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Molecular characterisation of the *Rhynchosporium commune* interaction with barley

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CONTENTS

1. Abstract

The interaction between *Rhynchosporium commune* and its host barley were studied to gain a better understanding of how the pathogen infects its host and to provide further characterisation of resistance in barley, using a combination of bioinformatics, transcript expression analysis, proteomics and confocal microscopy.

Expression analysis of potential effector sequences identified novel candidate effectors *Rc_10934, Rc_2091* and *Rc_2835* which showed the highest abundance during the biotrophic infection. A further two novel candidates Rc07_03591 and Rc07_02334 and a LysM domain containing protein (RcLysM3) were identified using a proteomic analysis of infected plant apoplast. Functional assays were used to characterise one of the LysM domain containing proteins indicating its potential involvement in the evasion of plant immune responses. Further analysis of the apoplast revealed some of the most abundant molecules that are present in *R. commune*'s infection toolkit. Cell wall degrading enzymes (CWDEs), virulence factors and proteins involved in detoxification were all highlighted as some of the key players of pathogenesis.

A R. commune strain expressing green fluorescent expressing (GFP) was used to characterise differences in pathogen growth and colony morphology in response to different genetic backgrounds of barley using lines carrying the *Rrs3* (Abyssinian), *Rrs4* (CI11549) and *Rrs13* (BC line 30) genes and barley landraces with uncharacterised resistance. This study also identified *R. commune* strains recognised by barley genotypes containing *Rrs3* (Abyssinian), *Rrs4* (CI11549), and *Rrs13* (BC Line30) resistance genes as well as two super virulent strains that overcome these resistances.

Rrs1 resistance was further analysed using comparative proteomics to identify proteins differentially expressed in resistant and susceptible cultivars. Pathogenesis related proteins - chitinase, glucanase and thaumatin-like protease, were identified in the barley apoplastic fluid and were shown to be upregulated during infection. In addition, serine carboxypeptidase and purple acid phosphatase proteins were identified that were novel to the barley resistance interaction but have been identified in other incompatible interactions as defence related proteins.

Asymptomatic growth of *R. commune* on the model dicotyledonous plant *Nicotiana benthamiana* was shown to be confined to the leaf surface making it a good model for characterisation of non-host interactions.

2. Introduction

Fungal plant pathogens represent a group of agronomically important microorganisms causing devastating diseases on some of the most important world crops. Among these pathogens, the fungus *Rhynchosporium commune* causes one of the most damaging diseases of barley worldwide. Barley was one of the first cultivated grains and is a major food source for developing countries and is known for its nutritional value and versatility. In addition, early maturation coupled with a high level of adaptability to stressful conditions allows it to grow in a wide variety of environmental conditions (Saisho & Takeda, 2011). Worldwide, barley production amounted to just under 150 million tonnes in 2015/2016 (http://www.statista.com/statistics/271973/world-barley-production-since-2008/). Barley crops infected with *R. commune* have lower yields and produce lower quality seeds. Across the globe there can be losses averaging 10% due to pathogen infection (Zhan *et al*., 2008). In the United Kingdom, around two thirds of the barley crop is used for animal feed and barley is a major element of the malting and brewing distilleries (Newton *et al*., 2011; Newman & Newman, 2006). Yield loss associated with the presence of this disease equates to £7.2 million a year, despite treatment (HGCA, 2013).

A relatively high genetic variation rate is a characteristic of this pathogen which has enabled it to overcome resistance genes deployed in attempts to control it (McDermott *et al*., 1988). However, utilising resistant cultivars is one of the most economically and environmentally beneficial methods of controlling the disease, providing a low input, cost effective strategy that can be used in combination with other control methods as part of an integrated disease management approach. There is a need to develop more effective and sustainable resistance to this pathogen and a deeper understanding of the molecular basis of host-pathogen interactions is a prerequisite.

The first research objective was to gain an understanding of how *R. commune* colonises its host and evades barley immunity. Pathogen proteins, termed effectors, are secreted by the pathogen to aid with the infection of the host plant. They can also be recognised by plants and can therefore activate a plant immune response resulting in resistance to the pathogen (avirulence proteins) (Jones & Dangl, 2006). Hence the identification of the pathogen proteins is a crucial first step in the discovery of barley resistance to *R. commune*. Many pathogen effectors that have been identified from fungal plant pathogens are typically secreted proteins that are often host specific, induced upon host colonisation and highly abundant during infection (Jonge *et al*., 2011; Zhu *et al*., 2013).

Initially, *R. commune* genome and transcriptome sequence data were used to predict a panel of candidate effector sequences (Avrova, unpublished). This was achieved by applying criteria to select for known features of fungal effectors. The NCBI BLASTp tool was used to compare the candidate effector sequences to the databases which identifies similarities to other sequences in the database. The Pfam online tool was used to detect any regions within the candidate effector sequences that could provide insights into their function. Sequences which were conserved between nine *R. commune* sequenced strains were selected. The durability of *R* genes will depend on how

quickly the pathogen can alter the effector which is being recognised, therefore sequences which are conserved are likely to be essential for the pathogen and less likely to be altered or deleted.

In order to select candidate genes which are highly expressed during a compatible interaction for further characterisation an infection time course using strain L2A on susceptible barley cultivar Optic was set up. Extraction of gene transcripts was carried out on each of the samples obtained from the infection time course. A further infection time course using a green fluorescent expressing (GFP) isolate, 214GFP, containing three replicates was also conducted. To finalise the research of effector identification, apoplastic fluid extracted from barley leaves inoculated with *R. commune* strain L73A was analysed to confirm the presence of candidate effector proteins during a susceptible interaction. Two time points were selected, 4 dpi (days post infection) - which represents the initial colonisation of the apoplast - and 7 dpi in which growth of the fungus would be well developed.

After prioritising candidate effectors it was necessary to determine if any were essential for pathogenicity and to gain an understanding of the function. In filamentous fungi, a common application to analyse the function of a gene is by its replacement or disruption with a marker gene for antibiotic resistance (Yang *et al*., 2004; Kück & Hoff, 2010; Chung & Lee, 2014). Targeted gene disruption was used to functionally characterise novel candidate effectors to determine if their function is essential for pathogenesis.

In contrast to candidate effectors that share no similarity to known effectors from other species, it was possible to use a different approach to predict the function of any genes that contained regions (domains) that are known to have a specific function. There have been many effectors from other plant pathogens which have been functionally characterised. An example is fungal effectors which contain a LysM domain and have been shown to bind chitin. Chitin is a component of the fungal cell wall and during infection plants can recognise fragments of fungal chitin and mount an immune response (Jones & Dangl, 2006). Therefore LysM containing proteins have been shown to play a fundamental role in the infection process of apoplastic pathogens through their ability to bind chitin and prevent host immune responses to the pathogen infection (Kombrink & Thomma, 2013). The ability to conceal chitin and protect the fungal cell wall within the apoplast is an effective strategy and there has been much research dedicated to the understanding of the LysM fungal protein effectors.

The second part of the research focused on identifying new barley resistance to *R. commune* which has become a top priority since the breakdown of *Rrs1* resistance occurred (Schürch *et al*., 2004; Zhan *et al*., 2008). However, due to the pathogen's high genetic variability, one of the biggest challenges is finding cultivars with longer lasting resistance (Zaffarano *et al*., 2006). Despite the economic importance of the disease no *R* genes have been cloned and the understanding of a resistant response is limited to the *Rrs1-AvrRrs1* interaction (Rohe *et al*., 1995). Evaluation of cultivar resistance has generally been scored using qualitative and subjective methods based upon the presence of visual disease symptoms on barley leaves after inoculation with the pathogen (Ayliffe *et al*., 2013). However due to the long asymptomatic phase of infection this approach fails to provide much insight into asymptomatic infection and how the pathogen is colonising the host. To investigate

the mechanisms of other barley resistant genotypes fluorescent confocal microscopy was used to visualise growth of *R. commune* during infection of barley lines containing *R* genes other than *Rrs1*. Analysis of lesion formation using a detached leaf assay was also assessed. During an incompatible interaction the pathogen was shown to be highly restricted in growth and a change in fungal morphogenesis characterised the *Rrs1* resistance response. The establishment of defence requires the fine regulation of a wide variety of apoplastic proteins which can act rapidly and effectively to restrict the pathogen's spread. Some studies have used proteomics to screen the apoplast for proteins involved in resistance, identifying extracellular enzymes involved in defence and cell wall metabolism (van der Westhuizen *et al*., 1998; Floerl *et al*., 2008; Delaunois *et al*., 2012). It has become evident that numerous approaches are required to obtain a more detailed picture of resistance and to gain a better understanding of the type of resistance barley confers against this pathogen. Furthermore, our knowledge is still limited regarding the mechanisms of other barley major *R* gene resistance to this pathogen. In addition, a comparative proteomics approach to identifying proteins present during an *Rrs1* resistant interaction may highlight some interesting proteins that can be used to assess the resistance of other barley genotypes.

Understandably, most of the research to date has focused on the narrow host range of *R. commune* due to the damage it causes as a pathogen (Zhan *et al*., 2008). Interestingly, a recent study conducted by King *et al*. (2013) showed *R. commune* to be pathogenic on Italian ryegrass, which was not previously classified as a host. In addition, *R. commune* can in fact grow asymptomatically on its host barley. No research has been conducted that has investigated the growth on non-grass species, despite the fact that many dicotyledonous plants like *Nicotiana benthamiana* have now been used for many years as model plants within the laboratory (Goodin *et al*., 2012). Plant species on which disease symptoms have not been observed are a non-host for a pathogen (Malcom *et al*., 2012). Non-host resistance (NHR) is more durable than host resistance but the mechanism of NHR to *R. commune* has not been previously addressed (Lee *et al*., 2016). There are multiple factors that contribute to NHR of non-adapted pathogens including induced and preformed plant defence mechanisms (Uma *et al*., 2011; Fan & Doerner, 2012; Stam *et al*., 2014). The development of a GFP expressing *R. commune* strain has been a valuable tool for understanding the mechanisms of the pathogen's growth during infection and in response to barley *Rrs1* genotypes (Kirsten *et al.,* 2011; Thirugnanasambandam *et al*., 2011). Hence, the growth of *R. commune* on a dicotyledonous plant can now be analysed.

3. Materials and methods

3.1. Plant growth

All plants used in this research were grown in a general compost mix from the James Hutton Institute (JHI) containing Intercept insecticide. Barley plants were grown under glasshouse conditions at 19^{o}C

with a 16-h day photoperiod for approximately 8-11 days. *N. benthamiana* plants were grown under glasshouse conditions at 22-24°C with a 14-hour day photoperiod for 4-5 weeks.

3.2. Culturing and storage of micro organisms

3.2.1. Fungi

R. commune strains from the culture collection at the JHI were grown on CZV8CM agar medium (Newton, 1989) at 17^{o}C in the dark. *Saccharomyces cerevisiae* strain FY834 and *Pichia pastoris strain* GS115 were grown from glycerol stock stored at -80^{o}C on Yeast Extract Peptone Dextrose (YPD) media at 28^{o}C for 2-3 days.

3.2.2. Bacteria

Escherichia coli cells (MAX Efficiency DH5α™ Competent Cells, Invitrogen) were grown overnight at 37^{o}C on Luria-Bertani (LB) agar medium with the addition of appropriate antibiotics.

3.2.3. Harvesting of fungal spores

R. commune conidia were harvested from approximately 14-day-old cultures by scraping the mycelial mat with a spatula following the addition of 5 mL of sterile distilled water (SDW). The suspension was filtered through glass wool or a filter unit containing 30 µm filter (Millipore). The suspension was centrifuged for 3 min at 1600 g and washed with SDW. This step was repeated three times. Spore concentration was measured using a haemocytometer.

3.3. *R. commune* inoculation of barley

3.3.1. Detached leaf assay

The assay was performed as described in Newton *et al*. (1989). Inspection of lesion formation began at 10 days post inoculation (dpi) and measurements continued until the leaf segment became too chlorotic to assess.

3.3.2. Barley spray inoculation and trypan blue staining of *R. commune in planta*

Ten day old barley plants were inoculated with a suspension of *R. commune* conidia (10^6 spores/mL, 0.1% Tween 20) and kept in plastic boxes at 100% humidity for 72 h with the first 24 h in the dark. After 72 h the inoculated plants were kept at 80% relative humidity.

Leaf samples were taken before inoculation, and at 1, 2, 3, 4, 6, 8, 10 and 13 dpi. To allow for variation in infection, leaf sections from five plants were collected for each time point, frozen in liquid nitrogen and stored at -70 ^{o}C prior to RNA extraction. Additional inoculated plants kept for 22 days after inoculation showed high levels of infection (results not shown). Uninoculated plants remained symptomless. Leaf samples were also taken at 3, 4, 6, 8, 10 and 13 dpi for trypan blue staining (Koch & Slusarenko, 1990) and light microscopy, to confirm the stages of infection as conidia germination

and penetration (1-3 dpi), the biotrophic interaction with internal hyphae spreading under the cuticle (3-8 dpi), and a transition phase between biotrophy and necrotrophy (10-13 dpi).

3.4. Confocal laser scanning microscopy (CLSM)

Leaf segments inoculated with isolate 214-GFP were mounted onto a glass slide using double sided tape to secure the sample. 10-20 µL of silicone oil was pipetted onto the barley leaf surface and a glass cover slip was placed on top. The Leica SP2 confocal microscope, controlled via software Leica Confocal Software (LCS) was used to capture images of 214-GFP strain growth on barley and on *N. benthamiana* at an excitation of 488 nm and emission collection of 500-530 nm. At the same time the autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm.

3.5. Molecular biology protocols

3.5.1. DNA extraction, RNA extraction and cDNA synthesis

DNA extractions were conducted using the Qiagen DNeasy kit following the manufacturer's guidelines.

Total RNA was extracted from barley leaves, conidia prepared as described above and conidia germinated in SDW for 24 h using a Qiagen RNeasy Plant mini kit, following the manufacturer's protocol. The extraction of mRNA from inoculated leaf samples was carried out in accordance with Dynabeads® mRNA DIRECT™ Kit protocol (Invitrogen). Prior to cDNA synthesis, RNA samples were DNaseI treated using Ambion Turbo DNA-free™ DNA Removal Kit following the manufacturer's protocol. First strand cDNA for real-time RT-PCR was synthesised from 10-15 µg of total RNA or 150 ng of mRNA by oligo dT priming using the SuperScript® III Reverse Transcriptase (Invitrogen), following the manufacturer's protocol.

3.5.2. Polymerase chain reaction

Polymerase chain reactions (PCR) were carried out using the Biorad T100TM Thermal cycler. The PCR cycle was dependent on the Tm of the primers, template, amplicon size and type of polymerase used. All primers used are listed in Table 4.1.

3.5.3. Quantitative RT-PCR (qRT-PCR)

SYBR green qRT-PCR assays for gene expression analysis were carried out as described in Avrova *et al*. (2003). *R. commune* actin was used as a constitutively expressed endogenous control gene. Relative expression of *R. commune* transcripts was normalized against expression levels in conidia (assigned a relative expression value of 1.0) as described in Grenville-Briggs *et al.* (2008). Assays were repeated on two independent occasions, using cDNA from two independent infection time courses. Primer sequences are provided in Table 4.1.

3.6. Yeast re-combinational cloning (YRC)

YRC was conducted using the procedure described by Oldenburg (Oldenburg et al., 1997). Plasmids used in this study were constructed using standard techniques (Sambrook and Russell, 2001).

3.7. Transformation protocols

3.7.1. *E. coli* and yeast transformation

MAX Efficiency® DH5α™ Competent *E. coli* cells (Invitrogen) were used for all *E. coli* transformations and the procedure followed the guidelines provided.

Transformation of *S. cerevisiae* was based on the protocol detailed in Knop *et al*. (1999).

Pichia pastoris was transformed following the protocol described in the Invitrogen *Pichia* expression kit manual.

Strategies for analysing protein expression in selected clones are described in detail in the *Pichia* expression kit manual (https://tools.thermofisher.com/content/sfs/manuals/pich_man.pdf).

3.7.2. Electroporation transformation of *R. commune*

R. commune conidia were harvested using the previously described method. The pellet obtained from centrifugation was suspended in 10 mL of SDW with 10 µL of ampicillin and left in the dark for 24-48 hours at 17°C for the conidia to germinate. The conidial suspension was washed 3 times with 10 mL of 1 M sorbitol and centrifuged at 1600 g for 3 min. The pellet was re-suspended in 100 µL of 1M sorbitol, transferred to an ice-cold 2 mL Eppendorf tube containing 1 µg of DNA and mixed gently. The mixture was kept on ice for 5 min before being transferred to an ice-cold electroporation cuvette. The germinated conidia and DNA were electroporated at 1.25 kV and transferred into a 50 mL falcon tube with 10 mL of Potato Dextrose Broth (PDB), 1mL of sorbitol, 10 µL of 100 mg/mL ampicillin and placed onto a rolling shaker for 24 hours. The suspension was centrifuged at 700 g for 5 min and re-suspended in 2 mL of PDB and 1 mL of 1M sorbitol. The sample was plated onto CZV8CM agar medium, containing 100 µg/mL of hygromycin and ampicillin. After 2-3 weeks, antibiotic resistant colonies were transferred onto fresh medium containing antibiotics as stated above.

Table 4.1: List of primer sequences.

Primer Name	Sequence	Primer Name	Sequence
Rc_1097 F	ATCCTCAGCACCGCAACATC	Rc_11301 F	CCCCAGTTACAGGCCCAATT
Rc_1097 R	TCGCAGCAATCCACGAATT	Rc_11301 R	CACGTATCGCTTGATGAAACCA
Rc_1130 F	CTTCGCGGCCTGTGGAT	Rc_11752 F	CAACTCTTCTATCGATCGTTCTCATG

Rc_1130 R	TTGCAGCCAGCGTCACAAT	Rc_11752 R	CATACAGTCGTCCTCCTCACAGTCT
Rc_1176 F	CTCACACTCCTTCTATCTATGCATCTG	Rc_11935 F	ATATTGTTAAGAGCCTAGGGCAGAGT
Rc_1176 R	TGGGCATCCGTCATTCTTG	Rc_11935 R	TTTGTGTCGCACTTATAATGGATGT
Rc_2091 F	CATCACTCTTCCTCGCTTTCCTT	Rc_11976 F	TCCGTCGCCTCCACCAT
Rc_2091 R	TCCCCAGATGCGTGGTATTC	Rc_11976 R	CCGCGCAGTTGTTCCAA
Rc_2410 F	CTCGTGGTGCGCAATCC	Rc_12364 F	GGCCTGGAAAACCCTCAAG
Rc_2410 R	CGCTTGTGACCTTGCTTCAAG	Rc_12364 R	TCGGAGGCCAAGGGATTAC
Rc_2608 F	CCCGTTTCCACCAAATCATC	Rc_LysM1 F	CGCTCTAGCCTGTTCAGC
Rc_2608 R	CGGCCTCGTCTTCTTTCTCA	Rc_LysM1 R	CGATTGAGGTAAACACCACT
Rc_2835 F	CGCATGTCGAGTCACGTATGA	Rc_LysM2 F	GCAACTCTGGCAACTCAGG
Rc_2835 R	ACGAAAATCGACTTGGGACAA	Rc_LysM2 R	CAATAGCATCCGGATTCTTG
Rc_4755 F	CGGGAGGCCGAGACAAA	Rc_LysM3 F	CGCTTCTCTCCTAGCAGTTG
Rc_4755 R	CAGCGCCTTTTAGTACTTGATGAA	Rc_LysM3 R	CGATTTGAGTGTTTGCCGC
Rc_5049 F	CAATGAGAACGCAGACGAGAAA	Rc_LysM4 F	GGCAGATCTACTCTTAGGCTGC
Rc_5049 R	ACTTCCGGCCCTCAGTACCT	Rc_LysM4 R	GCTTAGTTGGGGTGTGGC
Rc_5109 F	TAAGCGCTGCATCAATCGAAT	Rc_LysM5 F	AGAACAGTCGTCATACCTGG
Rc_5109 R	GCCACCATTACCAGGGATACAA	Rc_LysM5 R	CTCAAATAGCGTCGTCTGAG
Rc_5673 F	CGAGAGAGGCCAATGCAAA	Rc_LysM6 F	CTTCGGATATGATGAAGAGTTGG
Rc_5673R	CACACATAAAGAGCTCAGCCTTGT	Rc_LysM6 R	GCAGTTGCAGTAGCAGTAACG
Rc_5783 F	GCCTTATCAGCCGCAATCA	Rc_LysM7 F	TGTAAGGTGGGATTCACG
Rc_5783 R	CTATGCAATGGCAACTAGCGTAA	Rc_LysM7 R	CACGGTCGTGTGCAATC
Rc_6721 F	CAGAGGCACCAAAATGCAAA	Rc_Chi F	CGATGTGGAATATCGCAGAC
Rc_6721 R	CGCCGCAGAAGATGTTGTTT	Rc_Chi R	GAGGCAAGGTGCTAGGA
Rc_7108 F	GCTCAAGCAGTCCCAGAAACA	Rc_CAZy F	CGGCAGAATTACACCATTGC
Rc_7108 R	TCGTGGGAATCGGATCCA	Rc_CAZy R	CCATTGTGAGCTTGCATCAAG
Rc_7354 F	CACTCCATTGCTTCAAAGTCTCCTA	Rc_2091 G1	GGGCTGCTACTGTAACCACTAGC
Rc_7354 R	GCCTCAATGACCGAGACAATTT	Rc_2091 G2	CCATTCATCCAAGAGCGCTT
Rc_7612 F	GCACACCTACTGCTGCTCTGAT	Rc_10934_G1	AGTCAGCCACATCCATGAGC
Rc_7612 R	TGGCGCTCCTTTTGGATTC	Rc_10934_G2	GCAATCTGAGGCTTTCTTGCA
Rc_8075 F	CGCAGCCTCCCAAGAAGA	Rc_2835_G1	ACCGAGCATGAAAGGCCAC
Rc_8075 R	CGGCCAATTCCCAAACTACAT	Rc_2835_G2	CGTCGCAACATCATCGAAAC
Rc_8731 F	TCCGGCCAGCCAGACTACT	Rc_2091_P1	GGAAGGGCGATCGGTGCGGGCCGTTTAAACGCCTAATCTACTCGACGCCG
Rc_8731 R	GAAGCGCTTGTCGGAACTG	Rc_2091_P2	TTGTGTCATGAATTAACAGTTAACGAATACTGAAGGGAATGAATGTGGTG
Rc_9760 F	GGTGGTTCTCCCAACAATTGTAA	Rc_2091_P3	TTAGTGTCAAACAGTCAAACCAGTTCTACGGGATTCCTCTAGCGACTGAG
Rc_9760 R	TAAACTCCCTCGGCAAGCA	Rc_2091_P4	TGGAATTGTGAGCGGATAACAAGTTTAAACCCTACTGCCAAGACATCCG
Rc_10317 F	CTGCAGTGCAAGCTGAAGAGA	Rc_10934_P1	GGAAGGGCGATCGGTGCGGGCCGTTTAAACACCAGGGAAAGCCTAGAAGG
Rc_10317 R	CATCGATCGCATCCTTCAGA	Rc_10934_P2	TTGTGTCATGAATTAACAGTTAACGAATACCAAGTGTCAGGCAATGTAACG
Rc_10900 F	GGCTCCGGTACATACAAGTTCTG	Rc_10934_P3	TTAGTGTCAAACAGTCAAACCAGTTCTACGCTGTCTACCCGGAGAGAAGG
Rc_10900 R	TTCCAAAACCAACTGCATTTTCT	Rc_10934_P4	TGGAATTGTGAGCGGATAACAAGTTTAAACAGCATCTTTCATACACGCAG
Rc_10934 F	CTCGGGCTTAGCACCTTGAC	Rc_2835_P1	GGAAGGGCGATCGGTGCGGGCCGTTTAAACTACCTCTGCACCATCGTACG
Rc_10934 R	TGCGGCATTCGCCTCTAT	Rc_2835_P2	TTGTGTCATGAATTAACAGTTAACGAATACCCTGCTTACGAAGTACGGAG
Rc_11163 F	TTCACAACATCCACCACTCTTCTC	Rc_2835_P3	TTAGTGTCAAACAGTCAAACCAGTTCTACGGATGACGAGTCCTGCTTTGG
Rc_11163 R	TGATGGCGAATATTCCATTGC	Rc_2835_P4	TGGAATTGTGAGCGGATAACAAGTTTAAACGGTTGTCCGCGTCTCTTAGTC

3.8. Proteomics protocols

3.8.1. Protein extraction

Leaves were placed into a mortar, covered with liquid nitrogen and ground to a fine powder. Extraction buffer in a 1:1 ratio of m/v was added and plant leaf material was further grinded ensuring no thawing occurred. Samples were centrifuged at 8000 g for 5 min at 4°C and the supernatant was transferred to 1.5-mL Eppendorf tube and used immediately for enzymatic and protein assays.

3.8.2. Protein visualisation

Samples were prepared using the NuPAGE® protocol. SYPRO ® Ruby Protein Gel Stain (Invitrogen) was used to visualise proteins. Standard western blotting procedure was conducted (http://www.biorad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf).

3.8.3. Proteome analysis of barley apoplast

Apoplastic fluid was extracted using vacuum infiltration as described with slight modifications (Vanacker, H *et al*., 1998; Bolton *et al*., 2008). 8-10 day old cotyledons were gently removed from the plant stem. Approximately 20 leaves were placed into a 2 L glass beaker and covered with SDW. A second smaller glass beaker was placed on top of the leaves to prevent them rising. Vacuum was applied until the leaves were completely infiltrated using a vacuum infiltrator/freeze drier (Edwards Modulyo). The infiltrated leaves were blotted dry with paper tissue and were rolled in muslin cloth and placed leaf tip first into a 20 mL syringe which was introduced into a 50 mL conical tube. The apoplast extract was collected by centrifuging at 1000 g for 15 min at 4°C. The fraction collected in the 1.5 mL Eppendorf tube was transferred to a new 2 mL Eppendorf tube and centrifuged again for 10 min at 1600 g at 4°C. The supernatant was decanted into a clean 1.5 mL Eppendorf tube and filter sterilised using 0.2-µm Millipore filter. The samples were concentrated to approximately 1/5th of their original volume and stored at -80°C.

3.9. Polysaccharide binding assay

A polysaccharide affinity precipitation assay was used to determine the affinity of LysM domain containing proteins to various polysaccharides: crab shell chitin, chitosan, xylan or cellulose (all from Sigma Aldrich), following the protocol described in de Jonge *et al*. (2010).

4. Results

Computer based prediction models helped to prioritise *R. commune* genes for further analysis. BLASTp search matched gene Rc_6721 to a putative aldehyde dehydrogenase from the fungal plant pathogen *Diaporthe* (*Phomopsis*) species disease complex. Aldehyde dehydrogenases (ALDHs) help to protect the pathogen against plant immune responses (Singh *et al*., 2012). Another eight candidate effectors matched hypothetical proteins from other fungi (Table 4.2). The remaining 13 candidate effectors did not match any sequences in NCBI database. Most of BLASTp matches were to protein sequences from the foliar fungal endophyte *Phialocephala scopiformis*. In addition, there were similarities between some of the candidates to hypothetical proteins from *Marssonina brunnea* an important fungus that causes Marssonina leaf spot on all species of poplar, the soil borne pathogen *F. oxysporum* and a fungal plant pathogen that causes root rot in flax and wheat *Microdochium bolleyi*. BLASTp searches revealed the presence of varying numbers of LysM domains within some of the sequences (Figure 4.1). Four LysM domain proteins identified (RcLysM1, RcLysM5, RcLysM7 and RcChi) contained one LysM domain, while RcLysM2 and RcCAZy contained two LysM domains. Similar to the well characterised Ecp6 effector from the tomato pathogen *C. fulvum*, RcLysM3 contained three LysM domains, whereas RcLysM4 and RcLysM6 contain five and four domains respectively. BLASTp results are detailed in Table 4.3. A total of 31 *R. commune* candidate effectors were selected for transcription profiling during infection.

To help further prioritise *R. commune* molecules involved in infection the level of their presence during infection was determined. The candidate effectors expression profiles were split into four groups, based on the infection stage in which their expression peaked (Figure 4.2). The largest proportion of candidate effector genes were upregulated at the biotrophic stage. Exactly half of the candidate genes were most highly expressed between 6-8 dpi when the fungus would have already established a mycelial network within the apoplast. All of the candidates within this group exhibited a similar profile - a gradual increase from 1-2 dpi with a distinct maximum between 3-6 dpi, continuing expression at 8 dpi and a subsequent decline (Figure 4.2).

Three genes, *Rc_10934, Rc_2091* and *Rc_2835*, were selected for further analysis as they were some of the highest expressed during infection. The selected candidates shared the same expression profile, inclining from 1 dpi with highest expression at 6 dpi before declining at 8 dpi and subsequently at 10 dpi (Figure 4.5 H, I & J). The increase in transcript abundance between the three candidates varied considerably. *Rc_2835* showed the highest level of upregulation, reaching a 1200-fold increase at 6 dpi compared to its level in conidia. At the peak of its expression, Rc_2835 transcript was almost as abundant as actin. Both Rc_10934 and Rc2091 were highly upregulated during barley infection compared to their levels in conidia, with a 150-fold and 25-fold increase respectively. At the peak of their expression, Rc_10934 and Rc2091 transcripts were 1.5 and 5.5 times as abundant as actin, respectively. All LysM fungal effectors were expressed at a time corresponding to the potential release of chitin fragments from the fungal cell walls into the apoplast

and thus may play a role in chitin sequestration (Figure 4.5, A-G), apart from LysM4 and RcCazy, which were not expressed during infection. In addition, expression at this stage of infection suggests other possible roles in the colonisation of the plant apoplast aiding in the protection against plant immunity like that of *C. fulvum* effector Avr4 (van den Burg *et al*., 2006).

Table 4.2. Sequence analysis of *Rhynchosporium commune* candidate effectors and homology to other fungal proteins.

Candidate gene Id	Protein length	Cysteines	Top BLASTp hit	Species
Rc_01097	103	8	hypothetical protein MBM_09244	Marssonina brunnea f. sp.
Rc_01130	157	14	No significant similarities	
Rc_01776	91	8	hypothetical protein FOCG_15424	Fusarium oxysporum f. sp.
Rc_02091	138	10	No significant similarities	
Rc_02410	149	6	No significant similarities	
Rc_2835	125	6	No significant similarities	
Rc_05049	194	4	No significant similarities	
Rc_05109	116	6	hypothetical protein LY89DRAFT_729122	Phialocephala scopiformis
Rc_5673	157	8	No significant similarities	
Rc_05783	121	6	hypothetical protein LY89DRAFT_579580	Phialocephala scopiformis
Rc_06721	104	8	putative aldehyde dehydrogenase	Diaporthe ampelina
Rc_07354	151	8	hypothetical protein LY89DRAFT_723264	Phialocephala scopiformis
Rc_07612	129	8	No significant similarities	
Rc_08075	160	6	hypothetical protein MBM_08646	Marssonina brunnea f. sp.
Rc_08731	145	8	hypothetical protein Micbo1qcDRAFT_180629	Microdochium bolleyi
Rc_10317	67	6	No significant similarities	
Rc_10933	137	8	No significant similarities	
Rc_10934	117	6	No significant similarities	
Rc_11163	126	4	hypothetical protein LY89DRAFT_730227	Phialocephala scopiformis
Rc_11301	191	7	No significant similarities	
Rc_11752	59	6	No significant similarities	
Rc_11935	93	5	No significant similarities	

Table 4.3: Amino acid sequence analysis of *Rhynchosporium commune* LysM domain proteins.

Sequence Id	Protein length, aa	Top BLASTp match	Species
RcLysM1	688	LysM domain-containing protein	*Colletotrichum graminicola*
RcLysM2	332	putative cell wall-associated hydrolase	*Marssonina brunnea*
RcLysM3	232	putative cell wall-associated hydrolase	*Marssonina brunnea*
RcLysM4	449	LysM domain-containing protein	*Colletotrichum tofieldiae*
RcLysM5	269	hypothetical protein	*Phialocephala scopiformis*
RcLysM6	672	LysM domain-containing protein	*Colletotrichum graminicola*
RcLysM7	164	carbohydrate-binding module family	*Glonium stellatum*
RcCAZy	317	hypothetical protein	*Marssonina brunnea*
RcChi	979	glycosyl hydrolase family 18	*Colletotrichum incanum*

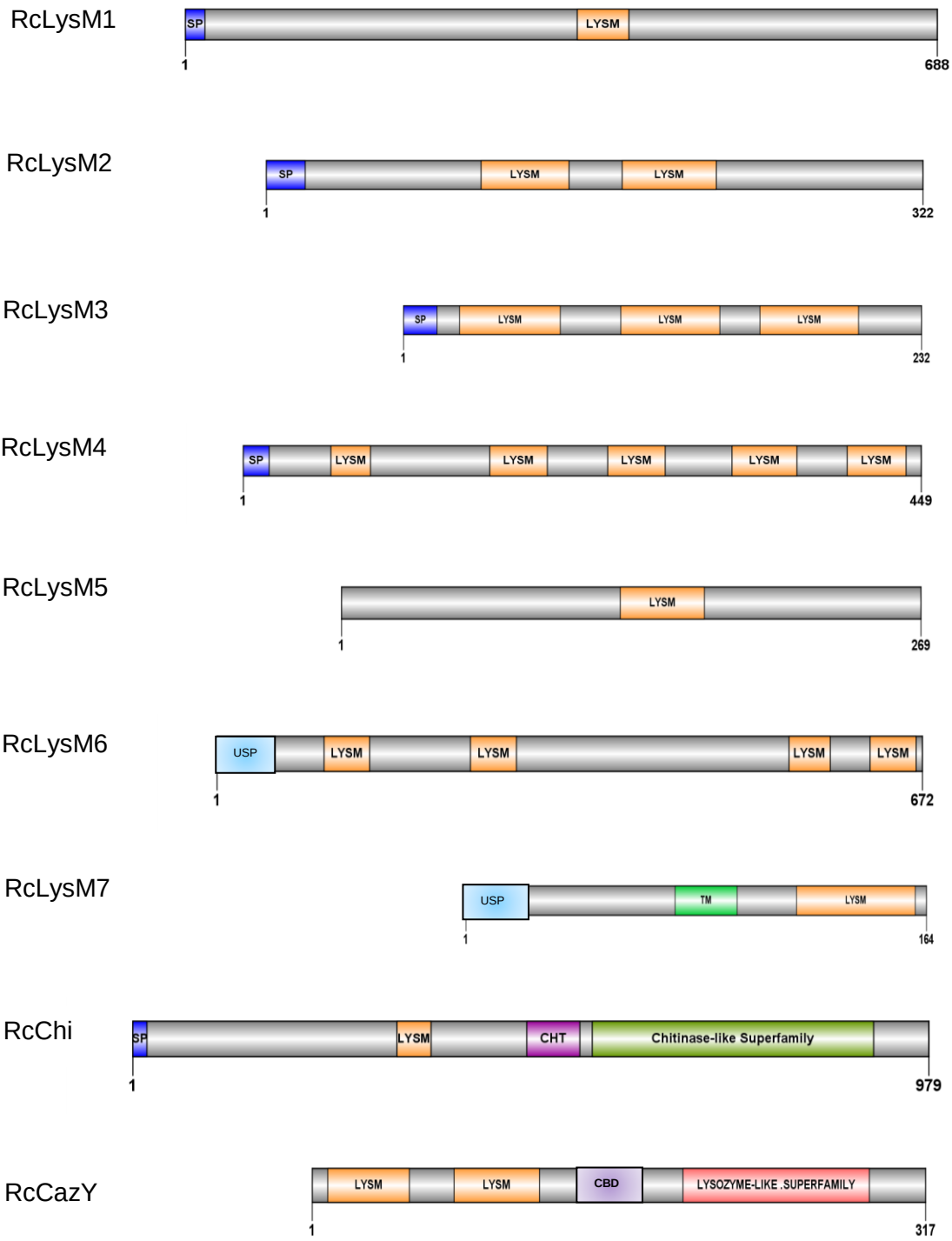

Figure 4.1: Schematic amino acid sequence diagrams of LysM domain proteins (not drawn to scale). Domains are highlighted as follows: LysM, orange; signal peptide (SP), blue; unconventional signal peptide (USP), light blue; transmembrane (TM), green; chitin binding domain (CBD), purple; Chitinase-like superfamily, yellow-green; and Lysozyme like superfamily, red.

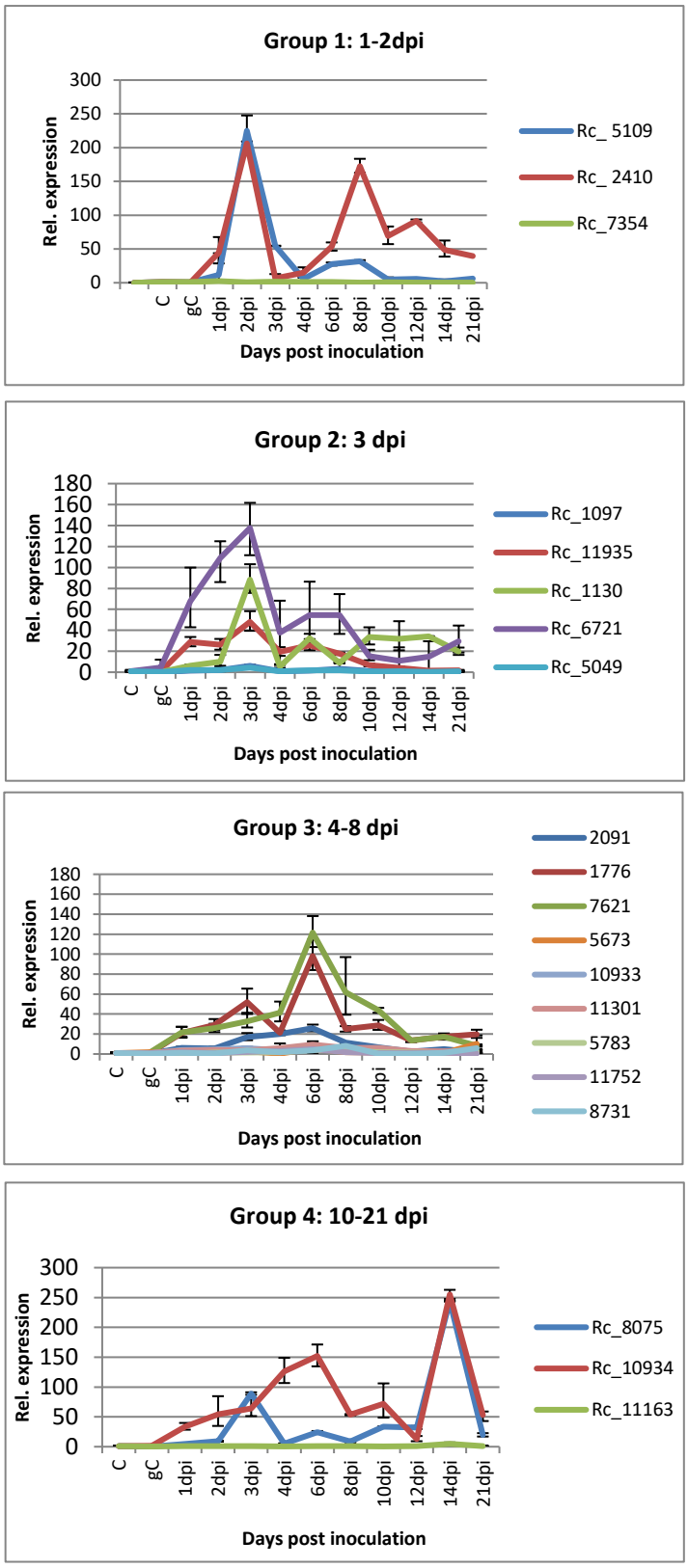

Figure 4.2: Relative transcript abundance of *Rhynchosporium commune* candidate effectors during infection of barley with *R. commune* strain L2A. Error bars indicate confidence intervals of the 3 technical repetitions.

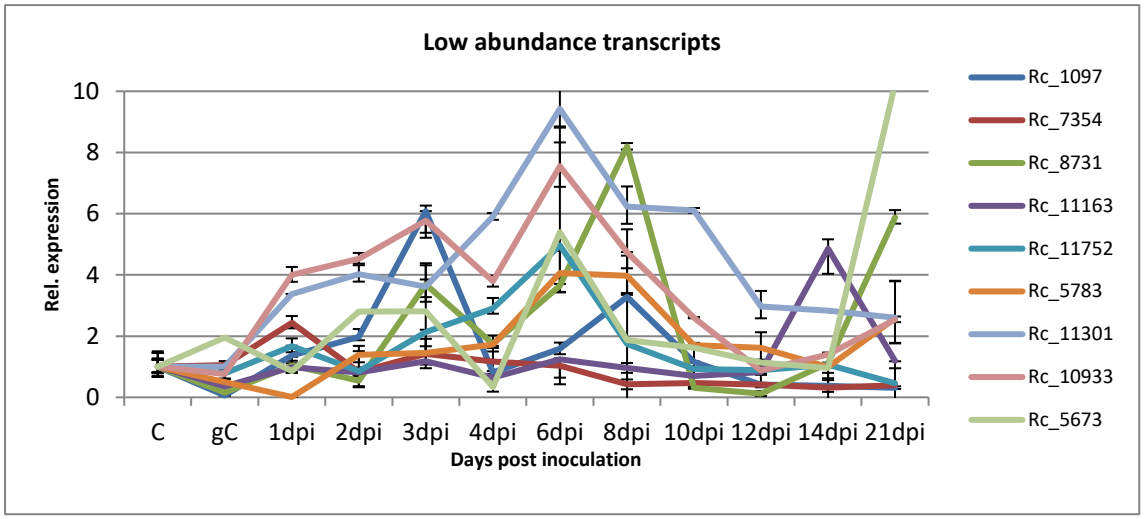

Figure 4.3: Relative expression of low abundance transcripts of *Rhynchosporium commune* candidate effectors during infection of barley with *R. commune* strain L2A. Error bars indicate confidence intervals of the 3 technical repetitions. Rc_7354 - Group1, 1-2 dpi; Rc_1097 – Group 2, 3dpi; Rc_5783, Rc_11301, Rc_10933, Rc_5763, Rc_8731 & Rc_11752 Group 3, 4-8dpi; Rc_11163 Group 4, 10-21dpi.

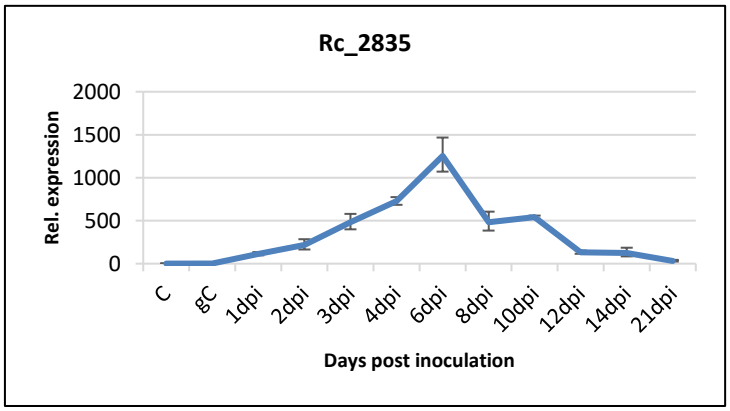

Figure 4.4: Relative transcript abundance of *Rhynchosporium commune* candidate effector Rc_2835 during infection of barley with *R. commune* strain L2A. Error bars indicate confidence intervals of the 3 technical repetitions.

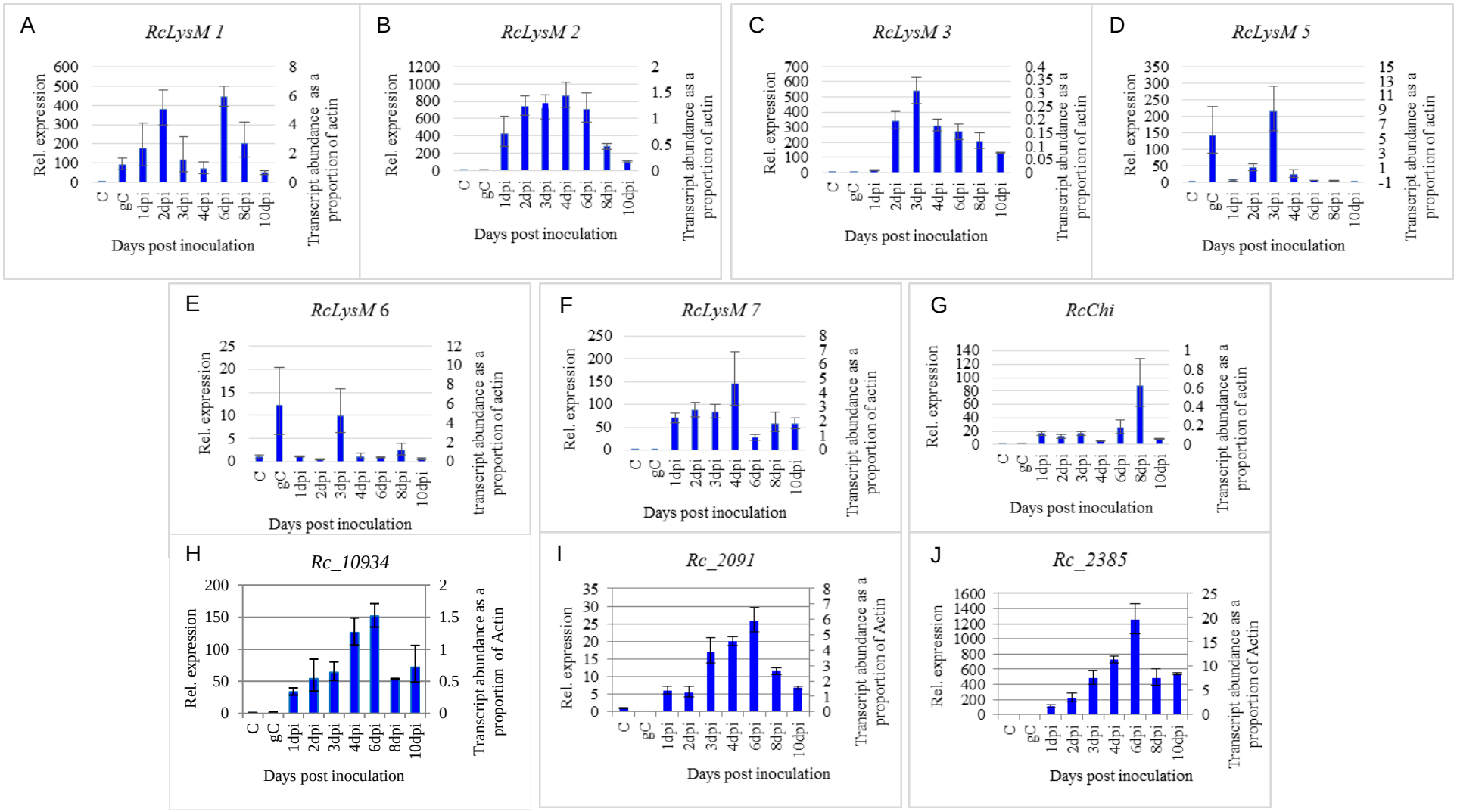

Figure 4.5: Relative expression of selected *Rhynchosporium commune* genes during infection of susceptible barley cultivar Optic with *R. commune* strain L2A. Error bars indicate the confidence interval for the average of technical repetition.

To finalise the research of effector identification, apoplastic fluid extracted from the barley leaves inoculated with *R. commune* strain L73A was analysed to confirm the presence of candidate effector proteins during a susceptible interaction. Two time points were selected: 4 dpi, which represents the initial colonisation of the apoplast, and 7 dpi, in which growth of the fungus would be well developed.

Plant cell wall degrading enzymes (CWDEs) were the most highly abundant proteins in the apoplast during infection. Enzymes involved in the breakdown of xylan (*Rc_07824*), lignin (*Rc_07699*), pectin (*Rc_03266*) and cellulose (*Rc_00972*) were identified (Table 4.4). This was not surprising as CWDEs play a significant role in pathogenesis with the ability to depolymerize the main structural polysaccharide components of the plant cell wall (Kubicek *et al*., 2014). Furthermore, amongst the most abundant proteins was a putative glucose-methanol-choline (GMC) oxidoreductase which has been suggested to be a lignocellulose acting enzyme (Couturier *et al*., 2015). Two different types of proteases were identified, a serine type carboxypeptidase and a subtilisin like protease (Table 4.4). In many cases, proteases are considered to be virulence factors of many pathogenic species (Hoge *et al.,* 2010).

Similar to many plant pathogens, *R. commune* secretes a probable catalase peroxidase at both 4 and 7 dpi with a high up-regulation of the protein at the latter time point of infection (Table 4.3). The importance of catalase peroxidases to circumvent the effects of plant defence have been highlighted in numerous studies (Zámocký *et al*., 2009). Catalase-peroxidase proteins are known to detoxify the products of the oxidative burst in the apoplast upon the triggering of plant immunity. Tanabe *et al.* (2011) demonstrated that one of the three catalase peroxidase genes identified in *Z. tritici* plays an important role in pathogenicity. MgDCat-1 is also upregulated during infection and is most abundant at 8 dpi.

Although none of the candidate effectors from the research described in this chapter were detected in apoplastic fluid from barley leaves infected with *R. commune*, proteomics analysis identified four other potential effectors. These included two proteins which had been previously highlighted as candidate effectors but not in the original panel: *Rc07_03591,* which showed homology to an effector like protein from powdery mildew; *B. graminis f. sp. Hordei*; and *Rc07_02334,* a hypothetical fungal protein from the anamorphic fungus *Glarea lozoyensis*. The best BLASTp hit for *RC07_10338* was to EC13 protein from anthracnose leaf spot which has been shown to be expressed during the establishment of biotrophic hyphae (Kleemann *et al*., 2008). Lastly, protein *Rc_LysM3* which contained three LysM domains was also identified. Interestingly LysM domain proteins have been well characterised in several plant pathogens and shown to play a fundamental role in fungal pathogenesis (Kombrink, 2013).

Candidates *Rc_10934, Rc_2091* and *Rc_2835* were selected in the attempt to knock out the gene of interest and obtain a phenotype. Each candidate knockout was attempted three times and resulted in the creation of between 60 to 100 transformants for each attempt. Amplification and sequencing of the deletion cassette from the DNA extracted from the transformants confirmed its

successful integration into the fungal genome. Primers were used to amplify the deletion cassette and determine if the original gene was disrupted. A typical result of genotyping of the transformants is detailed in Figure 4.6. No candidate effector genes were knocked out and time restrictions limited any further continuation of the approach.

To confirm the chitin binding prediction RcLysM3 protein tagged with V5 peptide at the C terminus to allow detection, was produced in *P. pastoris* and affinity binding to a range of polysaccharides was examined. RcLysM3 co-precipitated with crab shell chitin and, interestingly, with chitosan but not with any of the plant cell wall polysaccharides, xylan or cellulose (Figure 4.7). RcLysM3 was also identified as a potential avirulence protein. A change in an amino acid at position 67 from a glutamic acid (Q) to glutamine (E) within the protein sequence of RcLysM3 was identified that correlated with a change in virulence/avirulence of 9 sequenced *R. commune* strains on cultivar La Mesita (Table 4.5). Isolate L43D carrying the E allele was avirulent on cultivar La Mesita. A detached leaf assay confirmed the lack of macroscopic symptoms. RcLysM3 sequence was analysed in a further four isolates L101B, L90A, L43A and L43B. Both L101B and L90A contained the Q allele whereas L43A and L43B contained the E allele (Table 4.5). The latter two were isolated from the same plant and are possibly the same strain as L43D. While both L101B and L90A isolates containing the Q allele were virulent on La Mesita in line with Q allele being a virulent allele, virulence testing of the isolates L43A and L43B contained the E allele on La Mesita still needs to be conducted to determine if the correlation is valid for these isolates.

To identify novel resistance, detached barley leaves were inoculated with a conidial suspension of *R. commune* to obtain a phenotype of each barley line. A total of nine sequenced strains were used to infect a set of barley lines containing the resistance genes *Rrs3, Rrs4* and *Rrs13*. Each assay included a very susceptible cultivar Optic as a control to determine isolates' aggressiveness. Inspection of lesion formation began around 10 dpi and lesion measurements continued until 21 dpi. All barley lines tested including Abyssinian (*Rrs3*), CI11549 (*Rrs4*) and BC Line30 (*Rrs13)* were shown to be susceptible to L77 and AU2 which were the most virulent in comparison to other strains (Table 4.5). Strain AU2 caused early lesion development, susceptible barley lines inoculated with strain L77 also developed lesions quickly and produced symptoms that were comparable to the highly susceptible control Optic, although lesions did take longer to develop on CI11549 which contains the Rrs4 gene. In contrast strains UK7, L32B, L43D, L73A and 214-GFP caused no lesions on barley plants containing *Rrs3* or *Rrs4* and *Rrs13* (Table 4.5). The lack of lesions may indicate that these strains contain avirulence genes recognised by *Rrs3* or *Rrs4* and *Rrs13,* or the presence of extra resistance to these strains which can be further assessed.

Table 4.4 Mass spectrometry apoplastic proteins identified, top BLASTp match and predicted biological function.

Protein Id	Best BLASTp match	Species	Biological function
RCO7_03591	CELP0025 Effector like protein	*Blumeria graminis f. sp. hordei*	unknown
RCO7_11633	subtilisin-like protease	*Colletotrichum incanum*	proteolysis
RCO7_07041	catalase/peroxidase HPI	*Phialocephala scopiformis*	response to oxidative stress
RCO7_07699	putative glucose-methanol-choline oxidoreductase	*Diaporthe ampelina*	oxidation-reduction process
RCO7_10338	EC13 protein	*Colletotrichum higginsianum*	unknown
RCO7_09037	serine-type carboxypeptidase F	*Aspergillus udagawae*	proteolysis
RCO7_04918	putative glycosyl hydrolase family 43	*Colletotrichum sublineola*	carbohydrate metabolic process
RCO7_02661	putative cell wall-associated hydrolase	*Marssonina brunnea f. sp. 'multigermtubi'*	unknown
RCO7_07332	carbohydrate-binding module family 6 protein	*Bipolaris zeicola 26-R-13*	unknown
RCO7_10779	GPI-anchored cell wall beta-endoglucanase	*Marssonina brunnea f. sp. 'multigermtubi'*	carbohydrate metabolic process
RCO7_00972	Glycosyl hydrolase family 6, cellulase	*Glarea lozoyensis ATCC 20868*	carbohydrate metabolic process
RCO7_10679	putative exopolygalacturonase B	*Marssonina brunnea f. sp. 'multigermtubi'*	carbohydrate metabolic process
RCO7_07191	putative glycoside hydrolase family 7 protein	*Botrytis cinerea BcDW1*	carbohydrate metabolic process
RCO7_03266	pectin methyl esterase	*Marssonina brunnea f. sp. 'multigermtubi'*	cell wall modification
RCO7_02334	hypothetical protein GLAREA_02918	*Glarea lozoyensis ATCC 20868*	unknown
RCO7_01317	hypothetical protein MBM_04331	*Marssonina brunnea f. sp. 'multigermtubi'*	unknown
RCO7_07824	putative endo-1,4-beta-xylanase B	*Pyrenochaeta sp. DS3sAY3a*	carbohydrate metabolic process
RCO7_07974	hypothetical protein V499_03635	*Pseudogymnoascus sp. VKM F-103*	unknown
RCO7_11478	Zn-dependent exopeptidase	*Glarea lozoyensis ATCC 20868*	proteolysis
RCO7_03061	fermentation associated protein	*Marssonina brunnea f. sp. 'multigermtubi'*	fermentation

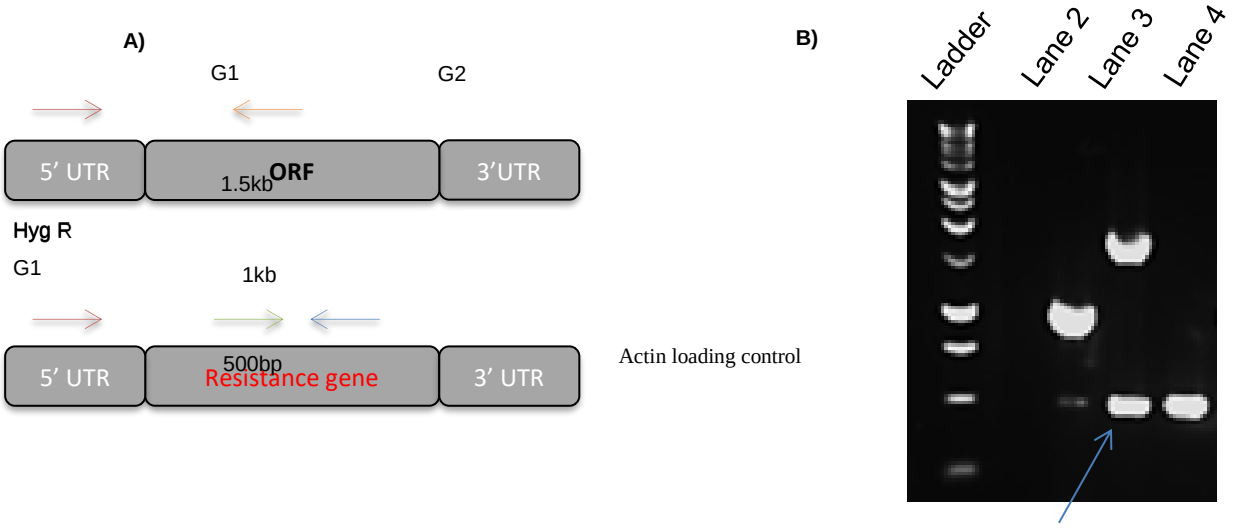

Figure 4.6: Genotyping of *Rhynchosporium commune* transformants. A) Primer locations used for genotyping strategy to determine targeted gene disruption and hygromycin resistance gene insert. Red_5' UTR G1. Orange- G2, wild type ORF reverse. Green hygromycin forward, Blue hygromycin reverse. B) PCR. Lane 1: 1kb ladder. PCR products produced using Lane 2: Hygromycin F&R primers. Lane 3: G1 &G2 primers – amplification of wild type. Lane 4: No amplification with G1and HYG R primers. Amplification of actin was used as a loading control.

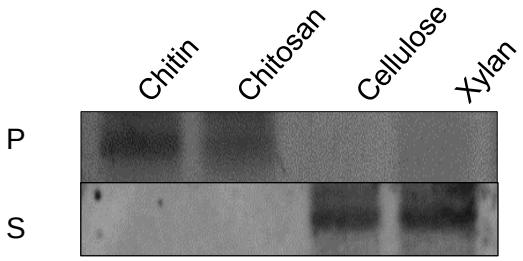

Figure 4.7 Protein gel showing RcLysM3-V5 protein co-precipitating in the pellet (P) of chitin and chitosan, but only present in the supernatant (S) of cellulose and xylan.

Table 4.4: Correlation of the Gln (E) and Glu (Q) allele with the virulence and avirulence of *Rhynchosporium commune* isolates on barley cultivar La Mesita.

	***R. commune* isolates**												
	13-13	214	L2A	L32 B	L43 D	L73 A	L77	UK7	AU2	101 B	90 B	L43 A	L43 B
La Mesita	V	V	V	V	A	V	V	V	V	V	V	?	?
RcLysM3 allele	Q	Q	Q	Q	E	Q	Q	Q	Q	Q	Q	E	E

Barley lines containing *Rrs4* and *Rrs13* showed a moderately high level of resistance in terms of lack of lesion formation to 5 out of 7 *R. commune* strains. Further analysis to determine how the fungus proliferates during asymptomatic infection was conducted using *R. commune* strain 214-GFP. Growth after 10 dpi was investigated to determine the extent of the mycelial network. In comparison to the susceptible barley line the amount of growth at 10 dpi was much less for BC Line30 carrying the *Rrs13* resistance gene (Figure 4.8 D). Although the growth was less, it followed the same pattern of growth as seen in a susceptible cultivar (Figure 4.8 H). Despite the fact that pathogen growth on *Rrs4* line Cl11549 was evident, the type of growth differed. Instead of the mycelium forming lines between the epidermal cells, the fungal growth was random. The mycelium did not travel far from the inoculation spot suggesting line Cl11549 is resistant to strain 214 (Figure 4.8 B).

Table 4.5: Virulence testing results of barley lines containing Rrs3, Rrs4 & Rrs13 resistance genes inoculated with *Rhynchosporium commune* strains L32B, L43D, L73A, L77, UK7, AU2 & 214-GFP.

Barley *R* gene	***R. commune* isolates**						
	L32B	**L43D**	**L73A**	**L77**	**UK7**	**AU2**	**214-GFP**
Rrs3							
	A	**A**	**A**	**V**	**A**	**V**	**A**
Rrs4							
	A	**A**	**A**	**V**	**A**	**V**	**A**
Rrs13							
	A	**A**	**A**	**V**	**A**	**V**	**A**

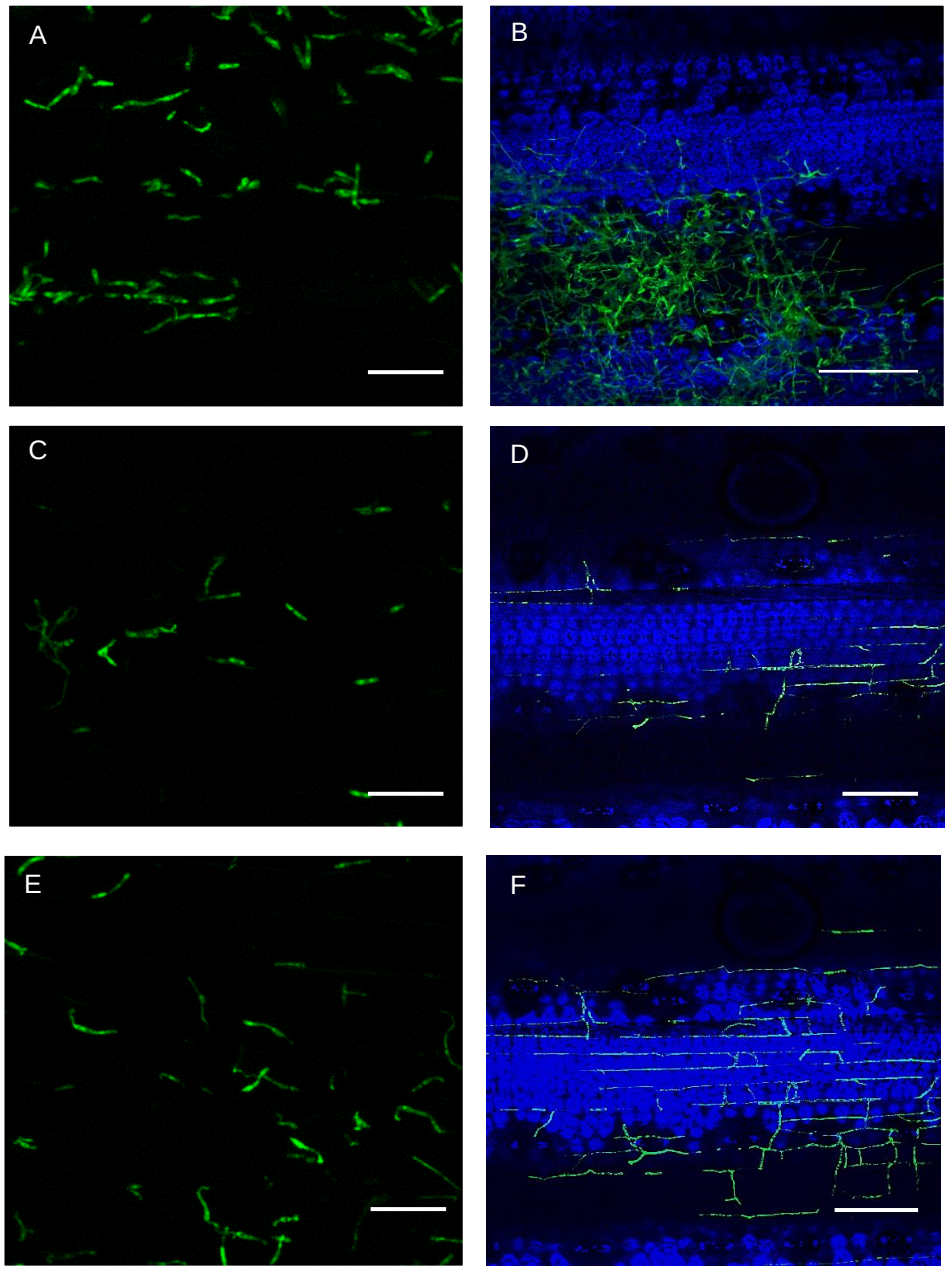

Figure 4.8: Confocal LASER microscopy images of *Rhynchosporium commune* strain 214-GFP infection on: A & B CI11549; C & D BC Line 30; E & F Optic. Scale bars A, C, E = 50µm & B, D, F = 100 µm.

In addition, a further two barley lines were analysed for asymptomatic growth. Syrian landraces were used to look at response to infection and were included in this research as they are genetically more diverse than cultivated barley which increases the chance of finding novel barley resistance. It was evident that the interaction between SLB 66_024 (unknown *R* gene) and 214-GFP was not compatible. The early stages of growth showed a similar pattern to a resistant line (Figure 5.2 B) and although there was quite a substantial amount of growth at 21 dpi, the mycelium did not grow along the epidermal cell walls (Figure 4.9 C). Instead, the growth was randomly dispersed. In contrast, growth of 214-GFP on SLB67-015 (unknown resistance) was established after 8 dpi (Figure 4.9 E) and continued throughout the assay resulting in a bidirectional mycelial

growth out with the inoculum spot (Figure 4.9 F). The pattern of growth was similar to a susceptible interaction.

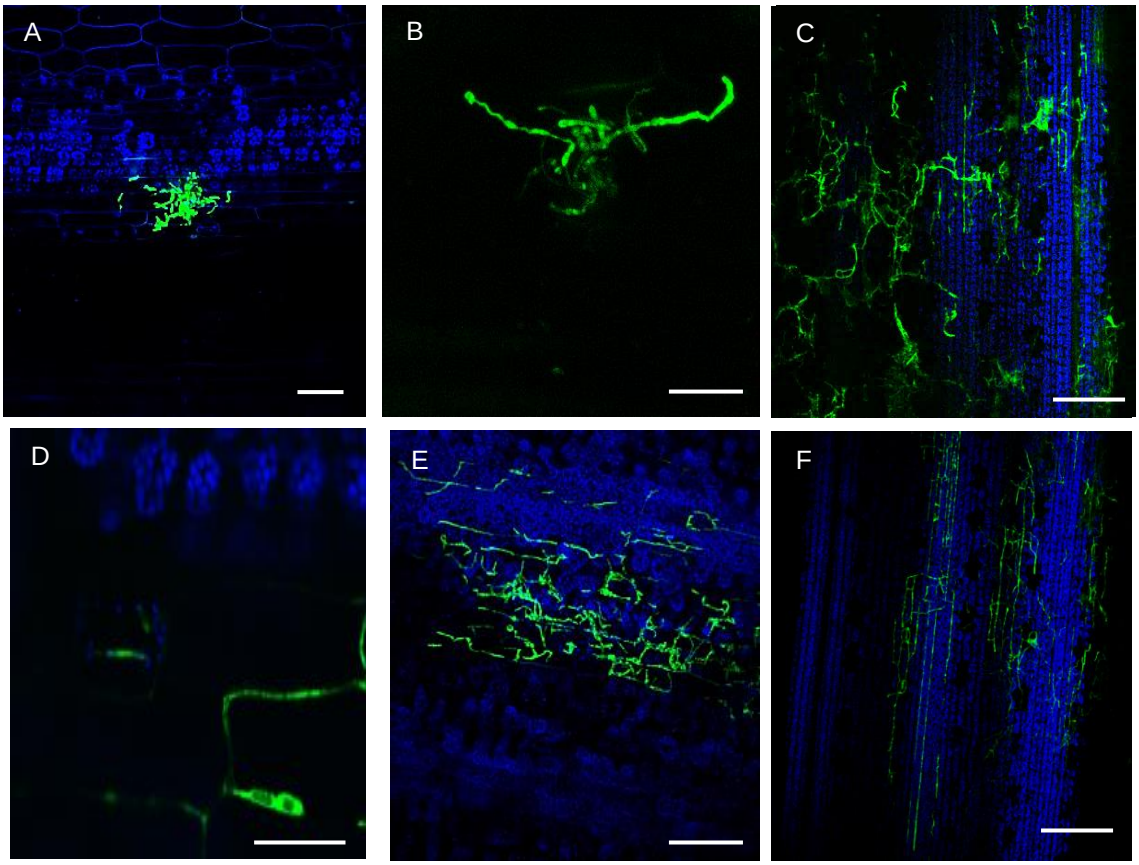

Figure 4.9 Confocal LASER microscopy images of *Rhynchosporium commune* strain 214-GFP infection on SLB 66-024 A-B and SLB 67-015 C-D at 3 dpi, 8 dpi and 21dpi. Scale bars A & B = 50µm, C, E, F = 100µm, D = 25µm.

Although microscopy can distinguish between lack of growth and the presence of morphological differences, to gain a better understanding of the molecules involved in resistance to *R. commune*, a quantitative proteomics approach was taken to determine the change in abundance or absence of proteins. Three biological experiments were used for the extraction of infected and uninfected apoplastic fluid. The infection of inoculated cultivars was analysed using *R. commune* strain 214-GFP. Leaf samples were viewed under a confocal microscope before taking samples for apoplastic extraction to confirm colonisation of the leaves of susceptible cultivars Optic and Atlas and restricted growth on the leaves of resistant cultivar Atlas 46. Growth of 214-GFP was as expected (Thirgnanasbanadam *et al.,* 2011) – Optic contained the highest level of colonisation whereas resistant Atlas 46 showed very restricted growth with random colony morphology, growth was identified on Atlas but not to the extent of Optic.

The intensity of each of the proteins was compared in 3 different cultivars, highly susceptible Optic, Atlas 46 which contains the *Rrs1* and *Rrs2* gene and the NIL Atlas which does not contain the *Rrs1* gene, uninfected and infected with *R. commune* strain 214-GFP at 4 dpi and 7 dpi. Four

proteins were highly abundant and showed a distinct increase in infected apoplastic fluid of Atlas 46 at 4 dpi. The remaining six proteins were most highly expressed in 7 dpi infected apoplastic fluid. This included an α-L-arabinofuranosidase involved in cell wall reorganisation which has been suggested as a putative defence related protein and was highly abundant in Atlas46 at 4 dpi infected apoplastic fluid (Figure 4.10). The protein has also been found in pathogens to aid with plant cell wall breakdown (Morant *et al*., 2008). In addition, pathogenesis related (PR) proteins which are well known to participate in complex plant defence responses to pathogens were also identified including glucan endo-1,3-beta-glucosidase- PR2 playing a role in the hydrolysis of fungal cell walls (Figure 4.10).

To investigate the possibility of *R. co*mmune growth on other plant species with the absence of any visual disease symptoms, *R. commune* inoculations were carried out on the model plant species *N. benthamiana*. Drop inoculations of spores from *R. commune* strain 214-GFP was carried out on leaves of *N. benthamiana* plants.

N. benthamiana plants with plasma membrane protein tagged with a red fluorscent protein were used to determine if any signs of damage were occurring inside the leaf tissue. At 5 dpi, microscopic anlaysis of *R. commune* revealed the germination of fungal conidia. By 9 dpi fungal mycelium had started to develop and the growth of the fungus from the original inoculation spot had increased. At 15 dpi there was a noticable increase in the amount of mycelium. From this point and until the last day of analysis the fungal mycelium did not grow in the same manner as it would on its host barley, outlining the epidermal cells. In fact the growth resembled that of an incompatible infection on barley with explorative hyphae growing in all directions (Thirugnanasambandam *et al*., 2011). The spread of the fungus did persist over time resulting in a sizeable colony by 28 dpi (Figure 4.11 A-C). The plant showed no evidence of plasma membrane deterioration, as would be seen during the late stages of infection in barley. The plant plasma membrane was unimpaired which was clearly evident at the later time point, 28 dpi (Figure 4.11). Throughout the entire experiment no macroscopic signs of infection were visible.

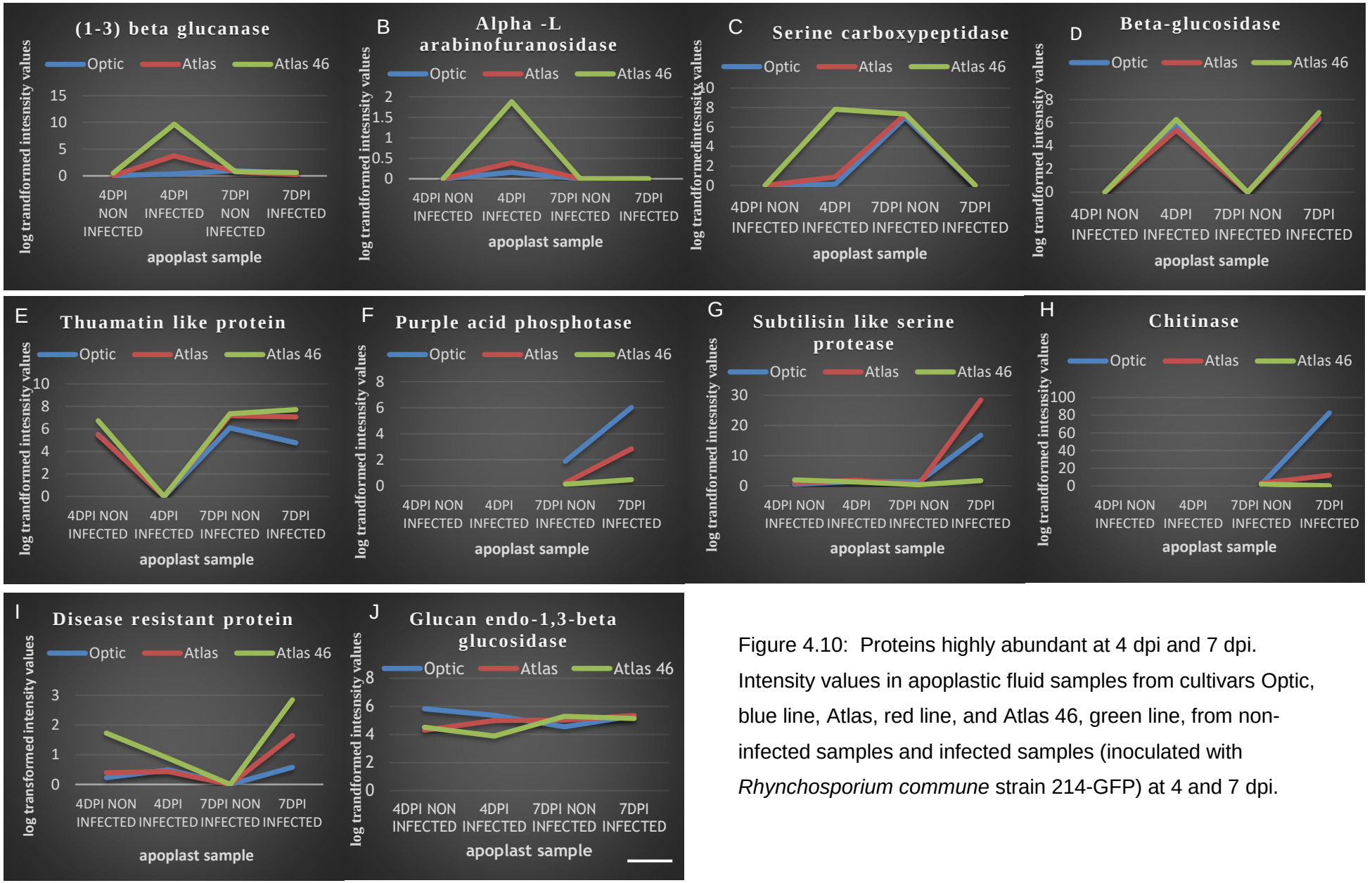

Figure 4.10: Proteins highly abundant at 4 dpi and 7 dpi. Intensity values in apoplastic fluid samples from cultivars Optic, blue line, Atlas, red line, and Atlas 46, green line, from non-infected samples and infected samples (inoculated with *Rhynchosporium commune* strain 214-GFP) at 4 and 7 dpi.

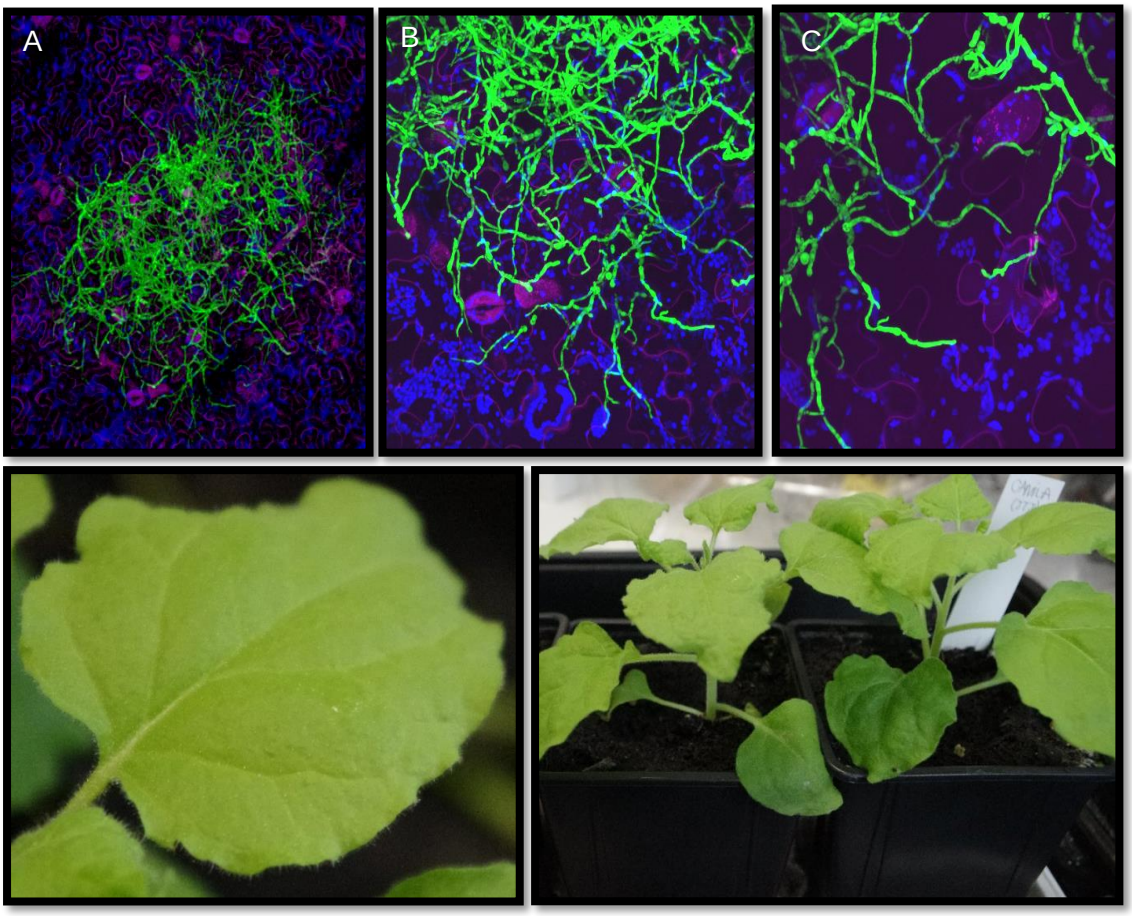

Figure 4.11: A-C Confocal microscopy images of *Rhynchosporium commune* strain 214-GFP on *Nicotiana benthamiana* line CB173 expressing red plasma membrane at 28 dpi. Orange arrow shows intact plant plasma membrane. *N. benthamiana* plants inoculated with *R. commune* strain 214-GFP D) at 9 dpi and E) 28 dpi, showing no macroscopic symptoms. Scale bars = 25 µm.

5. Discussion

As the global population increases rapidly, agriculture struggles to maintain the levels of crop production required for the immense rise in food demands. Plant pathogens have a high capacity to cause substantial disease levels on food crops, reducing the production and quality of food. Hence, greater emphasis to reduce the impact of crop disease is required. In many cases chemical treatments to limit or eradicate diseases are used, however the environmental impact of the applications can result in consequences to non-target organisms, pesticide drift and residues on food (Kilbrew & Wolff, 2010). Agriculture is faced with the challenge to maximise crop yields while decreasing negative environmental impacts. However, several factors influence the reduction of food security imposed by pathogens. The lack of well-developed diagnostic tools to identify asymptomatic pathogen infection can lead to severe disease implications later in the growing season. In addition, the level of disease severity can be overlooked due to subjective rather than quantitative methods

to detect pathogen biomass accumulation. Furthermore, experimental obstacles preventing the mapping and cloning of plant resistance genes in conjunction with the variation and vast amounts of evolving pathogen molecules, results in the lack of complete understanding of the mechanisms of resistance and pathogen infection. Therefore, the development of methods to identify pathogens, experimental research to gain an understanding of how the pathogen infects and the molecules involved in plant defence against pathogens will result in better understanding of how we can improve methods for diagnostics and predicting crop resistance durability.

As pathogens are known to use effector molecules to overcome plant resistance (Dangl & Jones, 2006), this study began with the exploitation of the genome and transcriptome sequences of *R. commune* to identify novel candidate effectors. The importance of effector discovery is high as the research into *R. commune* effector repertoire is still in its infancy. The expression profiling was effective in determining the timing and levels of gene expression that can be used to indicate the involvement of candidate effectors in pathogenesis. Extremely low efficiency of targeted gene disruption in *R. commune* limited the possibility of functional characterisation. However new approaches are now being developed in fungi, which can be used in the future (Matsurura *et al*., 2015). The identification of RcLysM3 was an important discovery, indicating its high abundance within the apoplast. Further characterisation revealing chitin binding abilities and avirulence correlation indicates an essential nature for this protein. Revealing that effectors are essential for pathogenicity and potentially recognised by the host plant (Avr genes) is an important factor. Barley *R* gene resistance to *R. commune* has not proved durable; therefore the discovery of novel avirulence genes that are required for pathogenicity is a critical step to identify more durable forms of resistance to this devastating fungal disease.

Despite the identification of *AvrRrs1* recognition by the *Rrs1* over 20 years ago (Rohe *et al*., 1995), there is very little information on the intricate molecular mechanisms that occur in a resistant response. To characterise other types of resistance to *R. commune,* cultivars were selected containing different *R* genes to that of *Rrs1*. The virulence testing approach helped to prioritise barley lines for further analysis using the 214-GFP strain. Microscopic assessment of the extent of the growth and the colony morphology were used to distinguish between susceptibility and potential resistance to *R. commune*. This is one of the characteristics of *Rrs1* containing plants that has been previously highlighted. Although barley lines presenting no symptoms and a decrease in biomass, physically restricted growth and / or random colony morphology could be a sign of resistant interaction, it still remains difficult to determine the durability of the plant defence. It is possible that some *R. commune* strains develop much slower throughout the growing season but their accumulation in barley leaves may still have an impact on the crop yield although no research has looked into this possibility. In addition, a range of *R. commune* strains need to be used to distinguish the level of asymptomatic infection. The production of some other fungal strains expressing fluorescent proteins would be highly beneficial for future research, especially for highly virulent stains such as AU2. Partial resistance could also be potentially at play, as it is also characterised by

reduced growth of the pathogen. Again, there is a need to gain a better understanding of this type of resistance.

Due to the lack of evidence to allow full confirmation of resistant lines, a proteome approach to identify the key players in *Rrs1* resistance was conducted. Initial research began on the contents of apoplast and its importance in plant pathogen interactions was identified almost 30 years ago. However, only a few studies have focused on plant-pathogen interactions in the apoplast (Mehta *et al.*, 2008). The identification of known defence proteins present in all of the cultivars used indicated similar components of a basal defence mechanism. However, the abundance of defence proteins in the resistant line was slightly higher. Only the disease related protein and α-L arabinofuriodase were highly upregulated in comparison to Optic and Atlas suggesting a specific role in the *Rrs1*-controlled resistance. Some proteins which were down regulated in Atlas 46 might represent susceptibility factors for disease and therefore were not upregulated in a resistant response. Only one biological repetition was available for analysis due to the inefficient labelling, other repetitions would be required to provide rigidity to the results. Although the extraction of the apoplastic fluid is relatively laborious and in some cases protein identification can be limited, this work has identified some important plant molecules that could be further analysed for their potential use as markers of barley resistance to *R. commune.*

Major *R* gene resistance is an important factor of sustainable agriculture, although protection against several strains of a pathogen may be incompletely effective. However, the use of *R* gene pyramids may provide an alternative and more effective strategy to control various *R. commune* pathotypes (Zhan *et al*., 2012). More recently the investigation of NHR has become more prominent in the literature. NHR is only beginning to be understood but in contrast to major *R* gene resistance the response involves multiple pathways (Gill *et al.,* 2015) and is known to provide simultaneous resistance to many pathogens. The results of this research have indicated *N. benthamiana* as a non-host. Furthermore, the inability of *R. commune* to infect non-grass species highlights the importance of crop rotations for prevention of the build-up of the pathogen inoculum in the field. Further identification and characterisation of components of NHR will provide an effective alternative for the future development of crops with a wide range of more durable resistance.

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