the reporter gene, leading to reporter gene transcription (SenGupta *et al.,* 1996).

6.2. Aims and Objectives

AIM: To create a sequence and structural model for the barley 28S rRNA.

OBJECTIVE: To use sequence information and ribosomal models available in the literature and online to create a model of the 28S rRNA for use in further experimental work.

AIM: To investigate the interaction of BEC1054 with RNA and DNA *in vitro.*

OBJECTIVE: To use DSF to determine whether BEC1054 interacts with RNA or DNA in a sequence or concentration specific manner.

AIM: To investigate the interaction of BEC1054 and JIP60 with rRNA.

OBJECTIVE: To use a Y3H assay to investigate the interaction of BEC1054 with rRNA; to investigate the interaction of JIP60 with rRNA.

6.3. Individual Contributions

The Differential Scanning Fluorimetry experiments were established with the help of the PhD student Giulia Bonciani. Giulia had:

1. determined the melting point for BEC1054 in sodium acetate buffer
2. found that BEC1054 was destabilised by RNA corresponding to the rat SRL

I obtained the yeast-three-hybrid kit from the laboratory group of Dr Wickens, at the University of Wisconsin-Madison. Members of their laboratory group kindly provided help and feedback via email.

6.4. Results

6.4.1. Ribosomal RNA and ribosomal putative interactors

I created a model for barley 28S rRNA based on a wheat model from GenBank, using data from IBSC un-annotated 454BacContigs (Figure 27). The 28S rRNA model's secondary structures were predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi), and viewed using PseudoViewer (http://pseudoviewer.inha.ac.kr/). Subsections used for experimental work are enlarged in Figure 28 and Figure 29.

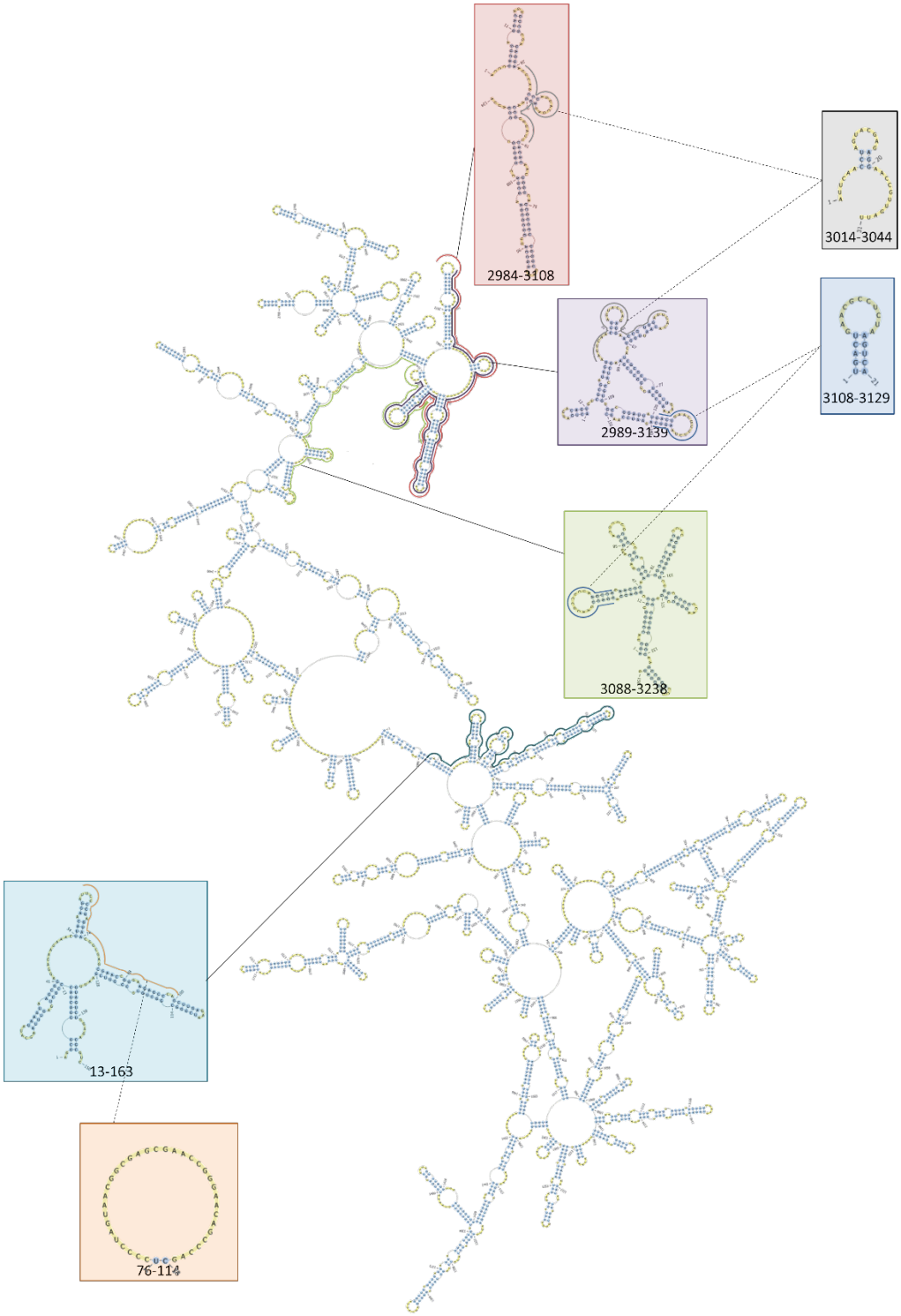


Figure 27: **Predicted secondary structure of the barley 28S ribosomal RNA.** A thermodynamic ensemble model was created for barley ribosomal RNA using Pseudoviewer (http://pseudoviewer.inha.ac.kr/), based on secondary structures predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Enlarged structures in coloured boxes highlight the sections used for experimental work; with their corresponding coloured lines marked onto the ribosomal RNA map. The numbers represent the bases, where "1" is the first 5' base.

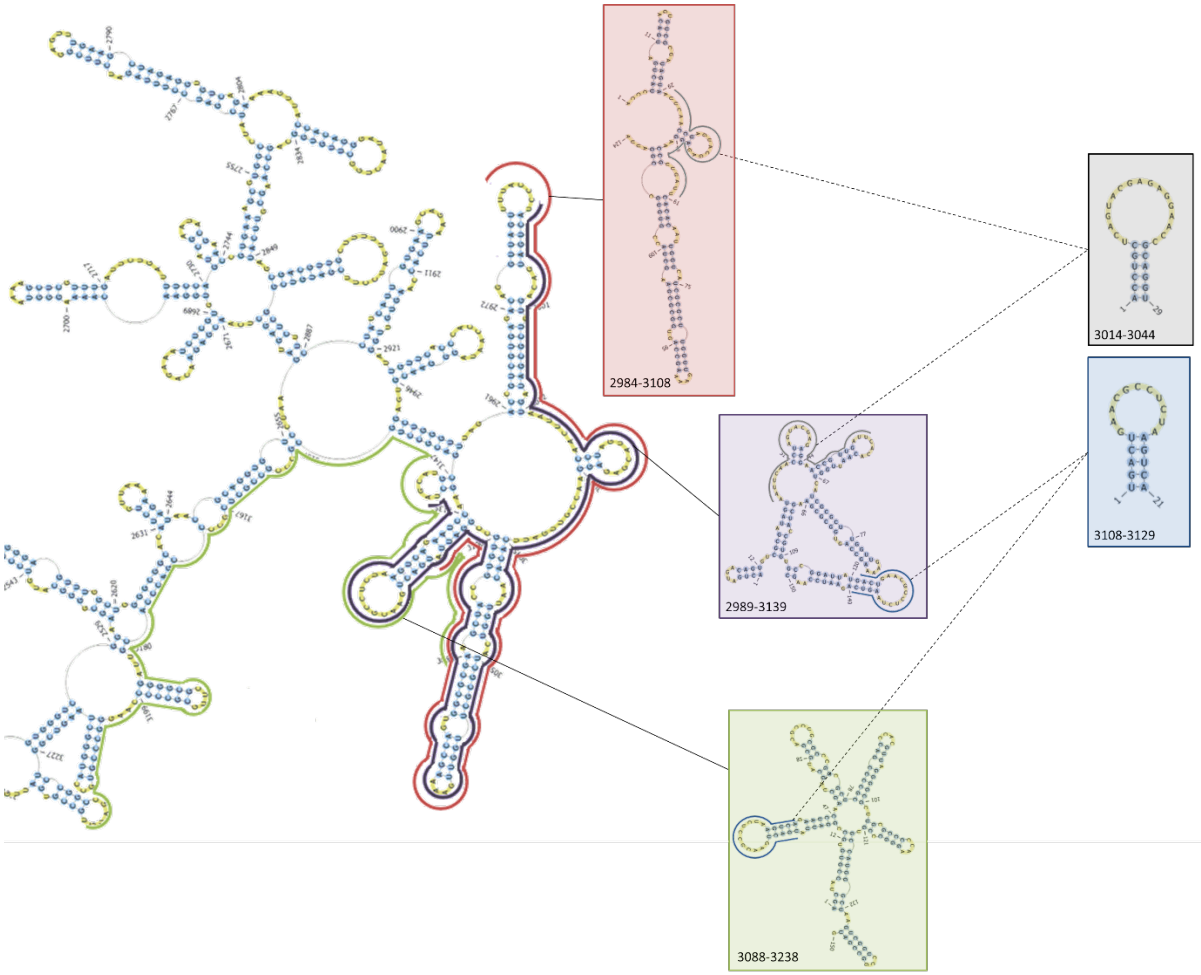


Figure 28: **Predicted secondary structure of the barley 28S ribosomal RNA subsection A.** A thermodynamic ensemble model was created for barley ribosomal RNA using PseudoViewer (http://pseudoviewer.inha.ac.kr/), based on secondary structures predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Enlarged structures in coloured boxes highlight the sections used for experimental work; with their corresponding coloured lines marked onto the ribosomal RNA map. The numbers represent the bases, where "1" is the first 5' base.

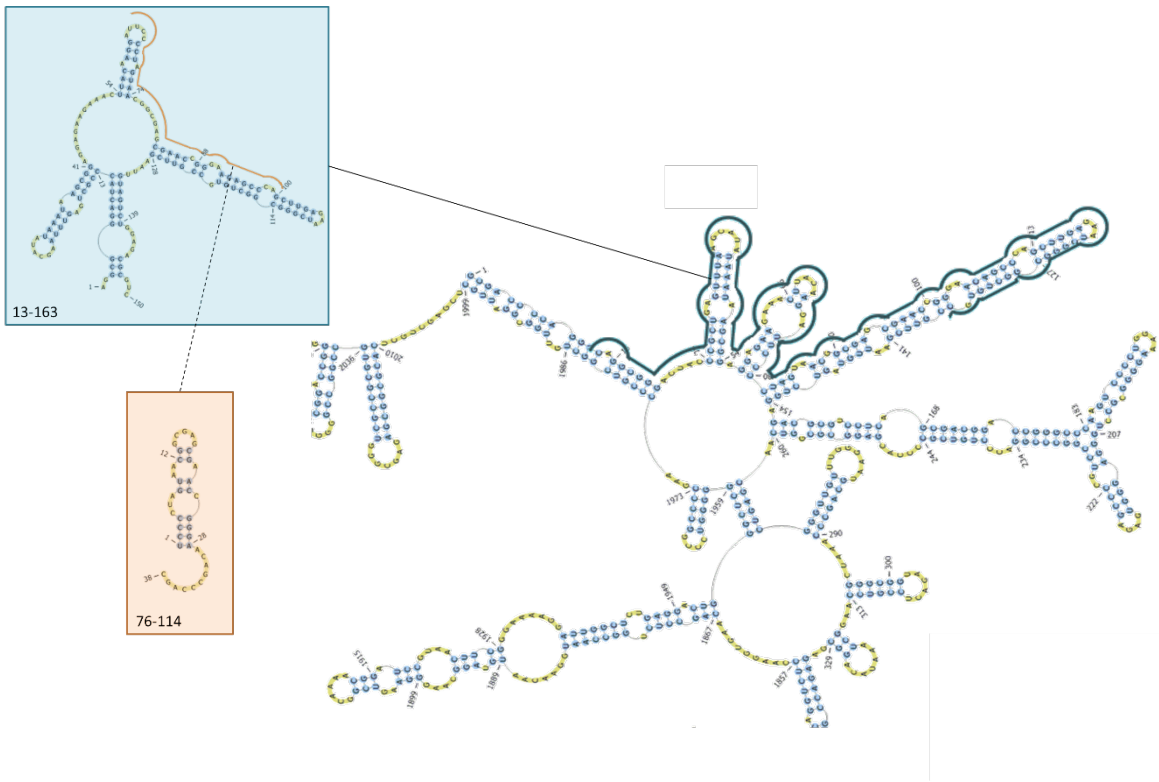


Figure 29: **Predicted secondary structure of the barley 28S ribosomal RNA subsection B.** A thermodynamic ensemble model was created for barley ribosomal RNA using PseudoViewer (http://pseudoviewer.inha.ac.kr/), based on secondary structures predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Enlarged structures in coloured boxes highlight the sections used for experimental work; with their corresponding coloured lines marked onto the ribosomal RNA map. The numbers represent the bases, where "1" is the first 5' base.

6.4.2. Differential Scanning Fluorimetry (DSF)

I synthesized three RNA fragments (Table 6) *in vitro* from primer templates (Figure 30): rat 28S SRL, barley 28S SRL (bases 3014-3044 on the barley 28S model), and barley section 76-114. These regions were selected as they contain the SRL, a section of RNA predicted to form a stem-loop close to the SRL, and a section of RNA from close to the 5' end of the ribosomal RNA (where the SRL is close to the 3' end). The latter two were intended as controls, to determine whether BEC1054 was binding to stel-loop RNA structures, or to sequences from near to the SRL.

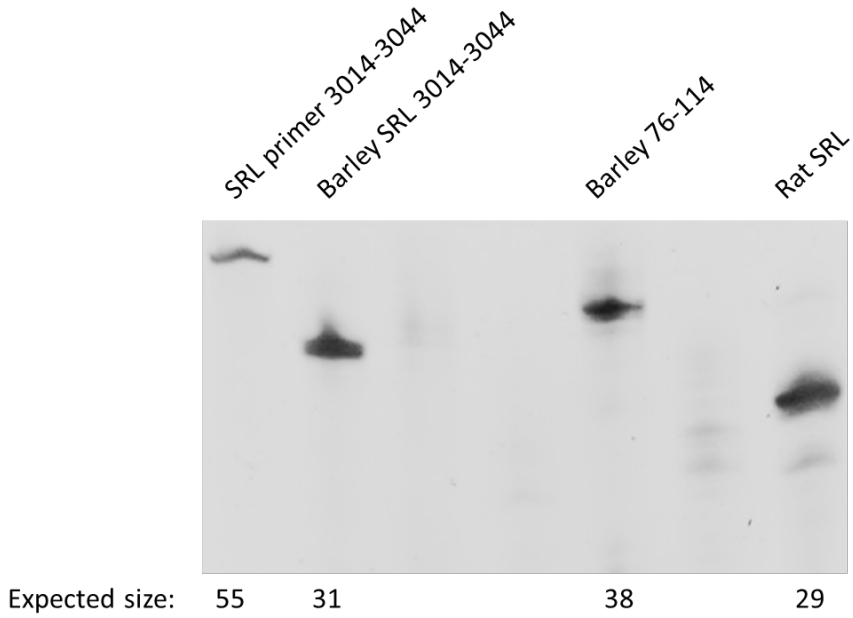


Figure 30: **Ribonucleic acid fragments synthesized by *in vitro* transcription.** RNA samples were synthesized using the MEGAshortscript kit (Applied Biosystems, Paisley, UK), and 100ng of the resulting product for each synthesis reaction run on 16% denaturing acrylamide gels with 7% urea and 1xTBE. Numbers above the gel represent the base number within the barley 28S ribosomal RNA model (with the first base at the 5' end numbered "1'). The abbreviations "SRL" refer to the sarcin-ricin loop, and "T7" to the T7 RNA synthesis promoter. Bands were visualized using SYBR Gold nucleic acid gel stain.

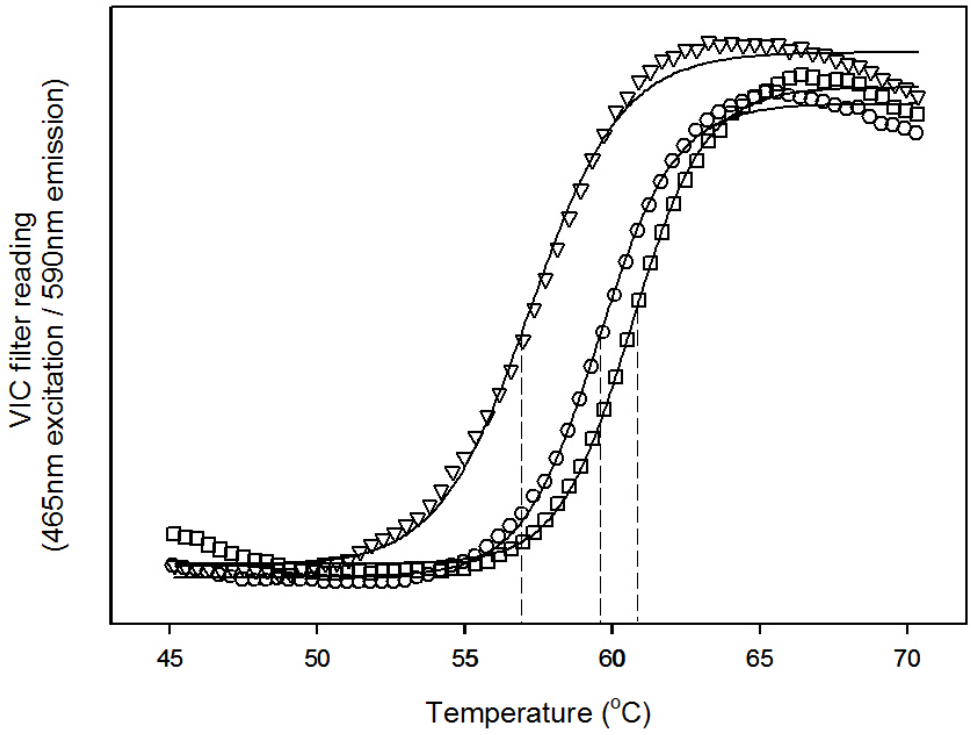


Figure 31: **Calculating the melting point of *Blumeria* Effector Candidate 1054 (BEC1054)**. Sigmoidal dose-response (variable slope) curves (shown as black lines) were fitted to melting curves for BEC1054 (circles), BEC1054 with 1 µM Barley SRL 3014-3044 +T7 primer (triangles) or BEC1054 with 20 µM Barley SRL 3014-3044 +T7 primer (squares). Dashed lines show the inflection point of the sigmoidal curves, which indicate the "melting point" of BEC1054.

I investigated the RNA and DNA-binding ability of BEC1054 *in vitro* through DSF (Figure 31), using synthesized RNA (Figure 32). A concentration-dependent decrease (Figure 32) in melting temperature was shown by BEC1054 with all three RNA ligands. The three interactions showing very similar decreases in temperature (Figure 32 D). Together, the results indicated that BEC1054 bound to all RNA species, but does not show any particular selectivity for the SRL RNA under the conditions used in this experiment.

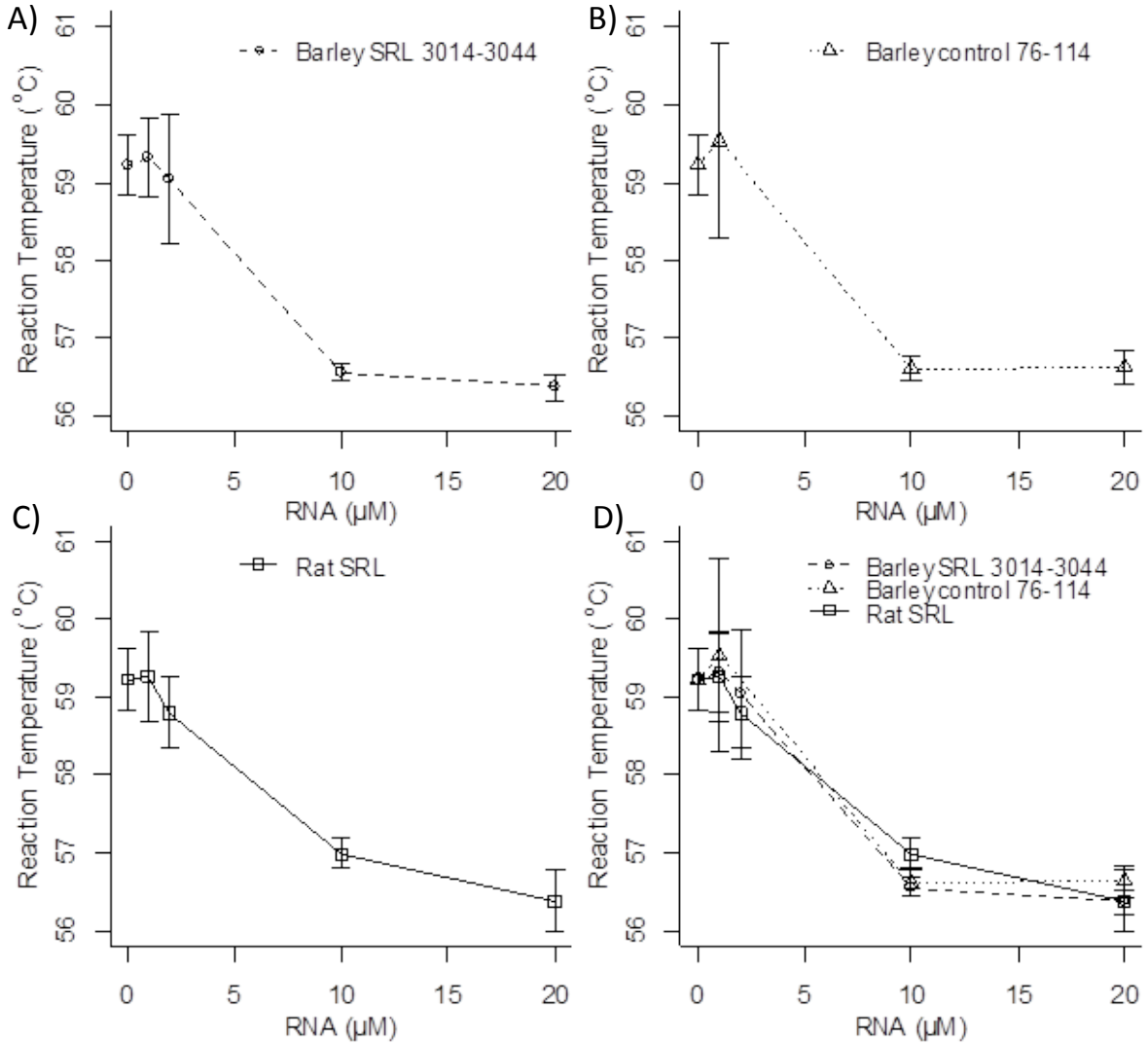


Figure 32: ***Blumeria* Effector Candidate 1054 (BEC1054) binds to RNA.** Graphs show the mean melting point for 10 µM BEC1054 with the ligands indicated on the bottom right, A) "barley SEL 3014-3044" indicates the barley 28S sarcin-ricin loop RNA bases 3014-3044; B) "Barley control 76-114" indicates a section of barley ribosomal RNA selected from within the 28S section; C) "Rat SRL" indicates the rat sarcin-ricin loop RNA; and D) shows the four graphs superimposed upo each other.

I investigated the ability of BEC1054 to bind single stranded DNA *in vitro* through DSF (Figure 33). Addition of HSP82 and the truly random primer NNNNNNNNNNNNNNNNNNNNNNNNNNNNN+T7 (where T7 is the RNA promoter required on DNA primers for the initiation of RNA synthesis) destabilised BEC1054, with increasing concentrations causing a greater degree of destabilization. In contrast, addition of three DNA primers for 28S ribosomal sections, "barley SRL 3014-3044+T7", "section 76-114+T7" and "rat SRL DNA+T7" (

Supplementary Table 22) caused a decrease in melting temperature for BEC1054 at low concentrations (i.e. they destabilised BEC1054)(Figure 32). Increasing the ligand concentration then *increased* the melting temperature of BEC1054, indicating that they stabilized it, and for the rat 28S SRL and barley SRL 3014-3044, the final melting point was just above that of BEC1054 alone (Figure 33). The synthesized RNA (section 76-114), used as a control, destabilised BEC1054 as before, but in a manner that was slight when compared with the shift caused by DNA, indicating that these results were not due to differences in experimental conditions.

I investigated the ability of BEC1054 to bind double stranded DNA *in vitro* (Figure 34). Double stranded DNA was created through mixing a primer encoding the T7 sequence, and a primer encoding the reverse complement of T7. As the concentration of double stranded DNA increased, the melting temperature decreased (i.e. BEC1054 was destabilised).

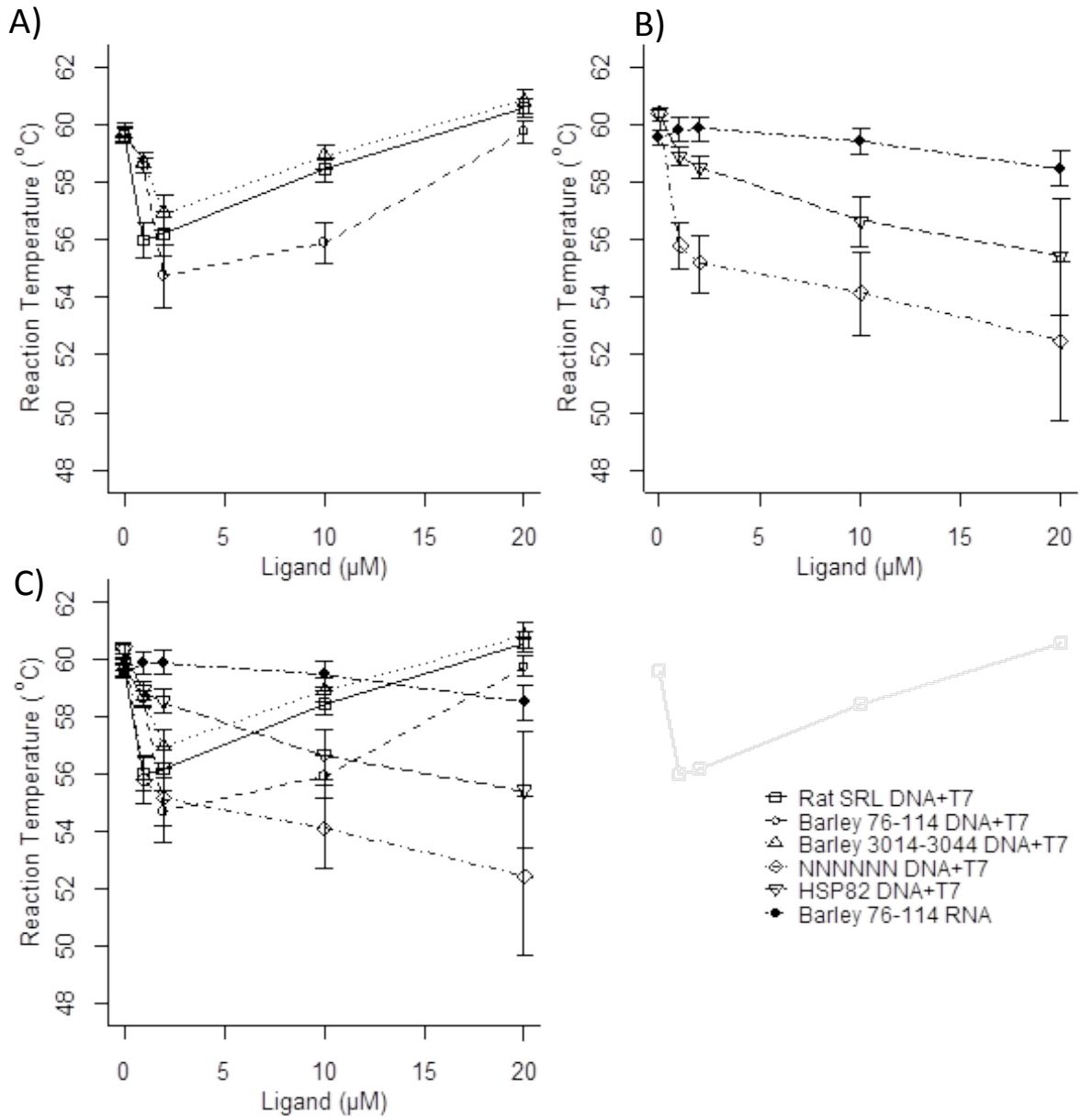


Figure 33: ***Blumeria*** **Effector Candidate 1054 (BEC1054) binds single stranded DNA.** Graphs show the mean melting point for 10 µM BEC1054 with the ligands indicated on the bottom right: "Rat 28S SRL DNA" is the primer for the rat 28S sarcin-ricin loop; "Barley 76-114 RNA" is a 5' section of barley ribosomal RNA from within the 28S subunit, "Barley 3014-3044" is the 28S region containing the sarcin-ricin loop; "NNNNNN DNA" is a random DNA primer with 30 "N" bases, and HSP82 DNA is a "forward primer" used to amplify heat shock protein 82; and in all cases "T7" represents the T7 RNA promoter required on DNA primers for RNA synthesis. Graph A) shows the DNAs with which BEC1054 decreased and then increased in melting point (Rat SRL DNA+ T7, Barley 76-114 DNA+T7 and Barley 3014-3044 DNA+T7); B) shows the DNAs and RNA with which BEC1054 decreased in melting point (NNNN DNA+T7, HSP82 DNA+T7 and Barley 76-114 RNA(; and C) shows the two graphs superimposed.

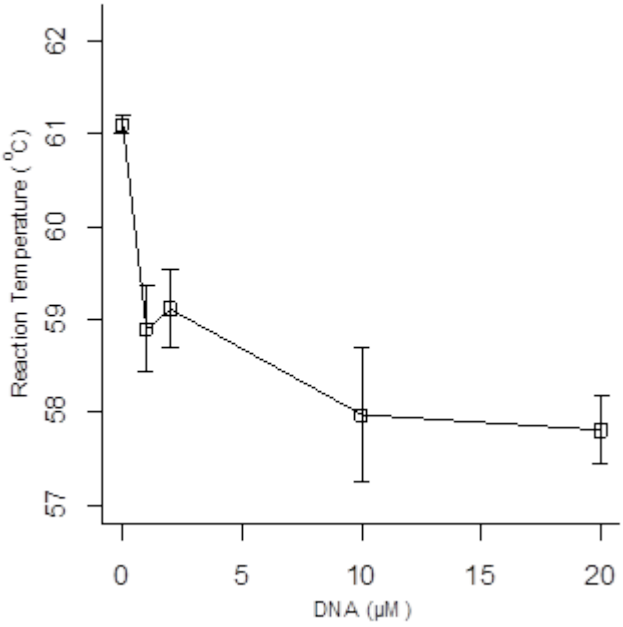


Figure 34: **Interaction of *Blumeria* Effector Candidate 1054 (BEC1054) with double stranded DNA.** The graph show the mean melting point for 10 µM BEC1054 with double stranded DNA, encoding the T7 RNA synthesis promoter and its reverse complement.

6.4.3. Yeast-three-hybrid (Y3H)

I used a yeast-three-hybrid system to determine whether BEC1054 interacted with RNA in a sequence-specific manner in yeast. The proteins BEC1054 and JIP60ml (Table 4) were expressed on the expression vector pDEST22, RNA was expressed on the pIIIA/MS2-2 vector, and the positive control protein on the pAD vector.

The vector pIIIA/MS2-2 contains a bacteriophage MS2 binding site, which is localised to the DNA binding site by a hybrid protein, encoded in the yeast chromosome, composed of the LexA DNA binding domain and the MS2 coat protein. The RNA sequence of interest is expressed on the pIIIA/MS2-2 plasmid, with a 3' tail of repeat MS2 sequences. Interaction between the RNA of interest and the prey protein leads to the localisation of the activation domain to the reporter genes (Figure 26). The plasmids pDEST22 and pAD both contain a transcriptional activation domain; when localised to

the reporter gene promoter, this will drive expression of the reporters *his3* and *lacZ* in the genome of yeast strain YBZ1 (SenGupta *et al.,* 1996, MacDonald 2001). A low transformation success rate, and poor growth, were observed for yeast transformed with BEC1054, as had happened for the Y2H assay (Figure 11).

As was the case for the Y2H assay, β-galactosidase activity indicates the level of expression of the *lacZ* reporter gene. The maximum conversion rate (V_i) of CPRG was again used as an indicator of a reaction (Figure 35), but this time between the bait RNA and the prey protein. Yeast lines containing pDEST22 and pIIIA/IRE-MS2 were used as the negative control. Further negative controls were comprised of BEC1054 or JIP60ml bound to the activation domain (pEXP22/BEC1054 or pEXP22/JIP60ml), and expressed with either a control rRNA section, or IRE (pIIIA/control_rRNA-MS2 or pIIIA/IRE-MS2). Interactions were performed using pEXP22/BEC1054 or pEXP22/JIP60ml, and pIIIA/SRL-MS2 or pIIIA/rRNA_control-MS2 (Table 7).

The β-galactosidase activity was higher than the negative control for almost all combinations of BEC1054 or JIP60ml with bait RNA (Figure 35), as can be seen by the elevation of the boxplots. This increase was statistically significant for all the lines which were elevated compared with the negative control (Supplementary Table 32). The only combination for which this was not the case was for BEC1054 with SRL rRNA, where the V_i was lower than the negative control, although the two were not significantly different (Supplementary Table 32). These results indicate that both BEC1054 and JIP60ml bind to RNA in yeast in a non-sequence specific manner except

for the interaction of BEC1054 with the SRL. The mean β-galactosidase activity was lower for BEC1054 than for JIP60ml in all RNA-prey combinations (Figure 35).

I further analysed the transformed yeast lines through plating onto media containing 10 mM 3AT, and lacking histidine. Growth on media containing 3AT indicates an interaction between the bait RNA and the prey protein. The strong positive control grew well on media containing 3AT, whereas the negative control did not. Both BEC1054 and JIP60ml formed a limited number of colonies on the 3AT media when co-transformed with plasmids encoding the SRL, but not with the other RNA controls. The selective media assay results indicate that BEC1054 and JIP60ml may interact in a sequence-specific manner in yeast.

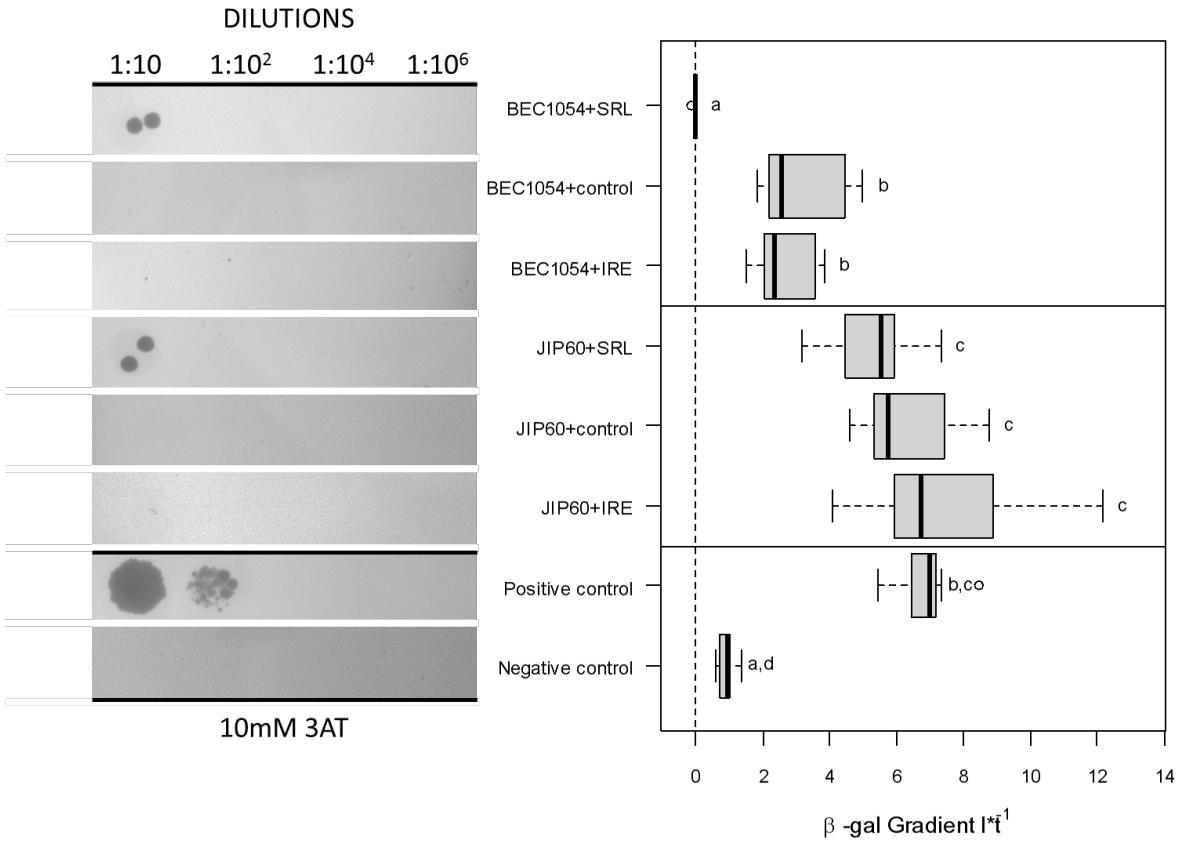


Figure 35: **Both fungal *Blumeria* Effector Candidate 1054 (BEC1054) and barley Jasmonate Induced Protein 60 (JIP60) show evidence of binding RNA in yeast.** A yeast-three-hybrid system was used to determine whether the RNase like BEC1054, and the barley RNase JIP60 bound RNA in a sequence-specific manner in yeast. For each yeast line under investigation, the name of the protein is given first, and the RNA second. The abbreviation "SRL" stands for ribosomal large subunit "Sarcin-Ricin Loop", "control" in the position of the RNA for a different section of the ribosomal large subunit, "IRE" for the RNA Iron Response Element, "positive control" for Iron Regulatory Protein 1 (IRP1) with the interacting IRE RNA sequence, and "negative control" for empty pDEST22 with pIIIA/IRE-MS2 . The proteins BEC1054 and JIP60 were expressed on the expression vector pDEST22, RNA was expressed on the pIIIA/MS2-2 vector, and the positive control protein on the pAD vector. **Left**: Growth promotion in the presence of 3AT (3-Amino-1,2,4-triazole) indicates an interaction. **Right**: The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. Games-Howell posthoc tests were used to determine whether the mean V_i (the maximum rate of conversion of the yellow substrate CPRG to the red product chloramphenicol red (and D-galactose)) was significantly different for different yeast lines (line 1 and line 2). Significant difference is indicated by the letters "a", "b", "c' and "d' ($p>0.05$), with bars labelled with different letters being significantly different. The circle for the positive control represents an outlier. Six independently transformed colonies were used for each yeast line.

6.5. Discussion

6.5.1. Barley 28S rRNA model

At the beginning of this study, no model for the barley 28S rRNA was available. I created a 28S rRNA model based on a wheat model (Armache *et al.,* 2010), using data from IBSC un-annotated 454BacContigs (Figure 27, Figure 28 and Figure 29). This model was used as the theoretical template for the design and synthesis of RNA fragments for DSF; and for the design of primers used, via PCR, to amplify the sections used in the Y3H assay.

6.5.2. Differential Scanning Fluorimetry (DSF)

The DSF assay is widely used in high-throughput screening for ligands which bind strongly to proteins (Cummings *et al.,* 2006); and to determine protein function (Carver *et al.,* 2005). I therefore used this assay to determine whether BEC1054 bound to nucleotides *in vitro.*

In this study, I found that BEC1054 interacted with RNA, decreasing thermal stability in a concentration-dependent manner (Figure 32). This result was not found to be sequence specific, as it interacted with three different RNA sequences in the same manner.

The decrease in thermal stability contrasted with the usual findings for equilibrium binding ligands, where the ligand increases thermal stability with increased ligand concentration (Niesen *et al.,* 2007, Cimmperman *et al.,* 2008). A thermodynamic model has been proposed, where the ligand binds to the denatured (unfolded) native protein; explaining the destabilization effect (Cimmperman *et al.,* 2008).

The RNA binding domain, (also called the ribonucleoprotein domain or RNA recognition motif (Clery *et al.,* 2008)) is, biochemically and structurally, the most studied RNA-binding domain (Maris *et al.,* 2005). The two effectors BEC1054, and its paralog BEC1011, possess the structure of an RNA binding domain, with four β-sheets and two α-helices (Clery *et al.,* 2008, Bindschedler *et al.,* 2011, Pedersen *et al.,* 2012). The RNA binding domain demonstrates a wide range of ligand recognition: it is able to bind with high or low affinity and specificity; to interact with varied lengths of RNA; to interact with multiple partners simultaneously; to interact with proteins; and to interact with DNA (reviewed by Clery *et al.,* 2008). It is therefore possible that BEC1054 may bind to both DNA and RNA *in vivo*.

In contrast with the RNA-binding results, BEC1054 was found to be destabilised, and then stabilized by a DNA ligand, with the final stabilization being significantly greater than BEC1054's initial melting temperature. Interestingly, this binding behavior was only seen for ribosome-related DNA sequences. It is not clear what these results mean, but they may indicate that BEC1054 is binding to ribosome related nucleotide sequences. To rule out whether this result could be due to the primer forming a primer-dimer at higher concentrations, and therefore no-longer interacting with BEC1054, I tested the interaction of BEC1054 with T7 double stranded DNA. I found that double stranded DNA destabilised BEC1054, and that the result was concentration dependent (Figure 34). Other proteins containing an RNA binding domain have been shown to bind to single stranded DNA (Ding *et al.,* 1999, Enokizono *et al.,* 2005). This would not,

however, explain why BEC1054 was stabilized at higher concentrations: this remains to be determined.

The RIPs α- and β-momorcharin have been shown to possess RNase (Mock *et al.,* 1996), DNase (Go *et al.,* 1992) (where nuclease activity is defined as the cleavage of a phosphodiester bond from RNA or DNA), and N-glycosidase (defined as the cleavage of the glycosidic bond linking an oligosaccharide to an asparagine side chain amide) activity (Fong *et al.,* 1996) in addition to their RIP activity. This DNase activity has also been shown for other RIPs, including (but not limited to): ricin A, phytolaccin and shiga toxin (Obrig *et al.,* 1985); alpha- and beta-pisavins (Lam *et al.,* 1998); luffin, cinnamomin and camphorin (Ling *et al.,* 1994) and trichosanthin (Li *et al.,* 1991). This DNA-degrading activity has been shown to contribute to the cytotoxicity of RIPs (Nicolas *et al.,* 1997).

It has been suggested that contamination with nucleases may have caused the DNase activity of some RIPs (Day *et al.,* 1998). Other studies have gone to great length to confirm that the alternative RNase activities seen are really related to the RIP in question (Fong *et al.,* 2000). The DSF assays performed in this study were done using BEC1054 purified from *E. coli*; and hence the results are unlikely to be due to RIPs from the plant or the fungus, as neither was present during the synthesis of BEC1054.

6.5.3. Yeast-three-hybrid (Y3H) and RNA interactions

Complex interactions occur within the host cell which cannot be mimicked *in vitro.* The interaction of proteins and RNA within cells can be influenced by factors including: intracellular localisation; ion concentrations; the relative concentrations of competing

proteins or RNAs; phosphorylation of the RNA binding protein; or the secondary structure of the RNA (which itself can be influenced through interaction with other RNA-binding proteins) (Huranova *et al.,* 2009). Performing RNA-protein interactions in yeast provides a compromise between *in vitro* and *in planta* assays. Yeast-N-hybrid systems allow the experiment to be performed in a eukaryotic cell, which provides a more realistic environment, whilst still providing an easily manipulatable and scorable system (Hook *et al.,* 2005) (when compared with *in planta* systems, which can be more costly in terms of both materials and effort).

The Y3H system has been used to investigate numerous protein-RNA interactions in yeast (SenGupta *et al.,* 1996, Sengupta *et al.,* 1999, Bernstein *et al.,* 2002, Hook *et al.,* 2005). In our study, a Y3H assay was used to investigate whether BEC1054 and JIP60ml bound to RNA in yeast (which is likely to represent closer to *in planta* conditions than DSF, which is *in vitro*). Our β-galactosidase assay results (Figure 35) demonstrated that BEC1054 did bind to RNA in yeast, as did JIP60ml. The mean β-galactosidase activity for BEC1054 was lower than for JIP60ml in all RNA-prey combinations. This result may indicate that BEC1054 is binding more weakly to RNA than JIP60ml; alternatively, it may further indicate that BEC1054 is affecting translation, as was hypothesised for the Y2H assay (see Section 4.5 for details).

When BEC1054 was paired with SRL RNA, the β-galactosidase activity was lower than the negative control (Figure 35). No relative β-galactosidase activity curve for the cleavage of chlorophenolred-ß-D-galactopyranoside (CPRG) into the red product chloramphenicol red (and D-galactose) occurred. The values varied around the level of

the blank (the readings for the buffer, without yeast lysate), with no clear trend. It is not clear what these results mean. They may indicate that BEC1054 is somehow affecting *transcription* of β-galactosidase when localised to the reporter gene; or that BEC1054 has a greater effect upon translation when paired with SRL RNA; or that some other reaction has taken place.

For the 3AT selective media assay, only the RNA-prey pairing of BEC1054 with SRL RNA, or JIP60ml with SRL RNA produced yeast colonies. In contrast with the β-galactosidase assay results, these indicate that the interaction of BEC1054 or JIP60ml with these RNA sections may be stronger than for the other RNA sections. The two proteins may therefore be binding to SRL RNA with a higher specificity than to the other RNA fragments; indicating that this may be the real RNA target. As mentioned previously, many RIPs have been shown to possess multiple activities, including DNase (Go *et al.,* 1992), RNase (Mock *et al.,* 1996) and N-glycosidase (Fong *et al.,* 1996) activity. The proteins JIP60ml and BEC1054 may well bind RNA, in the manner of trichosanthin, which transiently binds human immunodeficiency virus type 1 long terminal repeats before depurinating them (Zhao *et al.,* 2010). Other proteins may be involved in the interaction, and may help to determine the specificity beyond the binding of JIP60 and BEC1054 to RNA; and that these proteins would need to be present for the specificity to be more easily discerned (see Section 4.5 for further discussion of BEC1054 with multiple protein partners). Alternatively, BEC1054 may bind to a different RNA sequence which has not yet been identified.

6.6. Conclusion

The RNase-like effector BEC1054 was found to interact with oligo(poly)nucleotides both in yeast, and *in vitro.* Our *in vitro* results suggested that BEC1054 interacted with RNA in a non-sequence specific manner, and with DNA in a sequence-specific manner. My β-galactosidase results from yeast indicated that both BEC1054 and JIP60ml bound RNA, albeit very weakly; and the selective media assay indicated that this interaction may be specific for the region of the ribosome containing the SRL. Taken together, these results provide some evidence supporting our original hypothesis that BEC1054 may be binding to ribosomal RNA, as they show evidence for nucleotide binding activity *in vitro*, and evidence for BEC1054 binding to RNA *in vivo*.

6.7. Further work

AIM: To utilise the Y3H system to test a series of mutants of BEC1054, in an attempt to identify the nucleotides required for binding RNA.

OBJECTIVE: To create a series of BEC1054 mutants, and to screen them using the Y3H system, to determine whether they interact with RNA in yeast.

REASONING: The Y3H system provides a good compromise between a rapidly usable system for screening multiple protein constructs, and the use of an *in vivo* system, which provides a more realistic environment in which the reactions can take place. Identification of the amino acids required for the interaction of BEC1054 with RNA would provide us with a useful tool for further experimental work.

AIM: To screen a range of RNA targets for BEC1054.

OBJECTIVE: To create barley RNA library, and to screen it via Y3H with BEC1054, to determine whether BEC1054 interacts with any of the RNAs in yeast.

REASONING: Screening barley RNA library would allow us to identify additional RNA targets for BEC1054.

AIM: To investigate the binding of BEC1054 to RNA *in planta*.

OBJECTIVE: To utilise techniques such as RNA Fluorescence *In Situ* Hybridization (FISH) to determine whether BEC1054 interacts with ribosomal RNA in plants, and to utilise the aforementioned mutants of BEC1054, to help determine the amino acids required for binding RNA.

REASONING: *In planta* interactions would provide further validation of the interaction between BEC1054 and ribosomal RNA.

AIM: To determine whether BEC1054 binds to ribosomes *in planta.*

OBJECTIVE: To express BEC1054 with a fluorescent tag in *Nicotiana benthamiana*, to perform a ribosome extraction, and to determine whether the ribosomal pellet fluoresces. The ribosomal 40S 16-GFP could be used as a positive control, with a GFO only protein as the negative control.

REASONING: This would provide direct evidence for BEC1054 binding to the ribosome, and as such would help to validate our working hypothesis.

AIM: To interact 28S rRNA with BEC1054 *in vitro.*

OBJECTIVE: To extract ribosomes from barley seedlings using a sucrose gradient, and to then extract the RNA from the purified ribosomes. The ribosomal RNA could then be used for direct interactions with BEC1054 *in vitro*, for example protein NMR chemical shift mapping.

REASONING: This would provide further evidence for BEC1054 binding to the ribosomal RNA.

7. Expression of the *Blumeria* effector BEC1054 *in planta*

7.1. Introduction

Two strains of *B. graminis*, f.sp. *hordei* and f.sp. *tritici*, maintained within the host laboratory, infect barley and wheat respectively. They are each unable to infect the other host. *Triticum aestivum* cv. Fielder had previously been stably transformed with "wobble "BEC1054 (*wbec1054*) through Agrobacterium-mediated transformation at the National Institute of Agricultural Botany (NIAB, Cambridge). The wobble BECs are synthetic genes which do not possess a signal peptide, so that they remain within the plant cytosol, and which have silent "wobble" mutations which minimize the nucleotide sequence identity with the wild-type barley genes. The wobble mutations were added so that they would be insensitive to RNA interference directed silencing, in order to test complementation in the Host Induced Gene Silencing experiments (Pliego *et al.,* 2013).

Our hypothesis is that BEC1054 competes with RIPs by binding to rRNA (Kwon 2011), preventing them from cleaving a specific adenine base from the 28S rRNA sugar-phosphate backbone (Endo *et al.,* 1988b, May *et al.,* 1989, Funatsu *et al.,* 1991). The RIPs, such as JIP60, are N-glycosidases which depurinate the large subunit rRNA through the cleavage of an N-glycosidic bond. This causes a phosphodiester bond of the sugar-phosphate backbone to become exposed to chemical hydrolysis within the cell (Endo *et al.,* 1988a, Endo and Tsurugi 1988, Endo *et al.,* 1988b, Barbieri *et al.,* 1993). Here, I utilised these plants to determine whether expression of the fungal effector BEC1054 affected plant growth and yield, senescence or ribosomal degradation, and whether BEC1054 interfered with non-host resistance of wheat to *B. graminis* f.sp. *hordei*, or susceptibility to the adapted f.sp. *tritici*,.

Leaf senescence is a tightly controlled process, which involves large scale changes in gene expression (Guo and Gan 2005, Reinbothe and Reinbothe 2006, Zentgraf *et al.,* 2010, Christiansen and Gregersen 2014, Penfold and Buchanan-Wollaston 2014).

We hypothesized that the presence/absence of BEC1054 may affect chlorophyll degradation during senescence. Chlorophyll degradation can be used as an indicator of senescence. The breakdown of chlorophyll plays a vital role during the process, allowing nitrogen and other nutrients to be recycled, and preventing the accumulation of phototoxic intermediates of chlorophyll (for a review, see Hoertensteiner (2006)). Jasmonates were first reported to induce senescence in oat leaves (Ueda and Kato 1980). This induced senescence works in other monocot species including rice (Hung *et al.,* 2006), barley (Weidhase *et al.,* 1987a, Reinbothe *et al.,* 1992) and in dicots such as *Arabidopsis* (He *et al.,* 2002).

Plant transformation involves the insertion of "foreign" DNA, both from plants, and from non-plant organisms such as fungi, into plant cells (Barampuram and Zhang 2011). Transient or stable expression can result from the transfer of DNA into the plant cell. Stable expression is usually time-consuming, and involves the growth of a whole plant from tissue culture of treated cells or explants. The DNA is integrated into the host cell's DNA, and can therefore be inherited by subsequent generations (Hansen and Wright 1999, Barampuram and Zhang 2011). Multiple generations must be assessed for gene

copy number and protein expression to be accurately determined, causing stable transformation studies in crop plants to take years.

Transient expression occurs over a much shorter time frame than stable expression, usually days. It allows experimental results to be seen relatively rapidly, and thus facilitates screening of multiple proteins (Bernaudat *et al.,* 2011). Transient transformation methods are relatively difficult, although possible, in monocots as they are not natural hosts for *Agrobacterium tumefaciens* (Cleene and Ley 1976). Direct delivery methods, such as bombardment, are frequently used but result in a very low percentage of cells being transformed. The dicot *Nicotiana benthamiana* has been gaining popularity as a host for transient protein expression, as it can be efficiently transformed using *Agrobacterium* (Schob *et al.,* 1997, Goodin *et al.,* 2002, Voinnet *et al.,* 2003). Transient expression in *N. benthamiana* was used to determine whether the expression of BEC1054 or JIP60ml affected infection with the obligate biotrophic oomycete *Peronospora tabacina*, and whether JIP60ml or BEC1054 had an effect on cell death.

7.2. Aims and Objectives

AIM: To confirm the genotyping of wheat transformed with *wbec1054*.

OBJECTIVE: To utilise direct PCR to demonstrate the presence (or absence) of the BEC1054 transgene in wheat using the KAPA direct PCR system (KAPA Biosystems, Massachusetts, US).

AIM: To confirm the transcription of the *wbec1054* transgene in wheat.

OBJECTIVE: To utilise real-time PCR, to demonstrate the transcription of the *wbec*1054 transgene in wheat.

AIM: To determine whether expression of *wbec1054* in wheat has an effect on the phenotypic characteristics of the adult wheat plant.

OBJECTIVE: To measure a series of characteristics such as height, seed number and leaf number in the adult plant, to determine whether they are different between different homozygous null (-/-) and homozygous *wbec1054* (+/+) lines.

AIM: To determine whether expression of *wbec1054* in wheat affects the ability of *B. graminis* f.sp. *tritici* or f.sp. *hordei* to infect wheat

OBJECTIVE: To investigate the effect of the expression of *wbec1054* in wheat on infection with wheat and barley powdery mildew.

AIM: To determine whether expression of *wbec1054* or *jip60ml* in *Nicotiana benthamiana* has an effect on the ability of *Peronospora tabacina* to infect *N. benthamiana*.

OBJECTIVE: To utilise *Agrobacterium* to express JIP60ml and BEC1054 in *N. benthamiana*, and to infect the transformed leaves with *P. tabacina*.

AIM: To determine whether expression of *wbec1054* or *jip60ml* in *Nicotiana benthamiana* has an effect on cell death.

OBJECTIVE: To perform trypan blue staining on transformed leaves, to selectively colour dead tissues/cells.

AIM: To determine whether BEC1054 co-localises with any of the *N. benthamiana* cellular markers available in the host laboratory.

OBJECTIVE: To express *wBEC1054* with a C or N terminal GFP tag in *N. benthamiana*, and to determine through confocal microscopy whether it co-localises with mitochondria, early endosomes, late endosomes, or Golgi bodies.

7.3. Individual Contributions

Data was obtained with the assistance of the following undergraduate students, whom I supervised:

1. Peggy Luong
    a. re-confirming the presence of the fungal transgene in wheat
    b. re-confirming the transcription of the fungal transgene in wheat
    c. determining whether the presence of the *wbec1054* transgene affected infection of wheat with *Blumeria graminis* f.sp. *tritici*
2. Hannah Thieron
    a. determining whether the expression of *wbec1054* and *JIP60ml* affected infection of *Nicotiana benthamiana* with *Peronospora tabacina*
    b. determining whether BEC1054 or *B. graminis* f.sp. *tritici* infection affected ribosomal RNA degradation in wheat
    c. determining whether the expression of *JIP60ml* affected *N. benthamiana* cell death
3. Thomas Chandler
    a. determining whether BEC1054 or *B. graminis* f.sp. *tritici* infection affected chlorophyll degradation in wheat
    b. determining whether BEC1054 or *B. graminis* f.sp. *tritici* infection affected ribosomal RNA degradation in wheat

I performed analyses and processing for the collected data (both numerical and photographic), to ensure consistency in the statistical methods used. Confocal imaging was done with the help of Tolga Bozkurt.

7.4. Results

7.4.1. Determining the presence of the transgene

We used direct PCR (from a leaf template, instead of conventional PCR from extracted DNA) to confirm the presence of *wbec1054* in transgenic wheat. Homozygous T4 generation plants (produced via four generations of successive selfing) from the line 3.3.14 (+/+) exhibited the presence of the transgene when tested via PCR, and azygous control plants (3.3.12 (-/-)) did not (Figure 36). Genotyping of seeds from each generation indicated that the initial transformants had been homozygous, as the genes were found to be present in each successive generation.

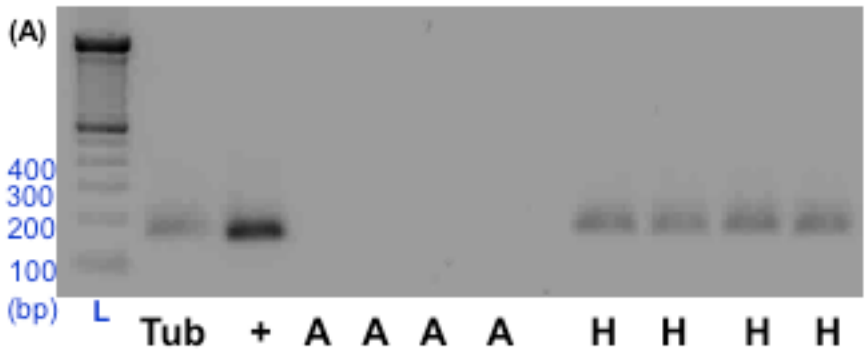


Figure 36: **Confirming the presence of fungal effector *Blumeria* Effector Candidate 1054 (BEC1054) in transgenic wheat**. Transgenic wheat had undergone *Agrobacterium*-transformation with BEC1054. The T4 generation plants (produced via four generations of successive selfing) exhibited the presence of the transgene when tested via PCR. The symbol "H" stands for homozygous (+/+), "A" for azygous (-/-), "Tub" for wheat tubulin, and "+" for a positive control conducted using the pCR8/BEC1054 plasmid. The wBEC genes have silent "wobble" mutations, which minimize the nucleotide sequence identity with the wild-type barley powdery mildew genes. Image reproduced with permission from (Luong 2014).

We extracted total RNA from one T4 plant for each of the lines 3.3.14 (+/+) and 3.3.12 (-/-), and used it as a template for cDNA synthesis. We performed end-point qPCR using the wobble BEC primers to confirm whether *wbec1054* was transcribed, as was found to be the case. Wheat lines containing *wbec1011* (1.16.14 +/+ and 1.16.3 -/-), a paralog of *wbec1054* were also tested.

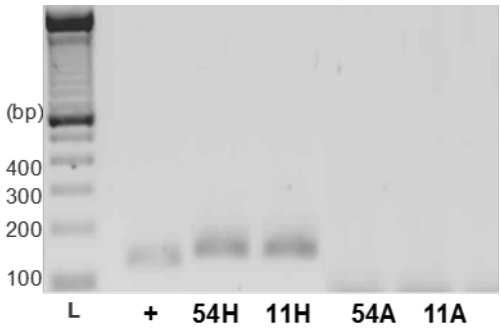


Figure 37: **Transcription of *wBEC1054* occurs in transgenic wheat.** Leaf material from transgenic wheat lines homozygous for *wbec1054*, or its paralog *wbec1011*, was used for RNA extraction and subsequently for cDNA synthesis. The cDNA was used as a template for endpoint PCR. The expected size of the products for *wbec1011* and *wbec1054* were 155bp and 184bp respectively. Where "54" indicates BEC1054, "11" indicates *wbec1011*, "A" azygous, "H" homozygous and "+" a tubulin positive control. The *wbec* genes have silent "wobble" mutations, which minimize the nucleotide sequence identity with the wild-type barley genes Image reproduced with permission from (Luong 2014).

7.4.2. Phenotyping plants azygous or homozygous for BEC1054

I assayed transgenic wheat lines from the T4 generation of lines homozygous for *wbec1054*, or azygous (3.3.14 and 3.3.12 respectively), to determine whether BEC1054 had an effect on the phenotypic characteristics of adult wheat. Azygous plant were obtained through selection from self breeding heterozygote transformed parent plants (Bonciani 2014). The characteristics assayed were: the number of leaves, maximum height, subcrown length, ear length (including whiskers), peduncle (internode 1) length,

the length of the three remaining internodes, the number of fertile tillers, the mass of the tillers, and the number of grains.

Bartlett tests were performed to determine whether the variance of the wheat phenotypic characteristics was homogeneous (Crawley 2005). This was found to be the case for the majority of the characteristics measured (Supplementary Table 33); but not for the leaf number; the length of internode three; the length of the ear; or the number of fertile tillers. Although the variance was found to be equal for the remaining characteristics, GLMs (Equation 15) were applied to each of the datasets for consistency (as opposed to using ANOVAs for those with equal variance). A "poisson" family structure was used to handle the count data; and all datasets were log transformed to account for overdispersion in the original models (the residual deviance was massively greater than the degrees of freedom). Post-hoc Games-Howell tests were used to determine which pairs of means differed significantly (Crawley 2005).

Equation 15: **A Generalized Linear Model Model used to determine whether the response variable (y) changed with the seed line under investigation**.

$$glm(log(y) \sim seed\_line, family = "poisson")$$

Statistical analyses were not performed for the subcrown, as many of the tiller subcrowns became detached during the drying out phase. This meant that it could not always be accurately determined which subcrown belonged to which primary tiller.

Summary models, displaying the mean measurements from the primary internode, were created for the *wbec1054* azygous and homozygous wheat.

I found the characteristics assayed were consistent between the homozygous and azygous wheat plants (Figure 38), i.e. there were no major phenotypic changes between those lines expressing BEC1054 and those without it. No significant differences were identified between the +/+ and -/- lines for any of the characteristics measured (Figure 38, Supplementary Table 34, Supplementary Figure 54,).

I did not perform statistical analyses for the subcrown, as many of the tiller subcrowns became detached during the drying out phase. This meant that I could not always accurately determined which subcrown belonged to which primary tiller. I created summary models for the *wbec1054* azygous and homozygous wheat (Figure 39). These models demonstrate visually that there is no difference in total height for the different primary tillers, and that there was no great difference between the lengths of the internodes or ear.

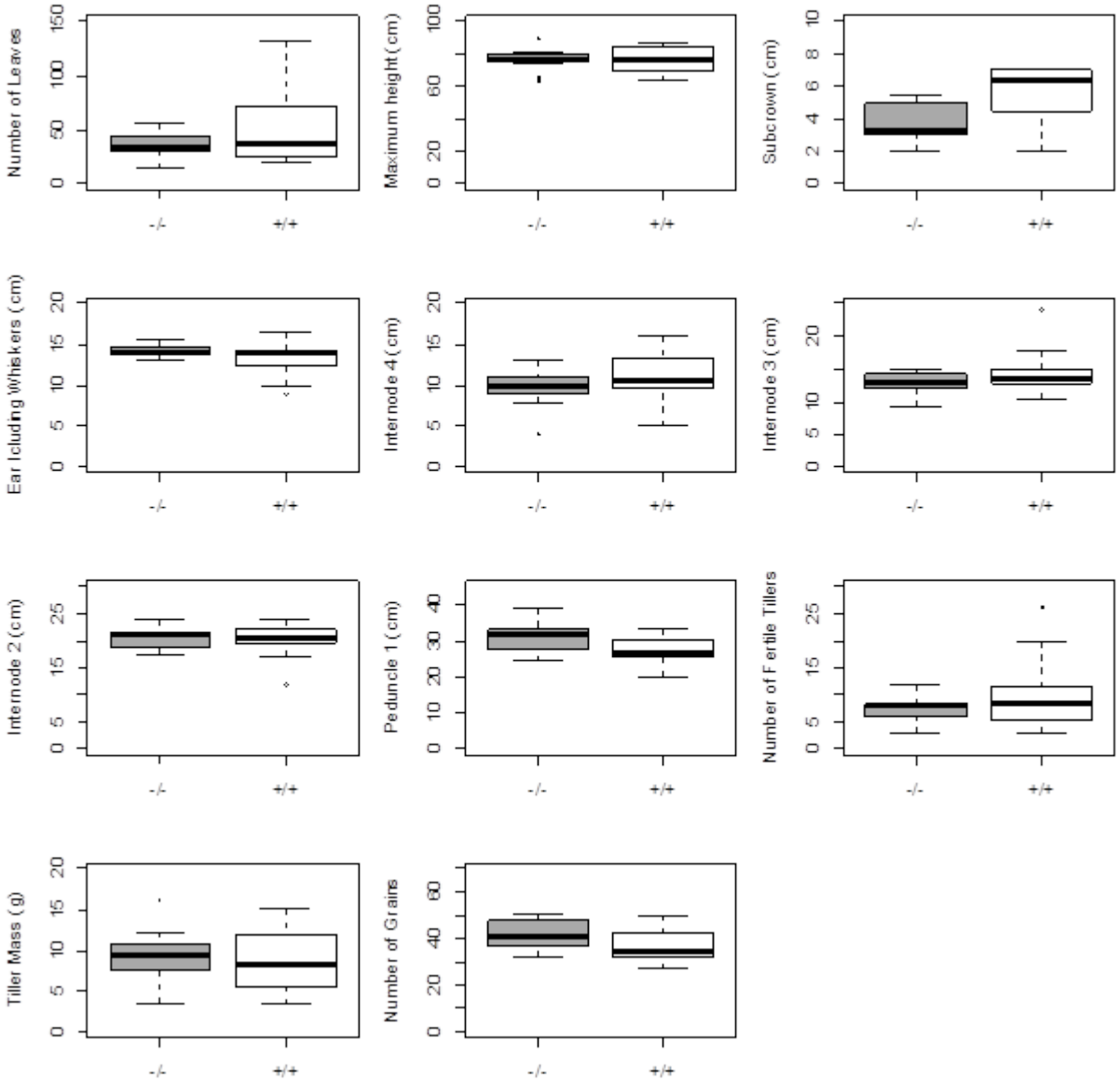


Figure 38: ***Blumeria* Effector Candidate 1054 (BEC1054) does not affect the phenotype of transgenic wheat.** Eleven phenotypic characteristics of wheat were investigated for the T4 generation of homozygous (+/+) wheat (line 3.3.14) transformed with *Blumeria* Effector Candidate BEC1054 (*wbec1054*) and for azygous (-/-) wheat (line 3.3.12). The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. Games-Howell post-hoc tests indicated that the characteristics investigated were not significantly different ($p>0.05$) for any wheat lines.

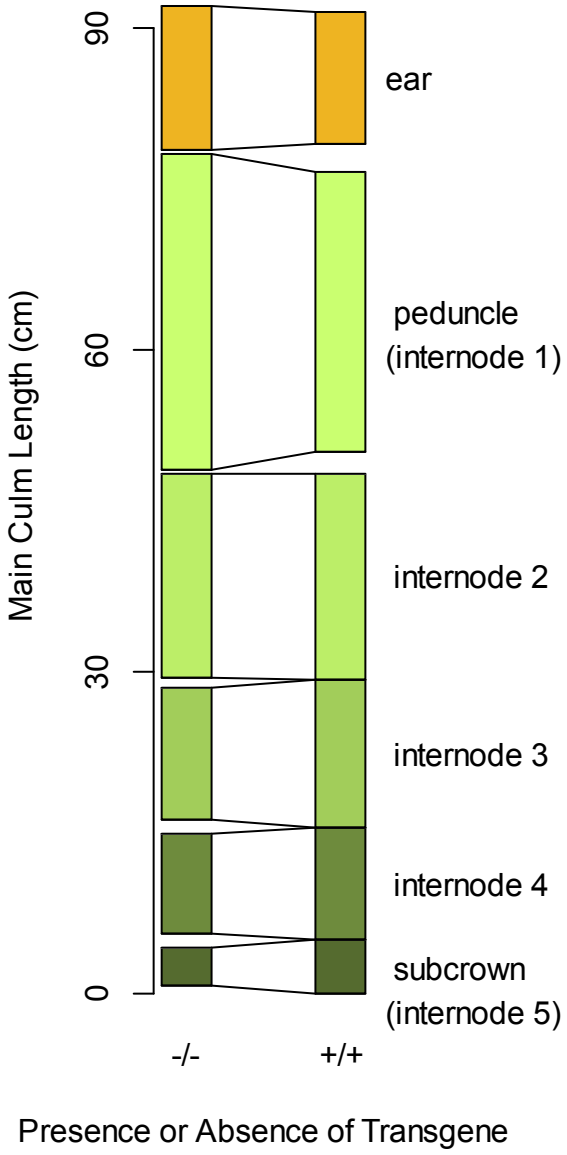


Figure 39: **Wheat main culm length is unaffected by the expression of *wbec1054*.** The two wheat models represent a summary of data collected for the T_4 generation of homozygous (+/+) wheat (line 3.3.14) transformed with *Blumeria* Effector Candidate BEC1054 (*wbec1054*), and for an azygous (-/-) wheat (line 3.3.12). The coloured bars for each internode and the ear represent the mean length of the primary tillers

7.4.3. Wheat infection assays

Transgenic wheat plants expressing the effector BEC1054 were assayed to determine whether this *B. graminis* f.sp. *hordei* effector affected the non-host resistance of wheat to

B. graminis f.sp. *hordei.* No haustoria were identified for wheat leaves inoculated with f.sp. *hordei*, indicating that BEC1054 was not involved in non-host resistance.

Transgenic wheat plants expressing BEC1054 were assayed to determine whether the presence of the effector affected the resistance of the host plant to *B. graminis* f.sp. *tritici* (Figure 40). The number of colonies, which formed epiphytic hyphae, was measured as a proxy for the presence of at least one functional haustorium. The presence of BEC1054 was found to increase the proportion of germinated conidia that formed at least one haustorium (propH), as can be seen by the elevation of boxplots labeled "+/+", when compared with the "-/-" from the same leaf segment and age. This increase was found to occur for plants of differing ages (three weeks or 11 weeks), and for different areas of the leaf. Furthermore, the propH was found to increase with distance from the leaf base, with the greatest mean propH at the leaf tip, as can be seen by the increasing elevation of the boxplots from leaf base to leaf tip (Figure 40).

A Generalised Linear Mixed Model (GLMM) was performed in R v 3.0.2. The count data for the number of germinated conidia with haustorium; or without haustorium; was bound as a single vector to create the response variable "y'. The age, genotype and segment (tip, middle or base) were set as fixed effects to account for the pseudoreplication of data from the segments (Equation 16). A binomial family was specified to account for the response variable being count data. Model simplification was performed, and non-significant interactions and factors removed, resulting in Equation 17. The linear hypotheses were then tested in a pairwise manner using the "multcomp" package in R.

Equation 16: **A maximal Generalised Linear Mixed Model before simplification.** The model contains all possible interactions for the explanatory factors "genotype", "age", and "segment'. The response factor "y" represents the number of germinated conidia with haustorium; or without haustorium bound as a single vector.

$$glmer(y{\sim}genotype * age * segment + (1|plant), family = binomial).$$

Equation 17: **A minimal Generalised Linear Mixed Model after simplification.** The model contains all possible interactions for the explanatory factors "genotype", and "segment'. The response factor "y" represents the number of germinated conidia with haustorium; or without haustorium bound as a single vector. All non-significant interactions and terms have been removed.

$$glmer(y{\sim}genotype + segment + (1|plant), family = binomial)$$

I found no significant two-or-three way interactions ($p>0.05$) for the interaction of the factors "genotype", "age" or "segment." Furthermore, I found no significant effect of age ($p>0.05$), i.e. although young plants also showed a higher propH than older ones, this effect was not statistically significant. In contrast, I found a significant difference for whether the lines were homozygous or azygous (0.13 and 0.09 respectively, $p<0.001$),

(Supplementary Table 36). A Tukey multiple comparison test was then conducted on the GLMM data, and it was determined that the propH was significantly different for the base, middle and tip ($p<0.005$), and that the propH was greatest at the leaf tip (Supplementary Table 36).

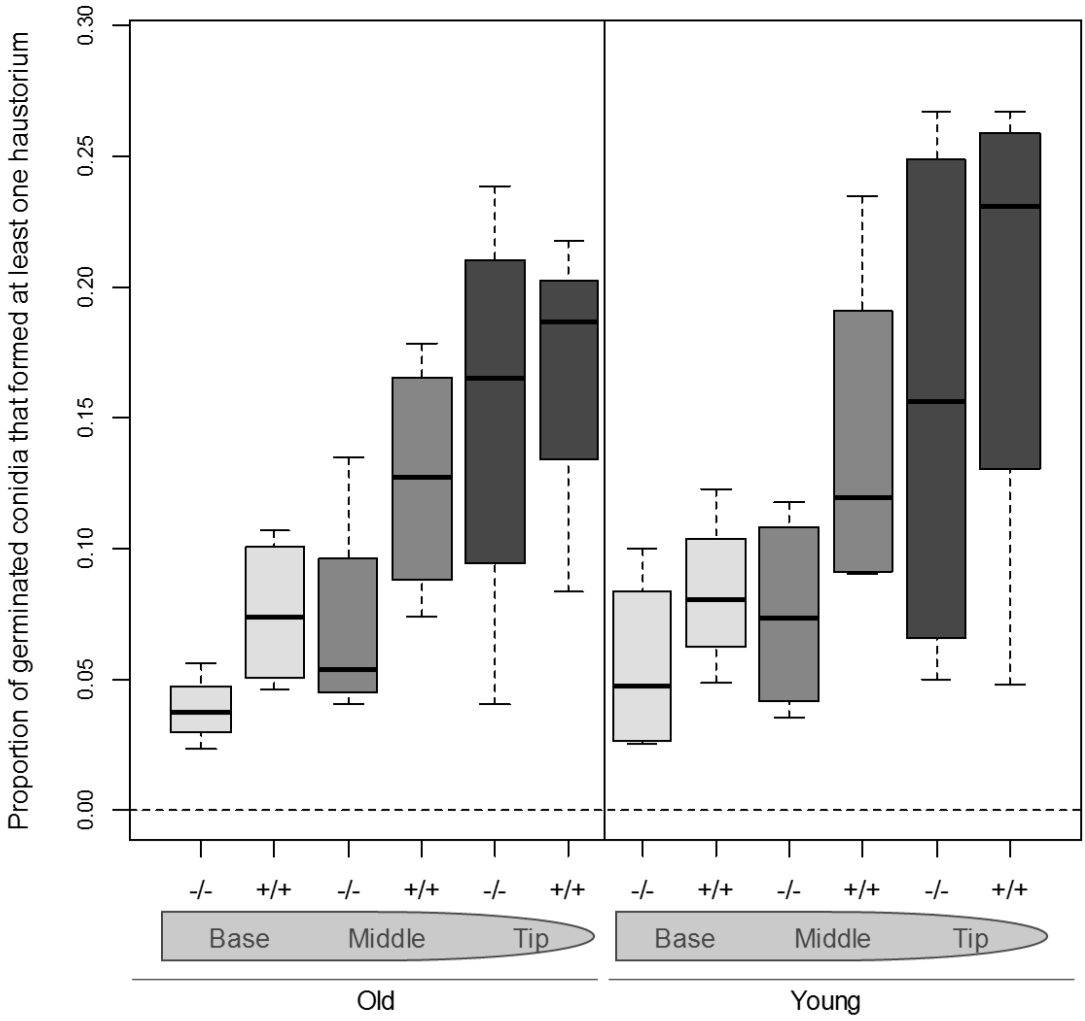


Figure 40: **Haustorial formation is increased in wheat by the non-host *Blumeria* Effector Candidate 1054 (BEC1054).** The mean proportion of germinated *Blumeria graminis* f.sp. *tritici* conidia which formed at least one haustorium was calculated for 2 cm leaf segments from the primary leaves of transgenic wheat homozygous (+/+ line 3.3.14) or azygous (-/- line 3.3.12) for *Blumeria graminis* f.sp. *hordei* effector BEC1054 (*wbec1054*). Old plants were 11 weeks old, young plants were three weeks old. The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars. Data utilised with permission from (Luong 2014).

7.4.4. Nicotiana infection assay

I used *Agrobacterium* lines to express GFP, BEC1054 with a C-terminal GFP tag, or JIP60ml with a C-terminal GFP tag in *N. benthamiana* (Table 8). We infiltrated four-week-old plants with *Agrobacterium,* (Section 3.5.3), with GFP on one side of the midrib, and either BEC1054-GFP or JIP60ml-GFP on the other. We then inoculated the leaves with *P. tabacina* sporangia at four points on their abaxial surface (Section 3.5.6). After 10 days, we collected 1 cm diameter leaf disks from each leaf infection site, removed the sporangia via washing, counted them, and used them to calculate the mean (Figure 41 top and middle). We then further calculated the proportion of sporangia in relation to the GFP only controls to account for leaf-to-leaf variation in levels of infection (Figure 41 bottom).

Expression of BEC1054-GFP increased the number of *P. tabacina* sporangia (Figure 41, top), as can be seen by the elevation of the BEC1054-GFP boxplot above zero. A total of 753 sporangia were counted from the samples, in contrast with 446 identified for GFP from the same leaves. In contrast, JIP60ml decreased the number of *P. tabacina* sporangia to almost zero (Figure 6, middle). In total, only six sporangia were identified from leaf samples transformed with JIP60ml, whereas 1775 were identified for the GFP control from the same leaves.

We observed a large variation for overall susceptibility (the number of sporangia, Figure 41, top). This can be seen through the wide spread of the data points, with average leaf counts varying from 1.75 to 64.75 for BEC1054-GFP, and 0.5 to 42.75 for its GFP control leaves. The paired data points for the leaf averages are also displayed in

Figure 41. For every pair of datapoints, a higher number of sporangia were observed with BEC1054-GFP than with its paired GFP control. The JIP60ml averages varied very little, due to the extremely low number of sporangia observed, and they displayed no obvious relationship to the results identified with the GFP control (i.e. the highest GFP-only leaf sporangia averages did not correspond with the leaf averages for JIP60ml for which spores were identified).

A GLM was performed to determine whether the number of sporangia 9 dpi varied with which plasmid had been agroinfiltrated (Equation 19).

Equation 18: **A Generalised Linear Mixed Model for the difference in *Peronospora* infection with agroinfiltration construct.** The explanatory factors "relative_difference" refers to the relative difference between the number of *Peronospora tabacina* sporangia identified from *Nicotiana benthamiana* leaf material infiltrated with the protein of interest, and the number identified from material infiltrated with GFP.

$$glm(relative\_difference \sim construct)$$

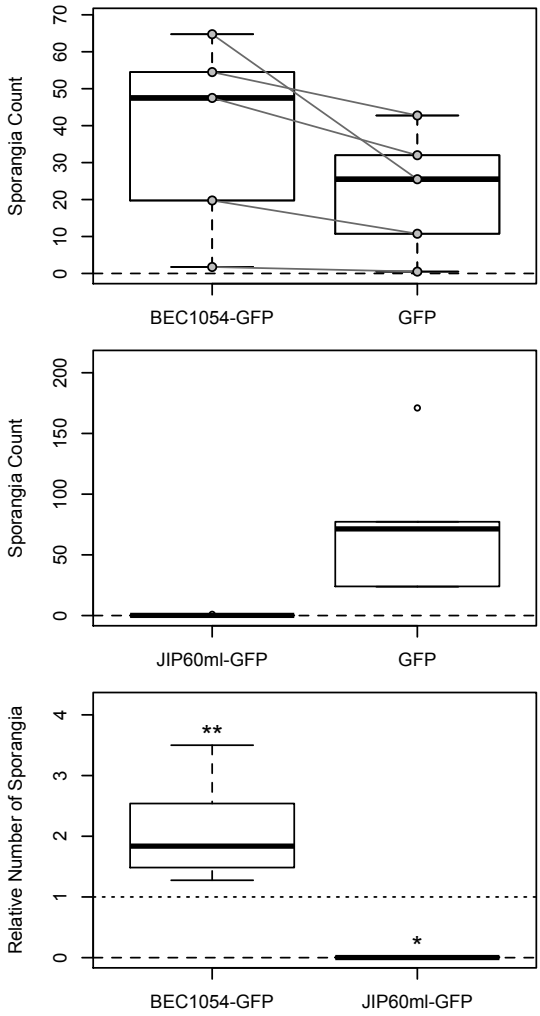


Figure 41: **Sporangia production is increased in wheat by the non-host *Blumeria* Effector Candidate BEC1054.** *Nicotiana benthamiana* leaves were infiltrated on one side of the midrib with *Agrobacterium* expressing GFP, and on the other side with *Agrobacterium* expressing BEC1054 with a C-terminal GFP tag, or barley Jasmonate Induced Protein 60 (JIP60) with a C-terminal GFP tag. Leaves were inoculated, within one hour of infiltration, with *Peronospora* sporangia on their abaxial surface. After 10 days, leaf disks were collected from each leaf, the sporangia removed via washing, counted, and used to calculate the mean. The top two graphs represent the leaf count data means for BEC1054-GFP, JIP60ml-GFP and their relative controls, whereas the bottom boxplot represents the proportion of sporangia in relation to the GFP only controls (where the GFP-only values, post normalisation, are equal to one). The paired means (calculated from two halves of the same leaf) are shown as linked grey circles on the top graph. Significantly more sporangia were found with BEC1054-GFP than with GFP only (biological replicates n=5, $p<0.001$), and significantly less for JIP60-GFP than with GFP only (biological replicates n=6, $p<0.05$). The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. Significant difference is indicated by "." for $p\leq0.1$, "*" for $p\leq0.05$, "**" for $p\leq0.01$ and "***" $p\leq0.005$. Data utilised with permission from Thieron, 2015.

I performed a Bartlett test, to determine whether the variance was homogeneous between the BEC1054-GFP and GFP lines. This was the case ($p<0.0001$). A GLM was performed (Equation 1), to determine whether the expressed protein (GFP, BEC1054-GFP or JIP60ml-GFP) had a significant effect upon the mean, which it did ($p<0.005$). Post-hoc Games-Howell tests were performed (Supplementary Table 42) to determine which pairs of means were significantly different.

Normalisation of the sporangia means against the GFP control (Figure 41, bottom) demonstrated that BEC1054-GFP increased the number of *P. tabacina* sporangia. All of the data points were elevated above one (where normalisation would make the GFP-only values equal to one).

7.4.5. Nicotiana benthamiana

Expression of JIP60ml-GFP induced cell death in *N. benthamiana*. Brown patches, corresponding to dead cells, could first be observed at ca. 5 dpi with *Agrobacterium*, with large numbers of cells having died by 9 dpi (Figure 42). Trypan blue staining was used to visualise dead cells. In leaves infiltrated with JIP60ml, the blue dye was seen to increase in strength towards the midrib, and brown patches of cells too dead to stain were seen towards the leaf perimeter. Infiltration with BEC1054 did not cause cell death. Areas of leaves infiltrated with *Agrobacterium* expressing BEC1054 did not contain greater numbers of blue or brown cells than non-infiltrated areas of the leaf.

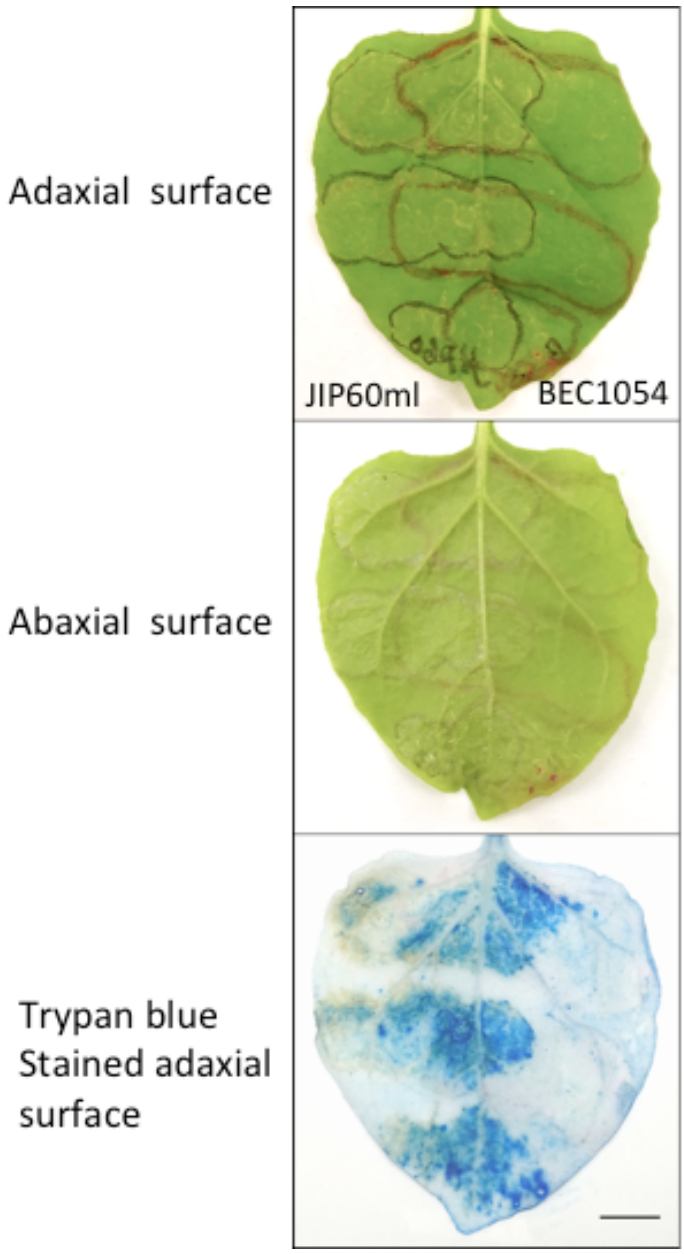


Figure 42: **Expression of *Blumeria* Effector Candidate 1054 (BEC1054) does not affect *Nicotiana benthamiana*'s response to Jasmonate Induced Protein 60 (JIP60).** Leaves were infiltrated with *Agrobacterium* expressing JIP60ml and BEC1054. Infiltrated areas are marked using black or red pen. Ten days after infiltration, the leaves were stained with trypan blue. Data utilised with permission from Thieron, 2015,

7.4.6. Chlorophyll breakdown does not appear to be affected by BEC1054

Seven day old seedlings of transgenic wheat, azygous (-/-) or homozygous (+/+) for *wbec1054*, were maintained uninfected, or infected with *B. graminis* f.sp. *tritici* once a day for three days to establish heavy infection across the wheat adaxial surface. We harvested primary leaves, and treated them with 45 μM methyl jasmonate (MeJA) or water (H_2O) for a further five days. Photographs of the treated leaves are shown in Figure 43.

Leaves treated with MeJA were yellower/ less green than those treated with water (Figure 43). This was the case for all leaf treatments (infected/uninfected and H_2O/MeJA). Infection led to the formation of "green islands" (Murphy *et al.,* 1997), visible on both H_2O and MeJA treated leaves as darker green patches surrounded by paler green/yellowing leaf tissue (Figure 43).

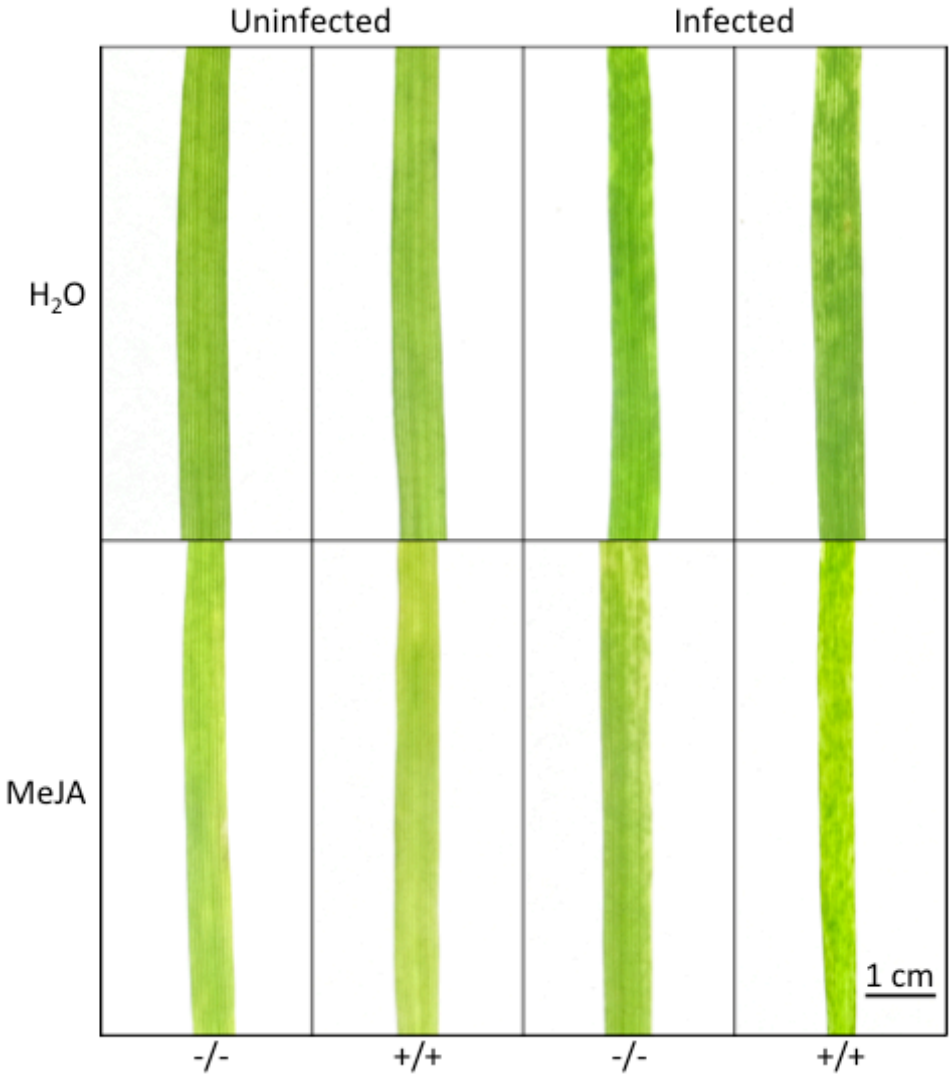


Figure 43: **Wheat primary leaf phenotypes following methyl Jasmonate (MeJA) treatment.** Seven day old seedlings of transgenic wheat, azygous (-/-, line 3.3.12) or homozygous (+/+, line 3.3.14) for *Blumeria graminis* f.sp. *hordei* effector (*wbec1054*), were maintained uninfected, or infected once a day, for three days. Primary leaves were harvested, and treated with 45 µM MeJA or water (H_2O) for a further five days. Data utilised with permission from Thieron, 2015.

We extracted total protein from the treated leaves, and determined the concentration of chlorophylls a and b using a spectrophotometer. The boxplots for the various treatments (with/without BEC1054, infected/uninfected and MeJA/H_2O) demonstrated very similar patterns for chlorophyll a and b (Figure 44), varying slightly around a value of ca. 0.5 µg/ml for chlorophyll a and 0.4 µg/ml for chlorophyll b.

I performed Bartlett tests to determine whether the variance of the datasets were normally distributed (Supplementary Table 37). I found this for the majority of the datasets, except for chlorophyll a treatment with MeJA/H_2O, which was significantly non-normal ($p<0.05$). Furthermore, the dataset for chlorophyll b treatment with MeJA/H2O was very weakly significant ($p<0.1$). All datasets were further analysed through GLM for consistency.

A GLM was performed for the factors H_2O/MeJA, infection status, and seed line. No significant two or three way interactions were found for either chlorophyll a or b ($p>0.05$) for any of the factors. The only factor found to be significant was treatment with H_2O/MeJA ($p<0.0001$). Games-Howell post-hoc tests were performed to determine whether any of the pairs of means were significantly different (Supplementary Table 38). There was a decrease in both chlorophylls a and b for uninfected plants treated with MeJA (as can be seen by the lowering of the boxes below those treated with water). This result was significant for line 3.3.14 (+/+) chlorophyll a ($p<0.05$), when compared with the H_2O treated lines. This decrease was also significant for lines 3.3.12 (-/-) and 3.3.14 (+/+) for chlorophyll b ($p<0.05$). This decrease was not present for infected

plants, with all of the infected plants for chlorophyll a and chlorophyll b being statistically similar.

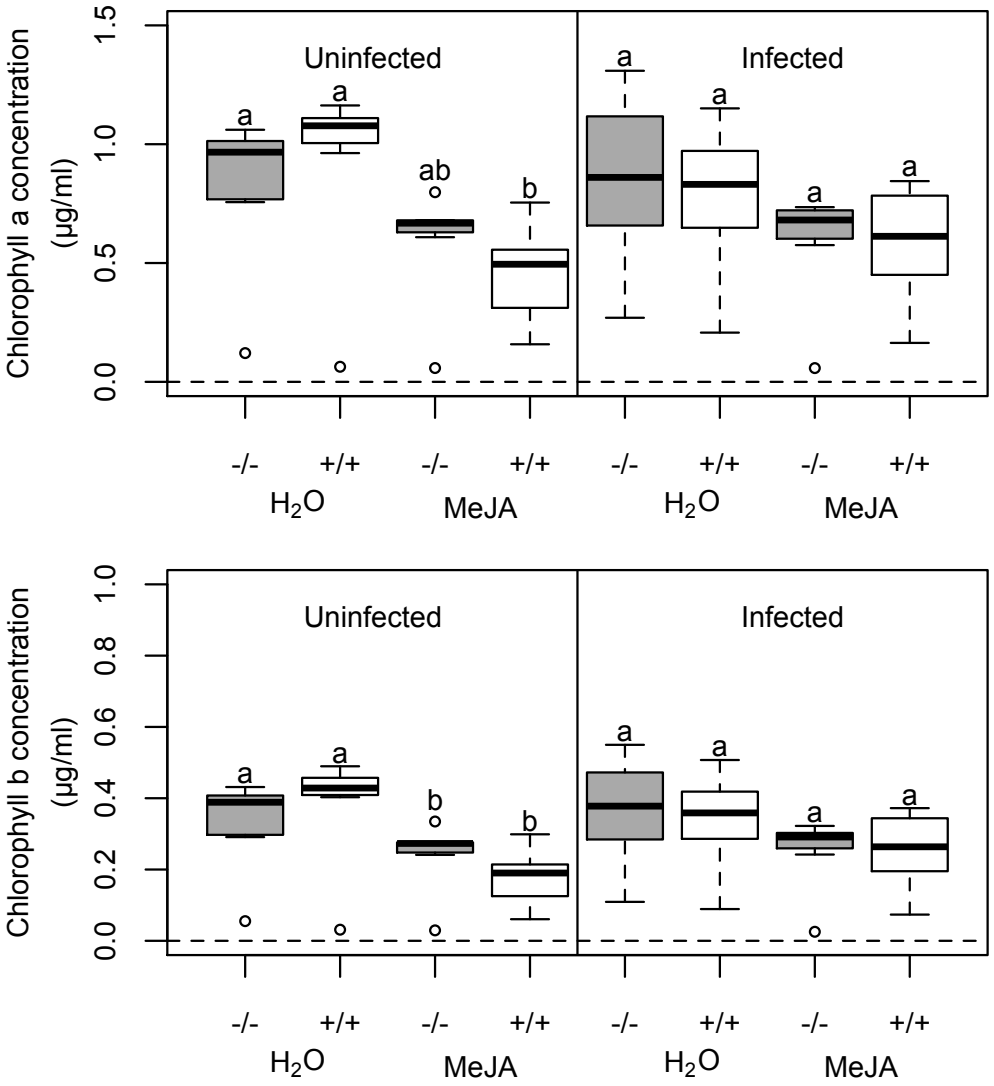


Figure 44: **Expression of BEC1054 does not affect chlorophyll a and b concentrations in wheat.** Seven day old seedlings of transgenic wheat, azygous (-/-, line 3.3.12) or homozygous (+/+, line 3.3.14) for *Blumeria graminis* f.sp. *hordei* effector BEC1054, were maintained uninfected or infected once a day for three days. Primary leaves were harvested, and treated with methyl Jasmonate (MeJA) or water (H_2O) for a further five days. Total protein was extracted from the leaves, and the concentrations of chlorophyll a and b measured spectrophotometrically. Significant difference was determined via post-hoc test for +/+ and -/- plants undergoing the same treatment. No significant difference was identified between any of the treatments ($p>0.05$). The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. The letters indicate significance, with identical letters being the same, and different letters being significantly different. Data utilised with permission from (Chandler 2015).

7.4.7. Ribosome inactivating proteins appear to be outcompeted by BEC1054

Seven day old seedlings of -/- or +/+ transgenic wheat were infected once a day for three days or maintained uninfected. Primary leaves were harvested, and treated with MeJA for a further five days. Ribonucleic acid was extracted from the primary leaves, and treated with 1 M aniline. The RNA was analysed on a Bioanalyzer Nano chip, an electropherogram produced and the area of the major RNA peaks measured (Agilent 2011; Section 0, and Figure 7).

I predicted that treatment of the depurinated rRNA with aniline *in vitro* would result in the formation of two fragments, one ca. 3027 nucleotides long, and one ca. 364 (based on the model shown in Figure 27), as aniline cleaves the sugar-phosphate backbone at site of the modified nucleotide(s) (Peattie 1979). A small peak was found to appear in MeJA-treated uninfected plants following treatment with aniline (Figure 45). The small peak was found to occur between the 2000nt and 4000nt ladder markers in the majority of samples, which fits this prediction. This peak will be referred to as the RNA "degradation peak" throughout the remainder of this chapter; and is shown enlarged in the top left of each electropherogram (Figure 45, middle). This peak was not observed when RNA was treated with water (instead of MeJA) (Figure 45, top), if the leaf material was infected with *B. graminis* f.sp. *tritici* (Figure 45, bottom), or if the RNA was not treated with aniline (Figure 45). If homozygous BEC1054 wheat leaf material was used, this peak was found to form intermittently and was reduced in size/area (when compared with the azygous control).

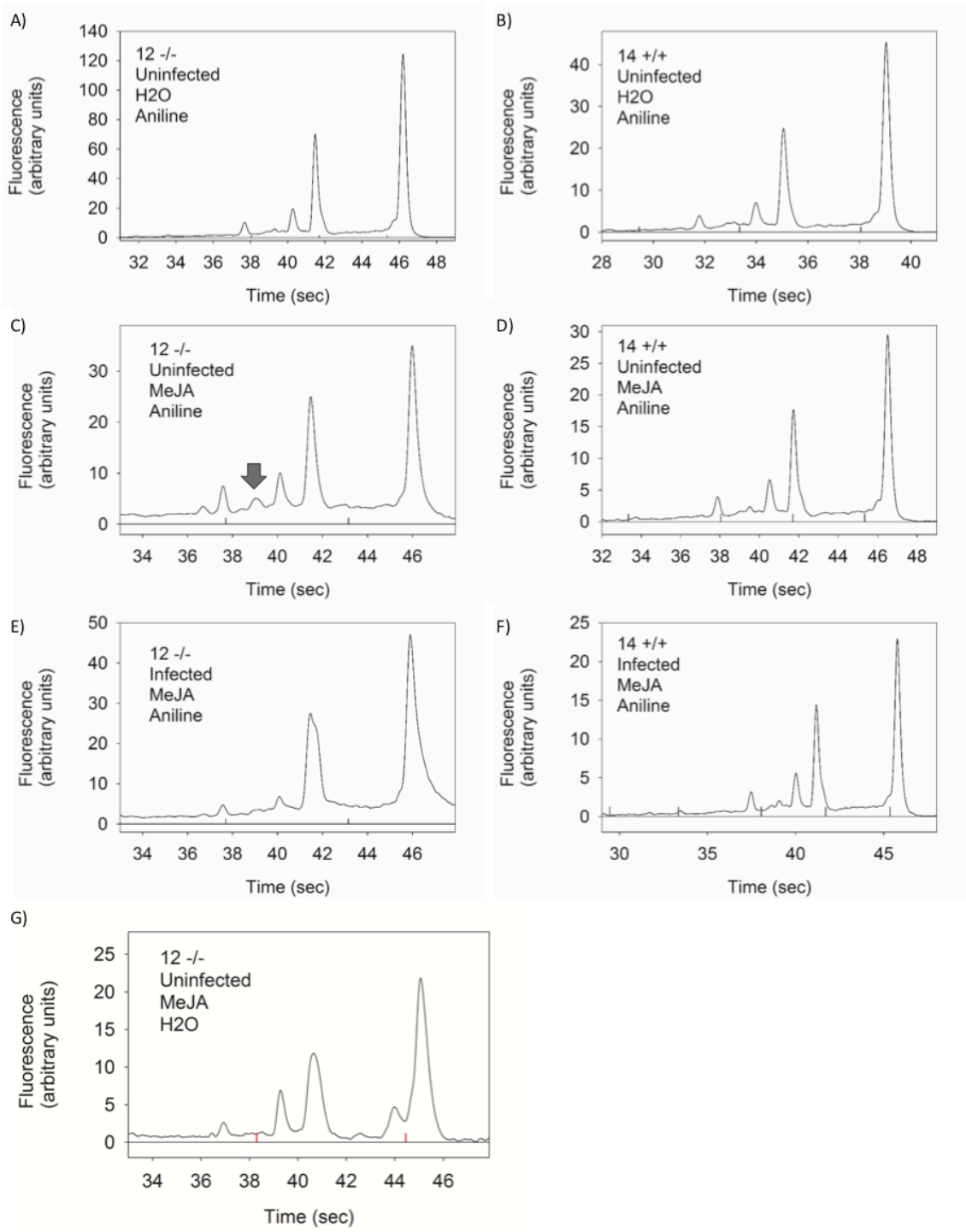


Figure 45: **Ribosomal RNA degradation peak analysis**. Seven day old seedlings of transgenic wheat, azygous (-/-, line 3.3.12 "12 -/-") or homozygous (+/+, line 3.3.14 "14 +/+") for *Blumeria graminis* f.sp. *hordei* effector BEC1054, were maintained uninfected or infected once a day for three days. Primary leaves were harvested, and treated with methyl Jasmonate (MeJA) or water (H_2O) for a further five days. Total RNA was extracted from the leaves, and an electropherogram produced using Bioanalyzer. Tick marks along the x-axis indicate the Bioanalyzer ladder peaks from right to left at 6000 nucleotides (nt) (this can be seen as the right most mark on samples A, B, D, F and G, 4000nt (the right most mark on samples C and E, 2000nt, 1000nt, 500nt and 200nt. Data utilised with permission from Thieron, 2015.

We calculated the relative peak area for the degradation peak, in relation to the area of the large (28S) and small (18S) ribosomal subunits (Figure 46 and Supplementary Table 41). A GLM was performed to determine whether infection, or the wheat line (homozygous or azygous) under investigation had an effect upon the area of a peak, termed the "degradation peak", which was observed following treatment of azygous plants with MeJA, and then subsequent treatment with aniline. The GLM was also used to investigate whether there was any interaction between the factors. The infection status had a significant effect (t=3.26, p=0.0038). The wheat line investigated did not have a significant effect upon the model (t=1.02, p=0.3186), but it did interact significantly with infection (t=2.3, p=0.0318), i.e. the infection status and wheat line together had an effect upon the degradation peak area. Simplification of the model lost this effect, so it was decided that the maximal model (Equation 18) should be kept.

Equation 19: **A maximal Generalised Linear Mixed Model for RNA peak data before simplification.** The model contains all possible interactions for the explanatory factors "infection" referring to infection with *Blumeria graminis* f.sp. *tritici*, and "wheat_line" referring to which seed line was used.

$$glm(set\_ca \sim wheat\_line * infection)$$

I performed Bartlett tests to determine whether the variance was homogeneous for the relative degradation peak area against the wheat line (3.3.12 or 3.3.14), infection, or both (Supplementary Table 40). None of the datasets showed homogeneous variance ($p<0.05$).

For uninfected primary wheat leaves, I found that the presence of BEC1054 decreased the degradation peak area, as can be seen by the lowering of the boxplot. This decrease was not significant ($p=0.0993$). It was, however, repeatable, occurring for both line 3.3.14 (pictured) and 3.3.7 (+/+) (Supplementary Figure 56 and Supplementary Table 39). For infected wheat leaves, the presence of BEC1054 increased the area of the degradation peak, as can be seen by the elevation of the boxplot. This change was not significant ($p=0.7389$). Infection of homozygous null wheat (-/-) with *B. graminis* f.sp. *tritici* decreased the area of the degradation peak, preventing its formation almost completely. This effect was significant ($p<0.05$).

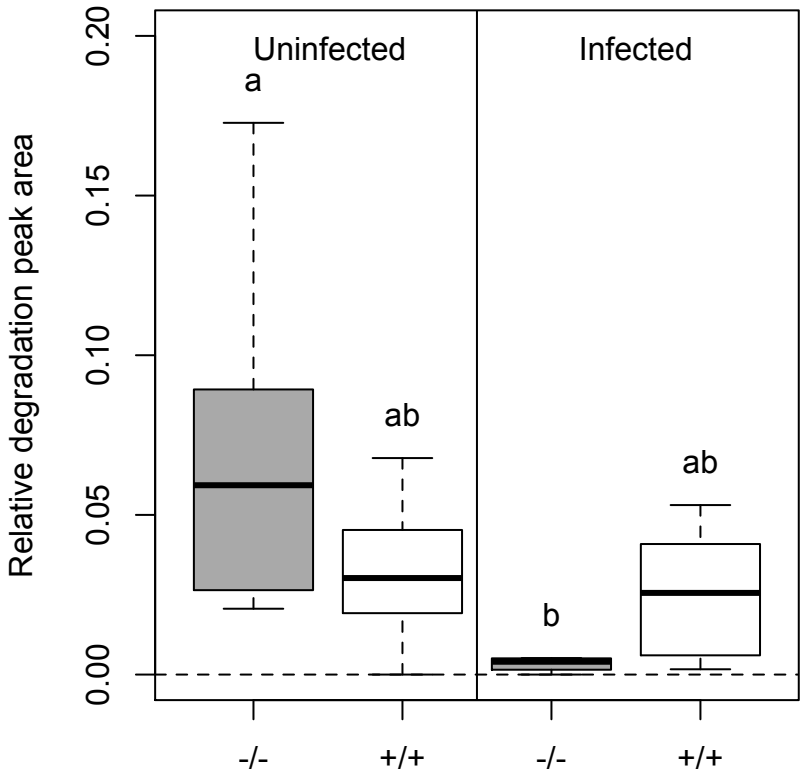


Figure 46: **Expression of BEC1054 decreases the formation of an RNA diagnostic peak**. Seven day old seedlings of transgenic wheat, azygous (-/-, line 3.3.12) or homozygous (+/+, line 3.3.14) for *Blumeria graminis* f.sp. *hordei* effector BEC1054 (*wbec1054*), were infected once a day for three days or maintained uninfected. Primary leaves were harvested, and treated with methyl jasmonate for a further five days. Ribonucleic acid was extracted from the primary leaves, and treated with 1 M aniline. The RNA was run on a Bioanalyzer Nano RNA chip, and the area of the major RNA peaks measured. The relative peak area was calculated for a putative ribosomal degradation peak, in relation to the area of the large ribosomal subunit (28S). The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. The letters indicate significance, with identical letters being the same, and different letters being significantly different. The number biological replicates, from left to right, was 6, 10, 5 and 4. utilised with permission from Thieron, (2015) and Chandler (2015).

7.4.8. Co-localisation of BEC1054-GFP with cellular localisation markers

The fluorescent signal of BEC1054 expressed with either a C-terminal or an N-terminal GFP tag, in *N. benthamiana,* was found to occur in the cytoplasm and the nucleus, with a much lower GFP signal also occurring in the nucleolus (Figure 47). Max scans, created through combining the maximum pixel intensity of the images obtained in a Z-stack, demonstrated that BEC1054's GFP tag signal occurred in faint puncta throughout the cytoplasm, and that these occurred for both N and C terminal fusions. These puncta were not co-localised with any of the RFP cellular markers available in the host laboratory, including early or late endosomes (ARA7 and ARA8), Golgi, or mitochondria (Geldner *et al.,* 2009).

I performed co-localisation experiments with 40S 16 with a C-terminal GFP tag and a cytoplasmic mitochondrial marker with an RFP tag. I found that in *N. benthamiana,* the GFP signal for 40S 16 was visible in the nucleus, very bright in the nucleolus, and was visible as puncta throughout the cytoplasm of the cell. This expression pattern matched that observed in wheat (Chapter 4 and Figure 16). Co-localisation was performed for 40S 16's GFP tag signal with an RFP mitochondrial marker (Figure 48). The GFP and RFP puncta occurred at the same points throughout the cytoplasm.

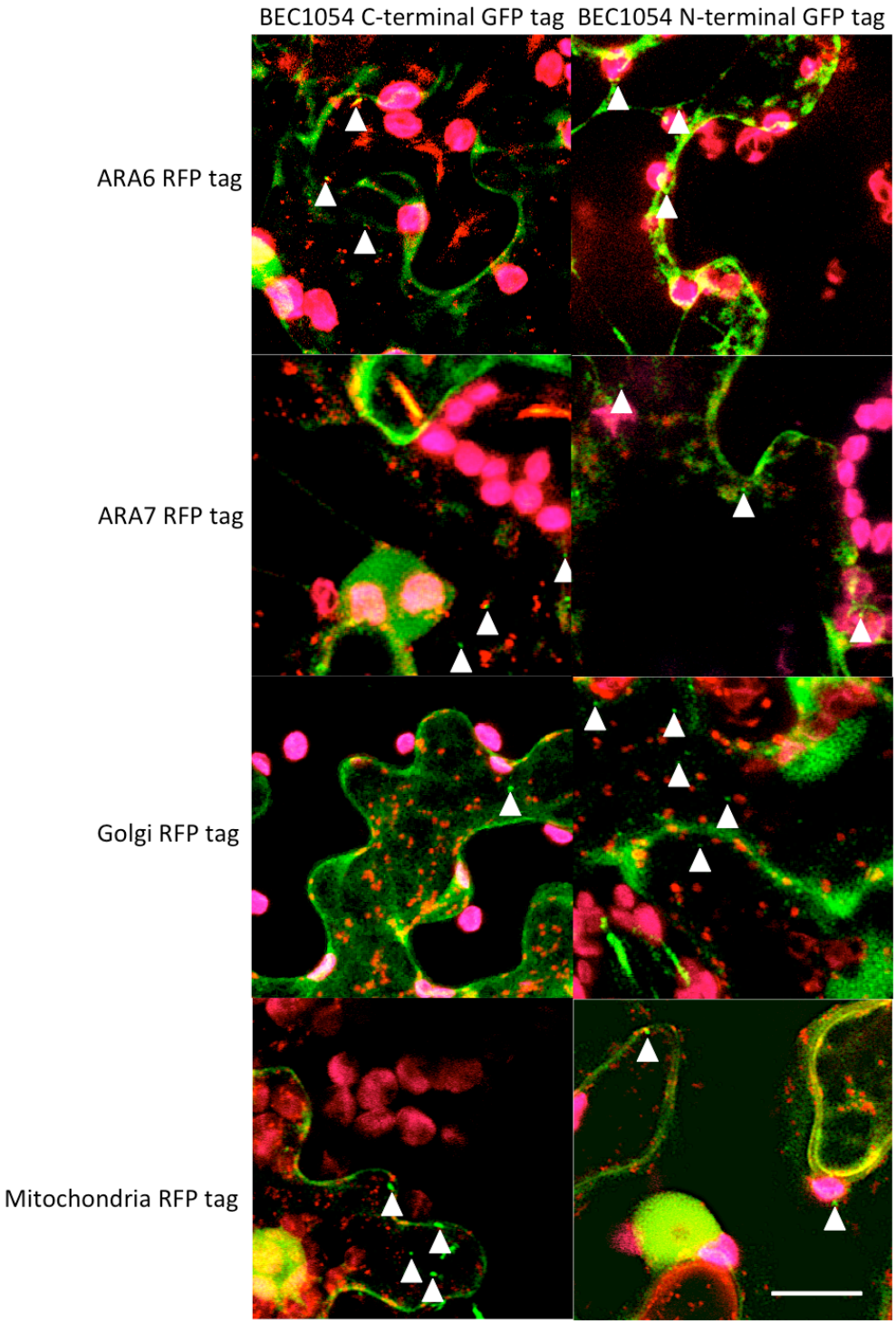


Figure 47: **Co-expression of *Blumeria* Effector Candidate 1054 (BEC1054) with multiple cellular markers.** Green fluorescent tags were expressed in *N. benthamiana* epidermal cells, fused to both the N and C terminal domains of BEC1054 (with BEC1054-GFP indicating a C-terminal tag, and GFP-BEC1054 indicating an N-terminal tag). White arrows indicate focal accumulations of BEC1054. Chloroplast autofluorescence is shown in pink. The red fluorescenr protein tagged cellular markers are shown in red.

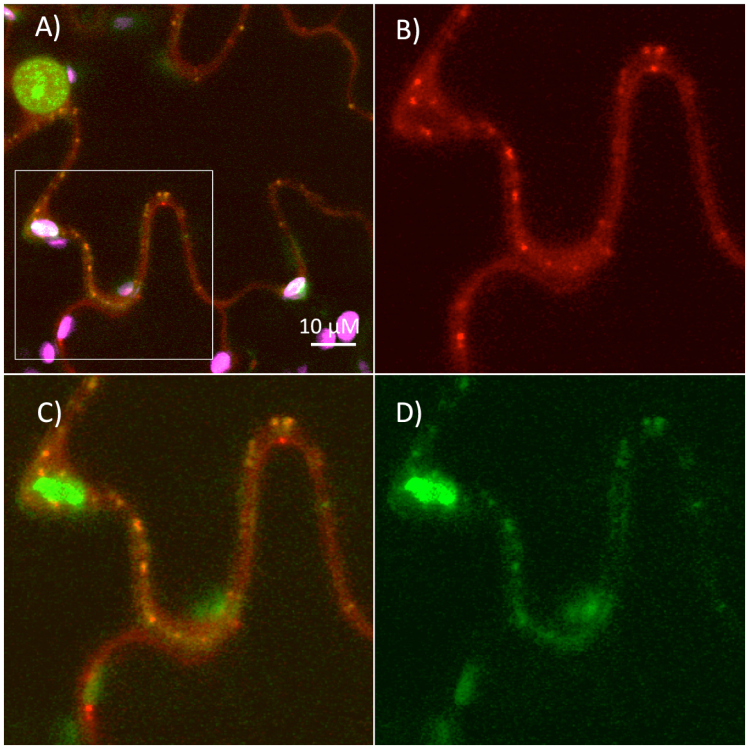


Figure 48: **Ribosomal subunit 40S protein 16 (40S 16) marks mitochondria and chloroplasts.** Co-expression of C-terminal labeled GFP, and an RFP labeled mitochondrial marker demonstrated that 40S 16 occurs as focal accumulations marking mitochondria. A) Merged colour overview, B) expanded RFP section, C) combined GFP and RFP sections, and D) expanded GFP section.

7.5. Discussion

7.5.1. Transgenic wheat phenotyping assay

Genotyping and qPCR determined that BEC1054 was present and was expressed in the T_4 generation of wheat line 3.3.14, and absent in line 3.3.12. These results reconfirmed those obtained previously (Bonciani 2014), demonstrating that the transgene was stable in line 3.3.14.

In a stable transformation system, genes are expressed throughout the life of the plant. It can therefore be important to determine whether any changes are due to the effect of the gene on development, or other essential processes. Furthermore, it is possible for the inserted DNA to interrupt a gene (Alberts *et al.,* 2002). The *wbec1054* was inserted via *Agrobacterium* mediated transformation, and the site of DNA insertion would have been random, making phenotyping of the transformed plants important.

Expression of BEC1054 in yeast had been shown to affect yeast transformation success and growth. I therefore suspected that stable expression of BEC1054 as a transgene in wheat may affect wheat growth and development. A randomized block design with six plots was used to investigate the phenotype of *wbec1054* homozygous and azygous plants, as per (Borrell *et al.,* 1993, Gasperini *et al.,* 2012), but with each block representing one potted wheat plant. This design was utilised to prevent any growth differences that would have occurred due to location within the growth chamber, i.e. if the floor were slightly sloped, which would have allowed some plants more water than others, or if there were a temperature gradient between the door and the ventilation system. Furthermore, all seeds were of the same age, originating from the same T_3

generation plant, and had been stored under the same conditions, helping to ensure that any physiological differences observed would have been caused by the presence (or absence) of the *wbec1054* transgene.

The height of wheat, the spike (head including whisker) length, number of leaves, number of fertile tillers, number of grains set per spike/ear, and grain yield are characteristics frequently assayed when assessing wheat plants (Borrell *et al.,* 1993, Hays *et al.,* 2007, Gasperini *et al.,* 2012, Fellahi *et al.,* 2013). The height of wheat is commonly determined using the primary tiller, with each tiller usually formed of hollow, round, jointed culms (stems). Each culm is usually comprised of five internodes, which are shortest at the base of the plant, and longest at the ear, with the peduncle being the longest (Gasperini *et al.,* 2012). The wheat models developed from the phenotyping assay demonstrated the same pattern for both homozygous (+/+) (Figure 39) and azygous (-/-) control plants. The boxplots and statistical analyses further demonstrated that there were no significant differences in the internode lengths, primary culm length, or any other characteristic measured (Figure 38). These results were reproducible, with a second homozygous line (3.3.7) showing the same characteristics (not shown). These results showed that BEC1054 did not have an effect upon plant growth, yield, or height under the experimental conditions used, and that the DNA had not disrupted an essential wheat gene.

7.5.2. Transgenic wheat and Nicotiana infection assays

In our experiments, no haustoria were observed on wheat inoculated with *B. graminis* f.sp. *hordei*. These results indicated that BEC1054 does not affect non-host resistance.

We used the proportion of *B. graminis* f.sp. tritici conidia that form haustoria on wheat as an indication of how successfully the pathogen infected the host plant. An expression analysis performed for BEC1054 demonstrated that it was transcribed at early stages during a *B. graminis* infection time-course in barley (Chapter 5 and (Pennington *et al.,* 2015)). These results indicated a correlation between *wbec1054's* expression and the establishment of early infection and/or haustorial formation. Our results demonstrated that the presence of the transgene *wbec1054* increased the proportion of conidia that formed haustoria (Figure 40) in both old and young plants. These results corroborate those identified previously, where it was shown in barley that silencing of BEC1054 led to a significant decrease in the virulence of *B. graminis* f.sp. *hordei* in its host, barley (Pliego *et al.,* 2013).

Monocot leaves, including wheat, mature along a basipetal (tip to base) gradient along the blade (Wernicke and Milkovits 1984, Itoh *et al.,* 2005). Furthermore, genes are expressed in a non-uniform manner along the longitudinal axis of mature leaves belonging to members of the Poaceae (Jiao *et al.,* 2009, Li *et al.,* 2010, Wang *et al.,* 2014). Li *et al.,* (2015) found that the leaf base of rice expressed at higher levels transcripts relating to secondary metabolism compared to the tips. Their data also revealed that transcription factors, such as WRKYs, were expressed differentially along the blade of the leaf, with 13 showing highest expression at the leaf base, and two highest expression at the leaf tip (Li *et al.,* 2015). The WRKY factors play roles in plants including disease resistance (Pandey and Somssich 2009), stress responses and hormone responses (Sakuma *et al.,* 2002, Chen *et al.,* 2012). The increase in infection seen in plants expressing *wbec1054* was strongest at the leaf base, and became reduced

towards the leaf tip (Figure 40). Together, the gradient in nutrient availability and in immune related transcripts helps to explain the gradient in infection seen with *B. graminis* f.sp. *tritici*. The difference in propH was higher between plants azygous or homozygous for BEC1054 at the leaf base than at the leaf tip. If other disease resistance factors such as RIPs, were more abundant at the base of the leaf (as were the WRKY transcription factors (Li *et al.,* 2015)), then BEC1054 may have played a greater role in preventing RIP induced cell death.

Agrobacterium was used to express BEC1054 and JIP60ml with a C-terminal tag in the dicot non-host *N. benthamiana* (Figure 41), which was then infected with the oomycete *P. tabacina*. *P. tabacina* causes blue mould, a downy mildew disease (Krsteska *et al.,* 2015). Our study demonstrated that the expression of BEC1054 increased the susceptibility of *N. benthamiana* to *P. tabacina*. A significantly greater number of sporangia were recovered from leaf material transformed with BEC1054-GFP than with a GFP only control. This suggests that that BEC1054 affected a conserved mechanism of the plant defense response, present in both monocotyledonous and dicotyledonous plants, and which favours both fungal and oomycete pathogens.

Far fewer sporangia were recovered from leaf material expressing JIP60ml-GFP than material expressing GFP (Figure 41). These results indicate that the JIP60ml construct (described in Section 3.4.1.3) was indeed active. The results shown (Figure 43) demonstrate that it caused extensive cell death by 10 days after agrobacterium infiltration. It is possible that the absence of sporangia was due to the death of the host cells, which are required by the biotrophic pathogen *P. tabacina*. Cell death caused by

JIP60ml first became visible at around 5 dpi, and was readily apparent by 10 dpi. The slow development indicates that it was not due to triggering of the hypersensitive response (which is notable for its rapidity) (Goodman and Novacky 1994), but instead was a more generalized cell death, possibly due to the ribosome cleavage activity of JIP60ml. This result is consistent with that for the BiFC assay, where two epidermal cells were identified expressing JIP60ml tagged with C-terminal YFP (Section 4.4.6), where we had hypothesised that JIP60ml was toxic, killing the majority of cells that it was expressed in, or that it affected translation of both the transformation marker and the YFP vectors. The asexual life cycle of *P. tabacina* takes five to seven days; with a second set of sporangia being produced around seven to 10 days (Krsteska *et al.,* 2015). The extensive cell death (Figure 42) caused by JIP60ml within this timeframe would have prevented the pathogen from establishing a successful biotrophic infection, and thus would have prevented the formation of sporangia.

7.5.3. Chlorophyll senescence assay

We induced senescence in wheat primary leaves, by floating them on MeJA (Hung *et al.,* 2006). After five days, we were able to detect a noticeable induction of senescence, both through the colour change of the wheat primary leaves from green towards yellow (Figure 43), but also through the decrease in the concentration of chlorophylls a and b in MeJA treated primary leaves (Figure 44). This change was significant for chlorophyll b from leaves homozygous and azygous for *wbec1054*, but only for chlorophyll a from homozygous leaves. There was also no significant difference between the chlorophyll a concentration for the homozygous and azygous leaves (Figure 44). The degradation of chlorophyll b is different from that of chlorophyll a. Degradation of chlorophyll b occurs first during senescence, with chlorophyll b being converted to chlorophyll a (Ito *et al.,*

1993, Ito *et al.,* 1996, Scheumann *et al.,* 1996). This may explain the clear degradation of chlorophyll b with MeJA treatment, and the result seen for chlorophyll a.

The decrease in concentration of chlorophylls a and b seen with MeJA treatment was delayed in plants infected with *B. graminis* f.sp. *tritici* (Figure 43). In compatible interactions, chlorophyll is maintained within viable host cells against a background of senescence, leading to the occurrence of "green islands" (Murphy *et al.,* 1997). Green islands can be observed in Figure 43.

7.5.4. RNA degradation assay

Differential expression has been found for jasmonate signaling related genes following *B. graminis* f.sp. *tritici* infection in wheat (Wang *et al.,* 2012), and following infection with the powdery mildew *Erysiphe necator* (Schw.) Burr in cultivated grapes (*Vitis vinifera*) (Weng *et al.,* 2014). An increase in jasmonate signaling would lead to the expression of defense related genes such as JIP60 in barley.

Jasmonates trigger or influence several processes in addition to senescence in a range of plant species including, but not limited to, rice spikelet development (Cai *et al.,* 2014), *Brassica* and *Linum* embryo development (Wilen *et al.,* 1991), and secondary metabolite biosynthesis (Gundlach *et al.,* 1992, Aerts *et al.,* 1994), storage protein accumulation (Staswick *et al.,* 1992, Mason *et al.,* 1993), wound responses (Farmer *et al.,* 1992, Xu *et al.,* 1994), and alteration of gene expression (Sembdner and Parthier 1993) including the induction of Jasmonate Induced Proteins (JIPs) (Weidhase *et al.,* 1987b). These JIPs possess a wide range of activities, for example JIP6 is a thionin-like protein (Andresen *et*

al., 1992), and JIP60 has been shown to be a ribosome inactivating protein (see Section 6.5 for further details).

Treatment of the depurinated rRNA with aniline *in vitro* results in the formation of two fragments, one ca. 3027 nucleotides long, and one ca. 364, as aniline cleaves the sugar-phosphate backbone at site of the modified nucleotide(s) (Peattie 1979). The small fragment, often used as an indicator of RIP activity, has previously been shown to occur in barley leaves after 72 h of MeJA treatment (Dunaeva *et al.,* 1999).

A diagnostic RNA peak, of between 2000 and 4000 nucleotides, was identified in the Bioanalyzer data, which occurred following MeJA treatment (Figure 45). The predicted size of the peak varied between samples/chips, but remained between these ladder markers in the vast majority of samples. Treatment with a control (H_2O, as opposed to MeJA) did not lead to the formation of this peak. One possibility is that this peak may represent the large fragment produced following RIP degradation of rRNA. Bioanalyzer RNA Nano chips are non-denaturing, so secondary folding may affect the running speed of RNA fragments (Agilent 2013).

Infection with *B. graminis* f.sp. *tritici* caused the diagnostic peak to disappear in control wheat plants azygous for *wbec1054* (Figure 45 and Figure 46). These results corroborate the idea that the fungus protects the plant ribosomes from degradation. The prevention of rRNA degradation would help to maintain a living plant cell as a food source for the fungus. The activity of RIPs, such as JIP60, prevents the binding of the eEF2/GTP complex to the ribosome, and thus inhibits protein synthesis at the

elongation step (Wool *et al.,* 1992); and it has also been suggested that RIPs may affect initiation (Osborn and Hartley 1990). Expression of JIP60 in *N. tabacum* indicated that JIP60 impaired elongation, as they found a shift towards polysomes in the polysome/monosome ratio (Gorschen *et al.,* 1997).

The presence of BEC1054 decreased the accumulation of the RNA seen as a "degradation" peak. Although this effect was not statistically significant, it was found to be repeatable, also occurring for the homozygous seed line 3.3.7. The RNA samples used were extracted from whole primary leaves. The wheat infection assay (Figure 40) demonstrated that a greater difference was seen in infection when looking at the leaf base, than when looking at the leaf tip. It may be that extraction of RNA from just the leaf base following treatment would allow a significant difference to be observed between the degradation peak areas for the azygous and homozygous lines (Figure 46). Alternatively, more than one of the RNase-like proteins associated with haustoria may be required for further protection of the ribosomes, as 120 are encoded by the *B. graminis* f.sp. *hordei* genome (Spanu, *pers. comm.*). It remains to be seen which of these explanations is correct.

7.5.5. Colocalisation of BEC1054 *in planta*

We were unable to identify the localisation of BEC1054 puncta *in vivo* (Figure 47). We found that the fluorescent signals for the GFP tag of BEC1054 did not co-localise with the RFP tags of early endosomes, late endosomes, mitochondria or Golgi (the markers utilised were developed by (Geldner *et al.,* 2009)), but instead occurred in other distinct puncta throughout the cell. The GFP signal for ribosomal protein 40S 16 co-localised

with the RFP signal for mitochondria (Figure 48), and in addition it occurred in bright spots in the chloroplasts.

The expression pattern of 40S 16 was similar to that of fluorescently tagged mammalian 40S proteins, which are also expressed in a granular/punctate manner throughout the cytoplasm, in the nucleus, and brightly in the nucleolus (Rugjee *et al.,* 2013). Rugjee *et al.,* (2013) found that the ribosomal proteins tested co-migrated with ribosomal subunit fractions, monosomes and polysomes, indicating that they had indeed bound to the ribosome. To date, we have been unable to find a fluorescently tagged plant ribosomal marker for confocal microscopy in the literature. Our results indicate that 40S 16 may provide such a resource.

7.6. Conclusion

Together, stable and transient expression systems provide a powerful toolset for investigating effector activity in plants. Stable expression, although more difficult/costly to achieve, allows the activity of the effector to be studied within the host. Transient expression can be utilised to rapidly screen multiple proteins for activity or expression. Here, we utilised transient expression to show that the *B. graminis* f.sp. *hordei* effector BEC1054, when expressed in *N. benthamiana,* or stably in wheat, affected the resistance of the host plant to the biotrophs *P. tabacina* and *B. graminis* f.sp. *tritici* respectively. Transient expression was also used to investigate the effects of BEC1054 and JIP60ml on plant cell death, where it was found that our JIP60 construct induced a cell death response. Furthermore, here we have provided evidence that infection with *B. graminis* f.sp. *tritici* prevented degradation of the ribosome by ribosome inactivating proteins; and partial evidence for BEC1054's ability to reduce this effect.

7.7. Further work

AIM: To determine whether the protection of ribosomal RNA by BEC1054 varies in a basipetal manner.

OBJECTIVE: To treat wheat leaves in the same manner as for the RNA analysis conducted above (Section 7.4.7), but to extract and analyze RNA separately from sections of the leaf, i.e. the base 2 cm and the tip 2 cm.

REASONING: Wheat infection was shown to vary in a basipetal manner, with the proportion of germinated conidia that formed at least one haustorium (propH) being highest at the leaf tip, and with the difference in this proportion for plants with and without BEC1054 being highest at the leaf base (Figure 40). The difference in formation of the diagnostic small peak may therefore be higher at the leaf base than at the leaf tip.

AIM: To determine the nature of the small peak.

OBJECTIVE: To create a much greater quantity of RNA, through the pooling of multiple samples, to run this RNA on a gel and on Bioanalyzer in parallel, and (if possible) to identify the band associated with the small peak through comparison of the two sets of results, excision of the band, extraction and identification of the RNA, possibly through mass spectrometry (Matthiesen and Kirpekar, 2009).

REASONING: If the band were identified, it could potentially be recovered and identified. If this fragment were the large one formed during RNA degradation by RIPs, this would lend further evidence to our working hypothesis.

AIM: To determine whether BEC1054 affects JIP60ml's necrosis inducing effect in *Nicotiana benthamiana*.

OBJECTIVE: To utilise a series of concentrations of *Agrobacterium* expressing JIP60ml, to identify a concentration at which the necrosis effect is weakly induced (i.e. only some cells show evidence of necrosis following trypan blue staining).

REASONING: This concentration of *Agrobacterium* expressing JIP60ml could then be utilised in conjunction with a range of concentrations of *Agrobacterium* expressing BEC1054, to determine whether BEC1054 has an effect upon this necrotizing effect. This concentration of *Agrobacterium* expressing JIP60ml could also be used to see more accurately whether JIP60ml has an effect on *Peronospora tabacina* sporangia formation, independent of its causing cell death (which prevents the establishment of infection by the biotrophic pathogen).

8. General summary: aims and objectives of this thesis

Chapter 4

AIM: To identify, and validate, protein interactors for *Blumeria* Effector Candidate 1054 (BEC1054) *in vitro*, in yeast and *in planta*

OBJECTIVE: To utilise protein-protein interaction methods, including affinity chromatography, yeast-two-hybrid and bimolecular fluorescence complementation to identify protein interactors for BEC1054

Chapter 5

AIM: To investigate the transcript abundance for Candidate Secreted Effector Protein (CSEP) family 21 across a *Blumeria graminis* infection time course

OBJECTIVE 1: To develop controls for qPCR normalisation of *B. graminis* and of the host plant, barley.

OBJECTIVE 2: To utilise quantitative Real-Time PCR (qPCR) to determine the relative expression of CSEP family 21 (which includes BEC1054), a conidia-specific gene (as a negative control), and putative host-plant interactors of BEC1054.

Chapter 6

AIM: To identify whether BEC1054 interacts with the large subunit ribosomal RNA (rRNA), particularly the Sarcin-Ricin Loop (SRL) *in vitro* or in yeast

OBJECTIVE: To utilise *in vitro* methods (Differential Scanning Fluorimetry) and in yeast (Yeast-Three-Hybrid) methods to determine whether BEC1054 binds to large subunit rRNA, or to the SRL

Chapter 7

AIM 1: To determine whether the presence of BEC1054 in wheat affects its phenotype, senescence, ribosomal degradation, or host/non-host infection

OBJECTIVE 1: To phenotype transgenic wheat homozygous or azygous for BEC1054

OBJECTIVE 2: To utilise chlorophyll degradation as a quantitative indicator of senescence, and to determine whether infection with *Blumeria graminis* f.sp. *tritici,* or expression of *wbec1054* affects senescence in wheat

OBJECTIVE 3: To determine whether infection with *Blumeria graminis* f.sp. *tritici,* or expression of *wbec1054* affects ribosomal RNA

OBJECTIVE 4: To determine whether the ability of *Blumeria graminis* f.sp. *tritici,* is affected by the presence/absence of the *wbec1054* transgene

AIM 2: To determine whether the presence of BEC1054 in *Nicotiana benthamiana* has an effect on *N. benthamiana* cell death, or the ability of *Peronospora tabacina* to infect *N. benthamiana*

OBJECTIVE 1: To utilise *Agrobacterium* to express JIP60ml and BEC1054 in *N. benthamiana*, and to infect the transformed leaves with *P. tabacina*.

OBJECTIVE 2: To perform trypan blue staining on transformed leaves, to selectively colour dead tissues/cells.

AIM 3: To determine whether BEC1054 co-localises in plant cells with any of the cellular markers available in the host laboratory.

OBJECTIVE: To express *wBEC1054* with a C or N terminal GFP tag, and to determine through confocal microscopy whether it co-localises with mitochondria, early endosomes, late endosomes, or Golgi bodies.

9. General discussion

During the first three months of my PhD, an undergraduate student (Seomun Kwon) came up with the initial hypothesis in which BEC1054 outcompetes Ribosome Inactivating Proteins (RIPs) by binding to rRNA (Kwon 2011). This idea formed the starting point of my project, around which I built much of my experimental work and literature searches. In addition, during the first year of my PhD, Giulia Bonciani was finishing a PhD investigating the activities of a number of effectors, including BEC1054 (Bonciani 2014), and had demonstrated that BEC1054 interacted with rRNA in a concentration dependent manner, and had helped contribute to the creation and genotyping of the transgenic wheat lines expressing BEC1054.

Dana Gheorghe used recombinant BEC1054 with an N-terminal 6x His tag as the bait to pull-down prey proteins *in vitro* from barley uninfected or infected primary leaves, or infected barley epidermis from primary leaves. Liquid Chromatography Mass Spectrometry (LCMS) was performed to identify the proteins bound to BEC1054. Analyses of the LCMS results were performed by Laurence Bindschedler, but the analyses were not completed until near the end of my second year.

9.1. Summary of key findings

I do not believe that BEC1054 is an avirulence (Avr) protein, nor that its plant partner is an R protein, i.e. I do not think that it interacts with plant proteins in a gene-for-gene manner.

Here, I have attempted to understand and interpret the activity of one effector, BEC1054, through investigation of its protein binding activity, its expression *in planta*, its RNA binding activity *in vitro* and in yeast; and through an analysis of its expression in wheat and *N. benthamiana*, both in relation to its effect upon infection, but also through its effect upon two of the key components of senescence: chlorophyll degradation and RNA degradation.

Within this chapter, I have tried to bring together the results from the diverse range of experiments performed, both by myself, by students under my supervision, by other members of our collaboration in the host laboratory and in other universities, and salient information from the literature. More detailed discussions for each individual experiment conducted can be found at the end of the appropriate results chapters. Key points have been integrated into Summary Figure 49 and Summary Figure 50.

9.1.1. BEC1054 and proteins

Multiple, complementary methods are required for the identification and validation of effector targets (Alfano 2009). Pull-down assays, and other *in vitro* approaches, can be utilised to identify putative targets for fungal effectors. In our experiments, BEC1054 was expressed with a polyhistidine-tag, purified, and bound with an affinity resin

containing a chelator such as Ni-nitrilotriacetic acid (Ni-NTA). Proteins were identified through LCMS, (Section 4.4.1), 247 of which were found to occur solely with BEC1054. Proportions analysis determined that significantly more ribosomal proteins were found associated with BEC1054 than with the glycosidase like BEC1005, or an empty Ni-NTA agarose beads (Table 1).

Nine protein targets from the LCMS screen were tested for further analysis, through Y2H and BiFC (Sections 4.4.4, 4.4.6 and 4.4.7). It was determined that BEC1054 interacted with PR5, PR10, GST, eEF1A(1) and eEF1G. The presence of significantly more ribosomal proteins within the LCMS screen, and the presence of ribosomal proteins within the Y2H and BiFC assays, indicated that BEC1054 may bind to the host ribosome (Pennington *et al.,* 2016). In addition to finding interactors for BEC1054, we identified an interaction occurring between GST and PR10 (Figure 18), where PR10 is from a family in which several members in differing species have been shown to have RNase activity (Liu and Ekramoddoullah 2006). In addition, PR10 has been shown in birch to be post translationally S-glutathiolated (Koistinen *et al.,* 2002). It is therefore possible that these proteins were pulled down as part of a complex.

9.1.2. BEC1054's expression

Obligate, biotrophic pathogens require that the host cells remain alive, and therefore that host immunity is suppressed during infection (Duplessis *et al.,* 2011, Spanu 2012). A previous study had highlighted the importance of BEC1054, and its paralogs BEC1011, in establishing a successful infection (Pliego *et al.,* 2013). Down-regulation of the two genes through Host-Induced Gene Silencing caused a reduction in haustorial formation (Pliego *et al.,* 2013). The controls developed for the qPCR assay allowed us to

obtain better resolution for our results than had previously been seen for the family 21 CSEPs (Pliego *et al.,* 2013); with an early expression peak being identified as occurring, in epiphytic material, for all of the CSEPs investigated (Chapter 5; (Pennington *et al.,* 2015)). In this study, we have shown that four members of CSEP family 21, including BEC1054 (CSEP0064), showed peaks in expression at stages representing penetration peg formation, and ca. 24-48 hpi, which represents the stages between haustorial formation and colonies becoming visible to the naked eye (Chapter 5; (Both *et al.,* 2005)). The role of the haustorium in delivering effectors to host cells has become increasingly apparent for filamentous pathogens, including in *B. graminis* (Bindschedler *et al.,* 2009, Godfrey *et al.,* 2010, Bindschedler *et al.,* 2011), rust fungi (*Uromyces fabae*) (Hahn and Mendgen 1997, Kemen *et al.,* 2005), and *Phytophthora infestans* (Whisson *et al.,* 2007).

The expression of the interacting proteins GST, PR5, PR10, eEF1A and eEF1G was investigated following infection with *B. graminis* (Chapter 5; (Pennington *et al.,* 2015)). Expression of the two elongation factors and GST was found to increase following infection, in a manner that overlapped with the peak in abundance for BEC1054. The two PR proteins were found to be reduced in abundance, when compared with pre-infection levels, at almost all time points (Chapter 5; (Pennington *et al.,* 2015)).

9.1.3. BEC1054 and RNA

Structural work performed by our collaborators confirmed the RNase-like fold of BEC1054 (R. Jones, *pers. comm*), which had been predicted using bioinformatics methods (Bindschedler *et al.,* 2011, Pedersen *et al.,* 2012).

In this investigation I demonstrated, through Differential Scanning Fluorimetry, that BEC1054 interacted with RNA *in vitro* (Chapter 6, Section 6.4.2) and that it was destabilised, and then stabilized by a DNA ligand. Interestingly, this binding behavior was only seen for ribosome-related DNA sequences. The RNA binding domains have been found to recognize a wide range of ligands, including DNA and RNA of varying lengths, with proteins, or with multiple partners simultaneously, and to interact with DNA (reviewed by Clery *et al.,* 2008). It is therefore possible that BEC1054 may bind *in vivo* to both DNA and RNA.

Interactions performed in yeast provide a compromise between *in planta* and *in vitro* assays: they are manipulatable/scorable whilst still being easier/cheaper to perform than the former, but provide a more realistic environment than the latter (Hook *et al.,* 2005). I demonstrated through the use of a β-galactosidase assay that BEC1054, and the RIP JIP60ml, bound to RNA in yeast (Figure 35). Only the RNA-prey pairing of BEC1054 with SRL RNA, or JIP60ml with SRL RNA produced yeast colonies on media lacking histidine, but containing 3AT (where growth on 3AT indicates a positive interaction) (Figure 35). Other RIPs, such as trichosanthin, have previously been shown to bind to RNA prior to depurination (Zhao *et al.,* 2010). The interaction of BEC1054 with SRL RNA resulted in β-galactosidase activity that was lower than the negative control (Figure 35). This result was found to be repeatable, but it was not clear what these results mean. They may indicate BEC1054 had a greater effect upon translation when paired with SRL RNA; that BEC1054 somehow affected *transcription* of β-galactosidase when localised to the reporter gene; or that some other reaction had taken place.

9.1.4. BEC1054 *in planta*

Wheat plants were transformed with *bec1054* (Section 7.4.1). Genotyping was performed via PCR to confirm the presence of the gene, and qPCR confirmed that it was transcribed (Section 7.4.1). Stable expression of *bec1054* in wheat, and transient expression through agroinfiltration in *Nicotiana benthamiana* were found to increase infection with the pathogens *B. graminis* f.sp. *tritici* and *Peronospora tabacina* respectively. The effect of BEC1054 on the infection of such diverse pathogens (a fungus and an oomycete), and on such diverse hosts (a monocot and a dicot), indicated that BEC1054 affected a conserved mechanism of the plant defense response.

The effect of BEC1054 on leaf senescence was investigated, using chlorophyll as a diagnostic measure (Section 7.4.6). We induced senescence in wheat primary leaves, by floating them on MeJA (Hung *et al.,* 2006). A significant induction of senescence was visible for both azygous and homozygous plants after five days (Figure 43), through the colour change of the wheat primary leaves from green towards yellow, and through the decrease in the concentration of chlorophylls a and b in MeJA treated primary leaves (Figure 44). There was no significant difference between plants azygous or homozygous for *bec1054*, i.e. BEC1054 did not have an effect on chlorophyll degradation. Infection with *B. graminis* f.sp. *tritici* was, however, found to delay the degradation of chlorophyll; with green islands around infected sites being visible on infected leaves (Figure 43).

Jasmonates trigger or influence several processes in addition to senescence in a range of plant species including induction of RIPs such as JIP60 (Weidhase *et al.,* 1987b). Following induction of RIPs with MeJA, treatment of the resulting RNA with aniline, and

analysis of the rRNA using Bioanalyzer, we found that a diagnostic peak was formed in the electropherogram for azygous plants treated with MeJA (Section 7.5.4). This peak was greatly reduced if the plants were treated with water (instead of MeJA), or if they were infected (Figure 45 and Figure 46). The disappearance of this peak following infection corroborates the idea that the fungus may be protecting the plant ribosomes from degradation.

The accumulation of cleaved RNA, seen as the diagnostic "degradation" peak, was found to decrease in plants homozygous for *wbec1054*, when compared with azygous plants. Although this effect was not statistically significant, it was found to be repeatable, also occurring for the homozygous seed line 3.3.7. These results indicate that wBEC1054 may have an effect on rRNA degradation. A greater number of the 120 RNase-like proteins associated with haustoria, produced by *B. graminis* f.sp. *hordei* (Spanu *et al.,* 2010) may be needed for the protection of the ribosome to be significant.

9.1.5. BEC1054's effect on translation

Throughout this investigation, a number of odd occurrences were identified for BEC1054. When expressed in yeast, in both the Y2H (line MaV203) and Y3H (line YBZ1) assay, BEC1054 was observed to decrease yeast growth and transformation success (Section 4.4.3 and Figure 11). This result was quantified for MaV203, and it was found that BEC1054 affected both the maximum growth rate and the maximum cell growth (Figure 11). For both the Y2H and Y3H assays, the maximum CPRG activity rate was found to be lower for negative controls containing BEC1054, than for the negative controls provided with the kits (Figure 12). This result was not due to the poor growth of yeast, as the results were normalised against the yeast cell count. In the BiFC assay,

the fluorescence of the YFP tag attached to BEC1054 could only weakly be observed, when compared with the other YFP tagged proteins (Figure 16); with only JIP60-YFP showing a similar level of weak fluorescence. If BEC1054 was binding to ribosomal proteins, it may have affected their efficiency/activity, whilst preventing it from being lost completely. The decrease in CPRG activity seen in the yeast assays, and the decrease in fluorescence observed with BEC1054, may indicate that BEC1054 was affecting translation. This could decrease overall levels of translation in the cell, including factors required for yeast to thrive, the translation of the β-galactosidase reporter, or the translation of further BEC1054-YFP in the BiFC assay.

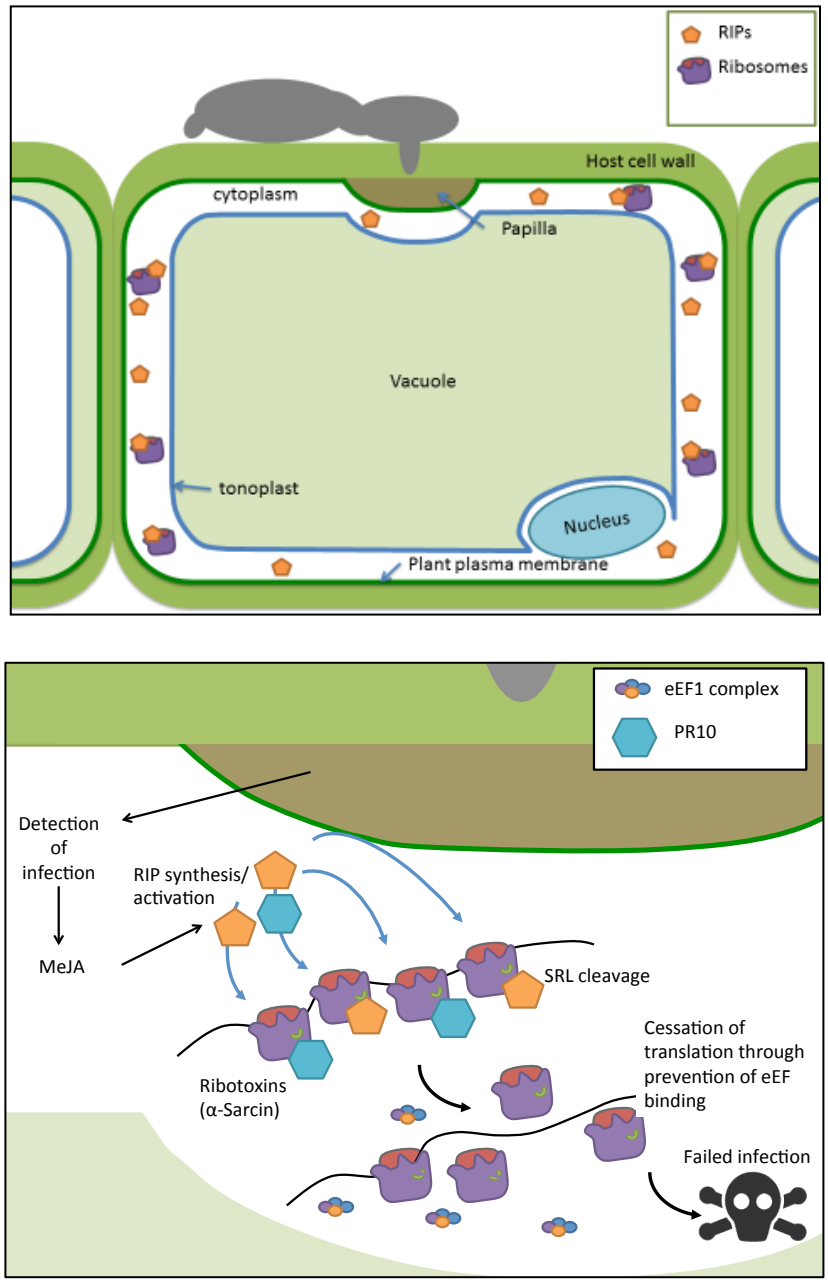


Figure 49: **An unsuccessful *Blumeria* infection, in which the invading fungal pathogen is detected by the plant, and its entry prevented. Top:** attempted cell penetration is detected, for example through the recognition of microbe associated molecular patterns by plant innate immune systems pattern recognition receptors, or through the recognition of avirulence genes by resistance proteins. Invasion is prevented through papillae formation (pictured), and/or through the hypersensitive response, which results in cell death. **Bottom:** An enlarged view of the interactions taking place between plant proteins and ribosomes. Following attempted infection, an increase in transcription of plant Ribosome Inactivating Proteins (RIPs), such as JIP60, occurs, and post-translational modifications are performed on inactive RIPs. Pathogenesis related protein 10 (PR10) is also hypothesized to be a RIP. The RIPs depurinate ribosomes at a specific base on the Sarcin-Ricin Loop (SRL). Cleavage of the SRL prevents the binding of the eukaryotic elongation factor (eEF) one complex, and leads to the cessation of protein translation, and subsequently to cell death.

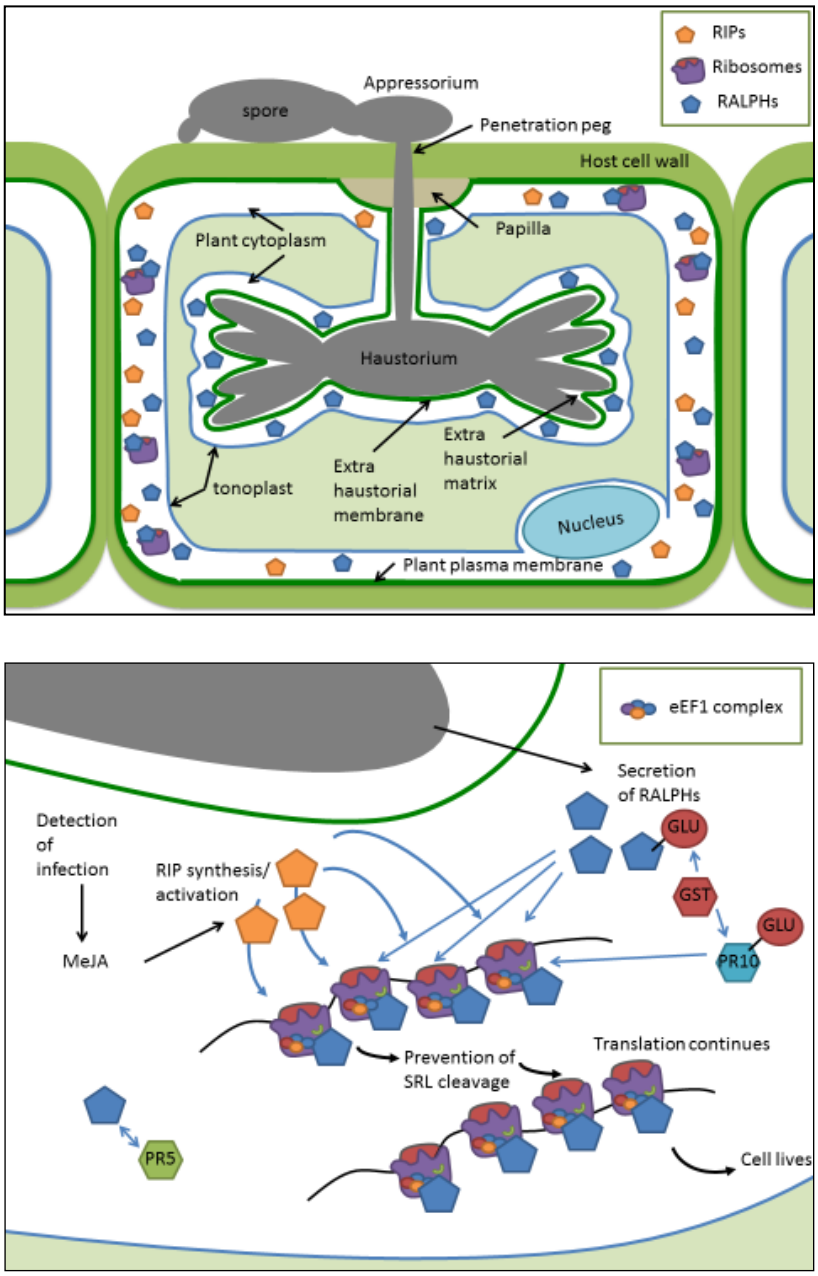


Figure 50: **A successful *Blumeria* infection, in which the pathogen successfully establishes infection, leading to disease.** The fungus penetrates the plant cell, forms a haustorium that assimilates nutrients from the host. **Top:** The haustorium is shown within the cell, and its actions in secretory warfare are indicated by its secretion of effectors such as the RALPHs (RNase-Like Effectors Expressed in Haustoria). **Bottom:** An enlarged view of the interactions taking place between plant proteins, fungal proteins and ribosomes. The possible mode of action of the RALPH *Blumeria* Effector Candidate 1054 (BEC1054), including all known protein interactions to date. The fungal effector BEC1054 is hypothesised to bind to ribosomes, preventing the cleavage of the SRL by RIPs. This is supported by experimental evidence that BEC1054 binds to eEF1A and eEF1G; and that it binds to nucleotides including ribosomal RNA. The plant glutathione-S-transferase (GST) interacts with both BEC1054 and pathogenesis related protein 10 (PR10), with it having been shown to S-glutathiolate the RNase PR10 in the literature. In addition, PR5 and BEC1054 were found to interact, but the role of PR5 in this model requires further investigation. Experiments expressing BEC1054 in transgenic wheat have indicated that it may protect ribosomal RNA.

9.2. Wider context

9.2.1. Ribosome inactivating proteins in medicine

Within this investigation, I have studied the activity of one effector, BEC1054. In our working hypothesis, for which we have been gathering increasing amounts of evidence, BEC1054 appears to be targeting/interacting with a key part of plant immunity, preventing the degradation of ribosomes, and thus helping to prevent the death of the host cell (Figure 50). Our chosen effector, BEC1054, is one of ca. 120 RNase-like protein effectors secreted by *B. graminis* f.sp. *hordei* (Pedersen *et al.,* 2012). The strong up-regulation of transcription of this effector, relative to conidia, and of related effectors (for example the other members of family 21), indicates its importance within the establishment of a compatible interaction (Chapter 5; (Pennington *et al.,* 2015)). The sheer number of these RNase-like effectors further highlights their key role within infection; as does the effect of BEC1054 on increasing infection in wheat, and BEC1054-GFP on increasing infection in *N. benthamiana* (Sections 7.4.3 and 7.4.4). The production of such large quantities of RALPHs, indicated that they could be playing a role in directly outcompeting RIPs.

In addition to plants, fungi have also been shown to produce ribosome inactivating proteins, for example the *Aspergillus* RIPs restrictocin, alpha-sarcin and mitogillin (Conde *et al.,* 1978), as have bacteria, for example shiga toxin from *Shigella dysenteriae* (Reisbig *et al.,* 1981) and the shigatoxigenic group of *Escherichia coli* (Mainil 1999). The widespread use of RIPs in plant defense makes it likely that other pathogens will also target or subvert this part of plant immunity. The fungus *Leptosphaeria maculans,* the causal agent of stem canker of oilseed rape (*Brassica napus*), secretes the effector

AvrLm4–7. Structural analysis of this protein found that it was topologically similar to RNA-recognition motif proteins, also possessing two α-helices and four β-sheets. This effector also possesses positively charged surface patches, indicating that it could bind to nucleotide ligands, although the authors could not detect DNA binding activity (Blondeau *et al.,* 2015). A greater understanding of BEC1054's activity may therefore help to suggest modes of action for other effectors that possess RNA-recognition motifs.

Expression of ribosome inactivating proteins in plants has been shown to cause resistance to a number of fungal and viral pathogens (Lodge *et al.,* 1993; Taylor *et al.,* 1994; Lam *et al.,* 1996; Moon *et al.,* 1997). Their expression in animals has suggested that they may be useful medicinal tools in humans (Kreitman 2006, Pastan *et al.,* 2007). Several RIPs have been used in the construction of immunotoxins and conjugates which target cancer cells (Flavell 1998, Flavell *et al.,* 2001, Rosenblum 2004). Multiple RIPs have undergone clinical trials, for example bouganin (Kowalski *et al.,* 2008) and gelonin (Borthakur *et al.,* 2013). They have also been shown to inhibit the replication of human immunodeficiency virus in clinical trials, but caused heavy side effects (Byers *et al.,* 1994). The therapeutic window of these toxins is relatively narrow (Shapira *et al.,* 2011), and they are associated with potentially life-threatening toxicities. Proteins which modify or moderate their activities may have far-reaching applications. If BEC1054 were to reduce, but not completely inhibit, protein translation then it too could potentially be used for similar treatments.

9.3. Overall conclusions

The fungal effector BEC1054 is an RNase-like protein associated with significantly more ribosomal proteins than the negative controls. I found that five proteins interacted with BEC1054 *in vitro*, in yeast and *in planta*: PR5, PR10, GST, eEF1A(1) and eEF1G, the latter two of which are associated with ribosomes. In addition, PR10 and GST were found to interact with each other in yeast and *in planta.*

Following the development and testing of qPCR control genes, I assayed the family 21 CSEPs to determine their transcript abundance across a *B. graminis* infection time course. All tested members of CSEP family 21 were identified in epidermal and epiphytic material. In epiphytic material, they showed an early peak in abundance, which had not previously been identified in the literature. In addition, all four CSEPs showed a second peak in abundance at 24-48h. In epidermal material, the CSEPs showed more diverse patterns of expression, with transcript abundance peaks occurring at ca. 24 for *csep0064 (bec1054)* and *0066,* which then decreased sharply to near the initial level by 48 hpi. In contrast, *csep0065* and *csep0264 (bec1011)* increased in transcript abundance more gradually, with a maximum at 48 hpi, followed by a gradual decrease in abundance by 120 hpi.

The RNase-like effector BEC1054 interacted with oligo(poly)nucleotides both in yeast, and *in vitro*. Both sets of reactions were found to be concentration dependent. My β-galactosidase results from yeast indicated that both BEC1054 and JIP60ml bound RNA; and the selective media assay indicated that this interaction may be specific for the region of the ribosome containing the ribosomal SRL.

Expression of BEC1054 in wheat or *N. benthamiana* affected the resistance of the host plants to the biotrophic pathogens *B. graminis* f.sp. *tritici* and *P. tabacina*. We found that the presence of *wbec1054* did not appear to affect chlorophyll degradation during induced senescence. We also provided direct evidence that infection with *B. graminis* f.sp. *tritici* prevented degradation of the ribosome by ribosome inactivating proteins; and partial evidence for BEC1054's ability to reduce this effect.

10. Acknowledgements

10.1. Laboratory members

I would like to thank:

- My supervisor, Pietro Spanu, for help, support, supervision and guidance
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- 36th New Phytologist Symposium Travel Grant (2015)

    - This funding helped to cover the majority of my travel and subsistence costs for my attendance at the 36th New Phytologist Symposium: Cell biology at the plant–microbe interface
- The British Society for Plant Pathology Travel Fund (2015)
- Fungal Conference Financial Aid (2015)
    - These two grants covered the majority of my travel and subsistence costs for my attendance at the 28th Fungal Genetics Conference, California, USA (17/05/2015 – 22/03/2015)
- New Phytologist Next Generation Scientists Symposium travel grant (2014)
    - This funding covered my costs for this symposium
- The John Stoney & Florence Stoney scholarship for academic excellence during the first two years of a PhD, administered by the British Federation of Women Graduates (BFWG) (2014-2015)
    - This award helped to cover the writing up phase of my PhD
- Deutscher Akademischer Austauschdienst grant (DAAD - German Academic Exchange Service) (2014)
- European Cooperation in Science and Technology Short-Term Scientific Mission grant (2014)
    - The DAAD and COST-STSM grants covered both living costs and laboratory expenses and materials during a four-month placement in Aachen, during which I completed the BiFC work
- PrimerDesign Gold Sponsorship (2014-2015)

    - This sponsorship included a seminar series for the Host University, reagents, housekeeping gene detection primers, and training in quantitative real-time PCR.
- Gregory Fund overseas conference funding
    - This funding allowed me to attend the XVI International Congress on Molecular Plant-Microbe Interactions in Rhodes, Greece (2014)
- BBSRC Scholarship for Imperial College London's 1+3 DTG (MRes/PhD) in Biology (2011-2015)
    - This funding covered both my Masters degree and three years of my PhD.

11. Additional outcomes of this PhD:

11.1. Journal articles arising from this investigation:

- Pennington, H. G, Gheorghe, D. M., Thieron, H., Luong, P., Chandler, T., Jones, R., Damerum, A., Morgan, S., Przydacz, M., Bonciani, G., Bindschedler, L. V., Cota-Segura, E., Bozkurt, T., Panstruga, R. and Spanu, P. D. Understanding the activity of BEC1054, a *Blumeria* RNase like effector. [In preparation]
- Pennington, H. G., Gheorghe, D. M., Damerum, A., Pliego, C., Spanu, P. D., Cramer, R., and Bindschedler, L. V. (2016). Interactions between the powdery mildew effector BEC1054 and barley proteins identify candidate host targets. Available online ahead of print: http://www.ncbi.nlm.nih.gov/pubmed/26813582
- Pennington, H. G., Linhan, L. & Spanu, P. D. (2015). Identification and selection of normalisation controls for quantitative transcript analysis in *Blumeria graminis*. *Molecular Plant Pathology*. 1(1),1-9

11.2. Conference presentations

- Pennington, H. G. (2015) *Barley proteins interacting with Blumeria RNase-like effector BEC1054.* [Presentation] Molecular Biology of Plant Pathogens, University of the West of England, 9th April.
- Pennington, H. G. (2014) *The proteins that interact with the Blumeria effector BEC1054.* [Presentation] 4th International Powdery Mildew Workshop, Imperial College London, 15th September.
- Pennington, H. G. (2014) *RNase like effectors and their putative interactors.* [Presentation] Molecular Biology of Plant Pathogens, Reading University, 26th August.

- Pennington, H. G. (2013) *RALPH effectors, RIPs and their mode of action - commonalities or coincidences?* [Presentation] 3rd International Powdery Mildew Workshop, University of Copenhagen, 30th August.

11.3. Scientific posters

- Pennington, H. G, Gheorghe, D. M., Thieron, H., Luong, P., Chandler, T., Jones, R., Damerum, A., Morgan, S., Przydacz, M., Bindschedler, L. V., Cota-Segura, E., Bozkurt, T., Panstruga, R. and Spanu, P. D. (2015) *Identifying the protein and RNA interactors of BEC1054* [Poster] 36th New Phytologist Symposium: Cell biology at the plant–microbe interface, 29th November.
- Pennington, H. G., Jones, R., Gheorghe, D. M., Cota Segura, E., Cramer, R., Bindschedler, L.V. and Spanu, P. D. (2015*) Identifying interactors of RNase like effector BEC1054* [Poster] 28th Fungal Genetics Conference, Pacific Grove, California, 20th March.
- Pennington, H. G., Jones, R., Cota Segura, E., Bonciani, G. and Spanu, P. D (2014) *Identifying interactors of RNase like effectors.* [Poster] New Phytologist next Generation Scientists Symposium, John Innes Centre, Norwich, 29th July.
- Pennington, H. G., Gheorghe, D. M., Smith, C. J., Cramer, R., Bindschedler, L. V., Luong, P., Wallington, E., Jones, R., Cota Segura, E. and Spanu, P. D. (2014). *RNase like effectors and their putative interactors* [Poster] XVI International Congress on Molecular Plant-Microbe Interactions, Agricultural University of Athens, 8th July.
- Pennington, H. G., Gheorghe, D. M., Smith, C. J., Spanu, P. D., Cramer, R., Bindschedler, L. V. (2014*). Investigating the interactors of the Blumeria Effector BEC1054.* [Poster] Graduate School Research Symposium, Department of Life Sciences, Imperial College London, 8th April.

12. Supplementary information

Supplementary Table 13: **Primer sequences used for initial gene amplification from barley or *Blumeria*.** The term "CSEP" stands for Candidate Secreted Effector Protein; and BEC for *Blumeria* Effector Candidate (Pedersen *et al.,* 2012). Primers marked with the symbol "*" were from (Pliego *et al.,* 2013) and ones marked with ** were from (Damerum 2013).

Protein	UniProt reference	Original GenBank Accession retrieved	GenBank accession for genes used in this study	FWD primer (5'-3')	REV primer (5'-3')
BEC1005	N1JK84			TTGAGCCAGGGATTTAACGCACAAGTAC *	TAATGGTTCAAAAGAATTGCAGG *
BEC1054 (CSEP0064)	N1JJ94			GCAGCTTATTGGGATTGTG *	CTAGCCCTCAACAAAAGTAC *
40S ribosomal protein S16	Q0IQF7	AK371280	KP293844	GCTGCTGTCCTCACCCGCCC	ACGGTAAGACTTCTGGAACCTCGC
Elongation factor 1 gamma	Q5Z627	AK248884	KP293852	GCGCTCGTTTTGCATTCGGGCAGC**	CTTGAAGCACTTGGCGTCCAGC**
Elongation factor 1a (colony 1)	Q9LN13	Z50789	KP293845	GGTAAGGAGAAGACTCACATC	TTTCTTCTTGATGGCAGCCTTGGTCACCTTGGC
Elongation factor 1a (colony 3)	Q9LN13	Z50789	KP293846	GGTAAGGAGAAGACTCACATC	TTTCTTCTTGATGGCAGCCTTGGTCACCTTGGC
Glutathione-S-Transferase	Q8H8U5	AK355502	KP293847	AGTTCGCTCGCTTTCC	CTCAATCCCAAGCCG
Malate dehydrogenase	Q6YWL3	AK364298	KP293848	GCATCATCATCTGCTACCATCAG	AACAGACGCCGCGGCTCCCTGCTGTTTG
Nucleoside Diphosphate Kinase	Q9LKM0	AK356457	KP293849	GCGGAGCAGACCTTCATCATGATC	AGCCTCATAGATCCAGTTGTGCTGGCTG
Pathogenesis-related protein 10	Q84QC7	AY220734	KP293851	GTCGCCGGCTGTGTCATCACCGAGCAGTG**	GACGTACTCGGCAGGGTGGGCGACCAGGTA**
Pathogenesis-related protein 5	O23997	AJ001268	KP293850	GCGTCCTCTCGTGTTGTC	TTCCTTATTGACCCAAG

Supplementary Table 14: **Primers used to create entry vector plasmids containing *Blumeria graminis* or barley genes.** In primers with the sequence "GGGGACAAGTTTGTACAAAAAAGCAGGCTTC, sections labelled in bold are the sequence for the start and end of genes. The sections not labelled in bold correspond to the start or end of the genes being amplified.

	Genes for N-terminal fusion (with a START codon and no STOP codon)		**Genes for C-terminal fusion (with no START codon, but with a STOP codon)**	
Gene name	**FWD START primer (5'-3')**	**REV primer (5'-3')**	**FWD primer (no START) (5'-3')**	**REV STOP primer (5'-3')**
Glutathione-S-transferase	ATGAGTTCGCTCGCTTTCC	CTCAATCCCAAGCCG	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**AGTTCGCTCGCTTTCCCCTG**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCACTCAATCCCAAGCCGCTTCTTTG**
Malate dehydrogenase	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGCATCATCATCTGCTACCATCAG**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**AACAGACGCCGCGGCTCC**	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**GCATCATCATCTGCTACCATCAG**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCAAACAGACGCCGCGGCTCC**
Pathogenesis-related protein 5	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGTCGCCGGCTGTGTCATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**GACGTACTCGGCAGGGTG**	GCGTCCTCTCGTGTTGTC	TCATTCCTTATTGACCCAAG
Pathogenesis-related protein 10	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGCGGAGCAGACCTTCATCATGATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**AGCCTCATAGATCCAGTTGTGCTG**	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**GTCGCCGGCTGTGTCATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCAGACGTACTCGGCAGGGTG**
Elongation factor 1 gamma	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGCGCTCGTTTTGCATTCGG**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**CTTGAAGCACTTGGCGTCC**	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**GCGCTCGTTTTGCATTCGG**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCACTTGAAGCACTTGGCGTCC**
Elongation factor 1a (colony 1)	ATGGGTAAGGAGAAGACTCACATC	TTTCTTCTTGATGGCAGCCTTGGTCACCTTGGC	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**GGTAAGGAGAAGACTCACATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCATTTCTTCTTGATGGCAGCCTTG**
Elongation factor 1a (colony 3)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGGTAAGGAGAAGACTCACATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TTTCTTCTTGATGGCAGCCTTG**	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**GGTAAGGAGAAGACTCACATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCATTTCTTCTTGATGGCAGCCTTG**
40S ribosomal protein S16	ATGGCTGCTGTCCTCACCCGCCC	ACGGTAAGACTTCTGGAACCTCGC	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**GCTGCTGTCCTCACCCGC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCAACGGTAAGACTTCTGGAACCTC**
Nucleoside diphosphate kinase	ATGGCGGAGCAGACCTTCATCATGATC	AGCCTCATAGATCCAGTTGTGCTGGCTG	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**GCGGAGCAGACCTTCATCATGATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCAAGCCTCATAGATCCAGTTGTGCTG**
BEC1054 (CSEP0064)	GCAGCTTATTGGGATTGTG	CTAGCCCTCAACAAAAGTAC	GCAGCTTATTGGGATTGTG	TCACTAGCCCTCAACAAAAGTAC
JIP60ml N-terminal domain	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGCTTTAGACAAAGTTGCTCCCATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**GTCCGCCATGTTGCTTCGG**	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**GCTTTAGACAAAGTTGCTCCCATC**	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCAGTCCGCCATGTTGCTTCGG**

Supplementary Table 15: **Primers used for amplification and modification of Jasmonate Induced Protein 60.** Primers labelled "*" were designed by Rhian Jones

	FWD primer (5'-3')	**REV primer (5'-3')**
JIP60 initial amplification primers	GCTTTAGACAAAGTTGCTCCCATCG	CCAAGGGTACTCCGACCATAAGATTG
N-terminal linker mutagenesis primers	GACCCGGTAAAGCAAGCAGTGGCG*	TAGCATAGCCTTGTTCCAGAGCGC*
Amplification of N-terminal domain	GCTTTAGACAAAGTTGCTCCCATCG	GTCCGCCATGTTGCTTCGGATGACT

Supplementary table 16: **Plasmids used to sequence YFP expression vectors**.

Plasmid name	**Sequence (5'-3')**
pESPYCE_FWD	CTGAGCAAAGACCCCAACGAG
pESPYNE_FWD	CTTCAAGGAGGACGGCAACATC
pUCSPYNE_FWD	TTGATGTGATATCTCCACTGAC

Supplementary Table 17: **Primers used for amplification of *Blumeria graminis* reference genes.** Table reproduced with permission from (Pennington *et al.,* 2015).

Accession	Gene	Abbreviation	FWD Sequence (5'-3')	REV sequence (5'-3')	Amplicon length	Efficiency
CCU82444	α-tubulin	*tuba*	GGTCACTACACTGTTGGTAAAGA	CCGAAGGAATGGAATACAAGAAAG	110	1.23
CAA35709	β-tubulin	*tubb*	GGAAACGCACCTACACTATACC	GCCGTGATGGAATTTAACTAACAA	94	1.19
CCU76638	actin	*actb*	CCCAATTTACGAAGGTTTCTCTC	TCAGCGGTTGTGGAAAAAGT	126	1.21
CCU80715	glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	GGAGCCGAGTACATAGTAGAGT	GGAGGGTGCCGAAATGATAAC	105	1.23
CCU82905	Histone 3	*h3*	GGAAACAACTCGCTTCTAAGG	GATTTTTGGTATCTTCTGATTTCAC	121	1.22
CCU80195	monoglyceride lipase	*mgll*	GCCCTACCAGCCGAAAAC	ATGCCTGATAATCCCTCTAACG	104	1.24

Supplementary Table 18: **Primers used for amplification of barley reference genes.** Table reproduced with permission from (Pennington *et al.,* 2015).

Accession	Gene	Abbreviation	FWD Sequence (5'-3')	REV sequence (5'-3')	Amplicon length	Efficiency
U40042	a-tubulin	*tuba*	CAACATACACCAACCTCAACAG	AACTCATTCACATCAACATTCAGA	100	1.23
AY145451	actin	*actb*	CTGTGCCCATTTATGAAGGATAC	GCTGAGGTTGTGAAGGAGTAA	127	1.22
X04133	ubiquitin	*ubq*	TTTGGTATTATTGAGGGTCTGATGA	TGCTGCTGGGGATGATGTT	130	1.25
AJ344078	adenosine triphosphatase	*h+-atpase*	TCTCAGGGTTCACAGGTCTT	CCGAACAGGTCCGTAATGG	93	1.24
X60343	glyceraldehyde-phosphate dehydrogenase	*gapdh*	CTGATTGAGAAGGCTGATGGAT	AGAGCAGGAGCGTCATTGA	128	1.25

Suplplementary Table 19: **Primers used for amplification of *Blumeria* Candidate Secreted Effector Protein (CSEP) family 21.** Table reproduced with permission from (Pennington *et al.,* 2015). Primers were from (Li 2014).

Accession	CSEP number	BEC number (where applicable)	FWD Sequence (5'-3')	REV sequence (5'-3')	Amplicon length	Efficiency
CCU83233	*csep0064*		GAAACGTTCGAGCTGCAGTA	TACAGCTCCTCCTTGCCAGT	149	1.24
CCU82938	*csep0065*	*bec1054*	GCTGCAGGGTTTTATCATGG	TCCAGACCAGCTTTCATTGG	114	1.23
CCU82934	*csep0066*		TGCCTTTAGTTGCTCACCAG	TTTCCCGGAAGCTGTTATTG	115	1.23
CCU83219	*csep0264*	*bec1011*	CGAGATGCAGCAGTATTTGC	TGCTCTCCTTGCCAGTTTTC	149	1.23
CCU76783	Conidia specific gene		GGGTTCATCGGGTCTTTTCT	AGTTGGGCCAAGGGTAAAGT	149	1.22

Supplementary Table 20: **Primers used for amplification of barley genes.** Table reproduced with permission from (Pennington *et al.,* 2015).

Accession	Gene	FWD Sequence (5'-3')	REV sequence (5'-3')	Amplicon length	Efficiency
KP293847	*gst*	TGCCAGGAATTACAAGGGTTT	GGTTATTATGCTCCAGTGAAGG	128	1.22
KP293850	*pr5*	CGCCGACCAACTACTCAATG	GGCAGGGCAGGTGAAGG	95	1.25
KP293851	*pr10*	GCCAGGGTGTTCAAGACAG	CGTCCAGCCTCTCGTACTC	142	1.22
KP293852	*eef1g*	GGCTGCTCCTGCTAAACC	AGGGGATTCTTGGGCTTAGG	117	1.23
KP293845	*eef1a*	GACAGGCGATCAGGTAAGGA	TGGGCTTGGTGGGAATCAT	91	1.23

Supplementary Table 21: **RNA synthesis primers.** "SRL" stands for the sarcin-ricin loop and "rRNA" for ribosomal RNA.

Name	**Primer (5'-3')**
Rat SRL +T7	ACCTGCGGTTCCTCTCGTACTGAGCAGGTCCCTATAGTGAGTCGTATTAAATT
Barley SRL 3014-3044 +T7	AATCAACGGTTCCTCTCGTACTAGGTTGAATCCCTATAGTGAGTCGTATTAAATT
Barley 76-114 +T7	GCTGGGCTGTTCCCGGTTCGCTCGCCGTTACTAGGGGACCCTATAGTGAGTCGTATTAAATT
Truly random primer +T7	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCCTATAGTGAGTCGTATTAAATT
T7 promoter	AATTTAATACGACTCACTATAGG

Supplementary Table 22: **primers used to amplify 28S ribosomal sections.** Numbers refer to the nucleotide number within the 28S ribosomal model. Sections in bold represent the start or end of the ribosomal section being amplified.

Bases	**FWD 5'-3'**	**REV 5'-3'**	**Reasons/features**	**Length**	**GC content (%)**
2989-3139	ACTAGTGGATCCCC C**ACTGATGACAGTGTCGCG**	CGACCTGCAGGCATG**GCTTGGATTCTGACTTAGAGG**	Contains SRL	150	49.3
13-163	ACTAGTGGATCCCC C**AGGCGGGACTACCCGCTG**	CGACCTGCAGGCATG**GACGCGTCTCCAGACTACAATTCG**	Negative control	150	54

Supplementary Table 23: **primers used to amplify *Blumeria* Effector Candidate 1054 (BEC1054), BEC1011 or wheat tubulin.** The abbreviation "*tubbw*" stands for wheat β-tubulin (Bonciani 2014).

Bases	**FWD 5'-3'**	**REV 5'-3'**
wbec1054	ACCTTCATCATCGGCTCCACCTTCT	AACCCTCGACGAAGGTGCACTTGT
wbec1011	CCGTGTTCGCGTTCAGCAAGGAAAA	CACGTAAAAATTCACGTGTTCGCCC
tubbw	ACCTGAACCACCTCATCTCG	CAAACCCGACCATGAAGAAG

Supplementary Table 24: **primers used for amplification and sequencing of *Agrobacterium* expression plasmids**

Name	**Sequence 5'-3'**	**Length (nt)**
pK7FWG2 FWD	CTCTATATAAGGAAGTTCATTTCATTTGGAGAGG	34
pK7FWG2 REV	AGGTGGCATCGCCCTCGCC	19
pK7WGF2 FWD	GCTGCCCGACAACCACTACCTG	22
pK7WGF2 REV	AGCGAAACCCTATAAGAACCCTAATTCCC	29

Supplementary Table 25**. Proteins identified interacting solely with *Blumeria* Effector Candidate 1054 (BEC1054) from the U36 Harvest database.** Full-length CDS barley sequences were obtained from UniProt using the UniRef90 identifiers. Putative interacting proteins for BEC1054, which underwent further investigation, are highlighted in grey, where dark grey indicates that they were identified with some of the negative controls (either "B05" (BEC1005) or in the absence of a bait protein), and light grey indicates that no interaction was observed with the negative controls. The letters "A", "B" and "C" represent the experimental conditions used, with "A" being 48h infected leaf epidermis magnetic NTA agarose beads, "B" being non-infected leaves and 1 ml NTA chromatography columns, and "C" being 7 day infected leaves with magnetic NTA agarose beads. The values present within the "B54" (BEC1054) columns indicate the number of times that a protein, with the same accession number, was identified in biological/technical replicates with BEC1054, but not with the negative controls (either "B05" (BEC1005) or in the absence of a bait protein). The values within the "Tot" (Total) columns indicate the number of times that a protein, with the same accession number was identified in all pulldowns of the same biological replicate. Where a larger value can be observed in the "Total" column than in the "B54" column, this indicates that the protein was identified with the negative controls.

HarvEST	UniRef	Protein description	B54 A	Tot A	BE54 B	Tot B	B54 C	Tot C	B54 ABC	Tot ABC
U36_14076	Q0IQF7	40S ribosomal protein S16	2	4			1	5	3	9
U36_1609	Q7XVZ0	40S Ribosomal protein S27	3	3					3	3
U36_2214	Q7XVZ0	40S Ribosomal protein S27	3	3					3	3
U36_32768	Q7XVZ0	40S Ribosomal protein S27	3	3					3	3
U36_15834	Q7XVZ0	40S Ribosomal protein S27	3	3					3	3
U36_49995	Q5I7L4	60S ribosomal protein L6			2	2			2	2
U36_21893	B6TA29	actin-related protein 2/3 (ARP2/3) complex 34 kDa subunit	3	3					3	3
U36_21421	Q2QXT1	acyl-CoA synthetase protein, putative	3	3					3	3
U36_41668	A6SI57	Alpha-ketoglutarate dehydrogenase E1 component	3	3					3	3
U36_31524	Q8LI32	amine oxidase family protein,					2	2	2	2
U36_50229	A6YH86	Ascorbate peroxidase	4	4					4	4
U36_21564	Q6EUS6	Aspartate aminotransferase	3	3					3	3
U36_545	Q2L9B8	ATP synthase subunit E, vacuolar					2	2	2	2
U36_19101	B8A7C1	ATP-dependent Clp protease			1	1	1	1	2	2
U36_50223	B8A7C1	ATP-dependent Clp protease			1	1	1	1	2	2
U36_51602	Q0JBL5	ATP-dependent Clp protease proteolytic subunit					2	2	2	2
U36_24974	C5YUG7	ATP-dependent Clp protease, Sb09g026620,			3	3			3	3
U36_15712	P04784	Chlorophyll a-b binding protein, chloroplastic					2	2	2	2
U36_49889	B4G249	dehydrogenase protein , putative, expressed	1	1			1	1	2	2

U36_45390	B4G249	dehydrogenase protein , putative, expressed	1	1			1	1	2	2
U36_18413	Q9LN13	Elongation factor 1-alpha , putative elongation factor Tu	2	4	1	5			3	9
U36_36305	Q9ZSW2	Elongation factor 1-alpha, putative elongation factor Tu					2	2	2	2
U36_17248	Q5Z627	Elongation factor 1-gamma 3	3	3					3	3
U36_35625	Q5Z627	Elongation factor 1-gamma 3	3	3					3	3
U36_41871	A7E7R3	Elongation factor 2	2	5					2	5
U36_69641	Q2H0S4	Elongation factor 2	2	5					2	5
U36_31227	C5YX02	eukaryotic translation inititation, Putative	2	2					2	2
U36_21911	P23225	Ferredoxin-dependent glutamate synthase, chloroplastic					2	2	2	2
U36_50246	Q10SI2	flavonol synthase/flavanone 3-hydroxylase, putative	1	1			1	1	2	2
U36_29611	Q10SI2	flavonol synthase/flavanone 3-hydroxylase, putative	1	1			1	1	2	2
U36_50247	Q10SI2	flavonol synthase/flavanone 3-hydroxylase, putative	1	1			1	1	2	2
U36_7525	Q655W2	glyceraldehyde-3-phosphate dehydrogenase, putative					2	2	2	2
U36_5312	Q8H8E0	GST, glutathione S-transferase					2	2	2	2
U36_49726	Q8H8U5	GST, IN2-1 homolog B					2	2	2	2
U36_18517	Q8H8U5	GST, IN2-1 homolog B					2	2	2	2
U36_20285	B6TLT1	HEAT repeat family protein, putative, expressed					2	2	2	2
U36_27828	Q6F2Y7	Heat shock protein 101			3	3			3	3
U36_57829	Q6F2Y7	Heat shock protein 101			3	3			3	3
U36_26803	P02276	Histone H2A.2.1	1	1	1	1			2	2
U36_23007	Q2QYB7	Homoserine dehydrogenase bifunctional aspartokinase/homoserine dehydrogenase	3	3					3	3
U36_22480	Q10CE4	hydroxyacid oxidase 1, Os03g0786100			1	1	1	1	2	2
U36_18976	A1C0L3	lipase, putative, GDSL-like lipase/acylhydrolase, UCW116	3	3					3	3
U36_35765	A1C0L3	lipase, putative, GDSL-like lipase/acylhydrolase, UCW116	3	3					3	3
U36_2649	Q7XDC8	Malate dehydrogenase, cytoplasmic, lactate/malate dehydrogenase, putative, exp	2	2					2	2
U36_20088	Q6YWL3	Malate dehydrogenase, lactate/malate dehydrogenase	4	6	1	3	2	2	7	11
U36_20089	Q6YWL3	Malate dehydrogenase, lactate/malate dehydrogenase	4	6	2	2	2	2	8	10
U36_20090	Q6YWL3	Malate dehydrogenase, lactate/malate dehydrogenase	4	6	1	3	2	2	7	11
U36_51035	Q6YWL3	Malate dehydrogenase, lactate/malate dehydrogenase	4	6	1	3	2	2	7	11
U36_51037	Q6YWL3	Malate dehydrogenase, lactate/malate dehydrogenase	4	6	1	3	2	2	7	11
U36_51036	Q6YWL3	Malate dehydrogenase, lactate/malate dehydrogenase	3	5	1	3	2	2	6	10
U36_10746	Q6YWL3	Malate dehydrogenase, lactate/malate dehydrogenase			1	1	2	2	3	3
U36_20290	Q93W16	miro protein, putative, expressed, Os01g0338000	1	1			2	2	3	3
U36_19	Q9LK	Nucleoside diphosphate kinase	2	10			1	1	3	11

006	M0									
U36_517	Q5JLV2	Os01g0894700 protein			1	1	1	1	2	2
U36_18259	Q10PG5	Os03g0235100 protein	3	3					3	3
U36_2386	O49866	Peroxidase	2	2			1	1	3	3
U36_21445	Q5I3E8	Peroxidase 10 (Fragment)	2	2			2	2	4	4
U36_19	O23997	PR5 Basic pathogenesis-related protein, thaumatin					2	2	2	2
U36_23528	Q5MBN2	PR5, Thaumatin-like protein TLP5	2	2			1	1	3	3
U36_9171	Q5MBN2	PR5, Thaumatin-like protein TLP5	2	2			1	1	3	3
U36_20861	Q84QC7	PR10, Pathogenesis-related protein 10 Bet v I family					1	4	1	4
U36_35919	Q84QC7	PR10, Pathogenesis-related protein 10 Bet v I family					1	3	1	3
U36_6026	Q84QC7	PR10, Pathogenesis-related protein 10 Bet v I family					1	4	1	4
U36_7717	Q84QC7	PR10, Pathogenesis-related protein 10 Bet v I family					1	4	1	4
U36_19133	Q0DZE5	protein kinase, pfkB family, putative, expressed	4	4					4	4
U36_30280	C5WYG0	Putative uncharacterized protein Sb01g019330	3	3					3	3
U36_6421	A3BW84	Putative uncharacterized protein, Os09g04310.1	1	1			1	1	2	2
U36_20346	Q6AV40	RAN binding protein, exportin-7-A, putative, expr					2	2	2	2
U36_21205	Q69U05	reductase, Os06g0666600 protein,	4	4					4	4
U36_11913	P26667	Ribulose bisphosphate carboxylase small chain PW9			2	2			2	2
U36_4226	Q10BX7	RNA recognition motif containing protein, Os03g0801800					2	2	2	2
U36_50575	Q7Y1F0	Serine hydroxymethyltransferase, mitochondrial?			2	2			2	2
U36_10414	D1IVV3	Shotgun seq. line PN40024, scaffold_44 Os05g36290.1	1	1	1	1			2	2
U36_18526	D1IVV3	Shotgun seq. line PN40024, scaffold_44, Os01g64630.4			2	2			2	2
U36_19915	B8B5H6	spermidine synthase putative	3	3					3	3
U36_47068	B8B5H6	spermidine synthase putative	3	3					3	3
U36_19660	Q6EZE7	Sucrose-phosphate synthase 2 (Fragment)	4	4					4	4
U36_19661	Q6EZE7	Sucrose-phosphate synthase 2 (Fragment)	4	4					4	4
U36_50671	Q6EZE7	Sucrose-phosphate synthase 2 (Fragment)	4	4					4	4
U36_35297	Q2R3T7	sulfotransferase domain containing protein	3	3					3	3
U36_15752	B4GMN3	tubulin/FtsZ domain containing protein, GL12416					2	2	2	2
U36_23924	Q94DY4	vacuolar protein sorting-associated protein 28	3	3					3	3
U36_14924	Q9AYE4	WD repeat-containing protein, Os03g0681700	4	4					4	4
U36_11862	Q6PWL8	WIN3, Wound-induced protein, vacuolar defense prot PR4e	1	1			2	2	3	3
U36_50018	Q6PWL8	WIN3, Wound-induced protein, vacuolar defense prot PR4e	2	2			2	2	4	4

Supplementary Table 26: **List of barley proteins grouped according to their UniRef90 descriptor as putative *Blumeria* Effector Candidate 1054 (BEC1054) interactors following identification by pull-down using the U36_Harvest database**. The letters "A", "B" and "C" indicate the experimental conditions, with "A" being magnetic NTA agarose beads and 48h infected leaf epidermis; "B" being 1 ml NTA chromatography columns and non-infected leaves; and "C" being magnetic NTA agarose beads and seven day Infected leaves. The numbers in the columns A, B and C indicate the biological replicates with which the protein was identified solely with BEC1054 (as opposed to with the negative controls). Where there are multiple numbers within a column, this indicates that the UniRef90 (a UniProt database identifier) is associated with several U36 (HarvEST database identifier) Expressed Sequence Tag (EST) entries.

UniRef90	Description	A	B	C	Total
Q9FNU8	26S protease regulatory subunit, putative			2	2
Q7GCH3	26S protease regulatory subunit, putative,			2	2
P42798	40S ribosomal protein S15a-1; 40S ribosomal protein S15a	2,1	1,1	1,x	4,2
Q0IQF7	40S ribosomal protein S16	1		1	2
B6TH42	60S ribosomal protein L9; ribosomal protein L6			2	2
Q2R480	6-phosphogluconate dehydrogenase, decarboxylating			2	2
Q9LHA8	70 kDa heat shock protein; DnaK family protein,		2	1	3
Q2R1G8	Acyl-coenzyme A oxidase 2, peroxisomal	2		3	5
P52894	Alanine aminotransferase 2 ; aminotransferase, classes I and II	3			3
Q8LI32	amine oxidase family protein, putative,			2	2
Q69UU3	aminotransferase, classes I and II, domain containing protein	1	1		2
Q7XN11	aminotransferase, putative, expressed			2	2
Q01859	ATP synthase subunit beta, mitochondrial n			2	2
A3BCW4	ATP synthase, putative, expressed			3	3
B8A7C1	ATP-dependent Clp protease		3	2	5
A3ATE4	ATP-dependent Clp protease		2		2
Q0JMN6	bifunctional 3-dehydroquinate dehydratase; Os01g0375200 protein			2	2
Q84MN8	bifunctional 3-phosphoadenosine 5-phosphosulfate synthase			2	2
P40880	Carbonic anhydrase, chloroplastic		2,2,1	x,x,1	2,2,2
Q9LEH7	Chitinase II	1		1	2
P04784	Chlorophyll a-b binding protein, chloroplastic			2	2
Q53PH9	DEAD/DEAH box helicase family protein, DSHCT domain containing protein			2	2
B6SKP8	Dynamin-related protein 1C	2			2
Q9LN13	Elongation factor 1-alpha ; elongation factor Tu	1	5		6
Q5Z627	Elongation factor 1-gamma 3	2			2
P17784	Fructose-bisphosphate aldolase cytoplasmic isozyme	1		2	3
Q40677	Fructose-bisphosphate aldolase, chloroplastic		1	1	2
P15737	Glucan endo-1,3-beta-glucosidase GII, glycosyl hydrolases family 17, putative	1		1	2
B6TLT1	HEAT repeat family protein, putative, expressed	1		2	3
Q334H8	Heat shock protein 101		2	1	3
Q08277	Heat shock protein 82		1	1	2

Q10CE4	hydroxyacid oxidase 1,		1	1	2
Q6YWL3	Malate dehydrogenase, lactate/malate dehydrogenase	3,1,1,1	3,1,1,1,1	3,1,1,1,1	9,3,3,3,2
Q84JH5	mannose-1-phosphate guanyltransferase, putative, expressed			2	2
Q9LKM0	Nucleoside diphosphate kinase	1		1	2
Q5JLV2	Os01g0894700 protein n		1	1	2
P35792	Pathogenesis-related protein PRB1-2; SCP-like extracellular protein, expressed	1		1	2
O49866	Peroxidase	1		1	2
Q5I3E8	Peroxidase 10 (Fragment)	1		1	2
C6ETB7	peroxidase Class III			2	2
Q5QNA5	phosphoenolpyruvate carboxylase, putative			2	2
Q9ZTS1	Probable methionyl-tRNA synthetase			4	4
Q7X5X9	proteasome subunit, putative, expressed			2,2	2,2
Q7X5X9	proteasome subunit, putative, expressed, UCRAM01			2	2
Q67UF5	protein disulfide isomerase PDIL2-3, expressed	2			2
Q657P0	Protein translocase subunit secA n, chloroplastic			2	2
Q6AV40	Putative RAN binding protein; exportin-7-A, putative			4	4
C5XR25	Putative uncharacterized protein, tetratricopeptide repeat		2		2
Q6PWL8	Putative vacuolar defense protein, WIP3 - Wound-induced protein	1		1	2
Q6AVA8	Pyruvate, phosphate dikinase 1, chloroplastic, PPDK1			2	2
Q7XGR3	RNA recognition motif containing protein, expressed	2			2
P46285	Sedoheptulose-1,7-bisphosphatase, chloroplastic			2	2
C5Y297	Serine hydroxymethyltransferase, mitochondria	1		1	2
B6U124	Succinate dehydrogenase flavoprotein subunit, mitochondrial	1		4	5
Q6EZE7	Sucrose-phosphate synthase 2	3			3
Q5MBN2	Thaumatin-like protein TLP5 , thaumatin, putative, expressed	1,1		1,1	2,2
Q6K6K7	ThiF family domain containing protei			2	2
Q53NR8	transport protein	2			2
B6T0F0	Trehalose-6-phosphate synthase	2			2
Q53M52	Tubulin alpha-2 chain ; tubulin/FtsZ domain containing protein			2	2
B9FIN1	tubulin/FtsZ domain containing protein,			2	2
C0PA67	tubulin/FtsZ domain containing protein, put		1	1	2
Q6H547	TUDOR protein with multiple SNc domains, putative,			2	2
Q9SAQ6	Ubiquitin (Fragment);ubiquitin family protein, putative, expressed			2	2
A1C0L3	UCW116, putative lipase ; GDSL-like lipase/acylhydrolase,	2			2
Q9LLR3	Vacuolar targeting receptor bp-80 ; vacuolar-sorting receptor	6			6
D1IVV3	Whole genome shotgun sequence of line PN40024 Vitis vinifera D1IVV3_VITVI LOC_Os05g36290.1		2	1	3
D1I3M9	Whole genome shotgun sequence of line PN40024 Vitis vinifera RepID=D1I3M9_VITVI LOC_Os05g49890.1	1		1	2

Supplementary Table 27: **A list of proteins identified with *Blumeria* Effector Candidate 1054 (BEC1054) from the International Barley Sequencing Consortium (IBSC) database through affinity pull-down and liquid chromatography mass spectrometry**. The letters "A", "B" and "C" indicate the experimental conditions, with "A" being magnetic NTA agarose beads and 48h Infected leaf epidermis; "B" being 1 ml NTA chromatography columns and non-infected leaves; and "C" being magnetic NTA agarose beads and seven day Infected leaves. The numbers in the columns A, B and C indicate the biological replicates with which the protein was identified solely with BEC1054 (as opposed to with the negative controls). At least two peptides were used to identify each protein in at least two independent biological replicates.

ISBC accession	Accession	UniProt	Protein description	A	B	C	Total
AK375372	AT3G14750	M0ZBG8_HORVD	unknown protein; Similar to A. *thaliana* protein FLX-Like 1	2			2
AK251411.1	AT1G67170	Not found	unknown protein; Similar to A. *thaliana* protein FLX-Like 2	2			2
AK370126	B9RLP7	F2E1V5_HORVD	Chaperone clpb, putative		3	2	5
MLOC_50979.1	B9RLP7	M0WFE2_HORVD	Chaperone clpb, putative		3	2	5
AK373131	B9SZ67	F2EAF6_HORVD	Wound-induced protein WIN1, putative (*Ricinus*); AK373131 BARWIN protein	1		2	3
MLOC_4512.2	B9SCC1	F2D006_HORVD	WD-repeat protein, putative	2			2
AK362773	B9S1U1	F2DFV6_HORVD	ATP-dependent clp protease ATP-binding subunit clpx, putative		1	1	2
MLOC_76454.6	B9S1U1	M0Z3M8_HORVD	ATP-dependent clp protease ATP-binding subunit clpx		3		3
AK250663.1	B9S5Y6	Not found	C20orf11, CRA + lisH motifs (in RAN binding prot and tubulin binding prot respectively)	2			2
AK362492	E4W3Z2	F2DF25_HORVD	Heat shock 70 kDa protein 5			2	2
AK251322.1	F2KPY6	Not found	S-adensyl L methionine (SAM) Methyltransferase type 11	2			2
AK248545.1	D4N8D8	Not found	Carbonic anhydrase		2		2
MLOC_4511.1	O03994	M0W8Z2_HORVD	Wound-induced protein; Barwin, PR4	1		2	3
MLOC_16610.1	Q9FS06	M0V101_HORVD	Cysteine proteinase inhibitor;	1	1		2
MLOC_55635.2	G7IUL0	M0WYF4_HORVD	Ser/thr protein phosphatase 2A 55 kDa regulatory SU B β isoform	3			3
AK370341	O49902	M0UIF1_HORVD	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase	2			2
AK355673	Q8S4P7	F2CVM0_HORVD	Thaumatin-like protein	2		1	3
AK371265	Q8S4P7	F2E541_HORVD	Thaumatin-like protein			2	2
MLOC_4305.2	G7L8A9	M0W3P1_HORVD	RNA-binding protein; nucleolysin TIAR like	2			2
AK371628	F2E654	F2E654_HORVD	Ubiquitin carboxyl-terminal hydrolase Deubiquitination enzyme/ peptidase	2			2
MLOC_69600.1	F2CQL5	M0YIA7_HORVD	Isocitrate dehydrogenase [NADP]			2	2
AK372321	G2HFQ0	F2E847_HORVD	Tubulin alpha-2 chain; GTPase activity			2	2
MLOC_65477.1	Q5I3E8	M0Y3W8_HORVD	Peroxidase 10	1		1	2
AK250662.1	D9YM16	Not found	Elongation factor 1-alpha EF-TU. GTP dependent binding of aa-tRNA			2	2
AK357263	C6H7S5	F2D056_HORVD	ATP-binding cassette protein, ABC transporter	2			2

Supplementary Table 28: **β-galactosidase activity datasets show non-homogeneous variance.** Bartlett's tests were performed to determine whether the β-galactosidase activity values for different yeast lines showed homogeneous variance. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01, "***" p≤0.005, and "****" for p≤0.001.

Protein a	Protein b	Bartlett's K squared	p-value	significance
BEC1054	GST	36.15	0.0000	****
BEC1054	MDH	22.60	0.0000	****
BEC1054	PR5	9.30	0.0256	*
BEC1054	eEF1G	5.53	0.1367	
BEC1054	40S 16	6.67	0.0831	.
BEC1054	eEF1a(1)	3.19	0.3626	
BEC1054	eEF1a(3)	17.75	0.0005	***
BEC1054	PR10	20.55	0.0001	***
BEC1054	NDPK	26.96	0.0000	****
Krev1	RalGDS	7.66	0.0535	.

Supplementary Table 29: **Fungal *Blumeria* Effector Candidate 1054 (BEC1054) interacts with multiple plant proteins in yeast. A CPRG galactosidase assay was used to quantify the interaction between BEC1054 and putative interacting plant proteins in a yeast-two-hybrid assay.** Yeast lines were lysed through freeze-thaw lysis, and the lysis supernatant added to buffer containing chlorophenolred-ß-D-galactopyranoside (CPRG). The names of the interactors are given first for the bait, and then for the prey, with a space referring to an empty plasmid, i.e. "BEC1054" is pEXP32/BEC1054 and pDEST22; whereas "BEC1054+eEF1G is pEXP32/BEC1054 and pEXP22/eEF1G Games-Howell posthoc tests were used to determine whether the mean V_i (the maximum rate of conversion of the yellow substrate CPRG to the red product chloramphenicol red (and D-galactose)) was significantly different for different yeast lines (line 1 and line 2). Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01, "***" p≤0.005, and "****" for p≤0.001.

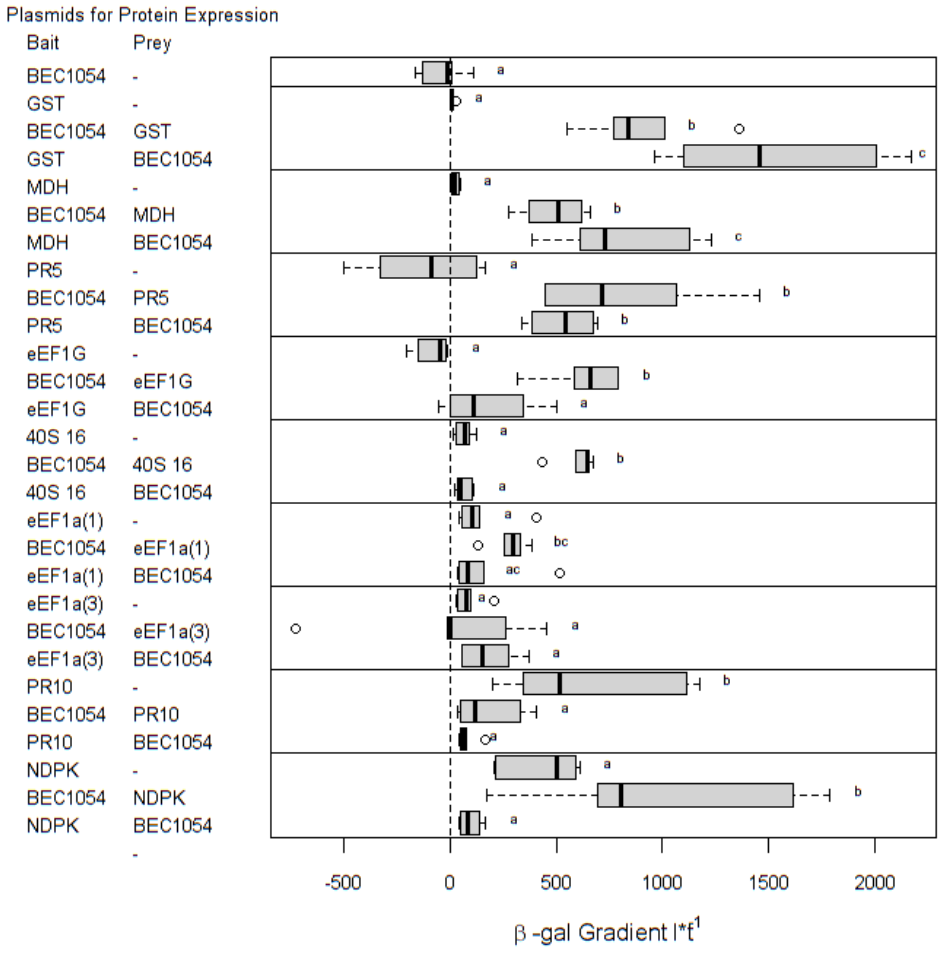
Line1	Line2	t-value	p-value	Significance
BEC1054	GST	0.27	0.9929	
BEC1054	BEC1054+GST	5.55	0.0001	***
BEC1054	GST+BEC1054	9.31	0.0000	****
GST	BEC1054+GST	5.28	0.0002	***
GST	GST+BEC1054	9.04	0.0000	****
BEC1054+GST	GST+BEC1054	3.76	0.0062	**
BEC1054	MDH	0.56	0.9416	
BEC1054	BEC1054+MDH	4.96	0.0004	***

BEC1054	MDH+BEC1054	7.90	0.0000	****
MDH	BEC1054+MDH	4.40	0.0015	***
MDH	MDH+BEC1054	7.34	0.0000	****
BEC1054+MDH	MDH+BEC1054	2.94	0.0371	*
BEC1054	PR5	0.56	0.9414	
BEC1054	BEC1054+PR5	5.66	0.0001	****
BEC1054	PR5+BEC1054	3.79	0.0058	**
PR5	BEC1054+PR5	6.22	0.0000	****
PR5	PR5+BEC1054	4.36	0.0016	***
BEC1054+PR5	PR5+BEC1054	1.86	0.2746	
BEC1054	eEF1G	0.49	0.9607	
BEC1054	BEC1054+eEF1G	7.57	0.0000	****
BEC1054	eEF1G+BEC1054	2.30	0.1306	
eEF1G	BEC1054+eEF1G	8.06	0.0000	****
eEF1G	eEF1G+BEC1054	2.79	0.0506	.
BEC1054+eEF1G	eEF1G+BEC1054	5.27	0.0002	***
BEC1054	40S 16	2.40	0.1090	
BEC1054	BEC1054+40S 16	15.29	0.0000	****
BEC1054	40S 16+BEC1054	2.31	0.1296	
40S 16	BEC1054+40S 16	12.89	0.0000	****
40S 16	40S 16+BEC1054	0.09	0.9997	
BEC1054+40S 16	40S 16+BEC1054	12.98	0.0000	****
BEC1054	eEF1A(1)	2.31	0.1295	
BEC1054	BEC1054+eEF1A(1)	4.16	0.0025	***
BEC1054	eEF1A(1)+BEC1054	2.48	0.0938	.
eEF1A(1)	BEC1054+eEF1A(1)	1.85	0.2797	
eEF1A(1)	eEF1A(1)+BEC1054	0.17	0.9981	
BEC1054+eEF1A(1)	eEF1A(1)+BEC1054	1.68	0.3598	
BEC1054	eEF1A(3)	0.95	0.7811	
BEC1054	BEC1054+eEF1A(3)	0.26	0.9936	
BEC1054	eEF1A(3)+BEC1054	1.68	0.3610	
eEF1A(3)	BEC1054+eEF1A(3)	0.68	0.9017	
eEF1A(3)	eEF1A(3)+BEC1054	0.73	0.8831	
BEC1054+eEF1A(3)	eEF1A(3)+BEC1054	1.42	0.5039	
BEC1054	PR10	5.14	0.0003	***
BEC1054	BEC1054+PR10	1.60	0.3986	
BEC1054	PR10+BEC1054	0.85	0.8318	
PR10	BEC1054+PR10	3.54	0.0103	*
PR10	PR10+BEC1054	4.29	0.0019	***
BEC1054+PR10	PR10+BEC1054	0.76	0.8720	
BEC1054	NDPK	2.52	0.086257	.

BEC1054	BEC1054+NDPK	5.44	0.000137	***
BEC1054	NDPK+BEC1054	0.68	0.901634	
NDPK	BEC1054+NDPK	2.92	0.039238	*
NDPK	NDPK+BEC1054	1.84	0.284746	
BEC1054+NDPK	NDPK+BEC1054	4.76	0.000644	***
BEC1054	Krev1+RalGDS-m2	3.25	0.0191	*
BEC1054	Krev1+RalGDS-Wt	12.14	0.0000	****
BEC1054	Krev1+RalGDS-m1	4.11	0.0028	***
Krev1+RalGDS-m2	Krev1+RalGDS-Wt	8.89	0.0000	****
Krev1+RalGDS-m2	Krev1+RalGDS-m1	0.85	0.8275	
Krev1+RalGDS-Wt	Krev1+RalGDS-m1	8.04	0.0000	****

Supplementary Table 30: **Glutathione-S-Transferase (GST) and Pathogenesis Related protein 10 (PR10) interact in yeast. A CPRG galactosidase assay was used to quantify the interaction between GST and PR10 in a yeast-two-hybrid assay**. Yeast lines were lysed through freeze-thaw lysis, and the lysis supernatant added to buffer containing chlorophenolred-ß-D-galactopyranoside (CPRG). The names of the interactors are given first for the bait, and then for the prey, with a space referring to an empty plasmid, i.e. "GST" is pEXP32/GST and pDEST22; whereas "GST+PR10 is pEXP32/GST and pEXP22/PR10. Games-Howell posthoc tests were used to determine whether the mean V_i (the maximum rate of conversion of the yellow substrate CPRG to the red product chloramphenicol red (and D-galactose)) was significantly different for different yeast lines (line 1 and line 2). Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01, "***" p≤0.005, and "****" for p≤0.001.

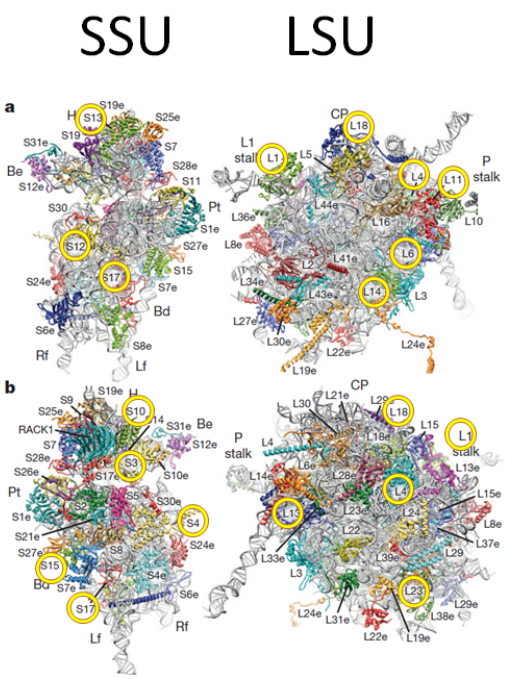
Line1	Line2	t-value	p-value	Significance
GST	PR10	5.18	0.0002	***
GST	GST_PR10	10.78	0.0000	****
GST	PR10_GST	9.65	0.0000	****
PR10	GST_PR10	5.61	0.0001	****
PR10	PR10_GST	4.47	0.0012	***
GST_PR10	PR10_GST	1.13	0.6740	



Supplementary Figure 51: **Yeast-two-hybrid shows the interaction of fungal *Blumeria* Effector Candidate 1054 (BEC1054) with multiple plant proteins.** The protein interactors abbreviations are: GST, glutathione-S-transferase; MDH, malate dehydrogenase; PR5, pathogenesis-related protein 5; eEF1G, elongation factor 1 gamma; and 40S 16, 40S ribosomal subunit protein 16; eEF1A(1), eukaryotic Elongation Factor 1 alpha; eEF1A(3), eukaryotic Elongation Factor 1 gamma; PR10, pathogenesis-related protein 10; and NDPK, nucleoside diphosphate kinase. A CPRG galactosidase assay was used to quantify the interaction between BEC1054 and putative interacting plant proteins in a yeast-two-hybrid assay. Yeast lines were lysed through freeze-thaw lysis, and the lysis supernatant added to buffer containing chlorophenolred-ß-D-galactopyranoside (CPRG). The names of the interactors are given first for the bait, and then for the prey, with a space referring to an empty plasmid, i.e. "B54" is pEXP32/BEC1054 and pDEST22; whereas "B54+eEF1A(1) is pEXP32/BEC1054 and pEXP22/eEF1A(1). Games-Howell posthoc tests were used to determine whether the mean V_i (the maximum rate of conversion of the yellow substrate CPRG to the red product chloramphenicol red (and D-galactose)) was significantly different for different yeast lines (line 1 and line 2). Significant

difference is indicated by the letters “a”, “b” and “c” (p>0.05), with bars labelled with different letters being significantly different. Six independently transformed colonies were used for each yeast line.

Supplementary Figure 52: **Three different eukaryotic elongation factor 1A from barley.** An alignment was produced using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) of three different eEF1A sequences amplified from barley cDNA.



Supplementary Figure 53: **40S and 60S proteins identified through *in vitro* chromatography with BEC1054.** "SSU" stands for small subiunit, and "LSU" stands for large subunit, Rf for the right foot, Pt for the platform, Lf dor the left foot, H for the head, CP for the central protuberance, Bd for the body and Be for the beak. Yellow circles highlight ribosomal proteins identified through *in vitro* chromatography, where a) is the interface view of the ribosomal subunit, and b) is the solvent view. Figure adapted from (Anger *et al.,* 2013).

Supplementary Table 31: **Analysis of RNA samples used for qPCR.** Where "hpi" stands for hours post inoculation; "conidia" for *Blumeria graminis* conidia; "barley" for *Hordeum vulgare* uninfected epidermal material; "epiphytic" for *B. graminis* epiphytic material; and "epidermal" for barley epidermal peels (containing B. *graminis* hyphae), and where the RNA Integrity Number is a value calculated using Bioanalyzer software to indicate the integrity of total RNA. Table reproduced with permission from (Pennington *et al.,* 2015)

Time point	Replicate	Concentration(ng/ul)	RNA Integrity Number
0hpi (conidia)	1	126.3	8.5
	2	133.1	8.3
	3	209.5	9.0
0hpi (barley)	1	456.4	9.2
	2	400.8	8.4
	3	480.0	8.8
4hpi epiphytic	1	27.1	7.0
	2	73.5	8.4
	3	38.5	7.9
6hpi epiphytic	1	219.0	8.6
	2	75.3	7.0
	3	79.7	8.2
16hpi epiphytic	1	39.0	8.0
	2	30.8	7.9
	3	24.9	7.0
24hpi epiphytic	1	12.3	8.0
	2	36.7	6.9
	3	74.9	8.7
24hpi epidermal	1	116.0	9.4
	2	220.0	9.0
	3	161.5	9.5
48hpi epiphytic	1	132.8	7.0
	2	66.6	7.5
	3	38.9	6.9
48hpi epidermal	1	210.0	8.3
	2	200.0	7.9
	3	120.9	9.4
72hpi epiphytic	1	369.8	8.7
	2	270.4	8.0
	3	155.0	9.2
72hpi epidermal	1	160.3	9.0
	2	160.0	8.0
	3	113.0	7.9
120hpi epiphytic	1	524.0	9.2

	2	230.5	8.6
	3	128.2	8

Supplementary Table 32: **Fungal *Blumeria* Effector Candidate 1054 (BEC1054) and Jasmonate Induced Protein 60 interact with RNA in yeast.** A CPRG galactosidase assay was used to quantify the interaction between BEC1054 and RNA sequences in a yeast-three-hybrid assay. Yeast lines were lysed through freeze-thaw lysis, and the lysis supernatant added to buffer containing chlorophenolred-ß-D-galactopyranoside (CPRG). The names of the interactors are given first for the protein, and then for the RNA, i.e. "BEC1054+SRL" is pIIIA/SRL-MS2. . The abbreviation "SRL" stands for ribosomal large subunit "Sarcin-Ricin Loop", "control" in the position of the RNA for a different section of the ribosomal large subunit, "IRE" for the RNA Iron Response Element, "positive control" for Iron Regulatory Protein 1 (IRP1) with the interacting IRE RNA sequence, and "negative control" for empty pDEST22 with pIIIA/IRE-MS2 . Games-Howell posthoc tests were used to determine whether the mean V_i (the maximum rate of conversion of the yellow substrate CPRG to the red product chloramphenicol red (and D-galactose)) was significantly different for different yeast lines (line 1 and line 2). Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01, "***" p≤0.005, and "****" for p≤0.001.

Line1	Line2	t-value	p-value	Significance
BEC1054+SRL	BEC1054+control	6.39	0.0000	****
BEC1054+SRL	BEC1054+IRE	5.46	0.0001	****
BEC1054+SRL	Positive control	14.88	0.0000	****
BEC1054+SRL	Negative control	2.03	0.2820	
BEC1054+control	BEC1054+IRE	1.19	0.7570	
BEC1054+control	Positive control	7.53	0.0000	****
BEC1054+control	Negative control	4.69	0.0007	***
BEC1054+IRE	Positive control	9.22	0.0000	****
BEC1054+IRE	Negative control	3.64	0.0096	**
Positive control	Negative control	13.38	0.0000	****
JIP60+SRL	JIP60+control	1.04	0.8360	
JIP60+SRL	JIP60+IRE	2.30	0.1770	
JIP60+SRL	Positive control	1.76	0.4190	
JIP60+SRL	Negative control	5.00	0.0003	***
JIP60+control	JIP60+IRE	1.26	0.7170	
JIP60+control	Positive control	0.68	0.9590	
JIP60+control	Negative control	6.08	0.0000	****
JIP60+IRE	Positive control	0.63	0.9690	
JIP60+IRE	Negative control	7.39	0.0000	****
Positive control	Negative control	7.03	0.0000	****

Supplementary Table 33: **Wheat phenotypic characteristics show variation in homogeneity of variance.** Bartlett's tests were performed to determine whether differing wheat phenotypic characteristics showed homogeneous variance. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01, "***" p≤0.005, and "****" for p≤0.001.

Characteristic	Bartlett's K-squared	Degrees of freedom	p-value	Significance
number of leaves	18.57	3	0.0003	****
Maximum height (cm)	0.55	3	0.9075	
Subcrown length (cm)	5.92	3	0.1154	
Internode 4	0.89	3	0.8274	
Internode 3	13.12	3	0.0044	***
Internode 2	3.64	3	0.3027	
Peduncle (1)	3.71	3	0.2947	
Ear length (including whiskers)	10.25	3	0.0166	*
Number of fertile tillers	9.74	3	0.0209	*
Tiller mass	0.75	3	0.8619	
Grain number	3.72	3	0.2928	

Supplementary Table 34: **Wheat lines homozygous and azygous for *Blumeria* Effector Candidate 1054 are not phenotypically different.** Games-Howell posthoc tests were used to determine whether the phenotypic characteristics listed beneath were significantly different for the T4 generation of homozygous (+/+) wheat (line 3.3.14) transformed with *Blumeria* Effector Candidate BEC1054 (*wbec1054*) and for azygous (-/-) wheat (line 3.3.12).. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005.

Phenotypic characteristic	Line 1	Line 2	t-value	p-value	Significance
Number of leaves	-/-	+/+	1.78	0.2992	
Maximum height (cm)	-/-	+/+	0.04	1.0000	
Internode 4	-/-	+/+	0.83	0.8390	
Internode 3	-/-	+/+	1.40	0.5082	
Internode 2	-/-	+/+	0.23	0.9955	
Peduncle (1)	-/-	+/+	2.59	0.0610	.
Ear length (including whiskers)	-/-	+/+	1.29	0.5719	
Number of fertile tillers	-/-	+/+	1.46	0.4699	
Tiller mass	-/-	+/+	0.36	0.9844	
Grain number	-/-	+/+	1.18	0.6415	

Supplementary Table 35: **The proportion of conidia which formed haustoria did not vary homogeneously in relation to differing factors investigated.** Bartlett's tests were performed to determine whether differing wheat factors showed homogeneous variance homogeneous variance. The abbreviation "combined" stands for a dataset where all the different factors were taken into account. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005. Data utilised with permission from (Luong 2014).

Set	Bartlett's k-squared	p-value	Significance
genotype	0.06	0.8131	
age	0.93	0.3358	
plant	24.77	0.0532	.
leaf segment	12.00	0.0025	***
combined	16.97	0.1087	

Supplementary Table 36: **The presence of the *Blumeria* Effector Candidate 1054 (BEC1054) transgene, and the location of sampling affect the mean proportion of conidia that formed at least one haustorium (propH).** Multiple Comparisons of Means were conducted, using Tukey Contrasts, to determine whether the mean propH values for homozygous and azygous plants transformed with BEC1054 were different, and whether the propH was affected by the location of the sampling (at the tip, middle or base of the leaf). Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01, "***" p≤0.005, and "****" p≤0.001. Data utilised with permission from (Luong 2014).

Line1	Line2	Estimate	Standard error	z-value	p-value	Significance
Homo	Azygous	0.66	0.10	6.35	0.0000	****
Middle	Base	0.59	0.11	5.41	0.0000	****
Tip	Base	1.09	0.16	6.71	0.0000	****
Tip	Middle	0.50	0.15	3.32	0.0025	**

Supplementary Table 37: **Chlorophylls a and b datasets show a mix of homogeneous and non-homogeneous variance.** Bartlett's tests were performed to determine whether the β-galactosidase activity values for different yeast lines showed homogeneous variance. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005. Data utilised with permission from (Chandler 2015).

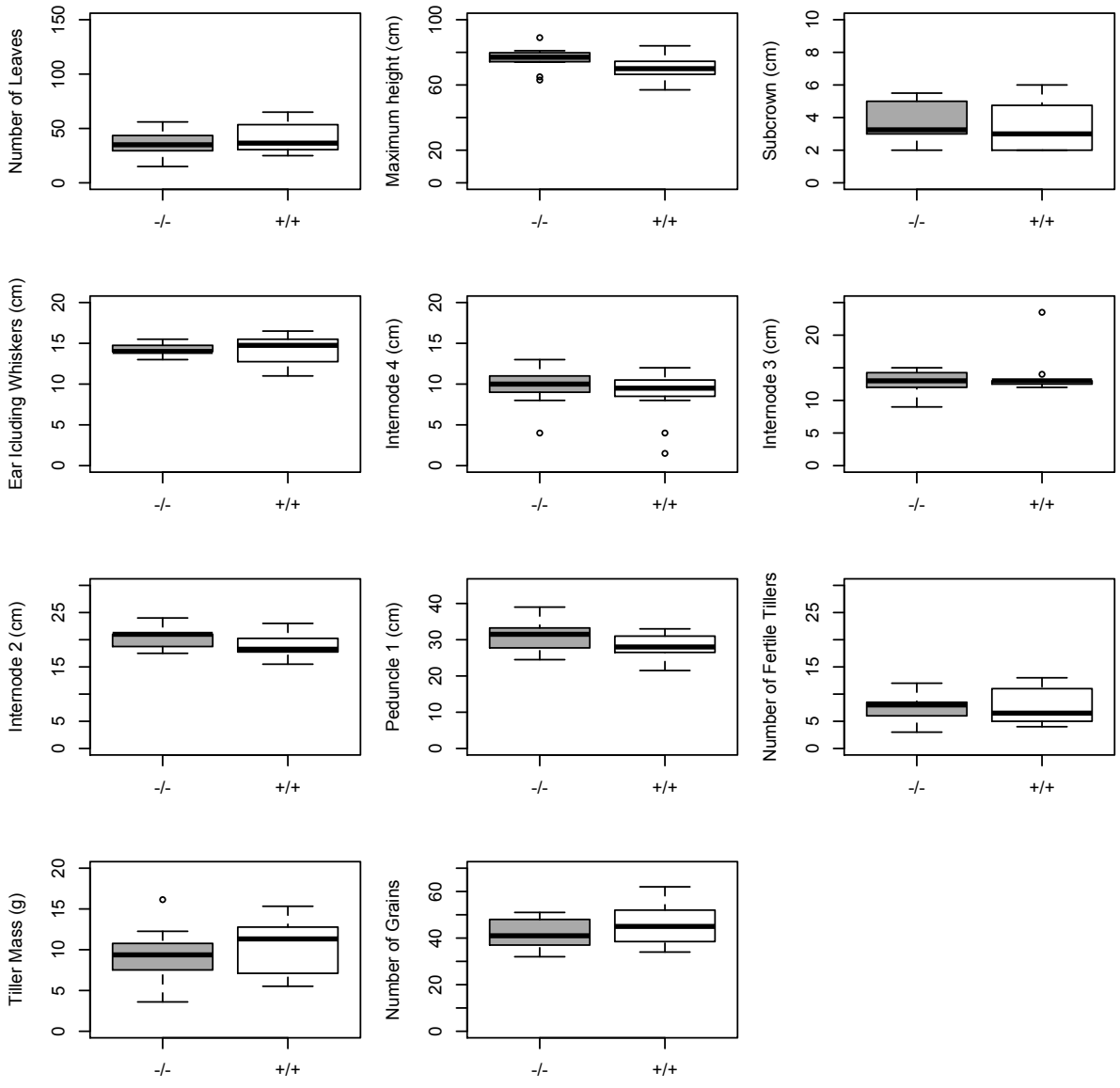
Dataset	Treatment	Bartlett's K-squared	p-value	Significance
Chlorophyll a	H2O/MeJA	4.57	0.0325	*
Chlorophyll b	H2O/MeJA	3.75	0.0527	.
Chlorophyll a	infection	0.53	0.4652	

Chlorophyll b	infection	0.21	0.6471	
Chlorophyll a	name	0.33	0.5647	
Chlorophyll b	name	0.46	0.4993	

Supplementary Table 38: **Treatment conditions affected the concentration of chlorophylls a and b.** Seven day old seedlings of transgenic wheat, azygous (-/-, line 3.3.12) or homozygous (+/+, line 3.3.14) for *Blumeria graminis* f.sp. *hordei* effector BEC1054, were maintained uninfected or infected once a day for three days. Primary leaves were harvested, and treated with methyl Jasmonate (MeJA) or water (H_2O) for a further five days. Total protein was extracted from the leaves, and the concentrations of chlorophyll a and b measured spectrophotometrically. Games-Howell posthoc tests were used to determine whether the chlorophyll a and b concentrations were significantly different under the treatments listed in the table below. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005. Data utilised with permission from Chandler, 2015.

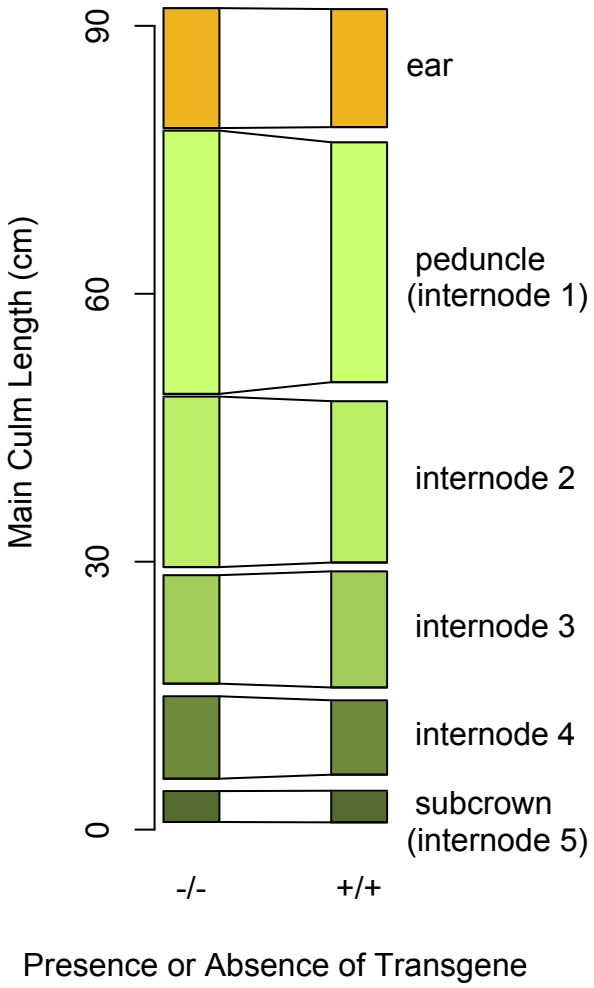
	Line 1	**Line 2**	**t-value**	**p-value**	**Significance**
Chlorophyll a	-/- uninfected H_2O	+/+ uninfected H_2O	0.83	0.8390	
	-/- uninfected H_2O	-/- uninfected MeJA	1.67	0.3600	
	-/- uninfected H_2O	+/+ uninfected MeJA	2.71	0.0522	.
	+/+ uninfected H_2O	-/- uninfected MeJA	2.50	0.0822	.
	+/+ uninfected H_2O	+/+ uninfected MeJA	3.54	0.0073	**
	-/- uninfected MeJA	+/+ uninfected MeJA	1.04	0.7252	
Chlorophyll b	-/- uninfected H_2O	+/+ uninfected H_2O	0.97	0.7676	
	-/- uninfected H_2O	-/- uninfected MeJA	1.59	0.4007	
	-/- uninfected H_2O	+/+ uninfected MeJA	2.79	0.0442	*
	+/+ uninfected H_2O	-/- uninfected MeJA	2.56	0.0723	.
	+/+ uninfected H_2O	+/+ uninfected MeJA	3.76	0.0042	**
	-/- uninfected MeJA	+/+ uninfected MeJA	1.20	0.6339	
Chlorophyll a	-/- infected H_2O	+/+ infected H_2O	0.53	0.9519	
	-/- infected H_2O	-/- infected MeJA	1.83	0.2810	
	-/- infected H_2O	+/+ infected MeJA	1.92	0.2423	
	+/+ infected H_2O	-/- infected MeJA	1.30	0.5687	
	+/+ infected H_2O	+/+ infected MeJA	1.39	0.5131	
	-/- infected MeJA	+/+ infected MeJA	0.09	0.9997	
Chlorophyll b	-/- infected H2O	+/+ infected H_2O	0.43	0.9733	
	-/- infected H_2O	-/- infected MeJA	1.82	0.2845	
	-/- infected H_2O	+/+ infected MeJA	1.83	0.2818	
	+/+ infected H_2O	-/- infected MeJA	1.39	0.5131	
	+/+ infected H_2O	+/+ infected MeJA	1.40	0.5095	

	-/- infected MeJA	+/+ infected MeJA	0.01	1.0000	

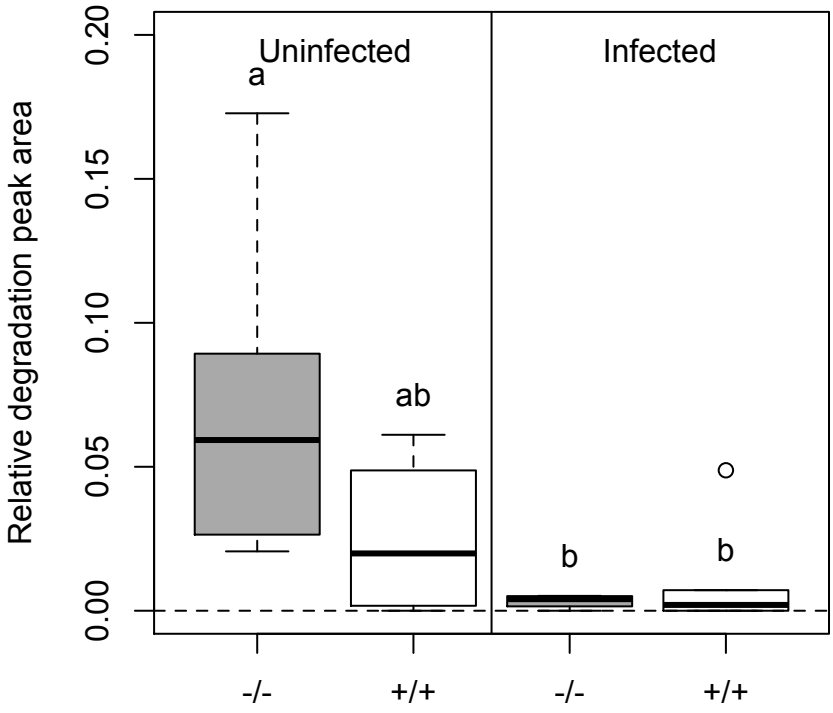


Supplementary Figure 54: ***Blumeria* Effector Candidate 1054 (BEC1054) does not affect the phenotype of transgenic wheat.** Eleven phenotypic characteristics of wheat were investigated for the T4 generation of homozygous (+/+) wheat (line 3.3.7) transformed with *Blumeria* Effector Candidate BEC1054 (*wbec1054*) and for azygous (-/-) wheat (line 3.3.12). The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles.

Games-Howell post-hoc tests indicated that the characteristics investigated were not significantly different ($p>0.05$) for any wheat lines.



Supplementary Figure 55: **Wheat main culm length is unaffected by the expression of *wbec1054*.** The two wheat models represent a summary of data collected for the T_4 generation of homozygous (+/+) wheat (line 3.3.7) transformed with *Blumeria* Effector Candidate BEC1054 (*wbec1054*), and for an azygous (-/-) wheat (line 3.3.12). The coloured bars for each internode and the ear represent the mean length of the primary tillers



Supplementary Figure 56: **Expression of BEC1054 decreases the formation of an RNA diagnostic peak**. Seven day old seedlings of transgenic wheat, azygous (-/-, line 3.3.12) or homozygous (+/+, line 3.3.7) for *Blumeria graminis* f.sp. *hordei* effector BEC1054 (*wbec1054*), were infected once a day for three days or maintained uninfected. Primary leaves were harvested, and treated with methyl jasmonate for a further five days. Ribonucleic acid was extracted from the primary leaves, and treated with 1 M aniline. The RNA was run on a Bioanalyzer Nano RNA chip, and the area of the major RNA peaks measured. The relative peak area was calculated for a putative ribosomal degradation peak, in relation to the area of the large ribosomal subunit (28S). The thick line denotes the median of each boxplot, the boxes represent the quartiles, maximum and minimum values are shown by the error bars, and outliers are indicated by circles. The letters indicate significance, with identical letters being the same, and different letters being significantly different. The number biological replicates, from left to right, was 6, 10, 5 and 4. utilised with permission from Thieron, (2015) and Chandler (2015).

Supplementary Table 39: ***Blumeria graminis* f.sp. *tritici* infection prevented the formation of a ribosomal degradation peak.** Seven day old seedlings of transgenic wheat, azygous (-/-, line 3.3.12) or homozygous (+/+, line 3.3.7) for *Blumeria graminis* f.sp. *hordei* effector BEC1054, were maintained uninfected or infected once a

day for three days. Primary leaves were harvested, and treated with methyl Jasmonate (MeJA) or water (H_2O) for a further five days. Total RNA was extracted from the leaves, and the major peaks measured using Bioanalyzer. Games-Howell posthoc tests were used to determine whether the relative degradation peak area was significantly different under the treatments listed in the table below. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005. Data utilised with permission from Thieron, 2015 and (Chandler 2015).

Set 1	Set 2	t-value	p-value	Significance
-/- uninfected	+/+ uninfected	2.01	0.2237	
-/- uninfected	-/- infected	2.97	0.0402	*
-/- uninfected	+/+ infected	3.00	0.0384	*
+/+ uninfected	-/- infected	0.88	0.8172	
+/+ uninfected	+/+ infected	0.67	0.9081	
-/- infected	+/+ infected	0.29	0.9909	

Supplementary Table 40: **Ribonucleic acid electropherogram peak area datasets show non-homogeneous variance.** Bartlett's tests were performed to determine whether the β-galactosidase activity values for different yeast lines showed homogeneous variance. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005. Data utilised with permission from Thieron, 2015.

Dataset	Bartlett's K-squared	p-value	Significance
Wheat line	9.27	0.0023	**
Infection	4.65	0.0311	*
Wheat line and infection	18.48	0.0004	***

Supplementary Table 41: ***Blumeria graminis* f.sp. *tritici* infection prevented the formation of a ribosomal degradation peak.** Seven day old seedlings of transgenic wheat, azygous (-/-, line 3.3.12) or homozygous (+/+, line 3.3.14) for *Blumeria graminis* f.sp. *hordei* effector BEC1054, were maintained uninfected or infected once a day for three days. Primary leaves were harvested, and treated with methyl Jasmonate (MeJA) or water (H_2O) for a further five days. Total RNA was extracted from the leaves, and the major peaks measured using Bioanalyzer. Games-Howell posthoc tests were used to determine whether the relative degradation peak area was significantly different under the treatments listed in the table below. Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005. Data utilised with permission from Thieron, 2015 and (Chandler 2015).

Set 1	Set 2	t-value	p-value	Significance

-/- uninfected	+/+ uninfected	2.44	0.0993	.
-/- uninfected	-/- infected	3.26	0.0182	*
-/- uninfected	+/+ infected	2.34	0.1202	
+/+ uninfected	-/- infected	1.42	0.4996	
+/+ uninfected	+/+ infected	0.29	0.9916	
-/- infected	+/+ infected	1.02	0.7389	

Supplementary Table 42: **Infiltration of *Nicotiana benthamiana* with barley and *Blumeria graminis* f.sp. *hordei* proteins affects production of *Peronospora tabacina* sporangia.** Games-Howell posthoc tests were used to determine whether the number of *P. tabacina* sporangia counted was significantly different for *N. benthamiana* leaf material infiltrated with *Agrobacterium* expressing either Green Fluorescent Protein (GFP), *Blumeria* Effector Candidate 1054 with a GFP tag (BEC1054-GFP) with a GFP C-terminal tag, or barley Jasmonate Induced Protein 60ml with a GFP C-terminal tag (JIP60ml-GFP). Significant difference is indicated by "." for p≤0.1, "*" for p≤0.05, "**" for p≤0.01 and "***" p≤0.005. Data utilised with permission from Thieron, 2015.

Line 1	Line 2	t-value	p-value	Significance
BEC1054-GFP	GFP	3.55	0.0093	**
BEC1054-GFP	JIP60-GFP	6.98	0.0000	***
GFP	JIP60-GFP	3.27	0.0156	*

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