Towards the identification of novel sources of resistance in barley to *Rhynchosporium commune.*

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Declaration of Authorship

This thesis has not been submitted in whole or in part to this or any other university for any degree and is original work of the author except where otherwise stated.

Signed: 

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Abstract

The deployment of resistance genes (R genes) in breeding programmes has been shown to be a useful strategy to reduce farmer's reliance on fungicides and offset the constant disease pressures on Ireland's primary crops. However, the durability of varieties with R genes can be tenuous due to the ability of fungal pathogens to rapidly evolve and overcome the resistant phenotype. Recently, the use of susceptibility genes (S genes) has emerged as a viable alternative. While the products of S genes can be exploited by pathogens to promote infection, crops with specific, non-functional S genes have reported strong pathogen resistance. Barley is the primary Irish tillage crop and current varieties are susceptible to the pathogen *Rhynchosporium commune.* The goal of this project was to investigate the barley – *R. commune* interaction and identify and characterise the activity of candidate barley S genes. To achieve this, an *in silico* analysis first identified 682 potential barley orthologues to known S genes. In parallel, the temporal transcriptomic response of barley to two diverse *R. commune* isolates identified 245 of these genes associated with a response to *R. commune*. Three of these genes were brought forward with the aim of validating functionality via transient knockdown. This project has delivered important insights into early signalling events in barley in response to *R. commune* and provided a fundamental dataset on the transcriptomic response of barley after *R. commune* infection that will support future investigations into the barley x *R. commune* interaction.

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1 Introduction

1.1 Importance of Barley in Ireland

Barley is the most important cereal crop in Ireland, grown on over 193,000 hectares (ha), producing 1,430 thousand tonnes of grain to the marketplace (CSO, 2021). The primary use of this grain is in animal feed production (87%), and the remainder is used by the malting industry and in food production (Teagasc, 2017). Spring barley accounts for 1,004,000 tonnes produced in Ireland, with winter varieties accounting for the remaining 426,000 tonnes. While winter varieties are higher yielding (8.3 tonnes/ha in 2020) due to their longer growing season compared to spring varieties (7.1 tonnes/ha in 2020) (CSO, 2021), spring varieties are often favoured due to lower costs associated with growing.

Winter barley requires a vernalisation period to initiate flowering, and as such, it is usually sown in late autumn. However, this can be a challenge as poor weather conditions can affect soil quality during the sowing season. Additionally, winter barley is at higher risk of diseases due to weather conditions, which are favourable to pathogens. Therefore, winter barley requires higher fungicide inputs compared to spring sown barley, which progresses more rapidly through the growth stages. A two-spray programme at half rates has been recommended for spring barley while winter barley requires a third application before flag leaf emergence (Collins and Phelan, 2020). Despite savings on materials costs, there are also challenges associated with spring barley such as delay to harvesting in the autumn due to inclement weather, need for higher seeding rates and ultimately a lower yield potential (Collins and Phelan, 2017).

While Ireland has a cool temperate climate and long day length suitable for barley growth, it is also an ideal environment for many barley pathogens including barley scald (*Rhynchosporium commune)*, ramularia (*Ramularia collo-cygni),* net blotch (*Pyrenophora teres),* and powdery mildew (*Blumeria graminis f. sp. Hordei, Bgh.)* (Walters et. al., 2012). Barley scald is one of the most significant diseases, present in all barley growing regions worldwide with the potential to reduce grain quality and induce yield losses of up to 40% (Xi

et. al., 2000). In Ireland, yield loss due to leaf scald of over 10% has been previously reported (Dunne, 2001).

1.2 Plant immunity

To protect against the range of pathogens they are exposed to, plants have developed a two-part innate immune system (Jones and Dangl, 2006). The first line of active defence occurs on the plant cell surface. Pattern-recognition receptors (PRRs) in the cell membrane are able to detect pathogen associated molecular patterns (PAMPs). This recognition event initiates signal transduction pathways and results in the onset of a defence response. PRRs are usually receptor like kinases (RLKs) or receptor like proteins (RLPs). PAMPs include highly conserved pathogen molecules such as bacterial flagellin or the fungal cell wall component chitin (Macho and Zipfel, 2014, Spoel and Dong, 2012). For example, in the model plant *Arabidopsis thaliana*, *CHITIN ELICITOR RECEPTOR LIKE KINASE* 1 (*CERK1*) encodes a PRR for chitin recognition (Miya et al., 2007). PRR-based recognition of these conserved molecular patterns activates PAMP triggered immunity (PTI). PTI responses include ion fluxes, production of reactive oxygen species (ROS), activation of downstream mitogen-activated protein kinase (MAPK) cascades, and the activation of immune hormone signalling pathways, such as salicylic acid (SA), jasmonic acid (JA) or ethylene signalling. In response to SA signalling in particular, the production of anti-microbial and pathogenesis related (PR) proteins contributes to protecting the plant against host and non-host pathogens (Spoel and Dong, 2012).

Adapted pathogens can bypass PTI through the delivery of effector proteins into the plant cells. Effector proteins can act in the cell cytoplasm, or in the apoplastic space, as well as in organelles including the nucleus, to supress the plant immune response. Effectors have been identified that facilitate different stages of infection, including effective penetration of host cells, inhibition of PRR activity and downstream PTI responses (Jones and Dangl, 2006). This is referred to as effector triggered susceptibility (ETS). The second line of plant immunity involves the detection of these effector proteins via intracellular immune resistance proteins (R proteins) and activation of so-

called effector triggered immunity (ETI). Most R proteins are nucleotide-binding leucine-rich-repeat containing proteins (NLR proteins), of which there are three functionally distinct groups: those with an N-terminal domain consisting of a coiled coil (CNL), those with an N-terminal Toll/Interleukin-1 receptor (TNL) and those with a RPW8-like CC domain (RPW8) (RNL) (Han, 2019). ETI is an accelerated and stronger PTI response and often (but not necessarily) culminates in a hypersensitive response (HR), which is characterised by programmed cell death of the infected cells (Jones and Dangl, 2006, Spoel and Dong, 2012). HR is also associated with the transfer of defence signals to uninfected neighbouring cells and organs resulting in a distal immune response referred to as systemic acquired resistance (SAR).

The Flor (1971) gene-for-gene model proposes that for each gene that initiates an immune response in the plant, there is a corresponding avirulence (Avr) gene in the pathogen. Avirulence genes usually encode for secreted proteins essential for pathogenicity (effector proteins), expressed at different stages of infection. In the gene-for-gene model, plants activate an immune response upon R protein mediated detection of a pathogen Avr product. Many models have been suggested regarding the underlying R protein perception of effectors. R proteins may directly recognise effectors, guard other effector targets and initiate response as a result of perturbations in these 'guardee' proteins, or act as decoys to prevent effectors from reaching their intended target (van der Hoorn and Kamoun, 2008).

The Guard Model proposes that R proteins 'guard' proteins that are targeted by pathogen effectors and react in response to pathogen induced changes in these 'guardee' proteins (Dangl and Jones, 2001, Mackey et al., 2003). For example, in *Arabidopsis*, AvrPphB SUSCEPTIBLE 1 (PBS1) is targeted by *Pseudomonas syringae* pv. *tomato* (*Pst*) effector AvrPphB. This effector binds to and cleaves PBS1, which then initiates RESISTANT TO *P. syringae* 5 (RPS5)-mediated resistance. In this case, RPS5 is the 'guard' protein, which initiates an immune response when its 'guardee' protein PBS1 is cleaved by the pathogen effector AvrPphB (Swiderski and Innes, 2001).

The decoy model proposes that so called ‘decoy’ proteins mimic effector targets to trap effectors in a recognition event (van der Hoorn and Kamoun, 2008). This model implies that the effector target guarded by R proteins is a decoy that mimics the operative effector target. For example, *Pst* effectors AvrPto and AvrPtoB target and inhibit RLKs involved in immune signalling. AvrPto and AvrPtoB are recognised by the tomato R protein PTO. PTO, when bound to a second host protein PRF, mimics these target RLKs, and on recognition of AvrPto and AvrPtoB initiate an immune response (Paulus and van der Hoorn, 2018).

The so-called ‘zig-zag’ model, proposed by Jones and Dangl (2006), pictures the events of plant microbe interactions and the resulting arms race (Figure 1.1), which comprises four phases. During the first phase, plant PRRs recognise PAMPs and initiate an immune response (PTI). The second phase is when the pathogen effectors bypass PTI and induce effector triggered susceptibility (ETS). Phase three only occurs when an effector is recognised by plant R proteins, resulting in ETI. As a result of increased selection pressures, the pathogen either sheds this recognised effector or expands its range of effectors, suppressing ETI and triggering ETS once more (Jones and Dangl, 2006). This in turn, drives the selection of novel host R-genes in the recognition of new pathogen effectors. This repetition results in a molecular ‘arms race’ between pathogen and plant (Andersen et al., 2016, Jones and Dangl, 2006).

A pathogen may also have more than one effector that evolves independently to alter the same host target through different mechanisms. In *Arabidopsis*, RPM1 INTERACTING PROTEIN 4 (RIN4), a regulator of both PTI and ETI, is targeted by at least two *Pst* effectors: AvrRtp2 and AvrRpm1. RESISTANCE TO *P. syringae* 2 (RPS2) and RESISTANCE TO *P. syringae* pv *maculicola* 1 (RPM1), two *Arabidopsis* R proteins, then detect effector induced perturbations of RIN4 and activate ETI (Kim et al., 2005). Alternatively, a single effector protein can manipulate multiple host targets. For example, the *Blumeria graminis f. sp. Hordei, (Bgh*) effector BEC1054 was shown to interact with BARLEY GLUTATHIONE-S-TRANSFERASE (GST), MALATE

DEHYDROGENASE (MDH) and PATHOGENESIS-RELATED PROTEIN 5 (PR5) *in vitro* (Pennington et. al., 2016).

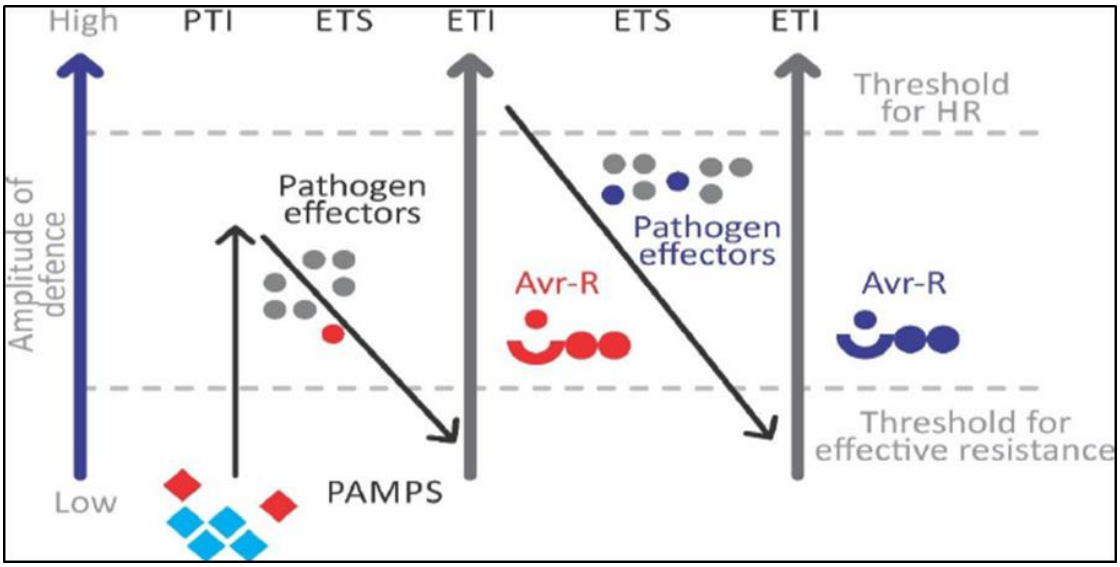


Figure 1.1: The zig-zag model. PAMPs are recognised by PRRs, which initiates PTI. Successful pathogens secrete effector molecules into the cell, inducing ETS. One effector (red) may be recognised by a plant R protein, activating ETI. Due to selection pressures, the pathogen may lose these (red) effectors recognised by R proteins, and other unrecognised effectors may evolve (blue). This 'arms race' between effectors and R-proteins continues as selection pressures change. Figure from Jones and Dangl, 2006.

Increasing evidence indicates that PTI and ETI are not two distinct processes and are in fact functionally linked. For example, ETI mediated resistance in *Arabidopsis* against *Pst* effector protein AvrRpt2 is compromised in PRR mutants (Yuan et. al., 2021). Additionally, pre-activation of ETI in *Arabidopsis* lines expressing the *Pst* effector protein AvrRps4 increased PTI-induced ROS production after PAMP treatment, while ETI activation alone did not (Ngou et. al., 2021). However, the zig-zag model (Figure 1.1) is still relevant to describe how host resistance can be overcome by adapted pathogens, and in turn, how selection pressures drive the evolution of new R genes and associated immune responses. This is particularly important in the context of crop breeding, as many crop varieties are initially highly resistant to important pathogens, but this resistance breaks down after years of commercial use.

For example, virulent *Zymoseptoria tritici* has also been detected on the previously resistant wheat cultivar (cv) Cellule (Kildea et. al., 2020). This is as a result of *Z. tritici* isolates undergoing rapid selection to evade detection by the wheat R gene *Stb16q* (Kildea et. al., 2021).

The purpose of models is to provide a representation of complex real-life systems. While the zig-zag model has been an extremely useful tool to illustrate events in plant-pathogen interactions, it has its shortcomings. For instance, while the four phases are described in a sequential manner, it is important to note that it is not a representation of a single interaction. Phases three and four occur as a result of selection pressures and as such occur on a population level. For example, the potato late blight pathogen *Phytophtora infestans* undergoes major population shifts in response to selective pressures. Particularly, the *P. infestans* strain 13_A2 was found to rapidly increase in field populations on both moderately resistant and susceptible potato cultivars (cvs). Genome sequencing of this strain revealed six novel effectors compared to the reference genome, including a homolog of Avr2 that could evade detection by potato resistance genes (Cooke et. al., 2012).

Furthering our knowledge of the plant immune system, particularly of components of PTI and ETI in a particular pathosystem is essential for developing breeding strategies for new resistant crop cultivars in the future.

1.3 *Rhynchosporium commune*

1.3.1 Taxonomy and origin

R. commune is a haploid ascomycete, with high genetic diversity and a relatively short life cycle; normally several generations occur in one growing season (Zhan et. al., 2008). It reduces tiller production and causes substantial lesion formation on the barley leaf blade (Figure 1.2 A, B), and can be isolated from leaves displaying lesions (Figure 1.2 C). First isolated from rye plants in the Netherlands and named *Marsonia secalis* Oud. (Oudemans, 1897), the pathogen was reclassified in 1901 as *Rhynchosporium graminicola* Heinsen due to its beak shaped conidia (Figure 1.2 D) (Heinsen, 1901). By 1921 it had been renamed *Rhynchosporium secalis* (Oud) J.J. Davis in compliance with

the International Rules of Nomenclature, and was characterised as infecting barley, rye, and other grasses (Avrova and Knogge, 2012, Davis, 1921).

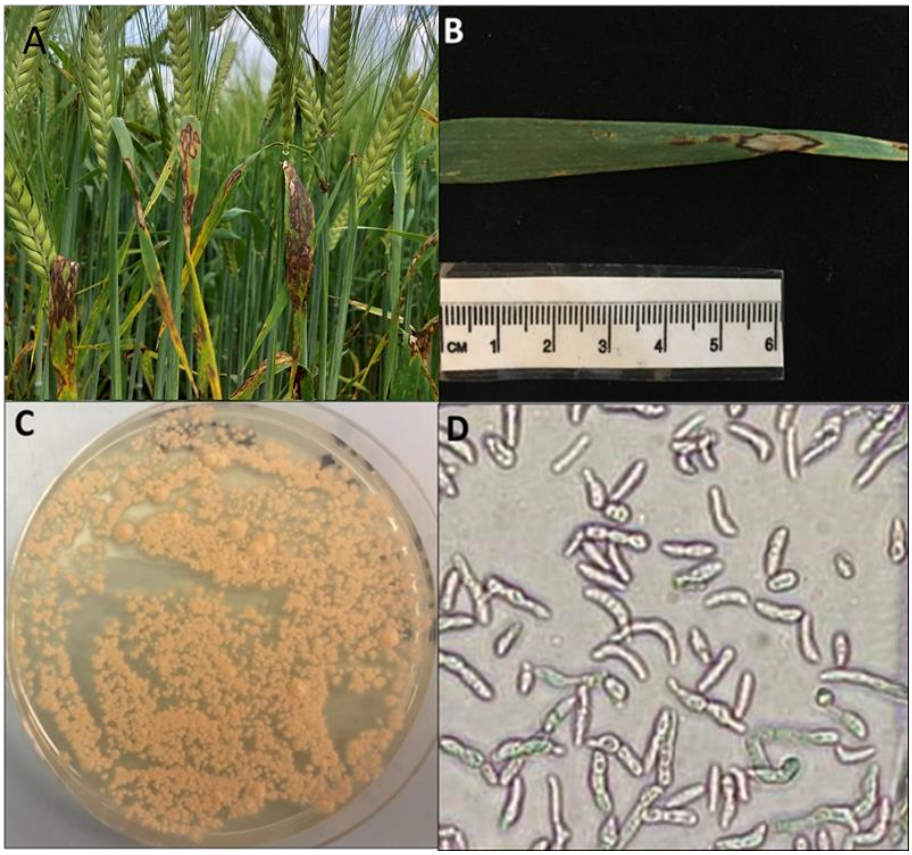


Figure 1.2: A) Leaf scald on susceptible winter barley, KWS Cassia, in the field (Oak Park, Co. Carlow, Ireland). B) Typical scald lesion on Cassia leaf. Lesions are typically light brown/grey, surrounded by a dark brown margin C) *R. commune* in culture. D) Typical *R. commune* spores.

Evidence for host specialisation by *Rhynchosporium secalis* was described by Zaffarano et. al., (2006). The same group carried out infection studies and showed that different *Rhynchosporium secalis* isolates specifically infected barley, rye or *Agropyron* species (Zaffarano et. al., 2008). Following this, based on phylogenetic analysis and their different hosts, *Rhynchosporium secalis* was re-classified into three distinct species (Zaffarano et al., 2011); *R. commune, R. secalis* and *R. agropyri. R. commune* is shown to infect barley, Italian ryegrass and other *Hordeum* species such as *Hordeum murinum* (King et. al., 2013). The name *R. secalis* was kept for isolates infecting rye and

triticale, while *R. agropyri* isolates specifically infect *Agropyron* species (Zaffarano et al., 2011). It has been proposed that the three species originated from a common unknown ancestor (Zaffarano et. al., 2008). *R. orthosporium* is a fourth distinct member of the *Rhynchosporium* genus, which is morphologically distinct from the other three *Rhynchosporium* species, producing cylindrical conidia instead of the typical beak shaped conidia of the other *Rhynchosporium* species. *R. orthosporium* specifically infects cat grass (*Dactylis glomerata*) (Caldwell, 1937).

The area of origin of a pathogen is generally accepted to be the centre of its highest genetic diversity (McDonald, 2015). As such, if *R. commune* originated as a true barley pathogen, high genetic diversity should be found where barley was first domesticated, in the Fertile Crescent of the Middle East. However, gene diversity analyses conducted by Zaffarano et al., (2006) suggest that this is not the case. In their study, 1,366 isolates from 31 field locations across five continents were analysed and showed highest allele richness in Scandinavia followed by Switzerland, with significantly lower allele richness in the Fertile Crescent and other regions tested. The lowest genetic diversity was observed in Syrian populations. These results are in agreement with the first recorded case of scald disease being reported in Europe (Oudemans, 1897). Furthermore, analysis into the nucleotide sequences of the *R. commune NECROSIS INDUCING PROTEIN 1 (NIP1)* avirulence gene identified 37 distinct haplotypes, with the highest haplotype diversity being found in Norway, and the least diversity being observed in Syria and Jordan (Brunner et. al., 2007). Altogether, these analyses suggest that barley is not the original host of *R. commune*, and instead that the pathogen originated in Northern Europe on a different host, possibly a different *Hordeum* relative or endemic grass species, before making a host switch sometime after the introduction of barley to Europe (approximately 8,000 years ago, Jones et. al., 2011; Zaffarano et al., 2006). However, while the original host of *R. commune* remains unknown, this proposed centre of origin is supported by the cool temperate climate in Northern Europe that is preferred by *R. commune*.

1.3.2 Genetic Diversity

The high genetic diversity of *R. commune* is proposed to be as a result of many factors, including large population size, high spontaneous mutation rate, gene flow and asexual reproduction (Goodwin et al., 1994, McDermott et al., 1989, Zaffarano et al., 2006). *R. commune* reproduces asexually via production of spores, which are produced directly from the hyphae. Given the high genetic diversity of *R. commune*, it is very likely that it also reproduces sexually, as sexual reproduction results in greater genetic diversity than asexual reproduction (McDonald, 2015), however, this has not yet been confirmed. Ascomycetes require two genetically distinct parents for sexual reproduction. The mating type is determined by Mating Type (MAT) loci, of which there are two distinct idiomorphs. Similar to its close relative *Tapesia yallundae*, the *R. commune* MAT1-2 locus contains a single gene encoding a protein with a high-mobility group (HMG) DNA-binding domain, while the MAT1-1 locus encodes a HMG domain protein with an additional DNA-binding alpha-box domain. Isolates of both these mating types have been identified in UK populations of *R. commune* (Foster and Fitt, 2003). However, the presence of MAT genes is not sufficient to confirm sexual reproduction as MAT genes have also been identified in the asexual fungus *Fusarium oxysporum* (Arie et al., 2000). Partial sequencing of the HMG and alpha-box domains in *R. commune*, showed that the frequencies of these two MAT loci were overall equal for a collection of 1101 *R. commune* isolates from 21 geographic locations globally. However, this equilibrium did not always hold when looking at diversity within specific regions, particularly in Australia and Switzerland (Linde et. al., 2003). Equal mating type distribution is consistent with sexual reproduction and random mating, and it could be argued that the sexual cycle is infrequent in populations with unequal mating type frequencies. Given the high genetic diversity of *R. commune* that is consistent with sexual reproduction, it is most likely that sexual reproduction does occur, however no sexual stage has been identified, and there has not been sufficient work done in this area to provide evidence to definitively confirm this.

1.3.3 Epidemiology

R. commune is likely to be a seed borne pathogen, but as seed infection is often symptomless, visual analysis of seeds is not sufficient to determine seed quality. Older studies by Skoropad (1959) and Kay and Owen (1973) show that under controlled conditions, seed borne inoculum can cause a transmission rate of 2-6% from symptomless seed. Symptomless seed infection can result in the long-range dispersal of *R. commune,* particularly with increasing demands for intensification of barley cultivation. Chemical treatment of seed can reduce disease incidence, however an ability to detect and quantify *R. commune* load on infected seed is important to further reduce spread of virulent *R. commune* strains and reduce reliance on current available chemicals.

Secondary spread of disease is likely to occur via splash dispersal of conidia (Figure 1.3). However, the extent of this is difficult to determine. Simulated rainfall experiments by Fitt et. al., (1986) show that water borne conidia in rain droplets can infect surrounding plants and upper leaves. Water droplets collected after simulated and natural rainfall over infected plants were both shown to contain *R. commune* conidia. However, the effect of rain dispersal is likely to be underestimated, as during natural rainfall and heavy simulated rainfall it was impossible to measure number of conidia per droplet (Fitt et. al., 1986). Additional variations in field conditions (e.g.: temperature, soil quality, changing rainfall intensity) mean that rain simulation experiments may not adequately describe field transmission of a pathogen. In an alternative approach to assess splash dispersal, Karisto et. al., (2022) inoculated the centre of experimental winter wheat plots with two *Z. tritici* isolates. After three months, quantitative PCR (qPCR) of leaf samples to detect the presence of *Z. tritici* showed a disease dispersal distance of up to one metre. However, there was no indication of whether the seed was tested prior to sowing, and if disease incidence was entirely as a result of splash-dispersal.

R. commune disease can be broken into two distinct phases that cover its hemibiotrophic nature, with an initial biotrophic asymptomatic phase, preceding the necrotic stage through which visual symptoms appear following

host cell death (Horbach et al., 2011). Infection commences with the arrival of conidia on the leaf surface, which produce hyphae that penetrate the cuticle via apressoria, rather than entering through the stomata (Jones and Ayres, 1972; Thirugnanasambandan et. al., 2011). Successful penetration is followed by subcuticular growth of hyphae along the cell walls (Thirugnanasambandan et. al., 2011). Throughout the infection, the pathogen is confined to the plant apoplastic space. Conidia form directly from hyphae, which then erupt through the leaf cuticle. During the asymptomatic growth phase, unlike other biotrophic pathogens such as *Bgh* (powdery mildew), *R. commune* does not produce feeding structures such as haustoria. Instead, it is thought to gain nutrients directly from host cells. Measurement of osmotic pressure and electrolyte leakage of cells of infected plants showed increased permeability of the host cells (Jones and Ayres, 1972), however the molecular mechanism behind this remains unclear. The increased permeability was greater in susceptible varieties compared to resistant varieties, but mycelium growth was also significantly reduced in the resistant variety (Jones and Ayres, 1972), making it difficult to draw clear conclusions.

The first sign of disease symptoms is observed by the collapse of mesophyll cells from extensive mycelia growth, resulting in classic scald lesion formation. Under field conditions, symptoms generally appear during the stem elongation phase (Zadoks Growth Stage (GS) 30-39, Zadoks et. al., 1974), and it is during this phase that rainfall likely contributes to splash-infection of upper leaves and surrounding plants (Figure 1.3).

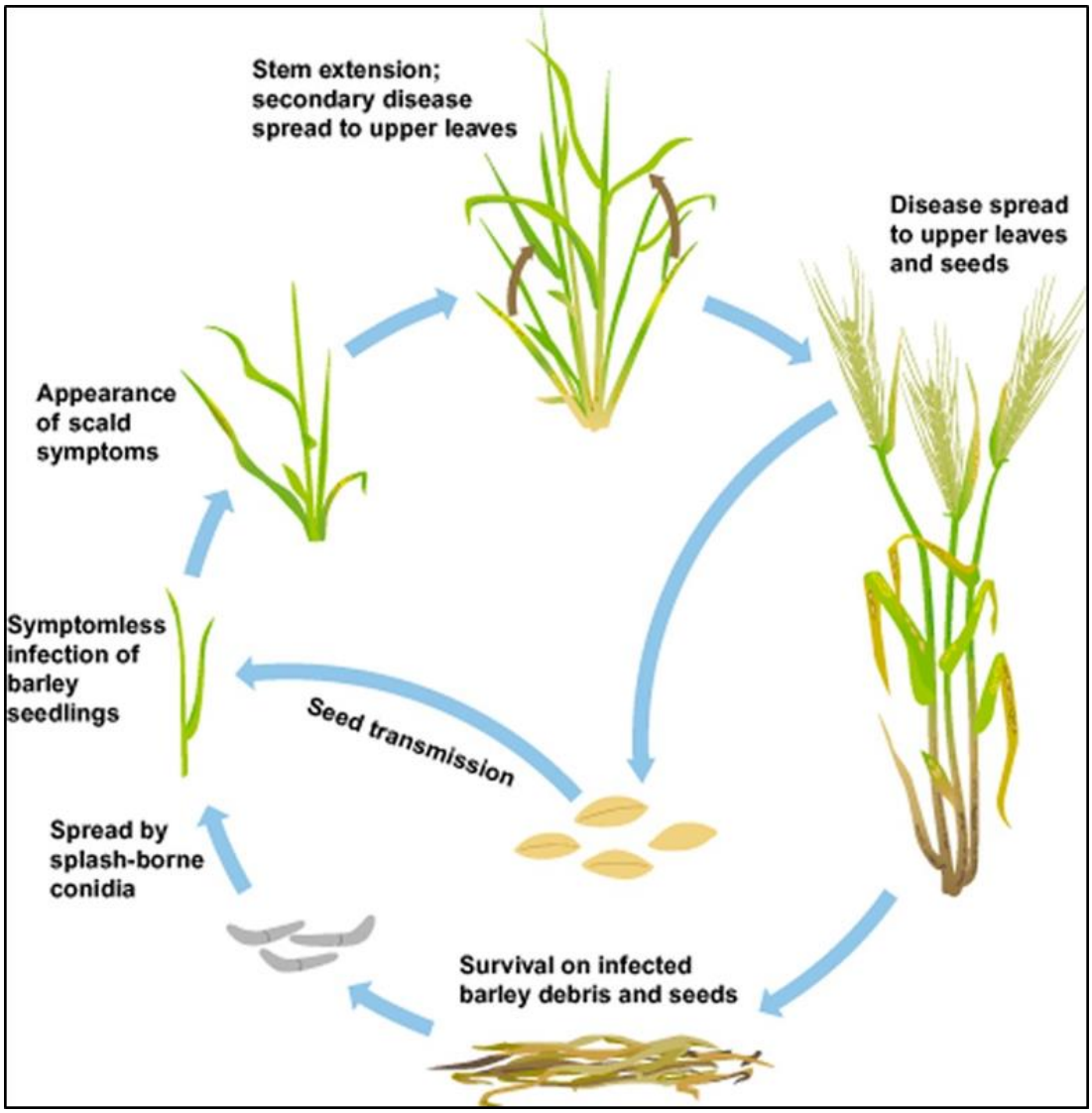


Figure 1.3: *R. commune* life cycle throughout the growing season (Figure from Avrova and Knogge, 2012). The primary source of inoculum is from infected seed and debris. Infected seed is usually symptomless, especially during seedling growth. Symptoms begin to appear during the stem elongation stage of growth, with the appearance of scald like lesions. This gives rise to a secondary source of inoculum via splash dispersal of conidia from lesions to upper leaves and seeds, and to neighbouring plants during rainy weather.

Based on older literature, the percentage of leaf area displaying scald like lesions on the flag leaf typically results in yield loss (r = 0.49) (James et. al., 1968), as a result of reduced photosynthetic area during infection. This is similar to other cereal pathogens such as the wheat pathogen *Z. tritici* which induces large necrotic lesions on the wheat leaf blade resulting in yield loss of up to 50% when the flag leaf is infected (King et. al., 1983).

A consistent challenge with the control of *R. commune* is detecting the pathogen in the field in the absence of visual symptoms. As a result of this situation, farmers apply fungicide programmes based on growth stage of the host, as opposed to presence / absence of disease. Multiple molecular diagnostic techniques have been developed to detect the pathogen at low levels in symptomless leaves and seeds. An enzyme-linked immunosorbent assay (ELISA) has been used to detect infection in symptomless leaves from naturally infected field trials and artificially infected plants grown in controlled atmosphere growth chambers (Foroughi-Wher et. al., 1996). Since then, more sensitive DNA detection methods based on polymerase chain reaction (PCR) have also been developed to identify infection in symptomless seed and leaves (Lee et. al., 2001; Fountaine et. al., 2007). The use of quantitative PCR (qPCR) involving detection of *R. commune* genomic DNA (gDNA) has revealed that colonisation actually occurs over most of the growing season in both resistant and susceptible barley cultivars (from a selection of 10 cultivars from the United Kingdom (UK) Recommended Growing list). *R. commune* DNA was detected as early as GS13 (three leaf stage). Interestingly, for some resistant cultivars, colonisation level of the pathogen was very high when compared to visual assessment of symptoms. For example, the increase in fungal biomass in resistant cultivar (cv) Leonie followed a similar pattern to susceptible cv. Vertige (Fountaine et. al., 2007). While disease pressure during these trials was reported to be low, there is no indication that seeds used were tested for *R. commune* prior to sowing, and therefore the level of inoculum available from seed on the different varieties is unclear. As the field trials were carried out according to the UKs Home-Grown Cereals Authority (HGCA) guidelines, it is likely that the seed had been chemically treated to reduce disease incidence, however this is not indicated in the text. Regardless, chemical treatment would not completely eliminate risk of disease.

1.4 Disease control

1.4.1 Integrated pest management

Integrated pest management (IPM) is a key concept introduced for control of crop pests. This was first defined in 1959 as a combination of biological and

chemical measures to control pest outbreaks (Stern et al., 1959). Since then, IPM has expanded to allow for the management of multiple pests simultaneously, through an integrated system of multiple control strategies. This includes prevention through agronomic practices, chemical and biological control, as well as forecasting disease pressures. A particular emphasis is placed on prioritising biological, physical and other non-chemical methods over chemical control, to reduce reliance on conventional pesticides (FAO, 2020). IPM is essential to combat issues such as pesticide resistance and environmental contamination (Ehler, 2006). However, these definitions of IPM are very broad, and difficulties often arise on how each measure should be applied to individual farms, given their different needs. Farmers often have different perceptions on what IPM includes and find difficulties in distinguishing what actions specifically fall under the banner of IPM (Kildea et al., 2019).

Current strategies to control the spread of *R. commune* involve a combination of chemical and biological mechanisms as well as agronomic practices. Infected straw and crop debris usually provide a major source of inoculum as well as increasing potential for splash dispersal during rainy weather (Fitt et al., 1986). As such, agronomic practices such as crop rotations and stubble management are key to reducing the amount of inoculum available in the following growing season. Delaying sowing date also has the potential to reduce disease pressure in barley. Walking the crops regularly to monitor for pests and disease is also recommended (Teagasc et. al., 2020).

1.4.2 Chemical Control

Chemical control involves the use of plant protection products such as fungicides to reduce disease incidence. Fungicides are commonly used as a foliar treatment sprayed over the crop canopy, but are also applied as granular products to soil, and in seed treatments (Poole and Arnaudin, 2014). Single site fungicides bind to specific protein targets and disrupt cellular processes and therefore can provide a high level of control at low doses. However, fungicide resistance can then arise through point mutations in genes encoding for target proteins. Multisite fungicides target a range of cellular processes (Lucas et al., 2015). Current fungicide programmes for barley involve the use

of demethylation inhibitors (DMIs or triazoles), quinone outside inhibitors (QoIs) or succinate dehydrogenase inhibitors (SDHIs), all of which are single site. Taking into account that over 193,000 ha of barley was grown in the last year (CSO 2021), over €16 million was spent on fungicide sprays on barley crops.

Benzimidazoles were previously used in the control of *R. commune*, by targeting β-tubulin in the pathogen. Specifically, benzimidazoles bind to β-tubulin and prevent microtubule assembly, resulting in mitotic failure (Butters et. al., 1999). However, by the early 1990's, resistant *R. commune* populations were reported in the UK (Locke and Phillips, 1995). A point mutation in the β-tubulin gene (E198A) was later attributed to resistance to benzimidazoles, reducing their binding efficiency to β-tubulin (Ma and Michailides, 2005). As a result, benzimidazoles are no longer recommended for control of leaf scald.

Resistance to DMI fungicides, which are currently used, has not yet been reported in *R. commune*. However, mechanisms of resistance have been identified in other crop pathogens such as *Bgh,* through a point mutation in the target site CYP51 - a cytochrome P450 demethylase (Wyand and Brown, 2005, FRAC 2012). Paralogs of the *CYP51* gene are present in *R. commune*; *CYP51A* and *CYP51B* (Hawkins et al., 2014). To date, no mutations in *R. commune CYP51* genes conferring resistance to DMI fungicides have been identified. However, eyespot pathogens *Oculimacula yallundae* and *Oculimacula acuformis*, both closely related to *R. commune*, have varying degrees of sensitivity to DMI fungicides. While many *CYP51* mutations have been identified in sensitive strains, no mutation in *O. yallundae* or *O. acuformis* has been directly related to DMI sensitivity (Albertini et. al., 2003).

The protein target for QoI fungicides which are currently used in the control of *R. commune* is MITOCHONDRIAL CYTOCHROME B. As this class of fungicide also has a single target-site mode of action, there is a significant risk of resistance development, as witnessed in the wheat - *Z. tritici* host-pathogen interaction. *Z. tritici* resistance to QoIs was first reported in 2002 (Fraaijie et. al., 2003). Resistance to QoIs occurs as a result of a point mutation in the mitochondrial cytochrome b (G143A). In 2008, a small number of

Rhynchosporium populations were identified with complete resistance to QoIs in France, with a number of isolates containing the G143A mutation. This mutation was also identified at low frequencies in the UK and Spain (FRAC, 2015). In Ireland, this mutation was found at four sites (Phelan et al., 2017), confirming that QoIs are vulnerable. Of significance, complete resistance to QoIs has been reported in other barley pathogens such as *Ramularia collo-cygni* (FRAC, 2012).

There is also concern that increased over-reliance on SDHIs will also result in pathogen resistance (Sierotzki and Scalliet, 2013), however there are no reported cases of SDHI resistant *R. commune* isolates to date. To preserve the efficacy of remaining chemistries, it is important that all necessary measures to reduce fungicide resistance are taken, such as only using fungicides when needed and using the lowest doses required for control. It can also be useful to use a mixture of active substances, so as not to over rely on a single activity and drive insensitivity (Corkley et. al., 2021).

Multisite fungicides are advantageous, as they have a lower risk of resistance occurring. As these chemicals interrupt multiple protein groups, their mode of action is not organism specific or site-specific. In contrast to fungicides with a target site specific mode of action, resistance to multisite fungicides cannot arise as a result of a single point mutation. Multisite fungicides generally form part of disease control programmes as a ‘back up’ to reduce overreliance on single site fungicides. However, in the EU, pesticide availability is reducing as a result of the Pesticide Authorisation Directive 91/414/EEC requiring a risk assessment for approval of the use of pesticides combined with Regulation (EC) 1107/2009 that requires all registered pesticides to meet very strict standards being approved for use (European Parliament, 2009). These increased restrictions have limited pesticide availability and will impact crop production in Ireland and the rest of the EU (Jess et al., 2014). In 2019, chlorothalonil, a major multi-site fungicide, did not meet these standards due to fears over health, environmental damage and groundwater contamination, and its use was prohibited in 2020.

This has increased the importance of applying an integrated approach to disease management. As such, there is a need to focus on increasing the resilience and efficiency of crop varieties, and include this in disease management strategies (Lin, 2011). Specifically, this highlights the importance of improving our understanding of host-pathogen interactions to identify key genes or signalling pathways involved in resistance or susceptibility to a particular pathogen (Stout and Davis, 2009). These genes or pathways can then be targeted to aid in breeding for cultivars with increased and durable genetic resistance.

1.4.3 Genetic control

The spread of crop pathogens is also controlled through breeding for resistant crop varieties. The two types of cultivar resistance are single gene (R gene) resistance (or qualitative resistance) which provides almost complete disease control but is often race-specific and can be overcome by emergence of new strains. In contrast, multi-gene resistance (or quantitative resistance), which offers partial control that is usually more durable as it is not race-specific, which is affected by disease pressure.

Identification of novel R genes plays a key role in breeding for disease resistance. Many R genes have been characterised in different plant species and are being used to develop disease resistant crop varieties in crop improvement research programmes (Gururani et. al., 2012). In barley, Andersen et al. (2016) identified 175 CNL genes, through identification of barley orthologs to *Arabidopsis* and rice CNL genes. The high frequency of splice variants and multiple exons have contributed to the rapid diversification of barley R genes (Andersen et al., 2016). Sixteen major R genes that confer resistance to *R. commune* have been identified in barley, as described in (Zhan et al., 2008). Studies involving R gene mediated resistance in the barley x *R. commune* pathosystem have focused primarily on the major resistant genes *RRS1* and *RRS2*. *RRS1* gives resistance to *R. commune* strains encoding the corresponding Avr allele *AvrRrs1*, which codes for a NECROSIS INDUCING PROTEIN NIP1. This protein is involved in the lesion forming stage of infection (Rohe et. al., 1995). Two other *R. commune* avirulence genes NIP2

and NIP3 have also been identified. These three NIP proteins are the only secreted effector proteins identified in *R. commune*. Expression analysis shows that *NIP1* is expressed in *R. commune* spores, while *NIP2* and *NIP3* are only expressed after inoculation of the host plant (Kirsten et. al., 2012). NIP1 and NIP3 stimulate plant plasma membrane localised H^+-ATPase, although the molecular mechanism involved remains unknown. The function of NIP2 also remains unknown (Kirsten et. al., 2012). More recently, distinct *NIP1* paralogs (*NIP1A* and *NIP1B)* have been identified in different *Rhynchosporium* species (Mohd-Assaad et. al., 2019).

In contrast to single R gene mediated resistance, quantitative resistance is often controlled by several genes, offering partial resistance to multiple strains (Zhan et. al., 2008). Regions of the genome containing the genes associated with quantitative traits are referred to as quantitative trait loci (QTLs). Barley cultivars with partial quantitative resistance still develop symptoms, however they may be less severe, or appear at later growth stages. As quantitative resistance does not rely on the recognition of specific avirulence genes in the pathogen, it does not impose the same selection pressures on the pathogen as qualitative R gene mediated resistance. Therefore, quantitative resistance can be viewed as more durable than qualitative resistance. Quantitative disease resistance is usually associated with a reduction in disease symptoms rather than complete absence of symptoms. This can be a result of host effects on latent period, reducing spore germination or production, but the molecular events involved in quantitative resistance are not as well described as major R gene mediated resistance in the barley x *R. commune* pathosystem.

Deletion or alteration of a pathogen Avr gene previously recognised by a host R gene triggers ETS. Therefore, single gene mediated resistance such as the *RRS1/NIP1* interaction is vulnerable to breakdown. In 614 *R. commune* isolates analysed, *NIP1* gene deletion was observed in 45% of strains. As a result, strains lacking this effector are overcoming *RRS1* mediated resistance. However, a *NIP1* deletion does not appear to affect fungal virulence, indicating the presence of other virulence genes. Interestingly *NIP2* deletion was only detected in 8% of strains, and *NIP3* deletion only in 0.3% of strains. It is hence

possible that these proteins are more essential to the pathogen (Schurch et. al., 2004).

In the barley x *R. commune* pathosystem, ‘resistance’ refers to the absence of visual disease symptoms. As such, it does not necessarily indicate a lack of pathogen colonisation (Zhan et. al., 2008). This is evident from work by Fountaine et. al., (2007) described earlier, in which pathogen DNA was detected in both resistant and susceptible barley cultivars across the growing season, despite the lack of visible necrotic symptoms. Additionally, infection time-course experiments using a green fluorescent protein (GFP) expressing strain of *R. commune* on resistant cv. Atlas46 and susceptible cv. Atlas showed that spores germinate normally in both the resistant and susceptible lines (Thirugnanasambandam et. al., 2011). In the susceptible line, hyphal growth resulted in an extensive network during the latent phase. In contrast, in the resistant line, while branched hyphae grew in the extracellular space, growth was limited and disorganised (Thirugnanasambandam et. al., 2011). In summary, resistant barley lines do not appear to stop *R. commune* colonisation, however, can impede hyphal development and growth. However, the molecular events involved in this resistance mechanism remain unknown.

Currently most commercial barley varieties only provide moderate levels of resistance to *R. commune*, and even the most resistant varieties (resistant rating = 8) on the Department of Agriculture, Food and the Marine (DAFM) recommended growing list still require fungicide treatments for control (DAFM 2022). This highlights the need to identify novel and durable sources of resistance that do not rely on race specific recognition of *R. commune.*

1.5 Breeding for host resistance

Domestication of crops is an evolutionary process in which humans have used wild species to establish new forms of crops with traits that met human needs, such as yield and edibility (Purugganan, 2019). During domestication, only seed from crops with the most favourable traits were selected for the next generation. As a result of this, genetic bottlenecks occur during domestication, specifically when only a subset of wild populations are selected for cultivation. This, combined with loss of genetic diversity through breeding programmes,

has led to a significant reduction of the genetic sources of resistance in modern cultivars compared to their wild relatives (Alqudah et. al., 2020). As such, wild relatives may be a useful resource for disease resistance. For example, Pikering et. al., (2006) demonstrated the transfer of a resistance allele from *Hordeum bulbosum* to generate a *R. commune* resistant hybrid line by crossing with susceptible barley cv. Emir. This line was then backcrossed with Emir, with resulting seed showing no visual symptoms under glasshouse conditions after infection with *R. commune*. However, during subsequent field trials disease pressure was reported to be low and visual symptoms were not reported.

Conventional breeding plays an essential role in crop improvement; however this method has drawbacks. Conventional breeding methods require examination of large populations over multiple generations, which is both time and labour intensive (Breseghello and Coelho, 2013). Response of cultivars in field trials are also influenced by environmental factors such as temperature, rainfall and challenge by other pests, highlighting the importance of testing new cultivars under field conditions.

Advancements in DNA sequencing technology and genetic engineering have increased our ability to breed for enhanced disease resistance. As the barley genome has been sequenced (The International Barley Genome Sequencing Consortium, 2012), candidate R genes can be identified through bioinformatics analysis or identification of molecular markers by comparing sequence data from different cultivars to the reference genome (cv. Morex). Marker assisted selection (MAS) breeding is used to enhance traditional breeding programmes. DNA markers that reveal sites of variation in different cultivars are identified and used to select parental genotypes in breeding programmes. This allows for selection of traits that are difficult to assess for in phenotypic assays (Edwards and Batley, 2010). As indicated in Section 1.4.3, quantitative resistance only provides partial resistance and while symptoms may appear, they may be less severe, develop later or have reduced spore production.

In experimental conditions, the resistance or susceptible phenotype of a barley cultivar is usually determined by the specific *R. commune* strain it is exposed to. Therefore, identification of resistance is strongly influenced by the genetic composition of the specific *R. commune* strain or population used in the trials. Natural inoculum contains a mixture of strains, as would occur under normal field conditions. This provides the ability to challenge cultivars with a number of virulence factors. Single strain experiments can give additional information by showing differential response of barley cultivars.

There have also been incidences where R gene resistance can be engineered in crops through the identification and characterisation of R genes from different cultivars. For example, the R gene *RPG1* which encodes an RLK confers resistance to many pathotypes of *Puccinia graminis* (cause of stem rust) and was cloned from barley cv. Morex, and efficiently transferred to the stem rust susceptible barley cultivar Golden Promise (Horvath et al., 2003). Transfer of R gene mediated resistance has also been demonstrated through the identification and characterisation of R genes from different plant species. One instance of this was demonstrated by Zhao et al., (2004) as the R gene *ZmRXO1* identified in maize, conferred resistance to *Burkholderia andropogonis* (cause of bacterial stripe in maize) in maize, and also to the non-host rice pathogen *Xoo* (cause of bacterial streak in rice). It was later shown that *ZmRXO1* is functional in rice for the control of *X. oryzae* (Zhao et al., 2005). This approach is not limited to the usage of R genes for disease resistance. Field inoculation of transgenic tomato lines expressing the *Arabidopsis* PRR *EF-TU RECEPTOR (EFR)* with *Ralstonia solanacearum* (causal agent of bacterial wilt) showed a reduction in disease severity over two years (Reduction of 66 % in 2015 and 23 % in 2016). However, disease incidence in non-transgenic lines was reported to be much lower in 2016 compared to 2015 (23 % and 82 % respectively), indicating low disease pressure in 2016. As such, the durability of these transgenic approaches is unclear (Kunwar et. al., 2018).

Another common breeding strategy to improve the durability of R gene mediated resistance is the stacking of multiple R genes that are effective against the same pathogen strains through different mechanisms. This can be

achieved by cross-breeding pre-existing R genes. In this case, it would offer more durable resistance as the pathogen would have to evolve multiple evasion strategies to establish infection (Dangl et al., 2013, Halpin, 2005). MAS and plant transformation have greatly improved the efficiency of selecting and stacking R genes. R gene stacking was demonstrated in potato cultivar Désirée, which is susceptible to the late blight pathogen *P. infestans* by combining three potato R genes *Rpi-sto1*, *Rpi-vnt1.1*, and *Rpi-blb3* using Agrobacterium-mediated transformation (AMT) (Zhu et. al., 2012).

Breeding for R gene mediated resistance has been the primary focus in plant breeding programmes aimed at improving disease resistance. However, the narrow genetic base of cultivated barley can result in genetic vulnerability (Muñoz-Amatraín et. al., 2014). Single gene resistance is at particular risk to crop pathogens with high genetic variation, such as *R. commune*. Any mutation in the avirulence gene leading to the non-recognition by the corresponding plant R gene will restore virulence, and render that particular cultivar susceptible, as illustrated by the zig-zag model. Often, new resistant crop cultivars are introduced, and soon becomes popular and are grown over a large area. However, when a new resistant *R. commune* isolate emerges, it can quickly spread due to lack of competition. This has been observed in Ireland in the spring barley cv Doyen. Doyen was classed with high resistance to *R. commune* and widely used in Ireland with no reports of severe disease up until 2006. In this year, severe disease levels were reported. By comparing cv Doyen resistance to other *R. commune* isolates which were isolated from barley cvs Lux, Tavern and Wicket, it was determined that the high disease levels observed on cv Doyen in 2006 was not as a result of seasonal pressures, but as a result of the emergence of a new isolate of *R. commune* (O'Sullivan et. al., 2007). This shows how resistant cvs can break down within a single growing season. It is possible that the new isolate had been present at a low frequency and built up in cv. Doyen due to lack of competition. As cv Doyen was very popular at the time due to its resistance rating, this race then increased and became widespread within a single growing season. In summary, there is an increasing need for more durable resistance strategies, which do not solely rely on R gene mediated resistance.

1.5.1 Susceptibility genes

Identification of pathogen effector targets has proved to be a useful tool in breeding for host resistance. Host genes encoding effector targets can be referred to as susceptibility (S) genes (Pavan et al., 2010, van Schie and Takken, 2014). More broadly, any plant gene that facilitates pathogen infection can be considered an S gene. For example, genes involved in negative regulation of the immune response are considered as S genes, as their activity in the absence of infection results in the suppression of immunity. These can be targeted by pathogen effectors and activated to increase susceptibility. When S genes become dysfunctional, for example through mutation, pathogens are impeded from colonising the plant, resulting in a resistant phenotype. In this case, by removing a specific S gene function, the pathogen loses a host factor required for a compatible host-pathogen interaction (van Schie and Takken, 2014, Zaidi et al., 2018). In summary, S genes are dominant genes whose impairment can result in recessive based resistance (Pavan et. al., 2010).

S-genes can be grouped into three different types, depending on how they facilitate pathogen infection. Type 1 are genes that allow for compatibility and aid early pathogen establishment. Mutations in these types of S-genes prevent pathogen penetration, conferring a non-host type of resistance (van Schie and Takken, 2014). For example, *ENHANCED DISEASE RESISTANCE 3 (EDR3)* in *Arabidopsis* is an S gene that was found in a screen to identify mutants with increased resistance to *Erysiphe cichoracearum,* a biotrophic pathogen that requires living host cells for colonisation. The *edr3* mutants showed increased cell death and reduced powdery mildew spore production as a result of nutrient deprivation. However, the *edr3* mutants also showed increased susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea* (Tang 2006).

Type 2 S genes are involved in the modulation of host defences to facilitate infection. Their targets include positive and negative regulators of plant immunity such as genes involved in controlling PTI (van Schie and Takken, 2014). JA and SA are two plant hormones involved in plant defence; JA is typically required for defence against necrotrophs and insects, while SA is

classically required for defence against biotrophs. The antagonistic interaction between JA and SA means that the JA pathway can supress the SA defence pathway and the SA pathway can supress the JA defence pathway (Turner et. al., 2002). In *Arabidopsis*, *bHLH*3, *bHLH13*, *bHLH14* and *bHLH17* supress the JA response (Huang et al., 2018). These genes encode for transcription factors and were shown to function redundantly. Quadruple mutants of these transcription factors reduced susceptibility to the necrotroph *Botrytis cinerea* in *Arabidopsis* (Song et. al., 2013). After inoculation of quadruple mutant *Arabidopsis* plants, the JA-regulated defence genes *ETHYLENE RESPONSE FACTOR 1 (ERF1), PLANT DEFENSIN 1.2 (PDF1.2), LIPOXYGENASE 2 (LOX2)* and *THIONIN 2.1 (THI2.1)* were highly induced upon pathogen perception (Song et. al., 2013). However, while having increased resistance to a necrotrophic pathogen, the quadruple mutant lines showed increased JA induced susceptibility to the hemibiotrophic pathogen *Pst*. Theoretically, any genes coding for proteins involved in the JA and SA signalling pathways (such as the *Arabidopsis* bHLH genes) can be considered S genes, given that their activity can be a benefit to either biotrophic or necrotrophic pathogens.

Type 3 S genes facilitate the post penetration needs of the invading pathogen, where pathogens use host machinery for their metabolic requirement and proliferation (van Schie and Takken, 2014). A classic example would be pathogenic viruses (such as potyviruses), which target and activate host genes involved in DNA or RNA replication to complete their life cycle. The plant machinery can also be exploited by pathogens for nutrition. In rice, SWEET proteins (SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTED) contribute towards *Xoo* susceptibility. SWEET11 acts as a sugar transporter, loading the apoplastic space with carbohydrates which then provide nutrition for the pathogen. *Xoo* secretes the PthXo1 effector into host cells where it directly interacts with the *SWEET11* promoter, specifically activating its expression. *pthXo1* mutants, show reduced pathogenicity as a result of reduced transcription of host *SWEET11*. In agreement with this, RNA interference (RNAi) of rice *SWEET* genes confers resistance to *Xoo* (Chen et al., 2010).

1.5.2 R genes Vs S genes

S genes can be described as dominant genes, whose impairment leads to recessive resistance traits. Mutations in dominant genes that would otherwise promote infection are thought to be more durable than dominant R gene mediated resistance. S gene mediated resistance does not rely on the recognition of a specific pathogen effector, and resistance is instead based on a 'loss of compatibility'. As such, this type of resistance cannot be overcome by changes in the pathogen population, either through expansion of effector repertoire or loss of existing effectors. Additionally, it could confer broad range resistance against many strains, as opposed to being strain specific, as it would not require recognition of specific effectors as observed with R gene mediated resistance. However, as S genes are involved in many primary functions including development, flowering and stress response, interruption of S gene function is usually accompanied with some form of fitness penalty, for example: reduced growth, reduced yield, or reduced tolerance to other biotic and abiotic stress (Van Schie and Takken, 2014). As such, not all S genes are suitable to be targeted for editing. For example, *MPK4* is a negative regulator of SA production in *Arabidopsis*, involved in both PTI and ETI. Removal of *MPK4* leads to a build-up of SA, conferring resistance to *Pst*. However, *mpk4* mutants also displayed dwarfism and spontaneous lesion formation (Petersen et al., 2000). Additionally, many S genes play central roles in immune signalling pathways such as hormone signalling, and removal of their function may lead to increased resistance to one pathogen but also increased susceptibility to another. It is important to first determine the function and suitability of an S gene for use in resistance breeding (Gawehns et al., 2013).

1.5.3 Engineering for S gene mediated resistance

One of the best described instances of S gene mediated resistance is the barley *MILDEW LOCUS O (MLO)* for *Bgh* resistance (Jørgensen, 1992). Powdery mildews are a group of disease causing fungi found to infect most staple crops worldwide. They are obligate biotrophs that infect living epidermal cells in order to reproduce. They show a high degree of host specificity, so that

the barley powdery mildew fungus, Bgh, can infect barley but does not grow on other cereal crops (Eichmann et al., 2010, Schweizer, 2007). *MLO* is a widely conserved plant gene involved in negative regulation of plant immunity. The 60-kDa MLO protein is predicted to have six membrane spanning helices and is thought to have a role in callose deposition (Jørgensen, 1992; Büschges et al., 1997).

Loss of *MLO* function in barley shows complete non-host resistance to otherwise virulent *Bgh*. A number of barley varieties with *mlo* loss of function mutations are used as a source of resistance in Europe (Jørgensen, 1992), and continue to show resistance after almost 30 years, illustrating the durability of this approach. Recessive *mlo* mediated resistance is characterised by rapid papillae formation and callose deposition at the encounter site of the pathogen, forming a barrier and preventing pathogen penetration, hence making this a type 1 S gene (Jørgensen, 1992, Skou et al., 1984). It has also been suggested that *mlo* mediated resistance to *Bgh* may also be a result of increased hydrogen peroxide production and cell death (Piffanelli et. al., 2002).

Orthologues of *MLO* have also been shown to act as S genes in other plant species, including *Arabidopsis* (Consonni et. al., 2006), wheat (Wang et. al., 2014) and oat (Reilly et. al., 2021). Indeed, due to the presence of MLO proteins in ancient plant lineages, it has been suggested that MLOs are required for other biological roles and were then exploited by powdery mildew pathogens to promote susceptibility. For example, *AtMLO7* has been described in the context of pollen tube reception, under the regulation of the receptor like kinase FERONIA (FER) (Kessler et. al. 2010). Interestingly, recessive mutations in *FER* also showed *mlo*-like increased resistance to the powdery mildew *Golovinomyces orontii* on the Landsberg erecta (Ler) *Arabidopsis* accession. This was shown by reduced penetration, as well as reduced colony establishment and conidiophore production (Kessler et. al., 2010).

However, there is a trade-off between durable resistance to powdery mildew and undesirable pleiotropic effects observed in barley *mlo* mutants.

Spontaneous necrotic leaf spotting observed in barley *mlo* mutants can cause reduced grain yield and quality (Jørgensen, 1992), due to reduced green leaf area intercepting sunlight. In *Arabidopsis,* both *mlo* and *fer* mutants show a spontaneous cell death phenotype as well as increased hydrogen peroxide production. In barley, mutations in *mlo* also result in increased susceptibility to other pathogens such as *R. collo-cygni*, as shown by McGrann et. al., (2014) under both laboratory conditions and in field trials (McGrann et. al., 2014). Additionally, Jarosch et al., (1999) showed that *mlo-5* mutants display increased susceptibility to the rice blast pathogen *Magnaporthe grisea* under laboratory conditions. Interestingly, functioning MLO may also reduce susceptibility to *R. commune,* as suggested in field trials conducted by Makepeace et. al., (2007). However, this was observed through a field evaluation, and the relative expression levels of MLO after infection was not examined in this study. Additionally, functioning MLO is also required for the colonisation of the beneficial root endophyte *Serendipita indica* due to the enhanced papillae formation impeding colonisation (Hilbert et. al., 2020).

Therefore, it is beneficial to know the role of the S gene in question - what the primary function of the candidate S gene is. Knowledge of the function of S gene candidates or their orthologues will give an insight into potential pleiotrophic effects that may arise as a result of reduced S gene function.

In recent years, the importance of S genes and their potential in resistance breeding has been highlighted. However, there is limited information available on S genes in barley outside of the *Bgh* pathosystem. As *R. commune* has a hemibiotrophic lifestyle compared to *Bgh* biotrophic lifestyle, there is potential to employ a similar S gene mediated resistance strategy in combination with other IPM control methods to ensure a more durable solution to the management of this pathogen.

1.6 Project Aims

The work described in this thesis aims to:

(1) examine the interaction between susceptible and resistant barley cultivars and the pathogen *R. commune* to identify a timeline of disease progression and corresponding host responses for selected *R. commune* isolates.

(2) identify candidate S genes in barley that promote *R. commune* infection through molecular and bioinformatics approaches.

(3) establish a transient expression system in barley to interrupt candidate S gene activity and examine the response of barley lines with reduced candidate S gene activity to *R. commune*.

1.6.1 Examining the interaction between barley and *R. commune*

The first aim is to examine the interaction between selected barley cultivars and Irish *R. commune* isolates to gain understanding of the disease timecourse of the selected isolates. There are many examples in the literature of studies to characterise colonisation of barley leaf tissue by different *R. commune* isolates. In the literature, a number of methods were established including field studies (Atkins et al., 2010), glasshouse or controlled atmosphere growth chambers (Al-Daoude et al., 2014, Jackson and Webster, 1976) and detached leaf assays (Newton et al., 2001, Thirugnanasambandam et al., 2011). The different protocols described combined with the high genetic variability across different *R. commune* strains can greatly influence the infection timecourse. Chapter 2 details the establishment of the most suitable and reproducible protocol for the inoculation of barley with the *R. commune* isolates used in this project.

Once a reliable and reproducible protocol had been established, inoculation experiments were carried out using two *R. commune* strains and two barley cultivars, one susceptible (KWS Cassia – referred to as Cassia for remainder of thesis, resistance rating = 4) and one resistant (KWS Infinity – referred to as Infinity for remainder of thesis, resistance rating = 7), which are both currently on the DAFM Winter Barley Recommended List (DAFM 2022). Assessment of visual symptoms over time and quantification of fungal biomass at several timepoints throughout the infection timecourse was then carried out. The aim was to assess the virulence and reproduction of the pathogen during latent and necrotic stages. Additionally, reverse transcription qPCR (RT-qPCR) on total RNA from infected leaf tissue was carried out with the aim to identify the expression levels of genes known to be involved in the plant immune response. This was necessary to assess how the different cultivars

were responding to the different isolates over time, but also to identify key timepoints in the infection process, including latent phase duration and onset of the host immune response.

1.6.2 Identification of candidate S gene targets

The second aim of this project is to identify potential barley S genes that may play a role in susceptibility to *R. commune*. This was done using an *in silico* approach and transcriptomics. The *in silico* approach consisted of sequence similarity searches of known S genes in other plant species (using an extensive list of S genes published by van Schie and Takken, 2014) to identify potential barley orthologues to these known S genes. The second approach involved global transcriptome analysis of infected barley to identify specific genes or pathways that are activated or repressed in susceptible or resistant cultivars, and in response to two different *R. commune* isolates at the key timepoints identified in the first project aim.

1.6.3 Functional analysis of identified S gene candidates

The third aim of this thesis was to analyse the role of candidate S genes in *R. commune* infection through knock down of S gene activity. Chapter 4 details attempts made to establish a transient expression system in three barley cvs Cassia, Infinity and Golden Promise using *Agrobacterium tumefaciens* leaf infiltration. Chapter 4 also describes the generation of an RNAi construct to knockdown S gene function to test the hypothesis that elimination of candidate S gene activity can result in a resistance like phenotype.

2 Establishing *R. commune* disease screening protocols to characterise infection process in barley cultivars.

2.1 Introduction

The life cycle and different stages of *R. commune* infection in barley are well characterised (Avrova and Knogge, 2012, Lehnackers and Knogge, 1990), however the specific molecular interactions of different *R. commune* isolates with barley cultivars are complex and not as well understood. As shown by Fountaine et al., (2007), resistance ratings of commercial barley cultivars do not correlate with infection levels determined by quantitative PCR (qPCR), indicating that the pathogen is capable of growing and completing its life cycle within the tissues of so-called resistant plants, without producing any symptoms. This can lead to the production of infected seed, which in turn contributes to the spread of disease across growing seasons. The barely response to early infection events between germination of conidia on the leaf surface and symptom appearance still remain largely unclear.

Many studies have been carried out to characterise colonisation of barley leaf tissue by different *R. commune* isolates using a variety of methods including field studies (Atkins et al., 2010, Topp et. al., 2019), glasshouse or controlled atmosphere growth chambers (Al-Daoude et al., 2014, Jackson and Webster, 1976) and detached leaf assays (Newton et al., 2001, Thirugnanasambandam et al., 2011). There is large variation in disease outcomes observed under similar experimental conditions. For example, Newton et. al., (2001) identified $1x10^5$ spores/ml as the optimum spore concentration for a detached leaf assay using three Scottish *R. commune* isolates and recorded lesion formation 8 days post inoculation (dpi) in susceptible barley cvs Maris Mink and Proctor. This observation is different to the detached leaf assay later described by Thirugnanasambandam et. al., (2011). However, in this work a GFP-tagged *R. commune* isolate was studied in the susceptible cultivar Atlas. In this cultivar, visible symptoms were not observed until much later, at 21dpi. This shows how *R. commune* isolate and barley cultivar selection can greatly affect disease progression.

There are also notable differences across the literature in the experimental conditions used in studying the barley x *R. commune* interaction *in planta*. Differences are observed in volume and concentration of the spore suspension used during inoculation, which ranges from 10^3 to 10^7 spores/ml in both whole plant and detached leaf assays, as well as variations in temperature (ranging 15-18°C) and relative humidity (RH) (ranging 80-100%) at which the plants are grown (Abang et al., 2006, Newton et al., 2001, Thirugnanasambandam et al., 2011, Xue and Hall, 1992). These factors, combined with the genetic differences in the *R. commune* isolates used in different labs can affect the outcome of infection and the duration of the asymptomatic period. This also highlights the need to optimise a protocol suitable for the specific isolates and barley cvs examined in this chapter.

The use of molecular diagnostic tools is well established for the detection of pathogens in barley and other crop species. While an ELISA has been successfully used to detect *R. commune* in asymptomatic barley (Foroughi-Wehr et al., 1996), genomic DNA (gDNA) detection by PCR is thought to be more sensitive (Pandey et. al., 2015). *R. commune* has indeed been detected in asymptomatic barley using standard PCR (Lee et al., 2001) and quantitative PCR (qPCR) (Fountaine et al., 2007) on gDNA. According to Fountaine et. al., (2007), *R. commune* DNA quantification carried out using a locked nucleic acid (LNA) probe assay is the most sensitive of three assays tested (SYBR-Green I, TaqMan probe and LNA probe), when measuring *R. commune* biomass accumulation in asymptomatic leaf tissue. The use of qPCR to monitor fungal biomass has also been demonstrated to be suitable for the detection of other crop pathogens, such as for the detection of *Fusarium graminearum* in asymptomatic wheat kernels (Horevaj et al., 2011), as well as multiple other *Fusarium* strains in infected wheat leaves, ears and grain (Waalwijk et al., 2004). qPCR methods have also been used in the diagnosis of the soybean rust pathogens *Phakopsora pachyrhizi* and *Phakopsora meibomiae* (Frederick et al., 2002).

Monitoring the response of the plant host is also important to understand plant/pathogen interactions. In this case, quantification of mRNA by reverse transcription qPCR (RT-qPCR) is a highly sensitive and commonly used tool

to quantify host defence related gene expression during pathogen infection. For example, RT-qPCR was used to determine the relative expression of the PTI marker gene *WRKY7* in tobacco and in potato treated with different PAMPs (flg22, chitin and Pep13) (Wang 2019).

Monitoring the expression of host defence related genes could provide valuable information to establish an efficient and standardized *R. commune* infection protocol, as well as to characterise the dynamic transcriptional changes of specific barley genes at early and late timepoints after infection. Compared to *Arabidopsis*, fewer genes in barley have been clearly established as markers for plant response to pathogens. Below are some genes that were identified as being of potential relevance to the barley x *R. commune* interaction.

SA signalling plays an important role in the response to biotrophic and hemi-biotrophic pathogens (Tanaka et. al., 2015). Despite this, the role of SA signalling in response to *R. commune* in barley is not documented. However preliminary work involving treatment of barley leaves with SA prior to *R. commune* infection has been described (Griffe, 2017). Inoculation of barley with *R. commune* isolate L73a showed delayed lesion formation in SA primed seedlings (8 dpi) compared to control (water) treated seedlings (6 dpi). Inoculation with a GFP tagged *R. commune* isolate also showed constricted hyphal growth in SA primed leaf sections at 6 dpi in a detached leaf assay (Griffe, 2017). This suggests that SA induced defence may play an important role during the early defence response to *R. commune.* As a result, genes associated with SA signalling or acting downstream of SA signalling, are potentially of interest to monitor barley response to *R. commune,* particularly during the biotrophic infection phase. For example, expression of *PATHOGENESIS RELATED PROTEIN 1* gene (*PR1*) has been shown to be induced by various pathogens in different plant species and is also associated with systemic acquired response (SAR) (Durrant and Dong, 2004). *PR1, PR5* and *PR9* have been shown to be expressed in the mesophyll of both resistant and susceptible barley lines within 24 h after infection *R. commune* isolate UK7. This activation is prolonged until 72 hpi in susceptible cv Atlas, which lacks the *rrs1* R gene compared to resistant Atlas 46 (*RRS1)* (Steiner-Lange

et al., 2003). The NONEXPRESSER OF PR GENES 1 (NPR1) protein is considered to be a master regulator of SA signalling and SAR, acting upstream of *PR* genes (Ding et. al., 2018). *NPR1* was also shown to be required for SA mediated suppression of JA responses (Sopel et. al., 2003). Therefore, monitoring *NPR1* expression is also likely to be of interest when studying barley x *R. commune* interaction.

SA also induces the expression of downstream transcription factors, such as WRKY33, that regulate important aspects of the plant's defence responses (Zheng et al., 2006). In *Arabidopsis, AtWRKY33* expression is significantly increased in *Arabidopsis* after pathogen infection, including *B. cinerea* and *Alternaria brassicicola* (Zheng et. al., 2006; Tao et. al., 2021), and is expressed at low levels in healthy plants. Disruption of *AtWWRKY33* results in increased susceptibility to the necrotrophic pathogens *B. cinerea* and *A. brassicicola* (Zheng et. al., 2006). Barley *WRKY43* and *WRKY50* have been identified as orthologues to *AtWRKY33* (Liu et al., 2014), and could potentially play a similar role in activation of immune response genes in the context of *R. commune* infection.

Another important regulator of plant defences against pathogens is the small molecule nitric oxide (NO), whose concentration is tightly regulated via both production and scavenging mechanisms (Shapiro, 2005). Plant hemoglobins, including HEMOGLOBIN (HB), scavenge NO under stress conditions (Groß et. al., 2013). In *Arabidopsis*, hemoglobins influence both the SA and JA/ethylene defence signalling pathways in response to hemibiotrophic *Pst* and necrotrophic *B. cinerea* pathogens (Mur et al., 2012). Monitoring *HB* expression in barley in response to *R. commune* could be relevant, not only because of the hemi-biotrophic lifestyle of the pathogen, but also because of the central role of NO signalling in plant defences against pathogens.

Previously, Al-Daoude et al., (2014) described the expression profile of *RAR1* in response to *R. commune* (isolate Rs46) infection of the resistant German barley cv Banteng. *RAR1* showed an increase of expression levels at 2 dpi, reaching its highest level at 5 dpi. Although this was shown using semi-quantitative RT-PCR as opposed to RT-qPCR, these results point to (i) a

potential role of *RAR1* in response to *R. commune*, and (ii) the fact that *RAR1* might be a good marker to monitor barley response to *R. commune*. *RAR1* encodes a zinc ion binding protein and is required for resistance signalling triggered by multiple R proteins, acting upstream of ROS production during ETI (Shirasu et al., 1999). The RAR1 protein interacts and forms a complex with SGT1 and HSP90 in barley during the immune response (Austin et al., 2002). SGT1 has also been shown to be a positive regulator of R gene mediated resistance (Austin et. al., 2002; Peart et. al., 2002). In *Arabidopsis,* there are two functionally redundant SGT1 proteins. Compared to wild type (WT) plants, *rar1 sgt1a* double mutants in *Arabidopsis* showed increased bacterial growth of *Pst* DC3000 expressing the effectors *avrRpt2, avrRpm1* or *avrPphB* at 3 dpi (Azevedo et. al., 2006), indicating that they are more susceptible. Additionally, the RAR1/SGT1 complex is required for barley MILDEW LOCUS A (Mla) mediated resistance to the powdery mildew fungus *Bgh* (Austin et. al., 2002).

BAX-INHIBITOR 1 (BI1) is a known conserved cell death regulator in animals and plants (Hückelhoven, 2004). Mutations in plant cell death regulators often show enhanced resistance to biotrophic pathogens, which require living host tissue to complete their life cycle or enhanced susceptibility to necrotrophic pathogens. Specifically, *BI1* expression was shown to increase after inoculation of susceptible barley cv Ingrid with *Bgh.* Additionally, virus and transient induced gene silencing (VIGS and TIGS) of barley *BI1* resulted in enhanced resistance to *Bgh* (Eichmann et. al., 2010). It has also been suggested at barley *BI1* functions downstream of *MLO,* as overexpression of *BI1* partially restored susceptibility to *Bgh* in *mlo* mutants (Eichmann et. al., 2006).

The best characterised S-gene in barley is *MILDEW LOCUS O (MLO)* which, as described in section 1.5.3, is involved in barley susceptibility to *Bgh* (Jorgenson 1992). Interestingly, it has also been suggested that active MLO may also reduce susceptibility to *R. commune* (Makepeace et al., 2007). This was determined in field trials and scoring disease incidence in cvs with and without functioning *MLO.* In addition, there is evidence that loss of *MLO* function can increase susceptibility to other barley pathogens such as

hemibiotrophic *Magnaporthe oryzae* (Jarosch et al., 1999), suggesting that *MLO* may not act as a susceptibility gene in all pathosystems.

2.2 Chapter Aims

The primary aim of this chapter was to identify the optimum growth conditions at which plants should be inoculated via different inoculation methods using a selection of Irish *R. commune* isolates and a number of barley cultivars. The barley cultivars were selected due to their diverse response to *R. commune* based disease response ratings (rr) as assigned by DAFM. These disease ratings are on a scale of 1-9, with 1 representing full susceptibility, and 9 representing full resistance. Different protocols were tested, including a pilot glasshouse study in spring barley against six Irish *R. commune* isolates, a detached leaf assay based on methods described in Newton et. al., (2001) in spring and winter barley against a single *R. commune* isolate. Finally, the use of a controlled atmosphere growth chamber proved the most reliable in producing a reproducible method to characterise the interaction between two *R. commune* isolates against susceptible and resistant winter barley cvs Cassia (rr = 4) and Infinity (rr = 7) (DAFM, 2022).

Once the experimental method had been optimised, the secondary objective of this chapter was to characterise the *R. commune* isolates in Cassia and Infinity to establish an infection timeline and identify significant timepoints during infection at which genome-wide gene expression changes could be monitored. The infection timecourse was established using three approaches:

(i) an assessment of the visual onset of symptoms to determine the timing of the latent and necrotic stages of the disease;

(ii) to follow the infection from the viewpoint of the pathogen, gDNA was collected from infected leaf tissue at multiple timepoints to quantify the accumulation of fungal biomass over time, using qPCR as described by (Fountaine et al., 2007);

(iii) to monitor the plant response to the infection, RT-qPCR was used to quantify the expression of defence related genes described above over the infection timecourse. These infection assays were carried out to identify the

appropriate sampling timepoints for a global transcriptome analysis study described in chapter 3.

2.3 Methods

2.3.1 Barley seed sterilisation

For the seedling assays described in section 2.3.2, barley seeds were first washed in 70% (v/v) ethanol for 3 minutes (min), then rinsed in sterile distilled water (SDW) three times for 1 min. Seeds were then washed in 25% sodium hypochlorite (v/v) for 20 min and rinsed in SDW until all the sodium hypochlorite was removed (8-15 washes). The seeds were air dried in sterile 9 cm Petri dishes under a laminar hood. Seeds were not checked for *R. commune* infection as they were sterilised prior to use.

2.3.2 Barley growth conditions

2.3.2.1 Glasshouse assay

Seeds of barley cultivars (cvs) Golden Promise, Propino, Tesla, Snakebite and Sanette (Table 2.1) were sown in 10 cm pots filled with John Innes #2 soil, two seeds per pot, and grown in a glasshouse at 18˚C under lights with a 16 hour light period until growth stage (GS) 13 (third leaf unfolded).

Table 2.1: List of barley cultivars and corresponding resistance ratings.

Cultivar	Rating	Resistance	Sowing	Reference
Cassia	4	Susceptible	Winter	DAFM, 2022
Golden Promise	5	Moderately susceptible	Spring	HGCA, 1990
Snakebite	5	Moderately susceptible	Spring	DAFM, 2012
Propino	6	Moderately resistant	Spring	DAFM, 2019
Tesla	7	Good resistance	Spring	HGCA 2015
Sanette	7	Good resistance	Spring	DAFM, 2017
Infinity	7	Good resistance	Winter	DAFM, 2022

2.3.2.2 Detached leaf assay

Sterilised barley seeds of cvs Propino, Golden Promise and Cassia (Table 2.1) were stratified on filter paper moistened with SDW at 4˚C in the dark for 7 days to synchronise germination of spring and winter varieties. Using forceps, sterile seeds were transferred into 15 ml falcon tubes with 2 ml 0.5X seed germination media (SGM) (Appendix 1), one seed per tube. The tubes were placed at 18˚C under 24 h light and grown until GS11 (first leaf fully unfolded).

2.3.2.3 Controlled atmosphere growth chamber assay

Sterilised barley seeds of cvs Infinity (Table 2.1) and Cassia were stratified on filter paper moistened with SDW at 4˚C in the dark for 7 days. Seeds were then transferred into 9 cm pots filled with John Innes #2 soil, one seed per pot. Plants were grown in controlled growth chambers (Snijders Micro Clima MC1750) at 18˚C, 80% relative humidity (RH) at a 16 h day photoperiod until GS13.

2.3.3 Isolation of *R. commune* field isolates

Leaves displaying scald lesions were collected from fungicide evaluation plots (i.e., no fungicide treatment control plots) of Cassia and Propino in Oak Park, Co. Carlow, Ireland (Table 2.2). Leaf sections were left to dry at room temperature for 48 h. Sections of the leaf displaying lesions were excised and washed under running water for 2 h. Lesions were then surface sterilised by placing in 70% (v/v) ethanol for 20 seconds (s) to remove the outer waxy layer of the leaf and transferred to 10% (v/v) sodium hypochlorite for 2 min. The leaf sections were then rinsed three times in SDW. The cleaned leaf sections were dried between two sheets of sterile filter paper. Using a scalpel, the lesions were cut into four sections and placed onto Czapek Dox agar (CDA) (Appendix 1) with chloramphenicol (50 mg/L) and streptomycin (50 mg/L). Plates were sealed with Parafilm and incubated at 18˚C in the dark for 14 days. This procedure was carried out to isolate the 2018 *R. commune* isolate OP18(9), used in this thesis (Table 2.2). The remaining *R. commune* isolates used in this thesis were provided by Dr. Kildea (Teagasc, Table 2.2). All were isolated using a similar protocol.

Table 2.2: List of *R. commune* isolates used in this chapter.

Isolate	Year Collected	Cultivar	Location
OP18(9)	2018	Cassia	Oak Park, Co. Carlow
OP16(27)	2016	Cassia	Oak Park, Co. Carlow
OP16(21)	2016	Cassia	Oak Park, Co. Carlow
OP16(22)	2016	Cassia	Oak Park, Co. Carlow
OP16(16)	2016	Cassia	Oak Park, Co. Carlow
L15	2015	Unknown	Lagganstown, Co. Tipperary
44.07	2007	Cassia	Oak Park, Co. Carlow

2.3.4 Fungal growth conditions

After two weeks of growth on CDA, sporulating mycelia were scraped off the initial isolation plates and transferred into a 1.5 ml microcentrifuge tube containing 1 ml SDW and vortexed for 5 seconds. 100 µl of the mix was pipetted onto ten freshly prepared CDA plates with chloramphenicol (50 mg/L) and streptomycin (50 mg/L) and spread using an inoculation spreader. Plates were sealed with Parafilm and incubated at 18˚C in the dark for 14 days.

2.3.5 Spore suspension preparation

R. commune spores were harvested by flooding two week old plates with 5 ml SDW and scraping the sporulating mycelia loose with an inoculation spreader. The suspension was filtered through a double layer of muslin cloth. The spore concentration was calculated using a Glasstic™ slide, with hemocytometer type grid to count spores and the concentration adjusted to $1x10^6$ spores/ml or $1x10^7$ spores/ml (dependent on experimental requirements) using SDW. For spray inoculation of seedlings (sections 2.3.6.1 and 2.3.6.3), the spore suspension was transferred into a handheld spray bottle with 0.1% (v/v) Tween 20 to act as a surfactant.

2.3.6 Barley x *R. commune* host-pathogen assays

2.3.6.1 Pilot glasshouse experiments to test inoculation protocol

R. commune spores from isolates 44.07, OP16(22), OP16(21), OP16(16), OP16(27) and L15 (Table 2.2) were harvested as described in section 2.3.5 with spore concentration adjusted to $1x10^6$ spores/ml. A mock treatment of SDW with 0.1% (v/v) Tween 20 was used as a control.

Two test screens were carried out in glasshouses. At GS13 (third leaf fully unfolded), 4-8 seedlings of barley cvs Propino, Tesla, Sanette, Snakebite and Golden Promise (Table 2.1) were spray inoculated until run off and covered with plastic bags for 48 h to maintain RH close to 100%. The seedlings were placed in a glasshouse at 18 °C, under lights to maintain a 16 h photoperiod. After 48 h the bags were removed. The seedlings were monitored every 2-3 days for disease symptoms, which were scored as percentage of leaf area displaying scald like lesions, and total loss of green leaf area as described by James (1971).

2.3.6.2 Detached leaf assay to quantify fungal biomass accumulation in barley.

Seeds of barley cvs Cassia, Propino and Golden Promise (Table 2.1) were grown as described in section 2.3.2.2. A 4-5 cm section of the first unfolded leaf was cut using a scalpel blade. Six leaf sections from 6 independent plants were placed into a 10 cm square petri dish containing 1% (w/v) agar with benzimidazole (100 mg/L). The cut ends of the leaf were sandwiched between cut strips of 1% (w/v) water agar (Figure 2.1) to minimize the potential for senescence. A brush was used to gently abrade the leaf surface at the two points of inoculation. 10 µl of a $1x10^6$ spores/ml suspension (*R. commune* isolate 44.07, Table 2.2) was pipetted onto the abraded area, with two inoculation points per leaf section. 10 µl of distilled water was used as a mock treated control. The plates were sealed using micropore tape and incubated at 18˚C for 10 days (16 h day photoperiod). Leaf sections were collected 10 days post inoculation (dpi) and freeze-dried for DNA extraction (section 2.3.7) and *R. commune* biomass quantification (section 2.3.9).

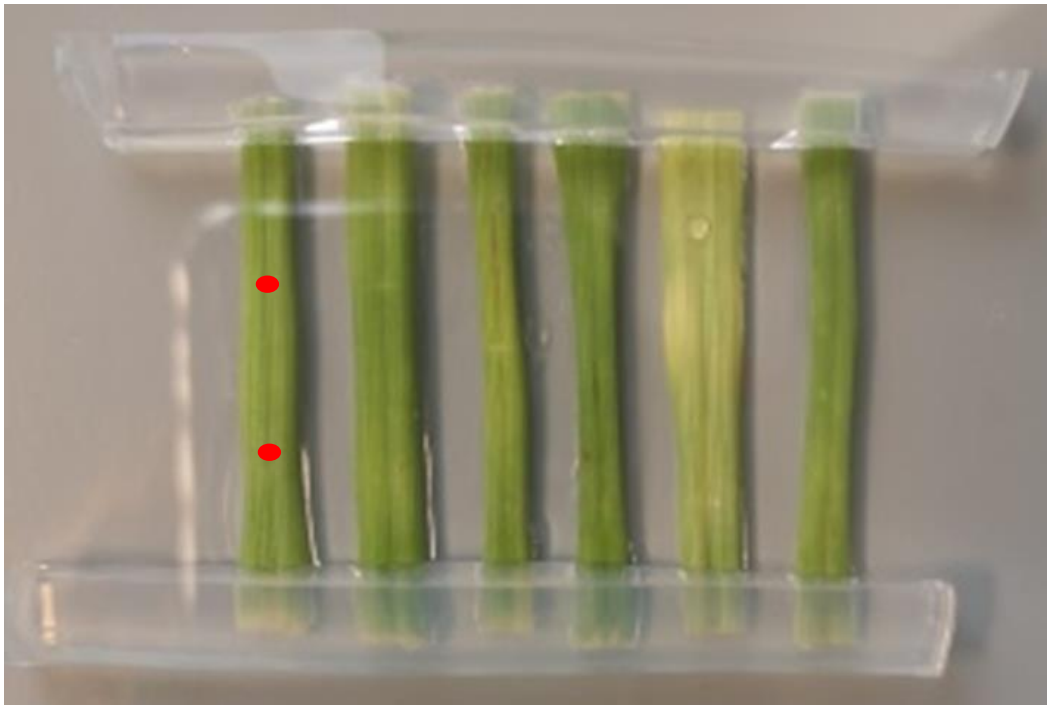


Figure 2.1: Detached leaf assay set up. 4-5 cm leaf sections of the first unfolded leaf from six individual plants were placed on water agar with the cut ends sandwiched between two layers of agar to slow down senescence and to hold leaf sections in place. Inoculation point indicated in red along the midvein.

2.3.6.3 Growth chamber assay to investigate fungal biomass accumulation and barley response to *R. commune.*

R. commune spores from isolates OP18(9) and 44.07 were harvested as previously described in section 2.3.5 and the spore concentration was adjusted to either $1x10^6$ or $1x10^7$ spores/ml. A mock treatment of SDW with 0.1% Tween 20 (v/v) was used as a control.

Eighteen Cassia and Infinity seedlings per treatment were spray inoculated at GS13 until run off and covered with plastic bags to keep RH at 100% to aid fungal penetration. After 48 h the plastic bags were removed, and seedlings kept at 80% RH. Disease was scored as the percentage of leaf area displaying scald like lesions and as total loss of green leaf area at 0, 2, 7, 10, 14, 17, 19 and 21 dpi for the $1x10^6$ spores/ml treatment. The second emerged leaf (leaf 2) was harvested at each timepoint except at 17 and 19 dpi and flash frozen

in liquid nitrogen and stored at -80˚C prior to nucleic acid extraction (2.3.7). Following this, a second analysis was carried out at a higher inoculation dose of $1x10^7$ spores/ml to increase disease incidence and elicit a stronger host response. Leaf 2 was collected for nucleic acid extraction at 0, 0.5, 1, 1.5, 2, 5, and 12 dpi.

2.3.7 Genomic DNA (gDNA) extraction

Two extraction processes were used: a manual CTAB-based approach for gDNA extraction from whole leaves (glasshouse and growth chamber assays) or from *R. commune* mycelia and an automated extraction system for gDNA extraction from leaf sections from the detached leaf assay.

For the manual CTAB method, 100 mg of frozen leaf tissue or 50 mg freeze dried *R. commune* mycelia was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was then transferred to a 2 ml microcentrifuge tube. A 1:10 volume of 2% (w/v) CTAB buffer (Appendix 1) was added to the ground tissue, mixed and incubated at 65˚C for 20 min. One volume of 24:1 chloroform: isoamyl alcohol (IAA) was added and the tube was vortexed for 30 s, followed by centrifugation at 11,000g for 10 min. The aqueous (upper) layer was removed and transferred into a fresh 1.5 ml microcentrifuge tube. 3 µl RNaseA was added and incubated at 37°C for 15 min to remove residual RNA contamination and the chloroform: IAA separation step was repeated. One volume of isopropanol was added to the supernatant, and the solution incubated at -20˚C for 1 h. The samples were centrifuged at 11,000g for 15 min to pellet the DNA. The isopropanol was removed, and the pellet washed twice in 70% (v/v) ethanol. Pellets were air dried and re-suspended in 50 µl SDW. DNA quality and quantity were determined on 1% (w/v) agarose gel at 60 V for 1 h and on a Nanodrop spectrophotometer, respectively.

For automated DNA extraction, leaf sections were freeze-dried at -50˚C for 48 h in 96 deep well plates and milled to a fine powder using steel 3 mm beads in a Retsch mixer mill. DNA extraction was carried out using the MagMAX Plant DNA Extraction Kit (Thermo Fisher Scientific) on the KingFisher™ Flex Purification System following manufacturer's guidelines. DNA quality and

quantity were determined on 1% agarose gel at 60 V for 1 h and on a Nanodrop spectrophotometer, respectively.

2.3.8 RNA extraction and cDNA synthesis

For RNA isolation, 150 mg of frozen tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich) or the RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's protocol. RNA quality and quantity were determined by visualisation of rRNA on a 1% agarose gel at 60 V for 45 min, and on a Nanodrop spectrophotometer, respectively. Comparison of RNA yield from both kits showed significantly higher RNA yield from the Sigma-Aldrich kit (t-test, $p < 0.05$) (Appendix 2, Supplemental figure S2.1), which was then used for all subsequent RNA extractions. Residual gDNA was eliminated using 2 µl gDNA wipeout solution from the Quantitect® Reverse Transcription Kit (Qiagen). 1 µg total RNA was then reverse transcribed using oligo-dT primers to produce cDNA for each sample using the Quantitect® Reverse Transcription Kit (Qiagen) according to manufacturer's protocol. cDNA was stored at -20˚C until needed.

2.3.9 Quantification of *R. commune* gDNA

DNA was extracted from 50 mg freeze dried mycelia from *R. commune* isolate 44.07 using the CTAB method as described in section 2.3.7. Total DNA concentration was determined on a Nanodrop spectrophotometer and adjusted to 100 ng/µl. A ten-fold serial dilution of the gDNA was prepared ranging from 100 ng/µl to 100 fg/µl. qPCR using a locked nucleic acid (LNA) probe (Table 2.3) was carried out in triplicate on each sample in the serial dilution to quantify the *R. commune cytochrome B* gene [Gene ID: DQ463419.1] as described by Fountaine et. al., (2007). Each well on a 96 well plate contained 1 µl template gDNA and 9 µl mastermix. The mastermix comprised of 1X PCR mastermix (Biorad), 0.4 µM forward primer (Rsrtpcr1F), 0.4 µM reverse primer (Rsrtpcr1R), 0.1 µM LNA fluorogenic probe (Rsrtpcr1P) and SDW. The sequence of the primers and fluorescent probe used are shown in Table 2.3. Volumes and component ratios were calculated and made up in master mixes to minimise the impact of pipetting. Amplification and detection

was carried out in a Roche LC96 lightcycler under the following conditions: 1 cycle at 94˚C for 10 minutes, followed by 45 cycles at 95˚C for 15 s and 60˚C for 1 min. The fluorescence from the probe was recorded at 60˚C for each cycle. Two negative controls (i) uninfected barley gDNA and (ii) a no-template control (SDW in the place of gDNA template), and one positive control (100 ng *R. commune* gDNA) were run simultaneously with experimental samples on each qPCR plate. The Cq value obtained was plotted against the concentration of *R. commune* gDNA to produce a standard curve (Appendix 2, Supplemental Figure S2.2). This process was repeated with each sample spiked with 100 ng of barley DNA to show that the presence of barley DNA did not interfere with the amplification of the *R. commune* cytochrome B region. Two biological replicates were completed in triplicate to generate the standard curves.

Fungal biomass accumulation in inoculated plants was determined similarly using the same qPCR method measuring the *R. commune Cytochrome B* gene but following gDNA extraction from inoculated leaf tissue (as outlined in section 2.3.7). Quantification of fungal biomass was determined in ng *R. commune* DNA / 100 ng total DNA extracted by comparing Cq values of experimental samples against the established standard curve.

Table 2.3: Primer and probe sequences for detection of genomic *R. commune* cytochrome B by qPCR (sequences from Fountaine et. al., 2007).

Primer/probe name	**Type**	**Sequence 5'→3'**
Rsrtpcr1F	Forward primer	ATGTGCTTCCTTATGGACAGATGT
Rsrtpcr1R	Reverse primer	ATTATTAACAGAAAAACCCCCTCAGAT
Rsrtpcr1P	LNA probe	/56-FAM/TAT G+AG+GTGCC+AC+AGT/3BHQ_1/

2.3.10 Barley gene expression analysis

Suitable reference genes for normalisation of RT-qPCR data are usually housekeeping genes that have constant and stable expression that is unaffected by the experiment. However, some studies have shown that it is important to identify suitable reference genes for a particular pathosystem. For example, the cycle of quantification (Cq) of 14 candidate reference genes in cucumber after infection with *Pectobacterium carotovorum* subsp*. Brasiliense (Pcb)* showed that commonly used reference genes were not expressed to the same level, and that some were not suitable for gene expression normalisation due to changes in Cq values after *Pcb* infection (Yuan et. al., 2022). Hence, to allow for the robust analysis of the relative expression of selected barley genes thought to be involved in barley immune signalling in response to *R. commune* infection, the suitability of five candidate reference genes were tested.

Candidate housekeeping genes for normalisation were selected based on their use in literature: *UBIQUITIN (UBI), ELONGATION FACTOR 2 (EF2), GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE (GAPDH), TUBULIN α* and *ACTIN* (Table 2.4) (Miricescu, 2019; Gines et. al., 2018; Ferdous et. al., 2015). The Cq values of these genes were examined for each treatment across all timepoints in *R. commune* and mock treated barley tissue using RT-qPCR to determine if expression was stable over the course of the experiment. *TUBULIN α* and *ACTIN* were selected for normalisation of target genes of interest.

Table 2.4: Barley housekeeping genes and primer sequences tested for normalisation of RT-qPCR expression data.

Primer name	Gene	Sequence (5' → 3')	Reference
HvActin_AM45_F1	Actin	GCAAGTGGCGTACTACT GGTATCGTTC	Miricescu (2019)
HvActin_AM46_R1	Actin	GGATCTTCATAAGGGAG TCCGTGAGAT	Miricescu (2019)
GAPDH_AM49_F3	GAPDH	GCTCACTTGAAGGGTGG TGCC	Miricescu (2019)
GAPDH_AM50_R3	GAPDH	TGATGGCATGAACAGTG GTCATCAGAC	Miricescu (2019)
UBI-F2	UBI	CCAGAAGGCTTAGAGGT GGCTTG	This study
UBI-R2	UBI	GCATCGCATTACAGGAG TAGGCG	This study
TubulinAlpha_F1	Tubulin *α*	TGGTCATTACACCATTGG CAAGGAGA	This study
TubulinAlpha_R1	Tubulin *α*	GTGTATGTTGGGCGCTC AATGTCA	This study
EF2_F2	EF2	CAAGAGAGTTGGGTCGT CTATCGC	This study
EF2_R2	EF2	CTCGGCCTGAGTTCGGA ACAC	This study

qPCR using cDNA generated from total RNA extracted from leaves of *R. commune* treated plants was carried out in 96 well plates, with each sample represented by the gene of interest and two reference genes (*TUBULIN α* and *ACTIN*). Barley target genes of interest are shown in Table 2.5. Individual reactions contained 1 µl cDNA, 1 µM of each primer and 1x Quantifast SYBR Green® PCR Master Mix (Qiagen) in a final volume of 10 µl. Plates were run on a Roche LC96 Lightcycler under the following conditions: initial heat activation at 95˚C for 5 min, followed by 40 cycles of denaturing at 95˚C for 10 s and a combined annealing/extension step at 60˚C for 30s. Data acquisition was at the combined annealing/extension step. The mean Cq value generated from the two reference genes was used to calculate the relative gene expression of target genes for each sample by calculating $2^{\Delta Cq}$.

Table 2.5: Barley target genes and primer sequences used for RT-qPCR.

Primer name	Gene ID	Sequence (5' → 3')	Reference
NPR1_F1	HORVU3Hr1G019230	TGCATTGAGATACTG GAGGAAGCTGA	This study
NPR1_R1	HORVU3Hr1G019230	CTCGCCAAAGCCAC TCGGT	This study
WRKY50_F2	HORVU3Hr1G088200	CCAAGAGGAGGAAA GACGGTGG	This study
WRKY50_R2	HORVU3Hr1G088200	GTGCACTTGTAGTA GCTCCTCGG	This study
WRKY43_F2	HORVU1Hr1G070250	AGTCGGCGGCTCGA ATTGC	This study
WRKY43_R2	HORVU1Hr1G070250	CTCAACTGGTGGGA GACTCGTAC	This study
RAR1_F1	HORVU2Hr1G097800	TGTTCTGACCATGG ATCACAGCC	This study
RAR1_R1	HORVU2Hr1G097800	GCAGCATCATGGTT ATCCTTCTCCTT	This study
BI1_F1	HORVU6Hr1G014450	AGTCTATGAGGAGC AGAGGAAGAGG	This study
BI1_R1	HORVU6Hr1G014450	GCGATGGCGGTTCC GACAA	This study
PR1_F2	HORVU7Hr1G033530	AACGATTTAGCTAGA GGGACCGAGC	This study
PR1_R2	HORVU7Hr1G033530	GACTGAATGTTCTGT GCTTATCGAGGTC	This study
SGT_F1	HORVU3Hr1G055920	TGATCGTATTGCTGA GGAGGCTAGC	This study
SGT_R1	HORVU3Hr1G055920	TTGTTCAATGCAGCA TCACCGTCA	This study
HB_AM51_F1	HORVU4Hr1G066200	CGGGAAGGAAGCCA TGTCTGC	Miricescu (2019)
HB_AM_R1	HORVU4Hr1G066200	TCTGCCTCGCCGAC GG	Miricescu (2019)
CERK1_F1	HORVU3Hr1G084510	GAGAAGCTGCTGTG GGAGTCC	This study
CERK1_R1	HORVU3Hr1G084510	ACCTCGTACATCGA CGGTCGT	This study
MLO_F1	HORVU4Hr1G082710	CCTCTCGTGATCCT CTTGTGTGT	This study
MLO_R2	HORVU4Hr1G082710	CGTTCTGGAACAAC GTCAGGTGT	This study

2.3.11 Statistical analyses

For gene expression analysis (section 2.3.10), expression of a gene of interest relative to mock treatment was calculated by the $2^{\Delta Cq}$ method. The mean relative expression for three plants was determined within a single biological replicate. Three biological replicates were carried out. An analysis of variance (ANOVA) was completed followed by Tukey pairwise comparisons to determine significant differences between *R. commune* and mock treated data at a given timepoint using R (version 4.0.2). In section 2.2.4, in which there were multiple *R. commune* treatments at different doses, a two-way ANOVA was carried out to determine significant differences in mean relative expression between *R. commune* treated and mock, and between different *R. commune* inoculation doses at a given timepoint.

2.4 Results

2.4.1 Pilot study of *R. commune* in barley in glasshouse conditions

In order to identify suitable conditions to study the barley x *R. commune* interaction, a pilot study was carried out in which a selection of barley cultivars were inoculated with Irish *R. commune* isolates collected between 2007 and 2016. Barley cvs were selected according to their resistance rating (rr) as defined by DAFM in Ireland and Home-grown cereals authority (HGCA) in the UK. In the first replicate of the pilot study, barley cvs Tesla, Sanette, Propino and Snakebite (Table 2.1) were selected, and spray inoculated with one each of the following *R. commune* isolates: OP16(27), OP16(16), 44.07, L15, OP16(21), OP16(22) (Table 2.2), as described in section 2.3.6.1.

Figure 2.2 shows the mean percentage green leaf area displaying scald like lesions in each cultivar for each *R. commune* treatment, and a mock (water) control. Data is for one biological replicate, consisting of 10 seedlings per treatment. Surprisingly, the more resistant cv, Tesla, showed higher levels of *R. commune* symptoms, an average of up to 20% of leaf area showing scald lesions on leaf 2 at 23 dpi after treatment with the 44.07, OP16(27) and OP16(22) isolates, and up to 15% on leaf 3 in response to all treatments. In comparison, the most susceptible cv Snakebite showed between 0 and 12% mean leaf area displaying scald lesions on leaf 2, and less than 10% mean leaf area on leaf 3 in response to all treatments. The OP16(16) *R. commune* isolate induced symptom appearance at 15 dpi, which is earlier than other isolates, where lesion area increased between 15 and 20 dpi in both leaf 2 and 3. This was recorded in all barley cvs. Unexpectedly, lesion appearance was recorded even on Mock treated samples. Particularly, for the resistant Tesla cv, over 30% of leaf 2 displayed lesions in the mock treated samples (Figure 2.2). This was higher than any of the *R. commune* treatments and is indicative of a problem with either the experimental design or with the conditions or seed used.

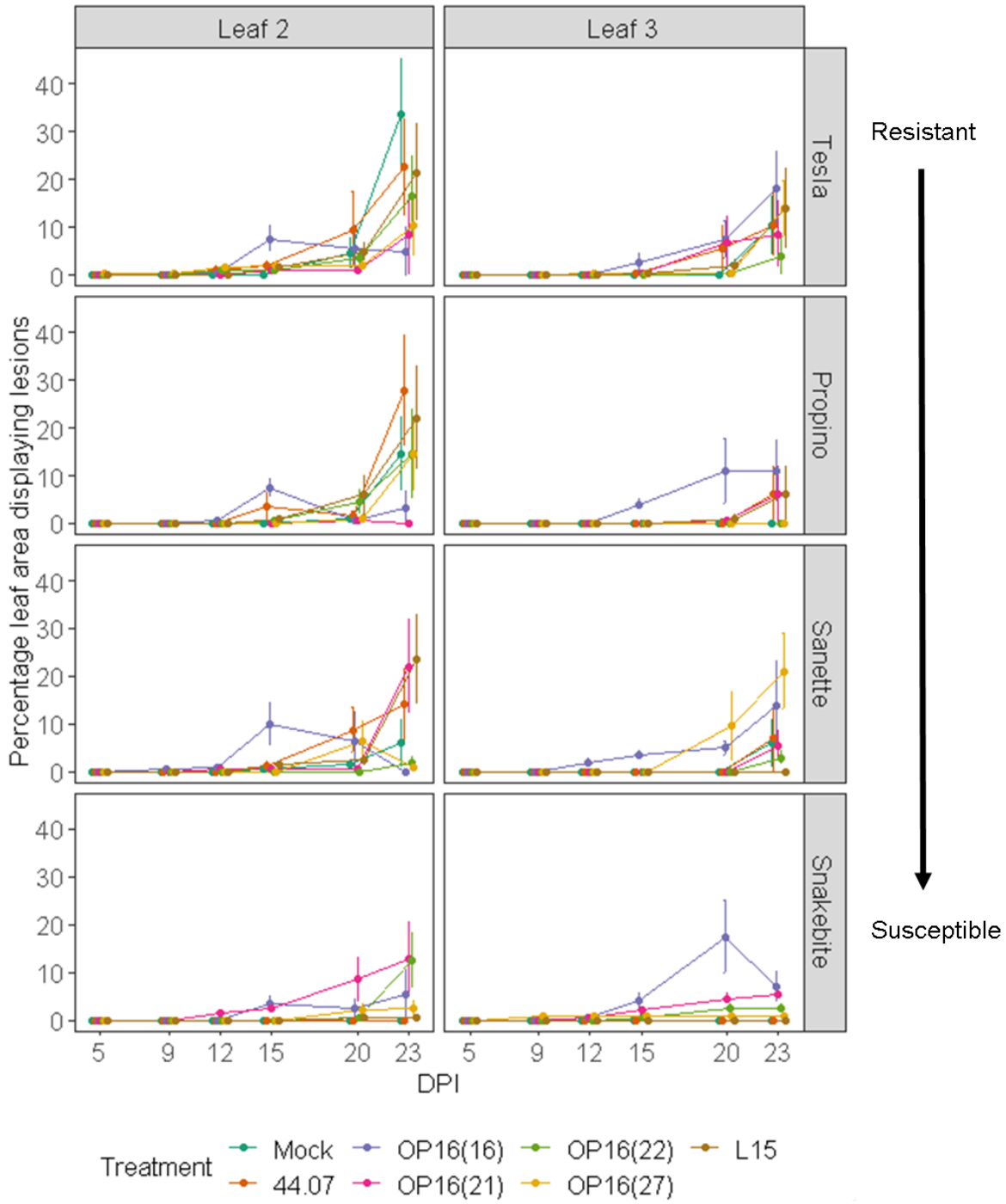


Figure 2.2: Percentage of leaf area displaying scald lesions after *R. commune* inoculation at 1 x 10^6 spores/ml of different barley cvs of increasing susceptibility to *R. commune*. Data is for one biological replicate, with each data point representing the mean leaf area of 10 seedlings. Error bars represent standard error of the mean (SEM).

In addition to scald lesions, barley leaf sections also showed other stress like symptoms including wilting, yellowing and necrosis after *R. commune* inoculation. This was also observed in leaf 2 of Tesla and Sanette mock

treated seedlings (Figure 2.3). By 21 dpi, it was difficult to distinguish between necrotic regions of the leaf and scald lesions. To account for all changes in leaf tissue after *R. commune* infection, total green leaf area was also recorded for each treatment and is shown in Figure 2.3. Loss of green leaf area was more severe in leaf 2 compared to leaf 3 for all cvs. Despite being the most susceptible cv, Snakebite showed the highest green leaf area on leaf 2 by 23 dpi. Loss of green leaf area in this cv was most severe in response to the OP16(16) isolate, with a mean loss of 75% in leaf 2 and 30% in leaf 3. In contrast, the most resistant cv, Tesla, showed a mean loss of green leaf area of up to 50% in leaf 2 at 23 dpi, in response to all treatments.

As with lesion formation in Figure 2.2, seedlings inoculated with the OP16(16) *R. commune* isolate displayed a reduction in green leaf area by 15 dpi, which is earlier than seedlings inoculated with the other *R. commune* isolates, where green leaf area began to decrease between 15 and 20 dpi in Sanette, and between 20 and 23 dpi in Tesla and Propino. Importantly, this data also shows that green leaf area was reduced in leaf 2 of Tesla, Sanette and Propino after inoculation with a mock treatment. This data hence points to problems with the growth conditions.

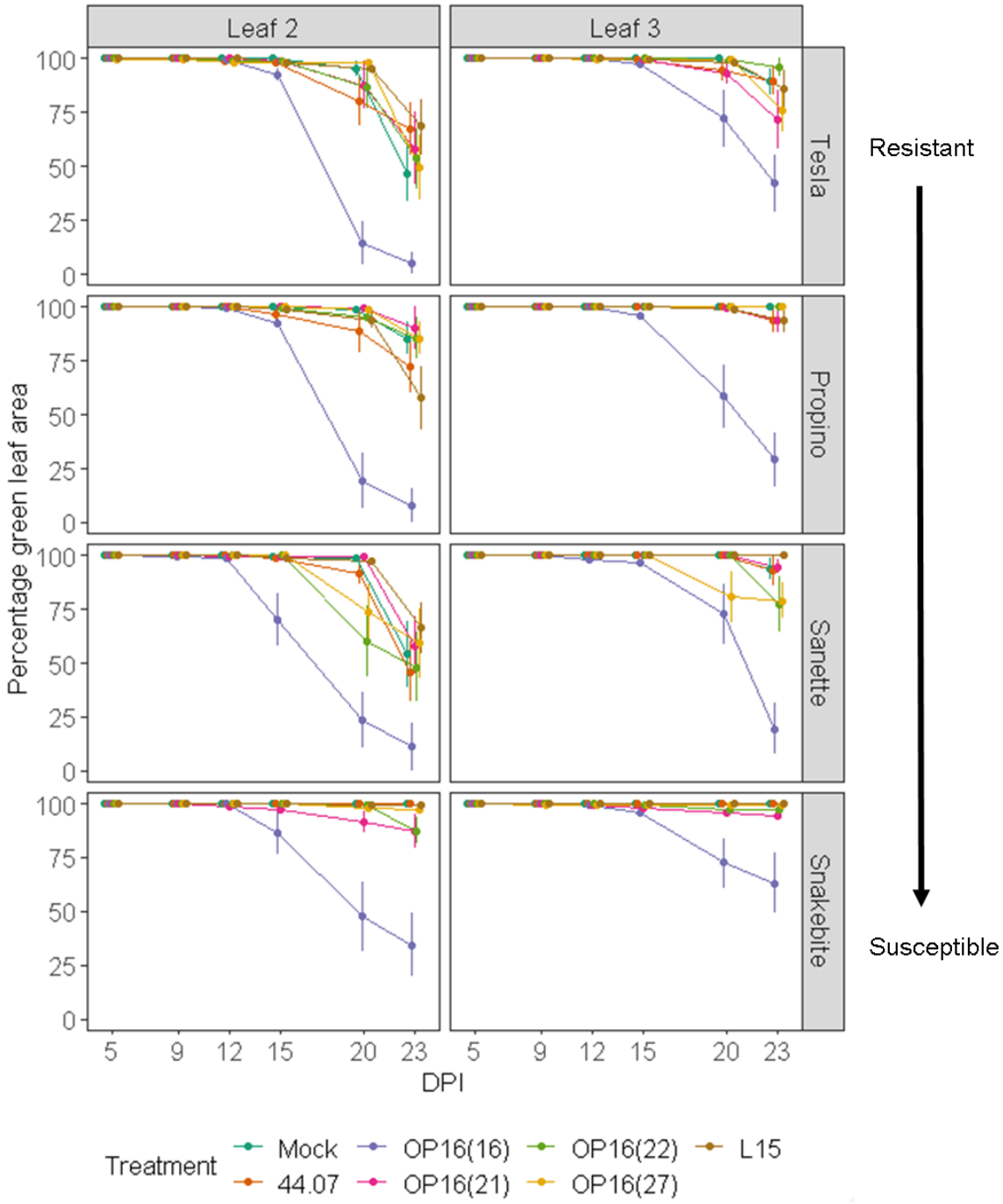


Figure 2.3: Mean green leaf area recorded as percentage after *R. commune* inoculation at 1 x 10^6 spores/ml of barley cvs of increasing susceptibility to *R. commune*. Data is for one biological replicate, with each data point representing the mean leaf area of 10 seedlings. Error bars represent SEM.

A second replicate of this pilot study was completed, however in this replicate, the *R. commune* isolate L15 was excluded due to contamination of a portion of the CDA plates, meaning that a spore concentration of 1 x 10^6 spores/ml

could not be achieved. Additionally, the barley cv Snakebite was excluded due to poor germination. The barley cv Golden Promise was introduced instead; similar to Snakebite, Golden Promise is also classified as susceptible to *R. commune.* Disease progression was also scored at 26 dpi instead of 23 dpi. As a result of these changes, data for the second replicate is presented separately. Similar to the first replicate, seedlings were spray inoculated at a concentration of 1 x 10^6 spores/ml as described in section 2.3.6.1. Similarly, disease incidence was scored as percentage leaf area displaying scald lesions (Figure 2.4) and loss of green leaf area (Figure 2.5).

Lesion formation was observed in resistant Tesla in response to all treatments (Figure 2.4). By 26 dpi, lesions covered on average up to 20% of the leaf area regardless of treatment. This is similar to lesion appearance by 23 dpi observed in the first replicate (Figure 2.2). Also similar to the first replicate, the more susceptible cv Sanette showed less symptoms compared to Tesla and Propino in leaf 3. Interestingly, despite being classed as susceptible, Golden Promise showed the least amount of lesions compared to the other cvs, with mean percentage leaf area displaying lesions observed being less than 20% in response to all treatments in leaves 2 and 3. However, as with the first replicate, other stress symptoms were observed including yellowing and necrosis in both *R. commune* and mock treated plants. Therefore, mean loss of green leaf area was also examined for the second replicate (Figure 2.5).

Loss of green leaf area in Tesla is most severe in response to the OP16(21) and OP16(27) in leaf two (46.67% and 32.5% green leaf area at 26 dpi respectively). However, there was large variation between seedlings of the same treatment. For example, in Tesla after inoculation with the OP16(27) isolate, by 26 dpi, 4 of the 6 seedlings showed complete loss of green leaf area on leaf 2 (0%) while the remaining two seedlings remained healthy (100% and 95% green leaf area).

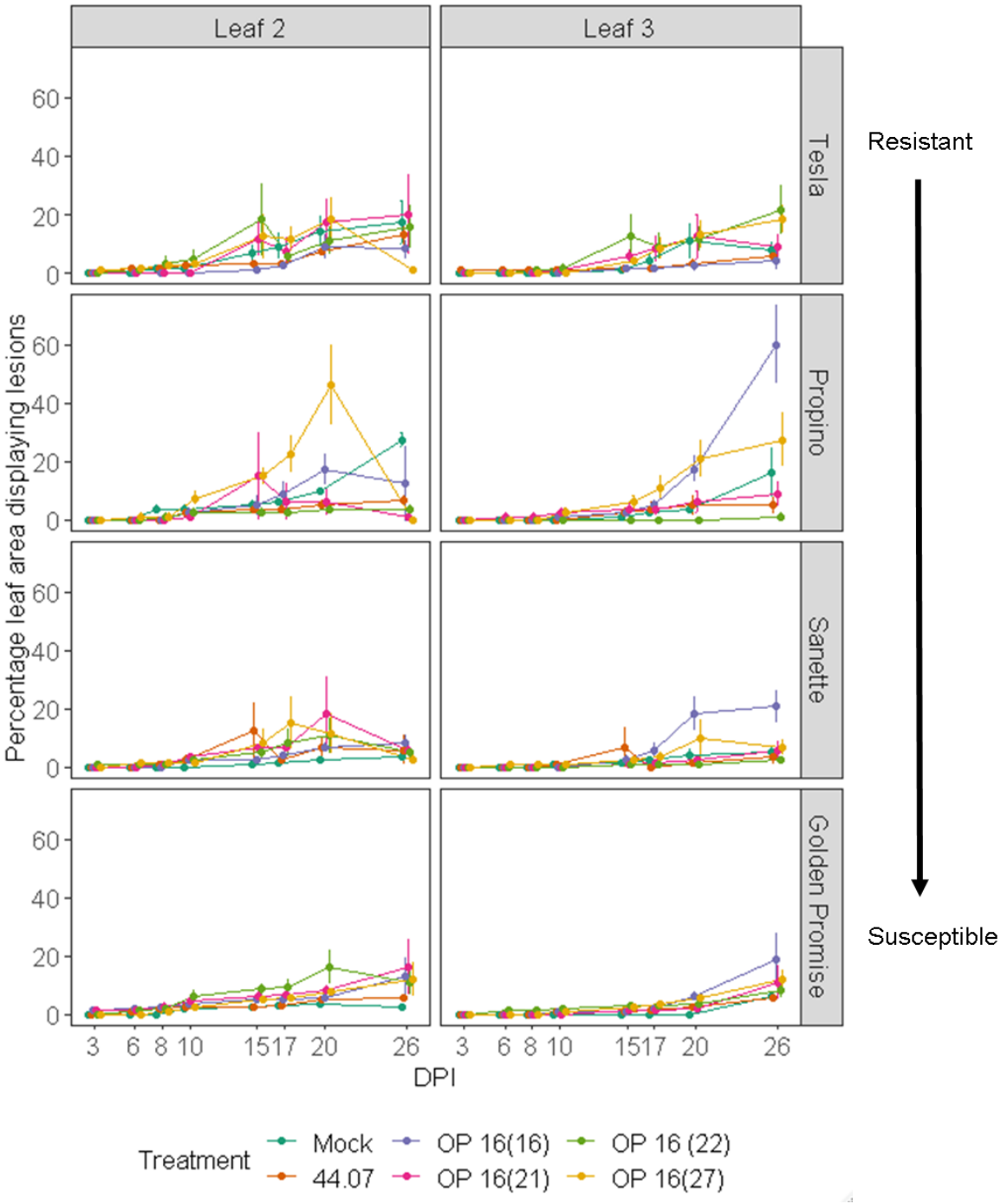


Figure 2.4: Mean percentage leaf area displaying scald lesions after *R. commune* inoculation at 1 x 10^6 spores/ml of barley cvs of increasing susceptibility to *R. commune*. Data is for one biological replicate, with each data point representing the mean leaf area. Number of seedlings inoculated: Tesla and Sanette n = 6, Propino and Golden Promise n = 8. Error bars represent SEM.

Propino showed greater loss of green leaf area in response to OP16(16) compared to the other cvs. (12.5% green leaf area remaining in leaf 2 at 26

dpi, and 40% remaining on leaf 3). However, similar to Tesla, this cv also showed severe loss of green leaf area in response to OP16(21) and OP16(27) isolates. In the more susceptible cv Sanette, inoculation with OP16(27) resulted in the greatest loss of green leaf area compared to the other treatments (15.8% remaining in leaf 2, and 47.5% on leaf 3). However, this was also variable between seedlings, with 3 of 6 seedlings showing complete loss of green leaf area in leaf 3 (0%) and the remaining 3 seedlings staying healthy (95-100% green leaf area). Golden Promise suffered the least loss of green leaf area after *R. commune* treatment, despite being the most susceptible cv. A loss of green leaf area between 10 and 25% was observed in all cvs by 26 dpi in mock treated samples.

These results indicated issues with growth conditions and experimental set up. As a result of problems such as potential cross contamination between *R. commune* and mock treated seedlings, inconsistencies between seedlings under the same treatment and issues experienced with temperature control in the glasshouse, it was not likely that data presented for the two replicates accurately depicted the interaction between the different cvs and *R. commune* isolates. Therefore, it was concluded that this method was not appropriate for analysis of host responses to *R. commune.* In the following sections, the use of a detached leaf assay and controlled atmosphere growth chambers are described, with the aim to overcome the problems encountered in the glasshouse.

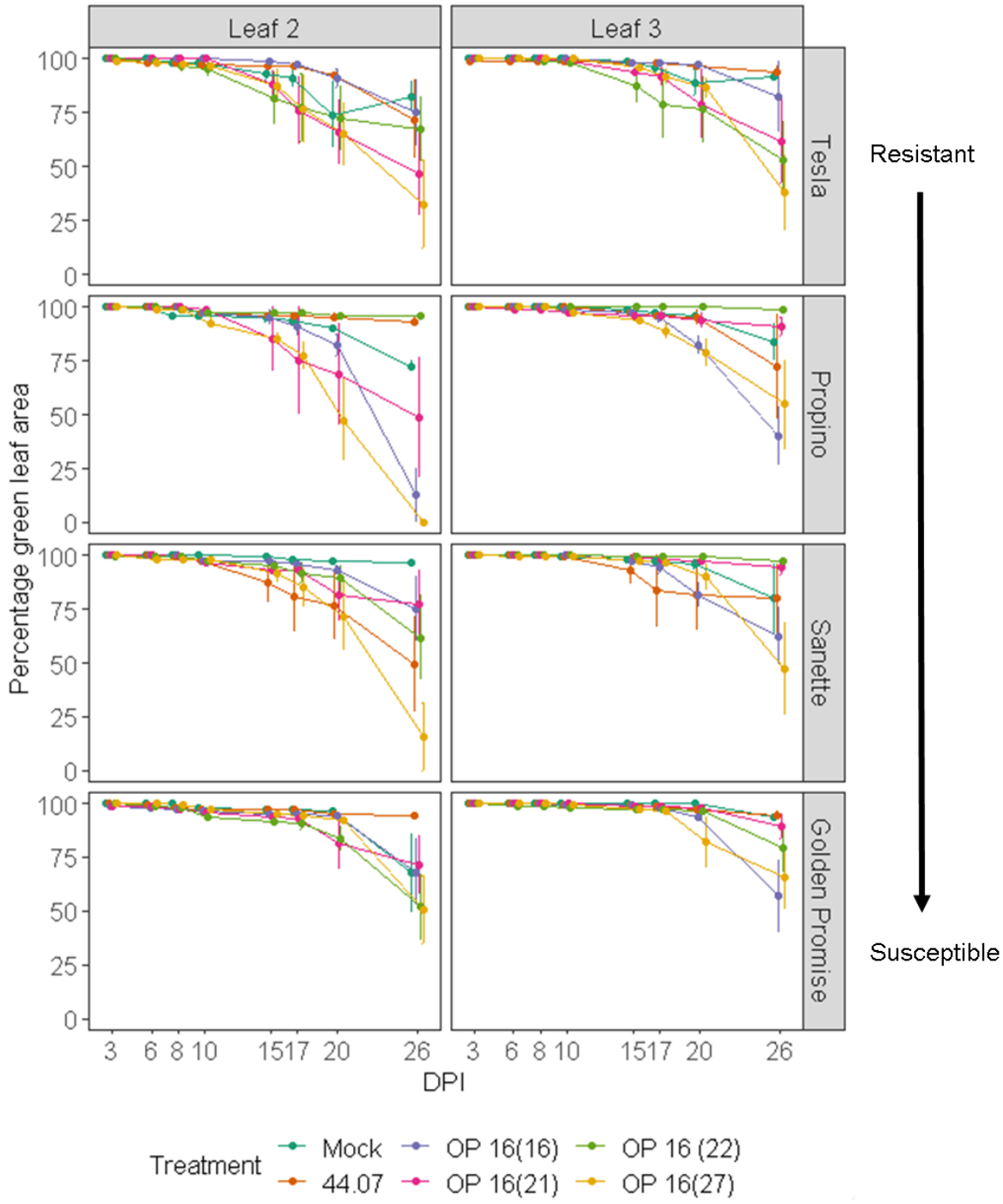


Figure 2.5: Mean percentage green leaf after *R. commune* inoculation at 1 x 10^6 spores/ml of barley cvs of increasing susceptibility to *R. commune*. Data is for one biological replicate. Number of seedlings inoculated: Tesla and Sanette n = 6, Propino and Golden Promise n = 8. Error bars represent SEM.

2.4.2 Quantification of fungal growth in barley using a detached leaf assay

In order to establish a method of examining *R. commune* proliferation in barley with complete control over external environment, as well as allowing to screen a larger number of technical replicates to determine variation between samples, a detached leaf assay was used as described in section 2.3.6.2. Leaf sections of Golden Promise, Propino and Cassia (Table 2.1) were inoculated with the 44.07 *R. commune* isolate (Table 2.2) at a dose of 1 x 10^6 spores/ml. This isolate was selected as due to its high spore production in culture. These cultivars were selected to include one resistant and one susceptible cv. Cassia was also included to monitor the 44.07 isolate in a winter cv. Additionally, the 44.07 isolate was originally isolated from Cassia plots in 2007. Lesion appearance was observed between 8 and 10 dpi. At 10 dpi, senescence was observed at the cut ends of the leaf sections particularly in Golden Promise. Senescence from the cut ends was less evident in Propino and Cassia. Leaf samples were collected, and lesions counted at 10 dpi (Figure 2.6).

The most severe symptoms after *R. commune* treatment were observed in Golden Promise. In Golden Promise samples inoculated with *R. commune*, a higher proportion of leaf sections showed two lesions (mean leaf sections =16.3 across 3 replicates), compared to Cassia (mean = 5.3) and Propino (mean = 5). Propino had the highest mean number of leaf sections with zero lesions at 10 dpi (28.3, compared to 22.6 in Cassia and 12 in Golden Promise). Notably, lesions observed in Golden Promise appeared larger than those in Propino. However, lesion size was not quantified as in many of the leaf sections it was difficult to accurately distinguish between senescence due to scald lesions and senescence from the cut ends.

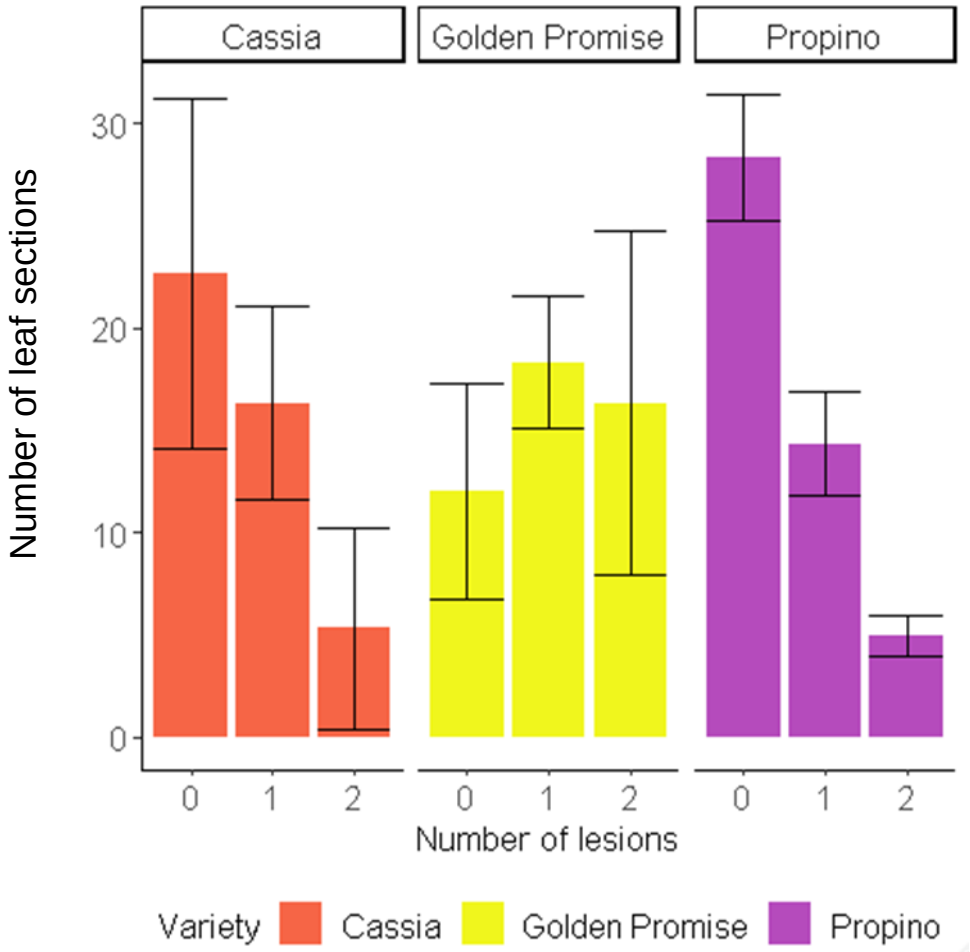


Figure 2.6: The number lesions on a single leaf section (totalling 144 leaf sections across three replicates) observed 10 dpi with 44.07 *R. commune* isolate at 1 x 10^6 spores/ml in Cassia (susceptible, 4) (red), Golden Promise (moderately susceptible, 5) (yellow) and Propino (Moderately resistant, 6) (purple).

DNA extraction was carried out on each of the leaf sections followed by qPCR to quantify fungal biomass accumulation at 10 dpi, using a standard curve (Appendix 2, Supplemental Figure S2.2). This was done to determine if there was a correlation between symptom appearance and fungal load. As shown in Figure 2.7, despite showing more severe symptoms (as indicated by the number of lesions) after inoculation with the 44.07 isolate, Golden Promise showed the lowest concentration of *R. commune* DNA (in ng) per 100 ng total DNA at 10 dpi. This could suggest that it takes less of the pathogen to result in lesion formation. A mixed effects ANOVA followed by Tukey's multiple comparisons test concluded that Propino shows a significantly higher

concentration of *R. commune* DNA per 100 ng DNA extracted compared to Golden Promise (p-adj = 0.004). At the same time, the Propino leaf sections remain intact with less severe symptoms appearing. This data suggests that despite Propino being a resistant barley cultivar, *R. commune* is still able to proliferate inside its leaf tissue, but without this resulting in severe visible symptoms. Interestingly, Cassia showed no significant difference in the level of *R. commune* detected in the leaf sections compared to Propino (Figure 2.7, p-adj = 0.13), despite a DAFM rr of 4 in Cassia, compared to Propino (rr of 6). This result correlates to similar lesion counts observed between Cassia and Propino in Figure 2.6. Indeed, while the rr against *R. commune* for Propino was 6 (moderately resistant) in 2019, this cv was previously categorised as resistant (rr = 7) up until 2012 (DAFM 2012), suggesting there might be a level of resistance breakdown.

The detached leaf assay solved previous issues experienced in the glasshouse, as leaf sections were kept in petri dishes sealed with micropore tape, and different treatments were kept separately. As the aim of this work was to investigate the barley x *R. commune* interaction from the host perspective in addition to fungal proliferation over time, barley gene expression analysis on genes thought to play a role in the immune response to *R. commune* would also be required. While the detached leaf assay is suitable for quantifying fungal proliferation and lesion formation, it may pose problems to study the barley response. For example, wounding responses from cutting leaf sections would not be differentiable from immune responses, as many features of these two stress signalling pathways are shared. Therefore, this chapter also focused on developing a different *in planta* assay.

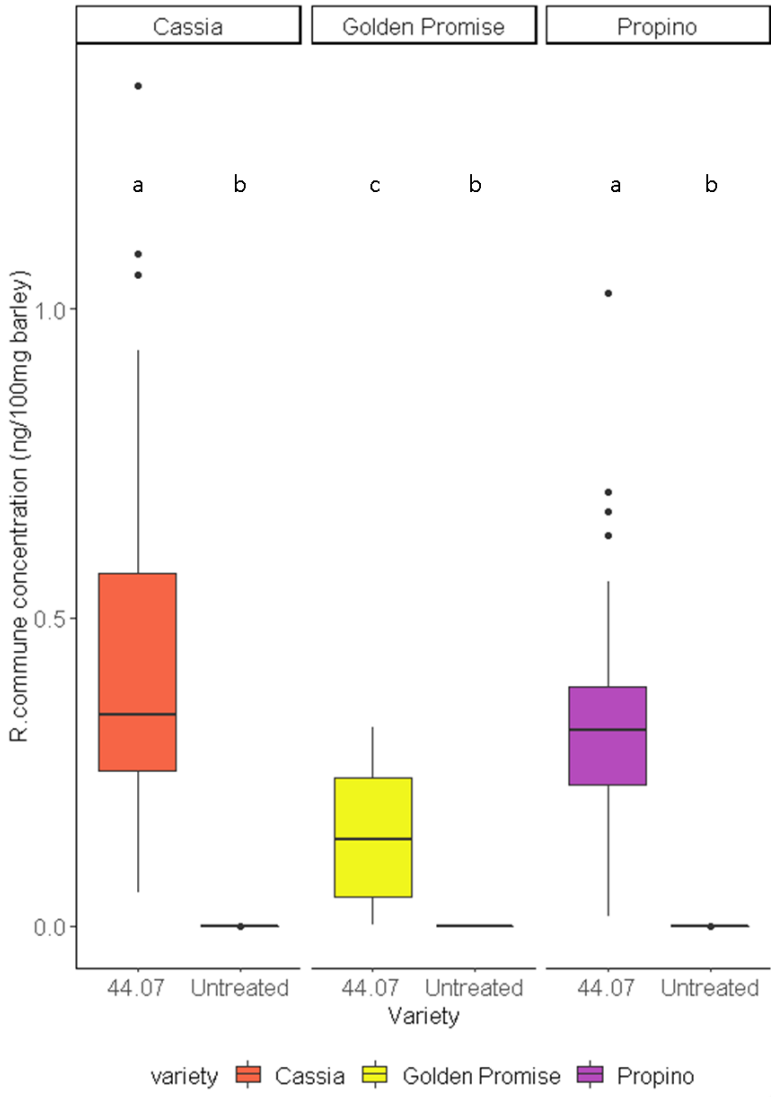


Figure 2.7: Quantification of *R. commune* gDNA as determined by qPCR and quantified against a standard curve in different barley cvs in a detached leaf assay, 10 dpi with 20 µl *R. commune* isolate 44.07 at 1 x 10^6 spores/ml. Untreated samples inoculated with 20 µl water. Letters indicate significant differences in *R. commune* concentration between all treatment x cv combinations, as determined by mixed effects ANOVA and Tukey's pairwise comparison test (p-adj < 0.05). Data representing three independent replicates, each consisting of 48 leaf sections.

2.4.3 Optimisation of conditions to investigate *in planta* fungal biomass accumulation and barley response to *R. commune*.

In order to more accurately examine the barley x *R. commune* interaction, it was necessary to optimise conditions for a disease screening assay in barley seedlings using controlled atmosphere growth chambers. This allowed for the

inoculation of whole seedlings similar to glasshouse screens (Section 2.4.1) to monitor disease progression and symptom appearance, while the use of controlled atmosphere growth chambers allowed for stricter control over temperature, potential contaminations, as well as RH, which can be increased to aid pathogen establishment. This assay could also be used to determine fungal biomass accumulation and to monitor gene expression changes without any additional stress responses being induced through wounding.

A screen using the barley cv Cassia (Table 2.1) was completed. This cv was chosen as it is classed as susceptible to *R. commune* (rr = 4), and the detached leaf assay (section 2.4.2) showed that it was susceptible to lesion formation and fungal biomass accumulation. This cv is also currently on the recommended growing list, unlike Golden Promise. Cassia seedlings were spray inoculated with two *R. commune* isolates at a 1 x 10^6 spores/ml: 44.07 which had previously shown virulence in Cassia through the detached leaf assay, and OP18(9) (Table 2.2). The OP18(9) isolate was also assessed to include a more recent isolate to determine any isolate specific responses in barley. OP18(9) was isolated from Cassia plots in 2018, while 44.07 was isolated from Cassia in 2007, which may also provide insight into changes in virulence in *R. commune* over time.

As in Section 2.4.1, disease was quantified as onset of visual symptoms, including percentage of leaf area displaying scald lesions, and total green leaf area. These measurements were recorded at 0, 2, 7, 10, 14, 17, 19 and 21 dpi. Six plants were examined at each timepoint, and three biological replicates were completed. Lesions began to appear between 14 and 17 dpi on leaf 2 (Figure 2.8). Lesion formation and cell death were more apparent in response to the OP18(9) isolate, compared to the 44.07 isolate (Figure 2.8). However, by 21 dpi, over the three replicates, 7 out of 18 leaves showed complete necrosis in response to the 44.07 isolate, and 8 out of 18 leaves in response to OP18(9).

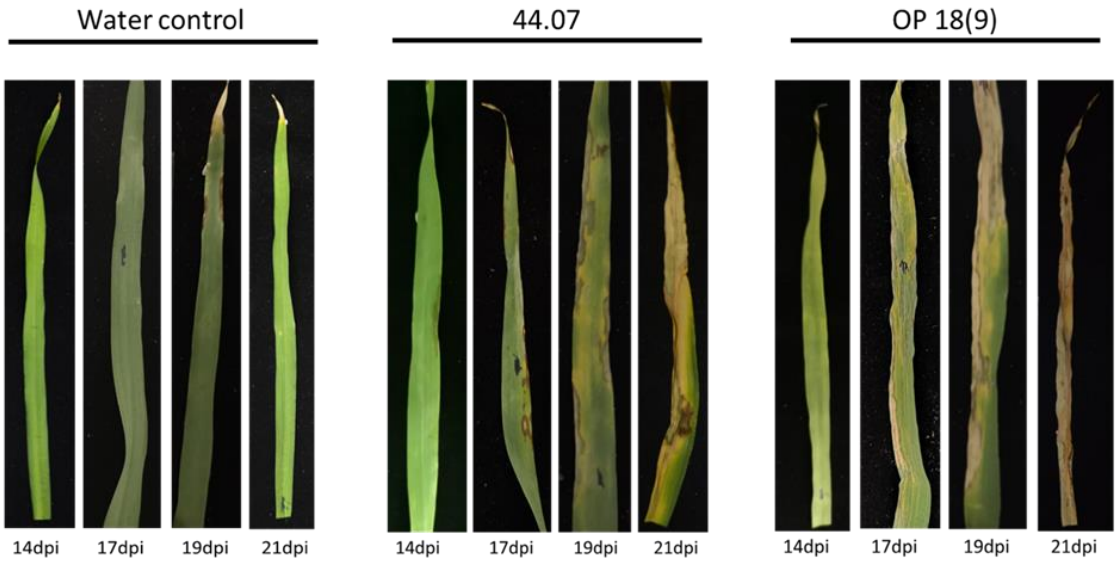


Figure 2.8: Onset of lesion formation on leaf 2 in susceptible cv Cassia between 14 and 21 dpi after inoculation with 44.07 or OP18(9) *R. commune* isolates at 1 x 10^6 spores/ml or mock (water) control. Images are representative of three replicates, each consisting of six individual seedlings.

To quantify the progression of symptoms in Cassia, mean percentage leaf area displaying scald lesions was recorded similar to section 2.4.1 (Figure 2.9). After recording symptoms leaf 2 and 3 were collected for nucleic acid extraction for quantification of fungal biomass accumulation and barley gene expression analysis. Therefore the data in Figure 2.9 does not represent repeat measurements from the same leaf over time. Lesions begin to appear on both leaves 2 and 3 between 14 and 17 dpi in response to both isolates (Figure 2.9). This is more pronounced in response to OP18(9) on leaf 2 and leaf 3 at 19 dpi however a two-way ANOVA indicated no statistically significant difference in lesion area between OP18(9) and 44.07 at any timepoint (p-adj > 0.05).

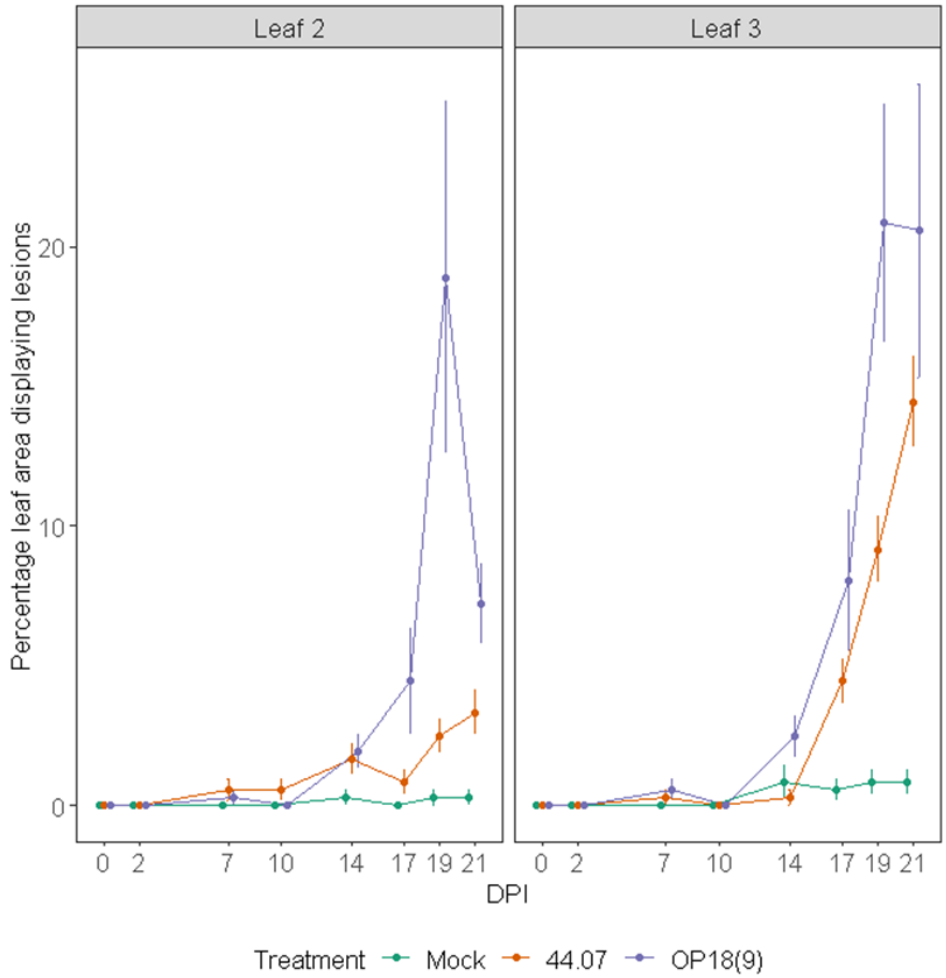


Figure 2.9: Mean percentage leaf area displaying scald lesions on Leaf 2 and 3 of cv Cassia after inoculation with *R. commune* isolates OP18(9) or 44.07 at 1 x 10^6 spores/ml. Mock treatment of water. Data for 3 biological replicates, each containing six individual plants per timepoint (n = 3), error bars represent SEM.

Similar to the pilot glasshouse experiments, seedlings displayed other stress symptoms such as yellowing, wilting and necrosis (Figure 2.8), but is not represented in Figure 2.9, which only quantified lesion area. Additionally, by 21 dpi, the presence of necrotic tissue made it difficult to distinguish between lesion boarder and necrotic regions. Hence, there is an apparent drop in mean leaf area displaying scald lesions between 19 and 21 dpi in response to the OP18(9) (Figure 2.9). In leaf 2, a drop of 11.6% leaf area displaying lesions is observed, and a drop of 0.25% is found in leaf 3. To complement these results, the mean loss of green leaf area in response to each treatment was also calculated (Figure 2.10). In both *R. commune* and mock treated seedlings,

small regions (< 1% of total leaf area) of leaves showed loss of green leaf area (as seen in Figure 2.8 water treatment at 19 dpi), particularly at the leaf tip.

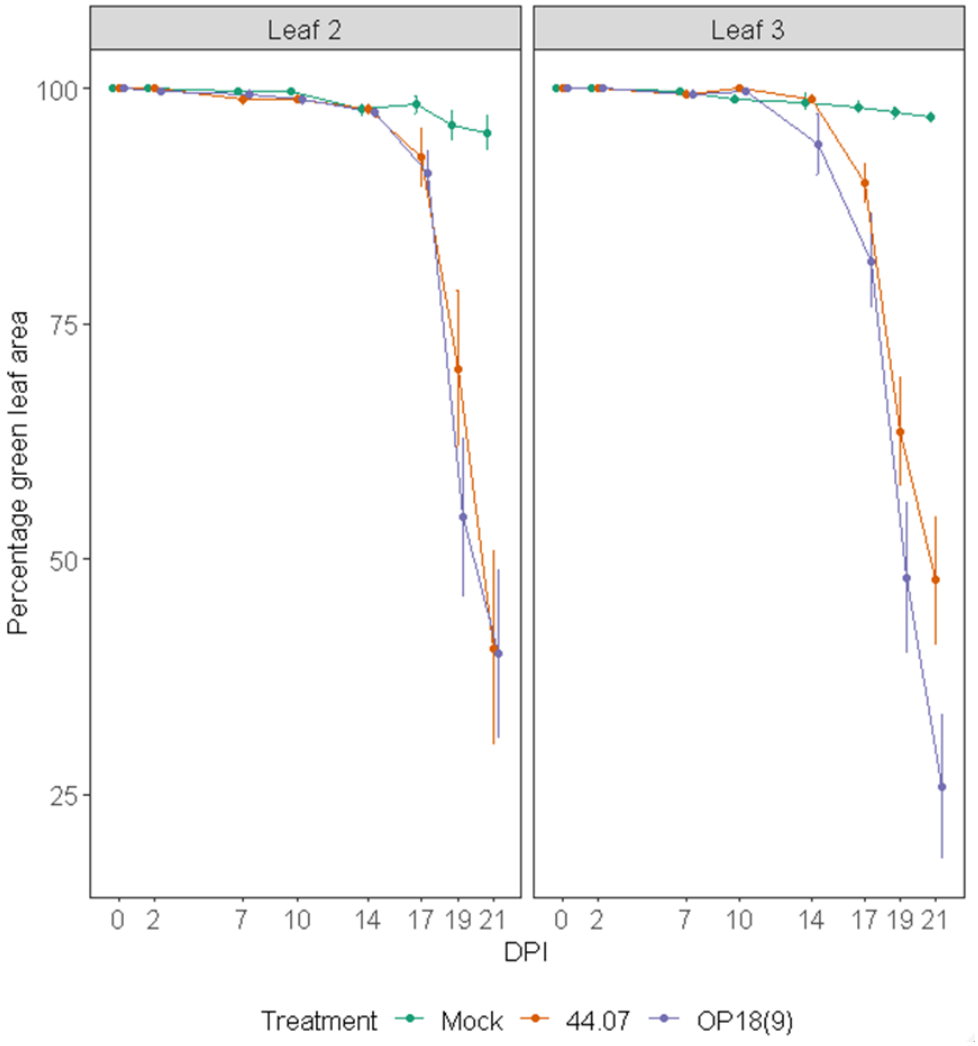


Figure 2.10: Mean percentage green leaf after *R. commune* inoculation of cv Cassia at 1 x 10^6 spores/ml. Mock treatment of water. Data for 3 biological replicates, each containing six individual plants per timepoint (n = 3), error bars represent SEM.

Both leaf 2 and leaf 3 began to show stress symptoms as observed by loss of green leaf area between 14 and 17 dpi in response to both *R. commune* isolates. In leaf 2, green leaf area is seen to reduce to similar levels in response to both isolates. While loss of green leaf area appears more severe at 21 dpi in response to OP18(9) compared to 44.07 at 21 dpi, a two-way ANOVA showed this was not a significant difference (p-adj > 0.05).

At each timepoint, tissue was collected from leaf 2 for gDNA isolation, followed by quantification of fungal biomass accumulation using qPCR and a standard curve, as described in section 2.3.9. No tissue was collected at 17 and 19 dpi due to limited plant numbers. gDNA of *R. commune* was detected in both 44.07 and OP18(9) treated samples at 0 dpi, which was expected since the 0 dpi samples were collected within 1 min after spray inoculation. *R. commune.* gDNA levels remain low until 10 dpi, after which levels started to increase. This corresponded to the timepoint at which symptoms appeared on the leaf surface. A tenfold increase in mean *R. commune* DNA concentration was seen between 14 and 21 dpi in 44.07, and a 7 fold increase between 14 and 21 dpi was observed in OP18(9) (Figure 2.11).

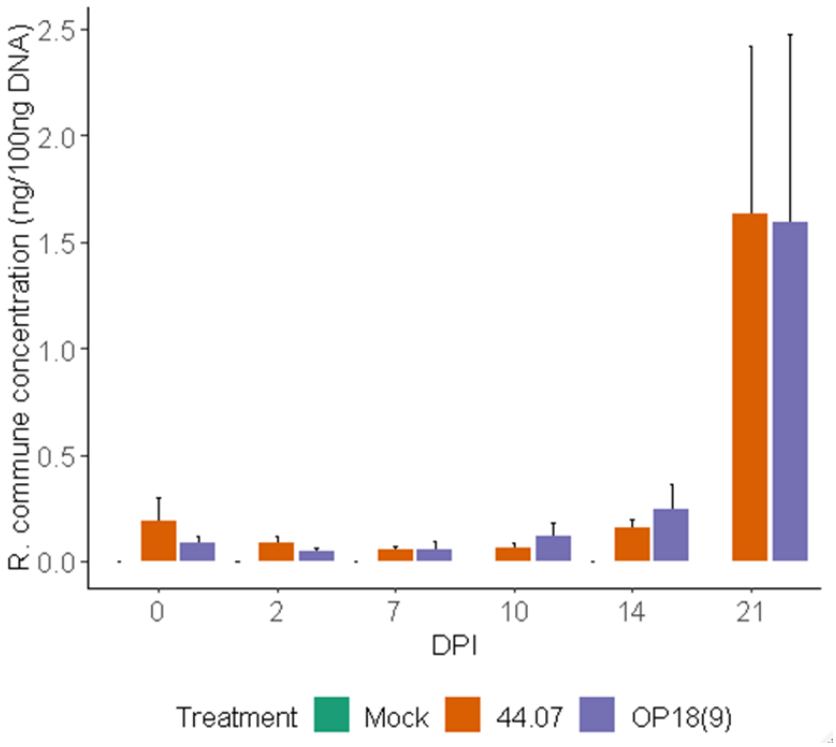


Figure 2.11: Temporal quantification of *R. commune* gDNA as determined by qPCR and quantified against a standard curve in Cassia (rr = 4) inoculated with OP18(9) (purple) or 44.07 (isolate) isolates or a mock water treatment (green). Error bars represent SEM, n = 3.

In order to gauge the response of Cassia (susceptible, rr = 4, Table 2.2), the expression level of genes thought to be involved in defence against pathogens (Table 2.5) was examined using RT-qPCR. Prior to this, the Cq values of five

candidate housekeeping genes were investigated for normalisation of expression data: *UBI, ACTIN, TUBULIN α, GAPDH* and *EF2* (Table 2.2). This was done to identify genes that showed stable Cq values in both *R. commune* and mock treated samples across the infection timecourse. The Cq values for each reference gene in response to each treatment are presented in Figure 2.12.

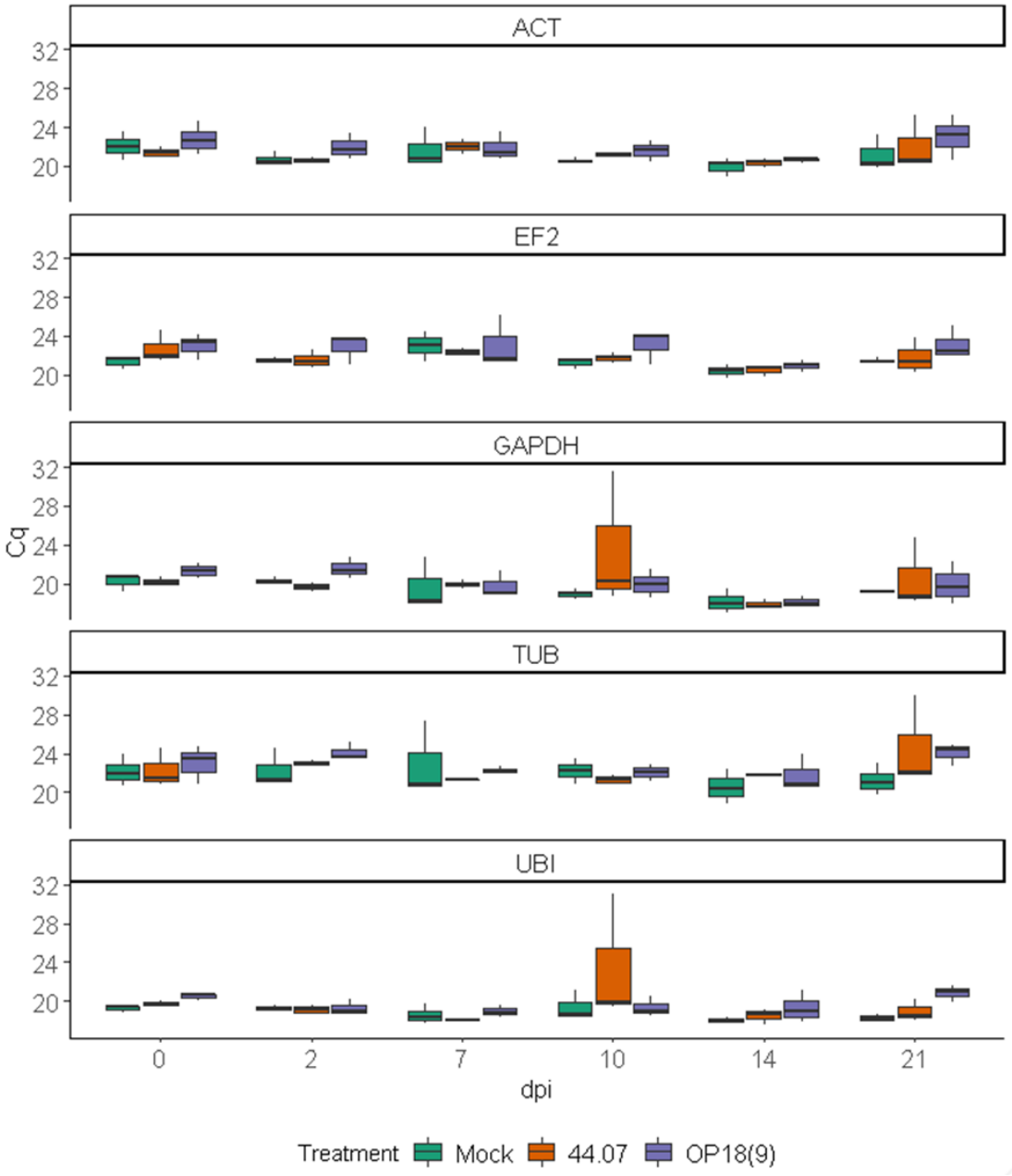


Figure 2.12: Cq values of candidate barley reference genes at each timepoint. Data is from three individual samples from one biological replicate. ACT: *ACTIN,* TUB: *TUBULIN α*, UBI: *UBIQUITIN,* EF2: *ELONGATION FACTOR 2*.

GAPDH and *UBI* showed large variation across samples in response to the 44.07 isolate at 14 dpi and therefore were not considered as suitable reference genes. While Cq values for *EF2* were stable across all timepoints for each treatment, amplification of gDNA was also observed with the primers used for this gene (data not shown). As Cq values were also stable across the timepoints for each treatment for *ACTIN* and *TUBULIN α*, the mean Cq of these two genes was used to calculate relative defence gene expression.

A subsequent gene expression analysis using RT-qPCR was completed identify defence related barley genes whose expression changed following inoculation with the 2 strains of *R. commune* in Cassia. *NPR1* relative expression in *R. commune* treated samples was comparable to that in mock treated samples until 14 dpi, when an apparent increase in mean relative expression was observed (Figure 2.13). However, this increase was not found to be significant in response to the OP18(9) treatment compared to the mock treatment. Specifically, over a 2 fold increase compared to mock was observed for the 44.07 treatment, whereas a 1 fold increase in mean relative expression was observed relative to the mock treated samples after inoculation with OP18(9) at 14 dpi, however this was also not found to be statistically significant.

Mean relative expression of *PR1* remained low in mock treated samples compared to *R. commune* treated samples (Figure 2.13). Following *R. commune* inoculation, mean relative expression increased after 10 dpi. However, there was high variation between replicates (Figure 2.13). This observed increase in relative expression compared to mock treatment was found to be significant at 21 dpi in response to OP18(9), but not 44.07, despite an over 5 fold increase.

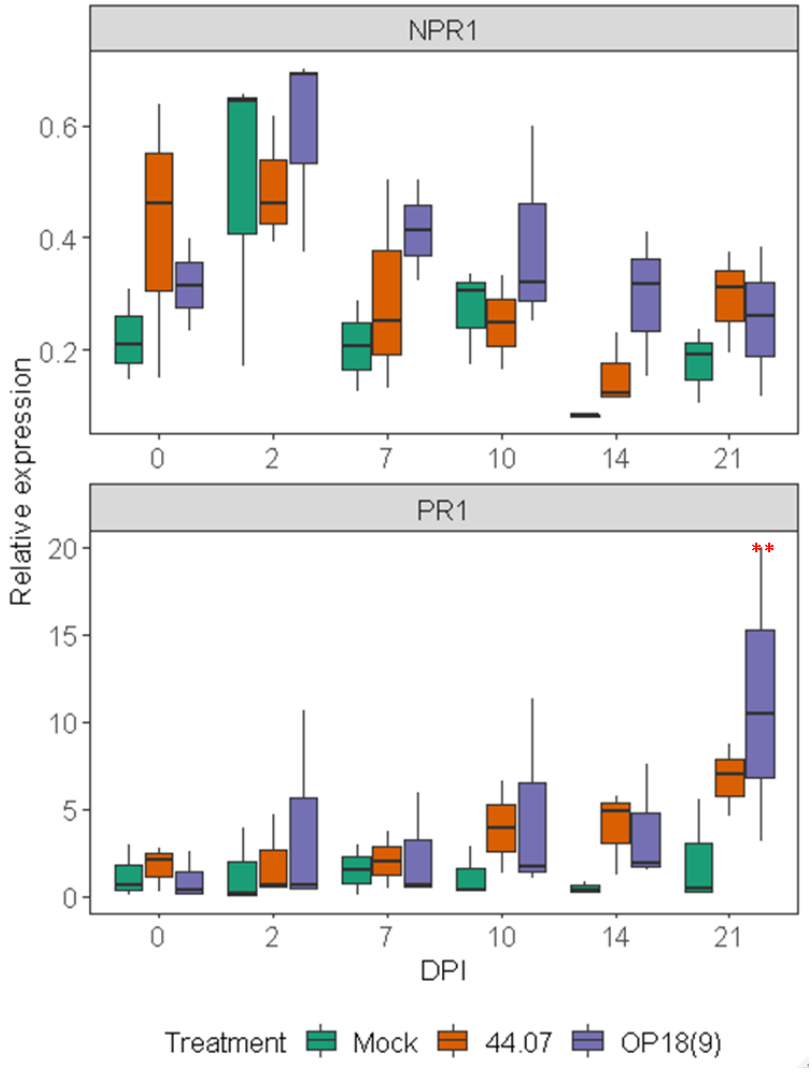


Figure 2.13: Expression of SA response genes *NPR1* and *PR1* relative to reference genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9) at 1 x 10^6 spores/ml. Significant differences between OP18(9) *R. commune* and mock treated samples at 21 dpi indicated, as determined by ANOVA and post hoc pairwise comparisons (n = 3) **: p-adj < 0.01

Assuming a level of functional conservation between *AtWRKY33* and its proposed barley orthologues *WRKY43* and *WRKY50,* the relative expression of these two barley transcription factors was also analysed. While in response to the 44.07 isolate, the relative expression of *WRKY43* appeared to increase between 7 and 21 dpi compared to mock treatment, this was not found to be statistically significant due to large differences observed between replicates (Figure 2.14). Similarly, relative expression levels of *WRKY50* remained low in all treatments until 21 dpi. The increase in *WRKY43* and *WRK50* expression

was stronger at 21 dpi in the presence of both strains but was more pronounced with OP18(9). Differences were nevertheless not statistically significant (Figure 2.14).

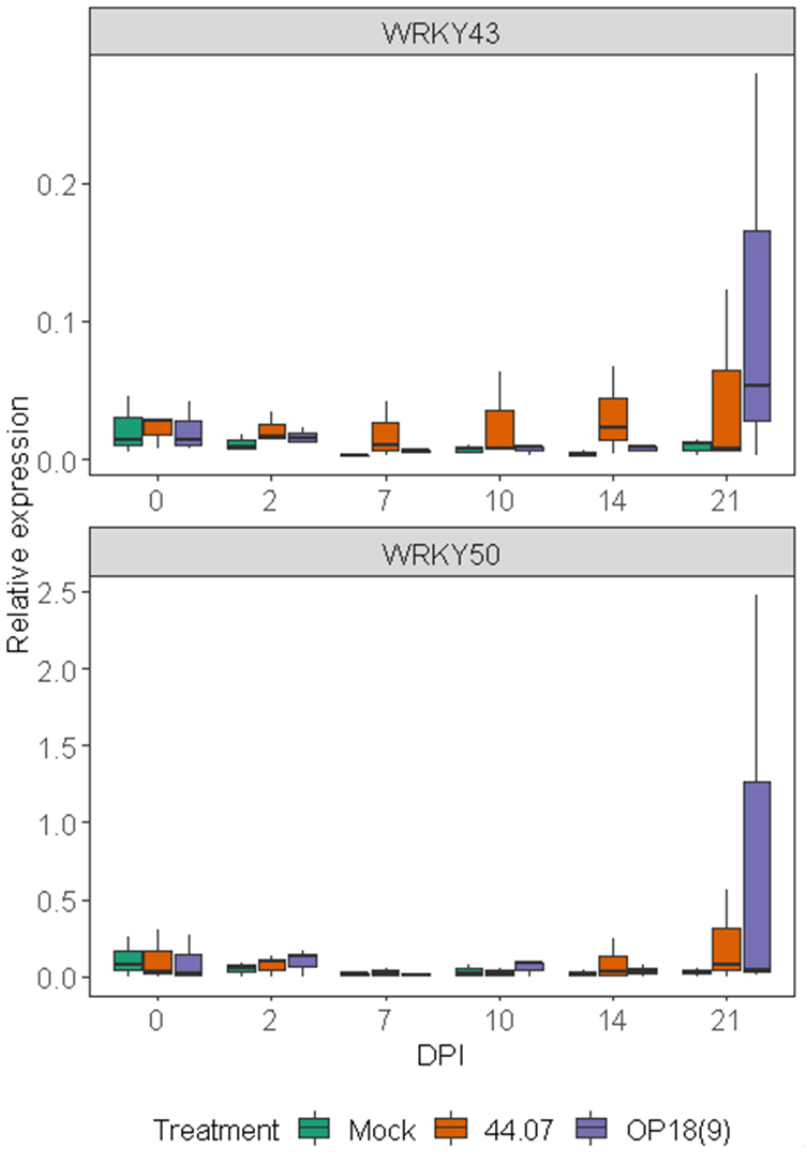


Figure 2.14: Expression levels of proposed orthologues to *AtWRKY33: WRKY43* and *WRKY50* relative to housekeeping genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9) at 1 x 10^6 spores/ml. No significant change in relative expression between *R. commune* treated samples and mock samples at any timepoint (n = 3).

The relative expression levels of two genes that have been shown to be involved in the barley x *R. commune* interaction: *RAR1* and *SGT1* (Austin et. al., 2002) were also investigated. However, there were no significant changes in relative expression of these two genes between *R. commune* and mock treated seedlings at any of the timepoints tested (Figure 2.15).

Due to its known role in cell death regulation, the expression of the *BI1* gene was also examined. It is possible that the activity of this gene may increase as *R. commune* transitions from the latent to the necrotic phase. However, while transcripts were detected at low levels across all timepoints, there was no change in expression after inoculation with either *R. commune* isolate (Figure 2.16).

The relative expression of *HB* was also investigated due to its role in NO and ROS signalling during early immune response. However, between 0 and 10 dpi, no *HB* transcripts were detected. Indeed, the majority of samples tested only showed amplification of *HB* within the last 6 cycles (of 40 cycles) in the qPCR, which may be a PCR artefact. While there was an apparent change in *HB* expression at 14 dpi in response to 44.07, and at 21 dpi in response to both isolates (Figure 2.17), this was only detected in two of the three replicates, and therefore was not found to be significant.

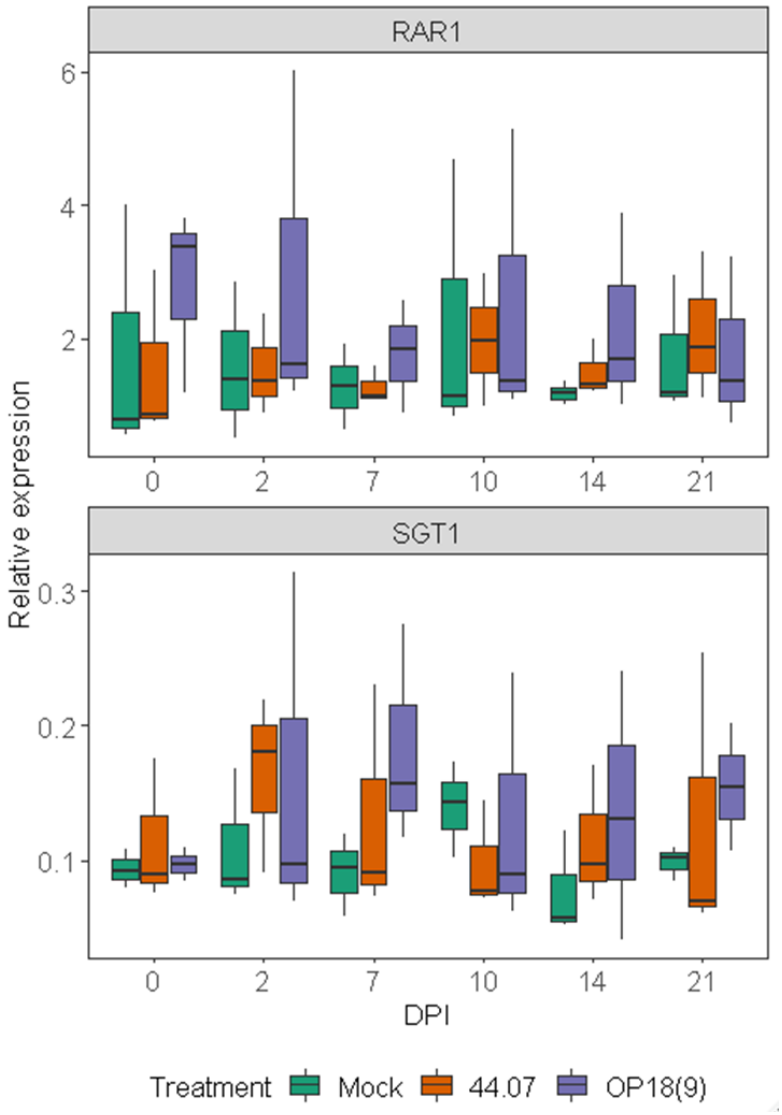


Figure 2.15: Expression levels *RAR1* and *SGT1* genes involved in R gene mediated immunity relative to housekeeping genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9) at 1 x 10^6 spores/ml. No significant change in relative expression between *R. commune* treated samples and mock samples at any timepoint (n = 3).

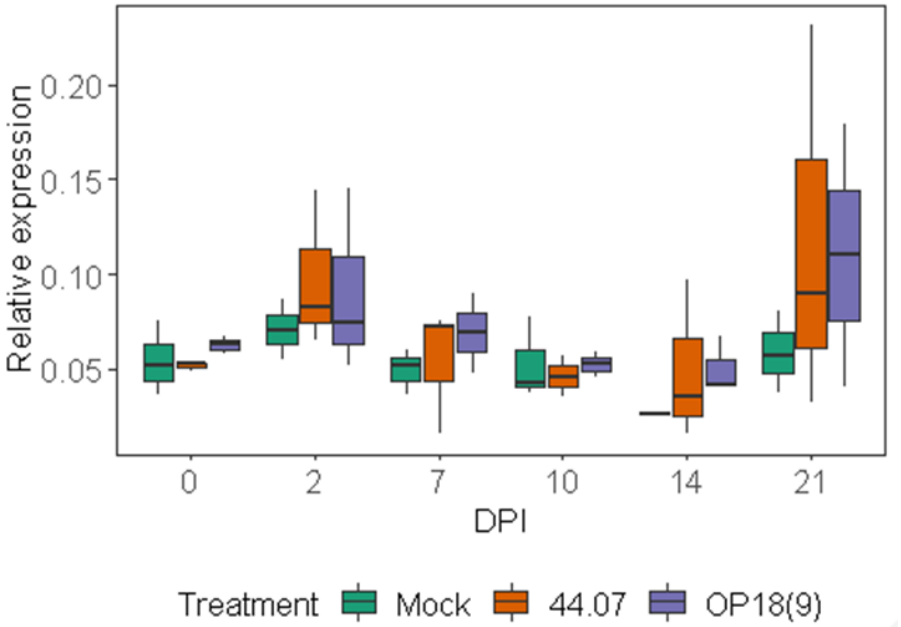


Figure 2.16: Expression of the conserved cell death regulator *BI1* relative to housekeeping genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9) at 1 x 10^6 spores/ml. No significant change in relative expression between *R. commune* treated samples and mock samples at any timepoint (n = 3).

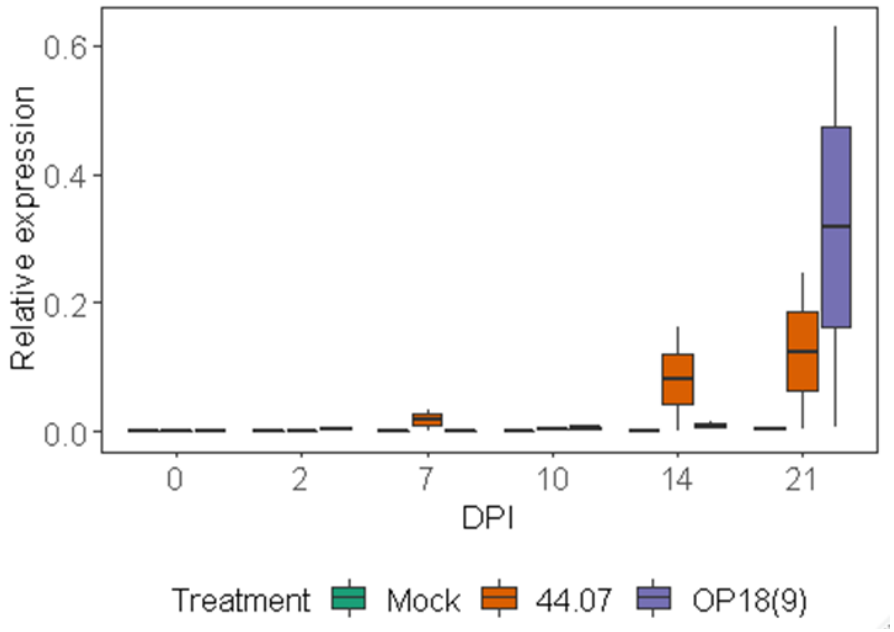


Figure 2.17: Expression of *HB* relative to housekeeping genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9) at 1 x 10^6 spores/ml. ANOVA showed no significant change in relative expression between *R. commune* treated samples and mock samples at any timepoint (n = 3).

Lastly, *MLO* is one of the best characterised S genes in barley. Although its role as an S gene has primarily been described in the barley x *Bgh* interaction,

it has been suggested that active *MLO* may reduce susceptibility *to R. commune* (Makepeace et al., 2007). Here, the relative expression of this gene in response to the two *R. commune* isolates was examined and found no statistically significant differences in relative expression between *R. commune* and Mock treated samples (Figure 2.18), despite an apparent increase in *MLO* expression at 21 dpi.

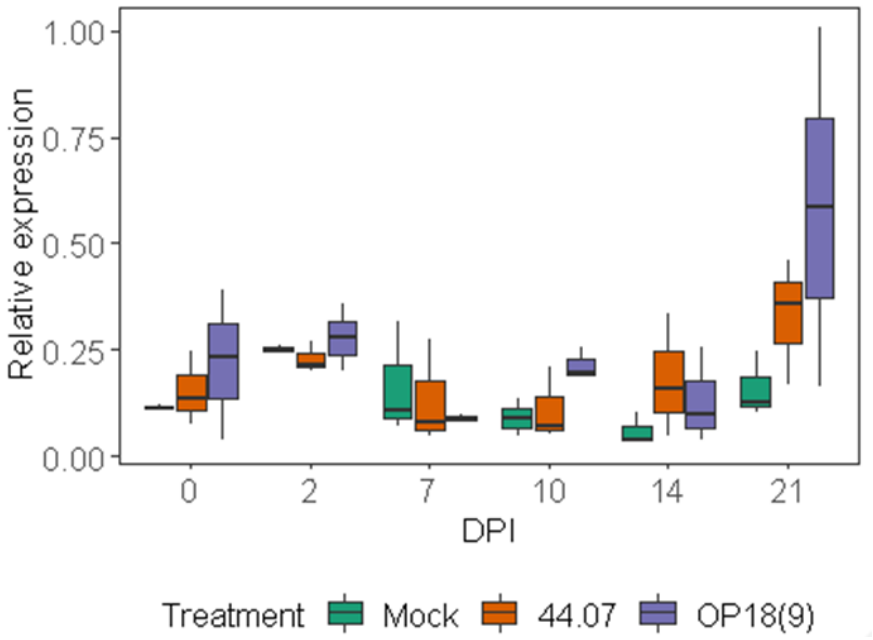


Figure 2.18: Expression of the known S gene *MLO* relative to housekeeping genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9) at 1 x 10^6 spores/ml. No significant change in relative expression between *R. commune* treated samples and mock samples at any timepoint (n = 3).

Of these genes tested, there were no statistically significant changes in expression in *R. commune* treated barley compared to mock treated barley between 0 and 14 dpi (Figures 2.13 – 2.18), with the exception of *PR1* which was found to be significantly expressed at higher levels in Cassia at 21 days after treatment with OP18(9), compared to mock treatment (Figure 2.13).

2.4.4 Optimisation of inoculation parameters to investigate barley response to *R. commune* isolates.

Given the low relative expression levels observed for each of the defence related genes and the absence of statistically significant changes in expression between mock and *R. commune* treated seedlings, it was hypothesised that a peak in expression at earlier timepoints might have been missed, especially because many of these genes are thought to be involved in early immune signalling. Hence, the expression of these genes between 0 and 2 dpi were next examined. The inoculation process was repeated in Cassia (Susceptible, Table 2.1), as described in section 2.3.6.3, with tissue from leaf 2 being collected at 0, 6, 12, 24, 36, 48 and 120 (5 dpi) hours post inoculation (hpi) for gene expression analysis. Despite the onset of visual symptoms observed after inoculation at 1 x 10^6 spores/ml, in section 2.4.3, no significant changes in expression of selected defence related genes were observed after *R. commune* treatment compared to mock. Therefore, to ensure a more robust host response, *R. commune* treatments with a ten-fold increase in dose from 1 x 10^6 spores/ml to 1 x 10^7 spores/ml were also tested.

At the earlier timepoints, no changes in *NPR1* expression were detected after inoculation with *R. commune* at 1 x 10^6 spores/ml (Figure 2.19). In contrast, inoculation at a 1 x 10^7 spores/ml rate resulted in an apparent 2 fold increase in relative expression of *NPR1* in response to OP18(9) at 6 hpi, and 2.5 fold increase at 12 hpi. However, variation between replicates meant that this was not found to be significant. Increased *NPR1* relative expression was also observed in response to the higher dose of the 44.07 isolate after 12 hpi until 36 hpi, at which time point, the difference was statistically significant (p-adj < 0.001). This increased expression at 36 hpi was not observed in response to the higher dose of the OP18(9) isolate (Figure 2.19).

Similar to *NPR1,* no changes in *PR1* expression were detected after inoculation with *R. commune* at 1 x 10^6 spores/ml (Figure 2.20). Interestingly, the expression of *PR1* was only shown to increase in response to the high dose of OP18(9) at 36 hpi compared to mock (Figure 2.20). This shows that activation of these SA response genes occurs during the early immune

response in an isolate dependent manner. No changes in *PR1* expression was detected in response to 44.07 (Figure 2.20).

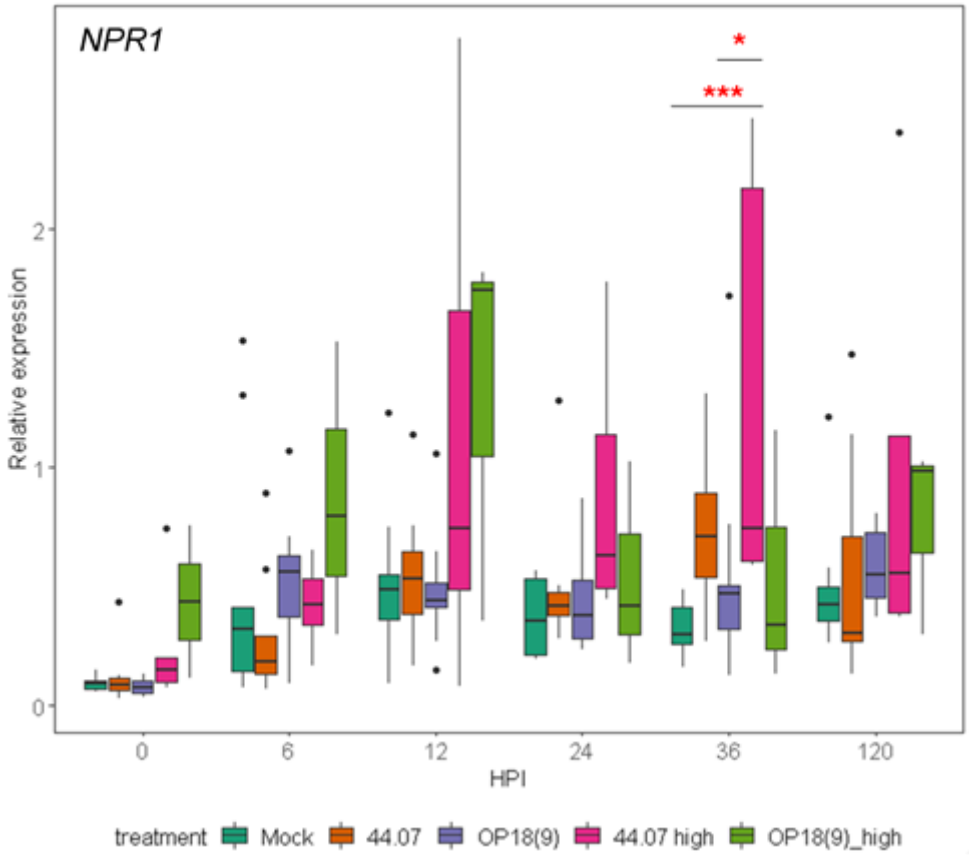


Figure 2.19: Expression of *NPR1* relative to reference genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9). Treatments marked 'high' are inoculated at a dose of 1 x 10^7 spores/ml. Otherwise, a 1 x 10^6 spores/ml dose was used. Significant differences between 44.07 *R. commune* treatment at 1 x 10^7 spores/ml and mock treatments at 36 hpi indicated: ***: p-adj < 0.001, and between different doses of the 44.07 *R. commune* isolate at 36 hpi indicated *: p-adj < 0.05. n = 3.

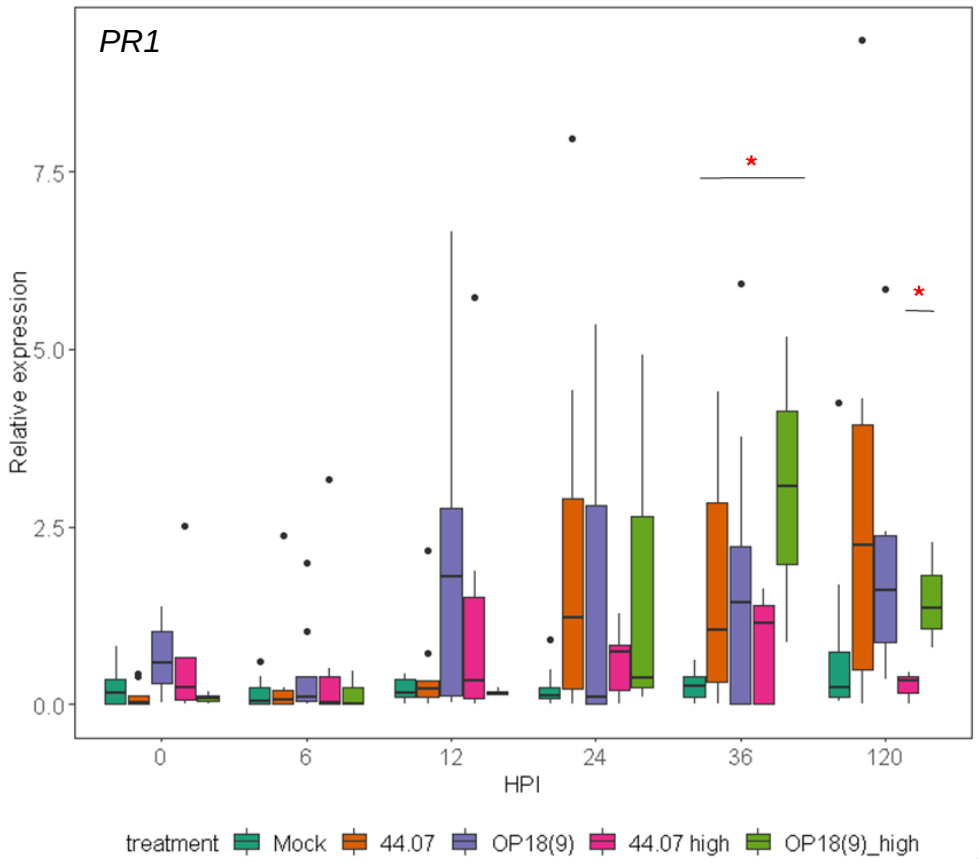


Figure 2.20: Expression of *PR1* relative to reference genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9). Treatments marked 'high' are inoculated at a dose of 1 x 10^7 spores/ml. Otherwise, a 1 x 10^6 spores/ml dose was used. Significant difference between OP18(9) *R. commune* treatment at 1 x 10^7 spores/ml and mock treatments at 36 hpi and between OP18(9) and 44.07 *R. commune* isolates at 1 x 10^7 spores/ml dose at 120 hpi indicated with *: p-adj < 0.05 (n = 3)

Despite up-regulation of *NPR1* and *PR1* at 36 hpi, the relative expression of *WRKY43* and *WRKY50* did not show any changes after *R. commune* infection at 1 x 10^6 spores/ml or 1 x 10^7 spores/ml compared to mock treatment at any of the earlier timepoints (Figures 2.21 and 2.22), and the apparent increase in *WRKY43* expression at 36 hpi in response to 44.07 was not statistically significant (Figure 2.21). No changes in *WRKY50* relative expression between *R. commune* and mock treated samples at the earlier timepoints, regardless of inoculation dose (Figure 2.22).

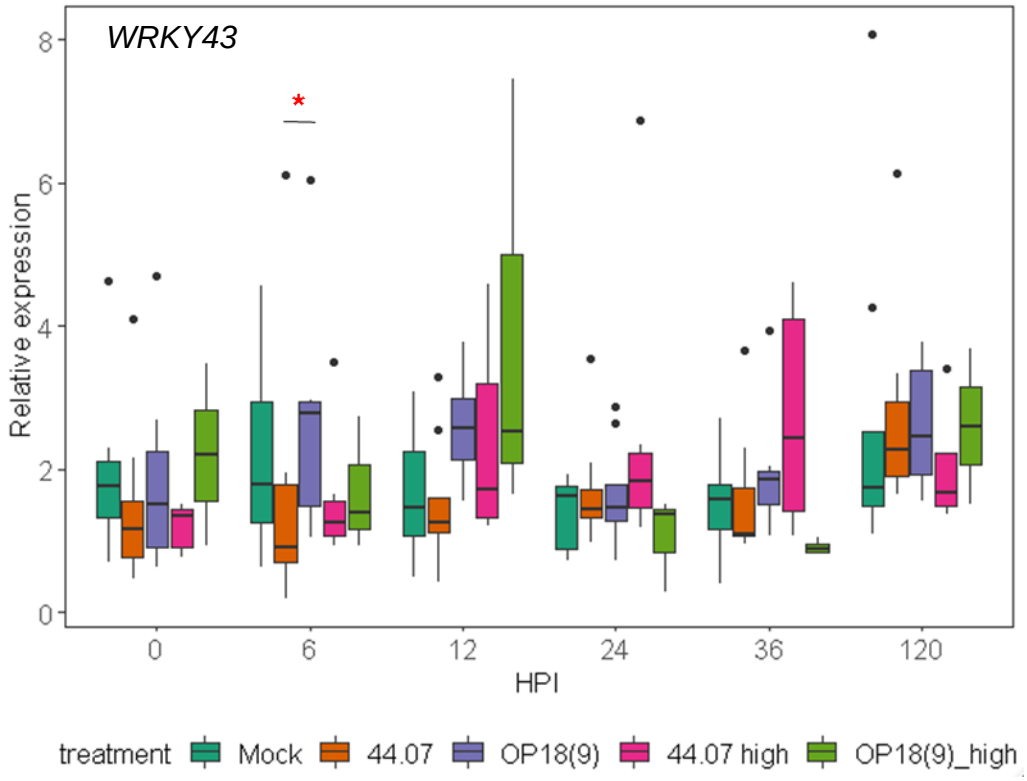


Figure 2.21: Expression of *WRKY43* relative to reference genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9). Treatments marked 'high' are inoculated at a dose of 1 x 10^7 spores/ml. Otherwise, a 1 x 10^6 spores/ml dose was used. Significant differences between OP18(9) and 44.07 *R. commune* treatments at 1 x 10^6 spores/ml at 6 hpi indicated with *: p-adj < 0.05 (n = 3)

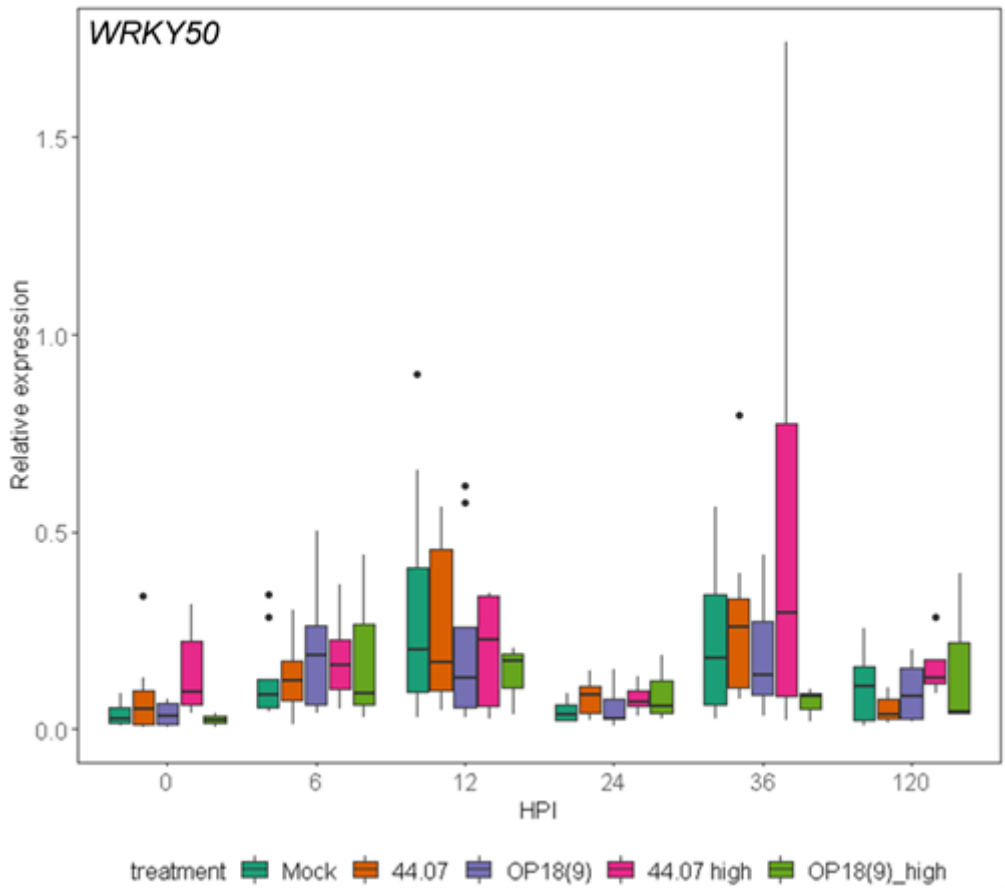


Figure 2.22: Expression of *WRKY50* relative to reference genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9). Treatments marked 'high' are inoculated at a dose of 1 x 10^7 spores/ml. Otherwise, a 1 x 10^6 spores/ml dose was used. No significant changes in *WRKY50* relative expression were detected between *R. commune* and mock treated samples (n = 3)

At the early timepoints, there were no changes in *RAR1* or *SGT1* relative expression detected between samples treated with *R. commune* at 1 x 10^6 spores/ml (Figures 2.23 and 2.24). Surprisingly, at 36 hpi, relative expression of both *RAR1* and *SGT1* appears reduced in response to the 1 x 10^7 dose of OP18(9) compared to all other treatments. This was not observed in response to the 1 x 10^7 dose of the 44.07 isolate. The reduction in relative expression of these genes compared to mock was not determined to be significant.

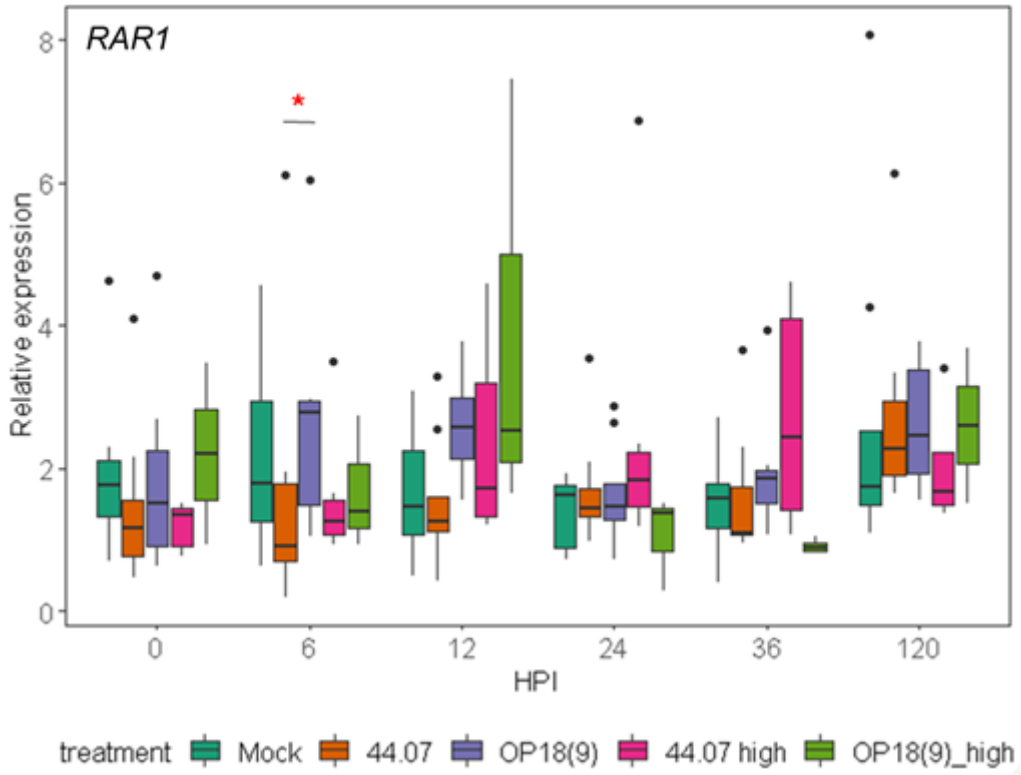


Figure 2.23: Expression of *RAR1* relative to reference genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9). Treatments marked 'high' are inoculated at a dose of 1 x 10^7 spores/ml. Otherwise, a 1 x 10^6 spores/ml dose was used. Significant differences between 44.07 and OP18(9) *R. commune* treatments at 1 x 10^6 spores/ml at 6 hpi indicated with *: p-adj < 0.05 (n = 3).

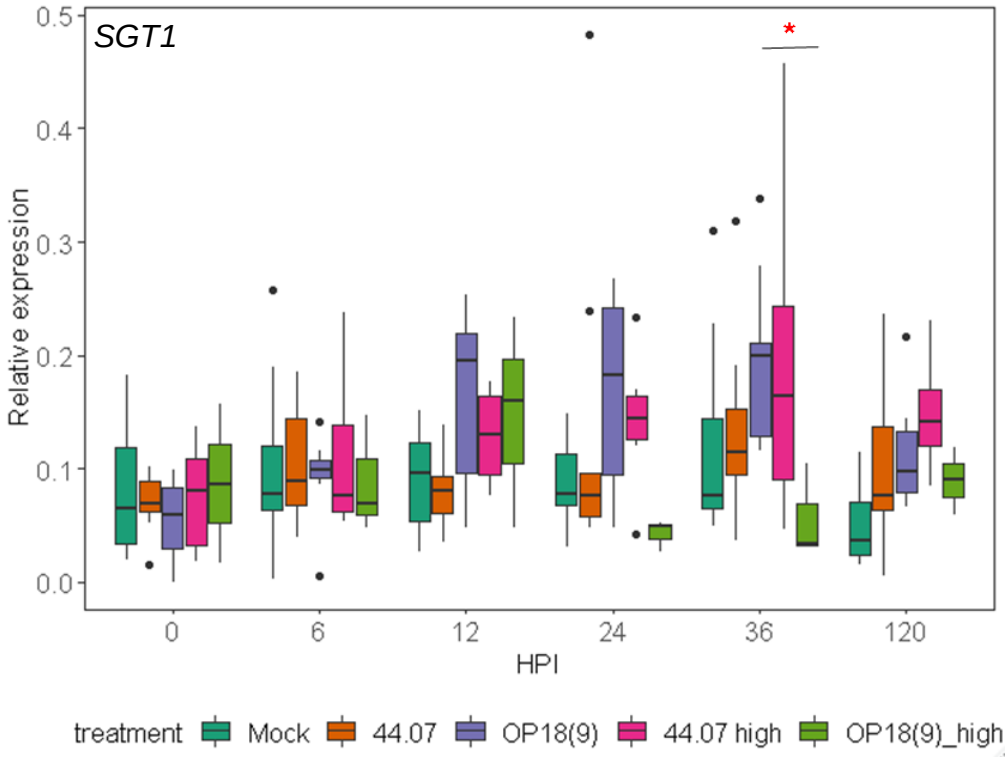


Figure 2.24: Expression of *SGT1* relative to reference genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9). Treatments marked 'high' are inoculated at a dose of 1 x 10^7 spores/ml. Otherwise, a 1 x 10^6 spores/ml dose was used. Significant differences between OP18(9) treatments at different inoculation doses at 36 hpi indicated *: p-adj < 0.05 (n = 3)

Inoculation with *R. commune* at a 1 x 10^6 spores/ml dose did not induce any changes in *HB* expression relative to mock treatment at the earlier timepoints (Figure 2.25). However, a higher pathogen load at 1 x10^7 spores/ml did result in an increase in relative expression at these timepoints. Specifically, an apparent 10-fold increase in *HB* expression in response to the higher dose of OP18(9) is observed at 12 and 24 hpi (Figure 2.25). This onset of increased *HB* relative expression is delayed in response to 44.07 at the same inoculation dose, and an apparent almost 4-fold increase is observed at 36 hpi. However, this increase in transcription of *HB* was only observed in 2 of three replicates. At 120 hpi (5 dpi), there is also an over 25-fold increase in *HB* expression in response to 44.07 high dose only (Figure 2.25). This suggests the role of *HB* in early immune response to *R. commune* may be isolate dependant.

Transcription remains low in untreated samples and samples inoculated with either isolate at 1 x 10^6 spores/ml at all timepoints (Figure 2.25).

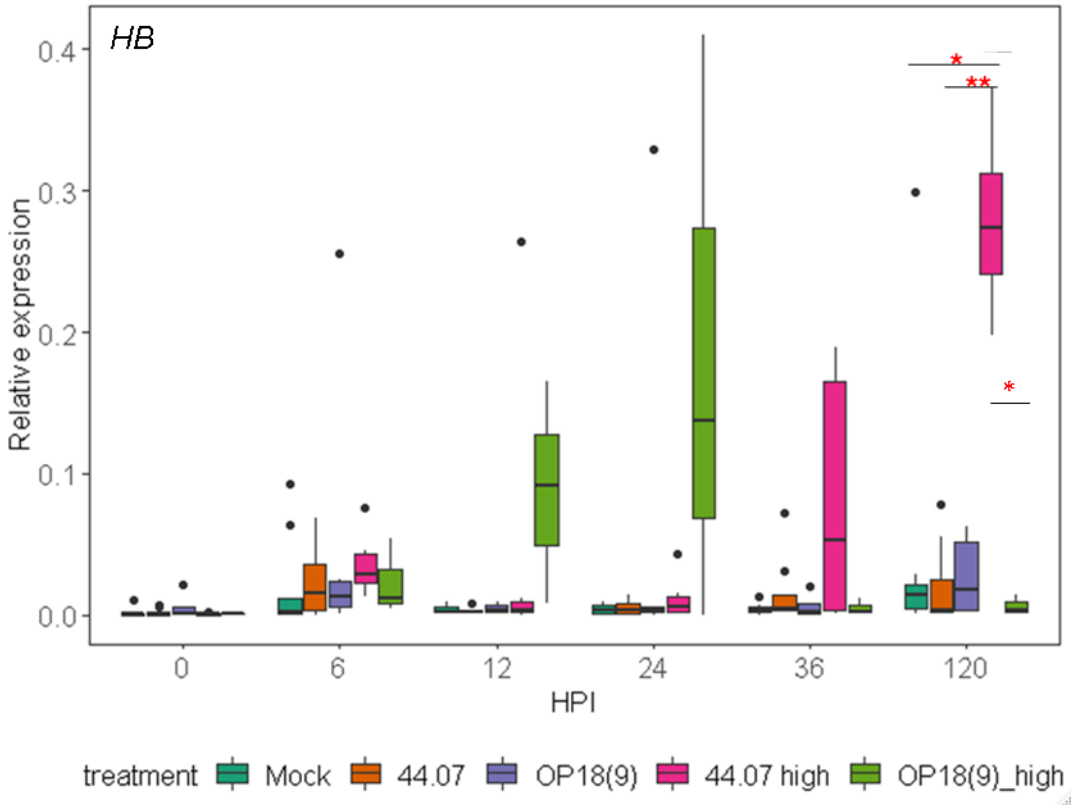


Figure 2.25: Expression of *HB* relative to reference genes in Cassia after treatment with *R. commune* isolates 44.07 and OP18(9). Treatments marked 'high' are inoculated at a dose of 1 x 10^7 spores/ml. Significant differences between 1 x 10^7 spores/ml dose of the 44.07 *R. commune* treatment and 1 x 10^6 spores/ml 44.07 *R. commune* treatment at 120 hpi indicated with ** (p-adj < 0.01). Significant differences between 44.07 *R. commune* treatment at 1 x 10^7 spores/ml and mock treatment at 120 hpi indicated with *. Significant increase in expression between 44.07 and OP18(9) treated samples at the 1 x 10^7 spores/ml dose also indicated with *: p-adj < 0.05, p-adj < 0.01, n = 3.

Although there was a level of variation between replicates, the higher inoculation load was found to induce a stronger response in Cassia in response to both *R. commune* isolates, particularly in *NPR1, PR1* and *HB* expression. As such, the remaining work in this chapter was completed using a 10^7 spores/ml inoculation dose. As significant changes in relative expression were detected in these genes at earlier timepoints, it was concluded that it is likely that key defence signalling is active during these stages.

2.4.5 Comparison of susceptible and resistant barley cvs in response to *R. commune.*

The experiments described in sections 2.2.3 and 2.4.4 indicated that the inoculation method on seedlings grown in controlled growth chambers was suitable to study the interaction of the 44.07 and OP18(9) *R. commune* isolates in Cassia, as both pathogen biomass accumulation and host responses could be quantified from leaf tissue. The secondary aim of this chapter was to identify the appropriate conditions for a global transcriptome analysis to identify candidate S genes that may contribute to barley susceptibility to *R. commune.* In section 2.4.4, it was determined that defence signalling pathways such as SA signalling are likely to be active at early infection stages (between 12-48 hpi). The aim of the global transcriptome analysis was to identify differences between one susceptible and one resistant barley cv, to determine any conserved defence responses and/or susceptible specific *R. commune* response genes. Therefore, prior to RNA sequencing (RNAseq), any changes in relative expression of *PR1, NPR1, WRKY43, WRKY50* and *HB* in the resistant cv Infinity was determined. Infinity was chosen as, it is a winter cv currently on the recommended growing list (DAFM, 2022). Unlike Cassia (rr = 4, Table 2.2), Infinity is classified as resistant to *R. commune* (rr = 7, Table 2.2) (Table 2.1).

The inoculation process was repeated in both Cassia and Infinity, maintaining the inoculation dose at 10^7 spores/ml. Tissue was collected for gene expression analysis at 0, 12, 24, 36, and 48 hpi to specifically focus on early defence responses. A final timepoint of 288 hpi (12 dpi) was also included for gene expression analysis, as this corresponds to the time at which symptoms appear in the susceptible cv, Cassia. In contrast, scald lesions were not observed on the resistant cv, Infinity, at 14dpi in response to either isolate after inoculation at 10^7 spores/ml concentration (Figure 2.26). Wilting and yellowing were also observed in Cassia at 17 dpi in response to both *R. commune* isolates. Interestingly, despite being classed as a moderately resistant cv, Infinity leaf sections displayed a susceptible phenotype at 17 dpi in response to the OP18(9) *R. commune* isolate, suggesting that this might be a more virulent isolate compared to 44.07. However more severe symptoms were

observed in susceptible Cassia in response to OP18(9) (Figure 2.26). In this instance, the Infinity x OP18(9) interaction appears to be a susceptible interaction.

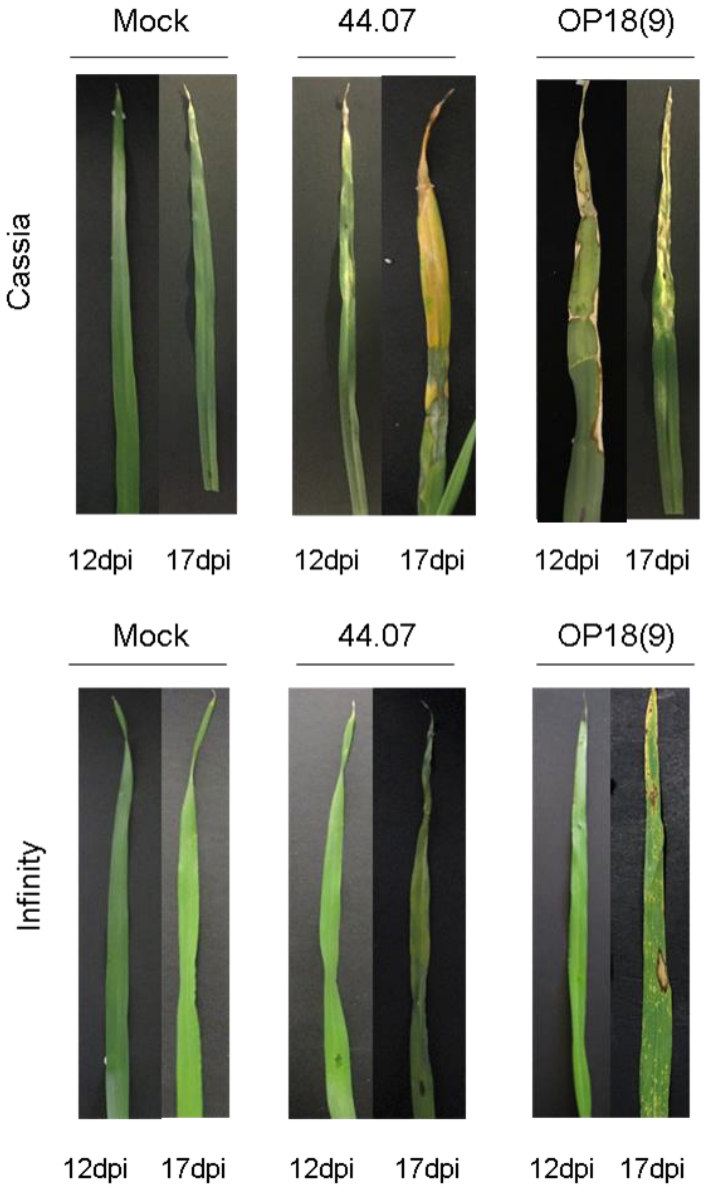


Figure 2.26: Onset of lesion formation on leaf 2 in susceptible cv Cassia and resistant cv Infinity at 12 and 17 dpi after inoculation with 44.07 or OP18(9) *R. commune* isolates at 1 x 10^7 spores/ml. Mock treatment with water with 0.1% Tween 20. Images are representative of three replicates.

Leaves from three individual plants were pooled for gene expression analysis. A peak in *PR1* relative expression was recorded at 12 hpi in response to 44.07 in Cassia where a tenfold increase is observed relative to mock (Figure 2.27).

A similar peak in expression was observed in section 2.4.4 under the same conditions (Figure 2.20). In Infinity, there was also an apparent 5 fold increase in *PR1* relative expression observed in response to the 44.07 isolate at 12 hpi, however this was not found to be significant. This response at 12 hpi was not recorded in seedlings inoculated with the OP18(9) isolate in either cv (Figure 2.27). There was also increased *PR1* relative expression in Cassia in response to the OP18(9) isolate at 36 hpi but not in response to 44.07. This suggests that the timing of *PR1* activation during early stages of *R. commune* infection in Cassia may be isolate dependent. This was not observed in Infinity.

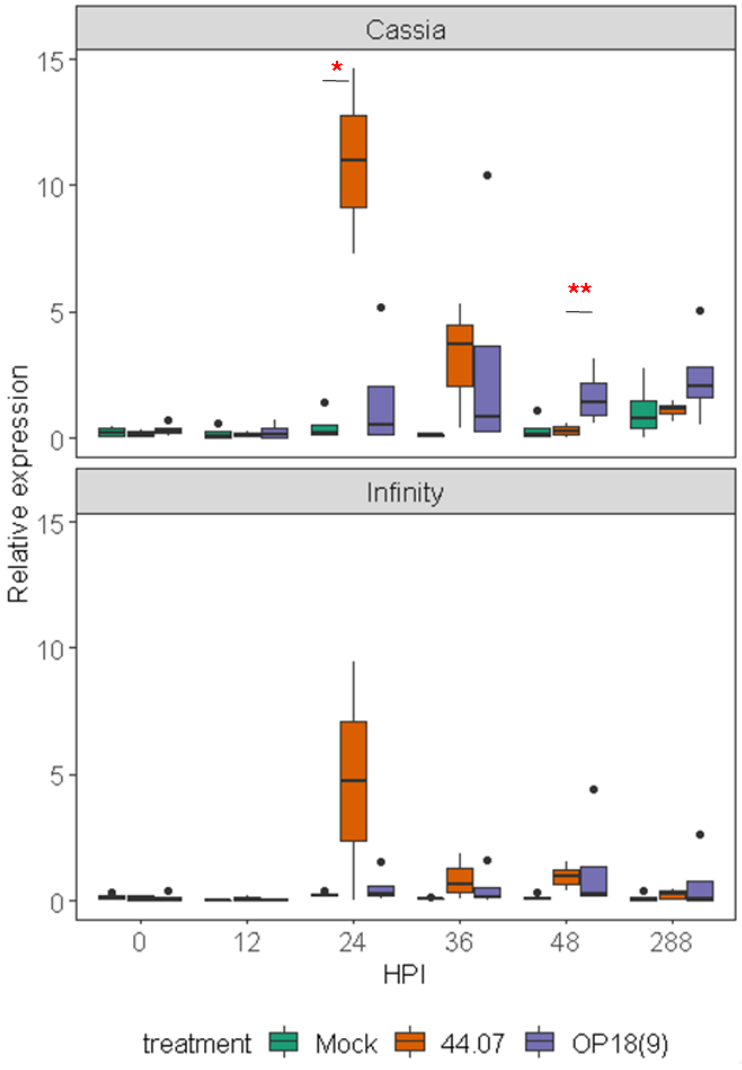


Figure 2.27: Expression of *PR1* relative to reference genes in Cassia and Infinity after treatment with *R. commune* isolates 44.07 and OP18(9) at a dose of 1 x 10^7 spores/ml. Significant differences between mock treated and 44.07 treated samples in Cassia at 24 hpi is indicated with a * (p-adj < 0.05). Significant difference in relative *PR1* expression after OP18(19) and 44.07 treatment in Cassia at 48 hpi indicated with ** (p-adj < 0.01) (n = 3).

Interestingly, relative expression of *NPR1* increased 4 fold at 12 hpi in response to 44.07 in Cassia, compared to mock treated Cassia (Figure 2.28). As such, increased *PR1* expression in Cassia at 12 hpi (Figure 2.27) may be as a result of *NPR1* mediated SA signalling, which in turn activates downstream *PR1.* This was not observed in Infinity. Similar to *PR1,* values for *NPR1* relative expression remain below 1 in response to all treatments in Cassia and Infinity at 0 and 12 hpi for all replicates. However, in Figure 2.19, when previously testing the two different inoculation doses in Cassia, increased *NPR1* expression was observed in response to 44.07 at the 1×10^7 dose at 36 hpi, which was not recorded in Figure 2.28.

An increase in *WRKY43* relative expression was recorded in Cassia in response to the 44.07 isolate at 24 hpi compared to mock. This was also obsered at 36 and 48 hpi however this was not found to be significant (Figure 2.29). An induction of *WRKY43* in Infinity at 24 hpi in response to 44.07 was also observed, but this was not as pronounced as in Cassia (8 fold increase compared to mock in Cassia, 6 fold increase compared to mock in Infinity), and not found to be significant (Figure 2.29).

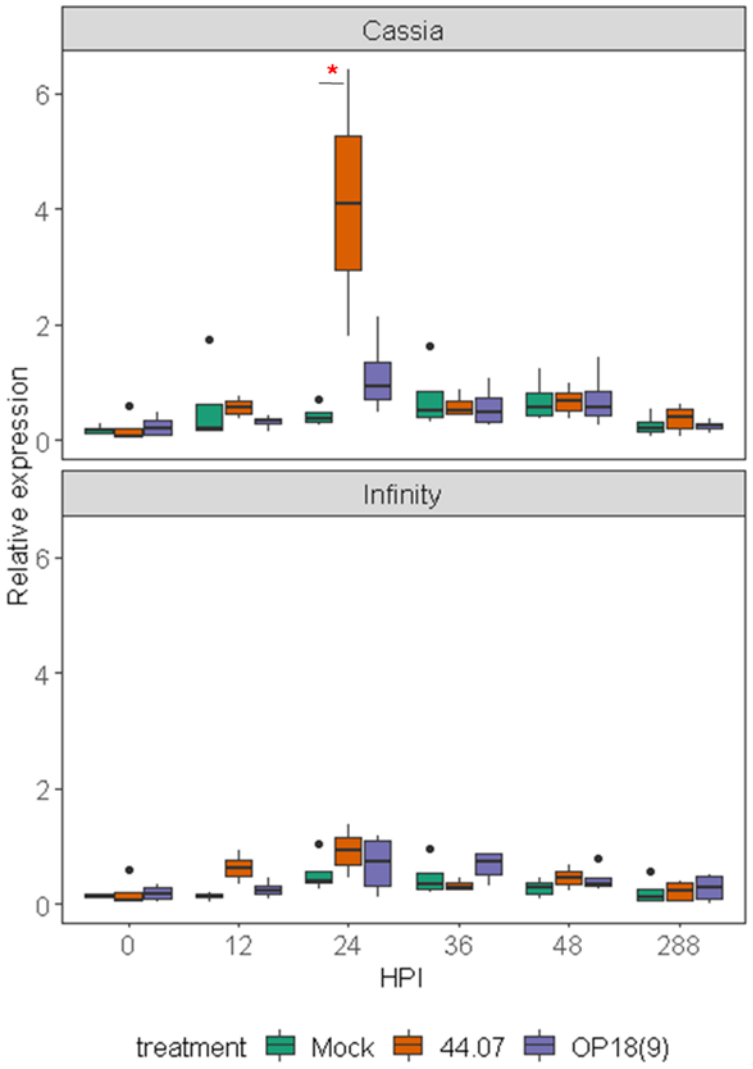


Figure 2.28: Expression of *NPR1* relative to reference genes in Cassia and Infinity after treatment with *R. commune* isolates 44.07 and OP18(9) at a dose of 1 x 10^7 spores/ml. Significant differences between mock treated and 44.07 treated Cassia at 24 hpi is indicated with a *: (p-adj < 0.05, as determined by two way ANOVA, n = 3). No significant changes in expression observed in Infinity after *R. commune* treatment.

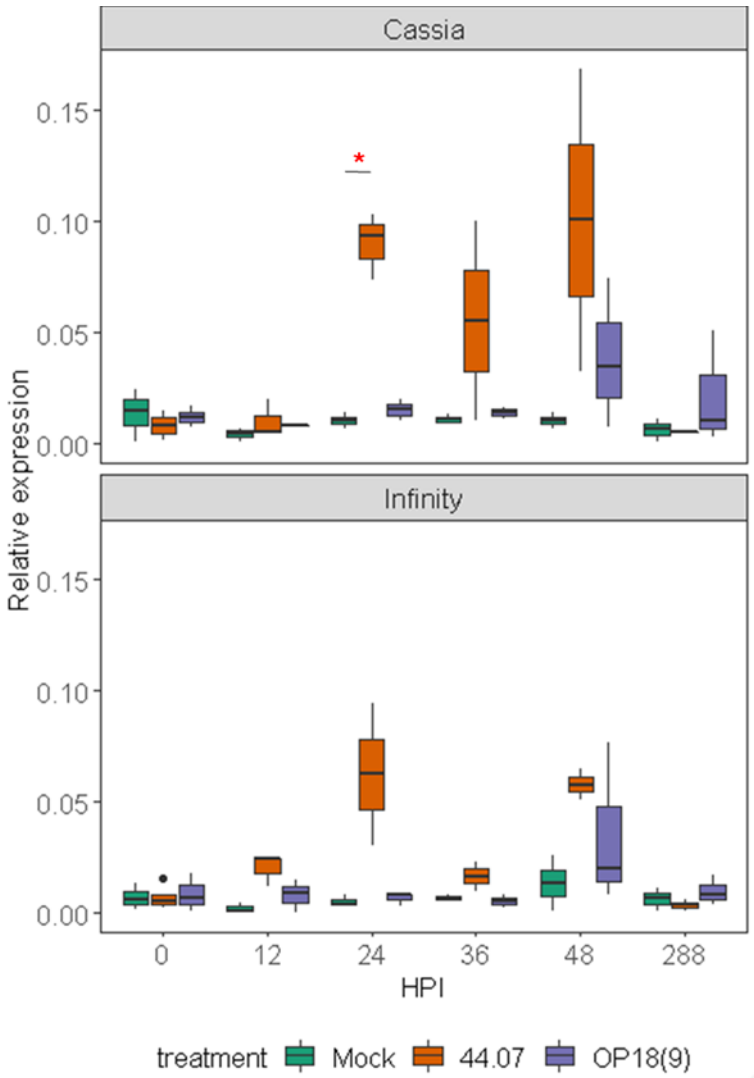


Figure 2.29: Expression of *WRKY43* relative to reference genes in Cassia and Infinity after treatment with *R. commune* isolates 44.07 and OP18(9) at a dose of 1 x 10^7 spores/ml. Significant differences between mock and 44.07 treated Cassia at 24 hpi is indicated with a * (p-adj < 0.05, as determined by two way ANOVA, n = 3). No significant changes in expression observed in Infinity after *R. commune* treatment.

An apparent 0.7 fold increase of *WRKY50* expression in response to 44.07 relative to mock was observed in Cassia at 24 hpi and 2.2 fold increase at 48 hpi (Figure 2.30). Also, at 48 hpi in Cassia a 1.5 fold increase in *WRKY50* relative expression compared to mock was recorded in response to the OP18(9) isolate. However, this increase in expression at these timepoints was not significant (Figure 2.30). This is similar to Figure 2.21, where there was no change in *WRKY50* expression after treatment with either isolate at 10^7 spores/ml at any timepoint in Cassia. Changes in *WRKY50* expression

between *R. commune* and mock treated samples in Infinity were also not detected (Figure 2.30).

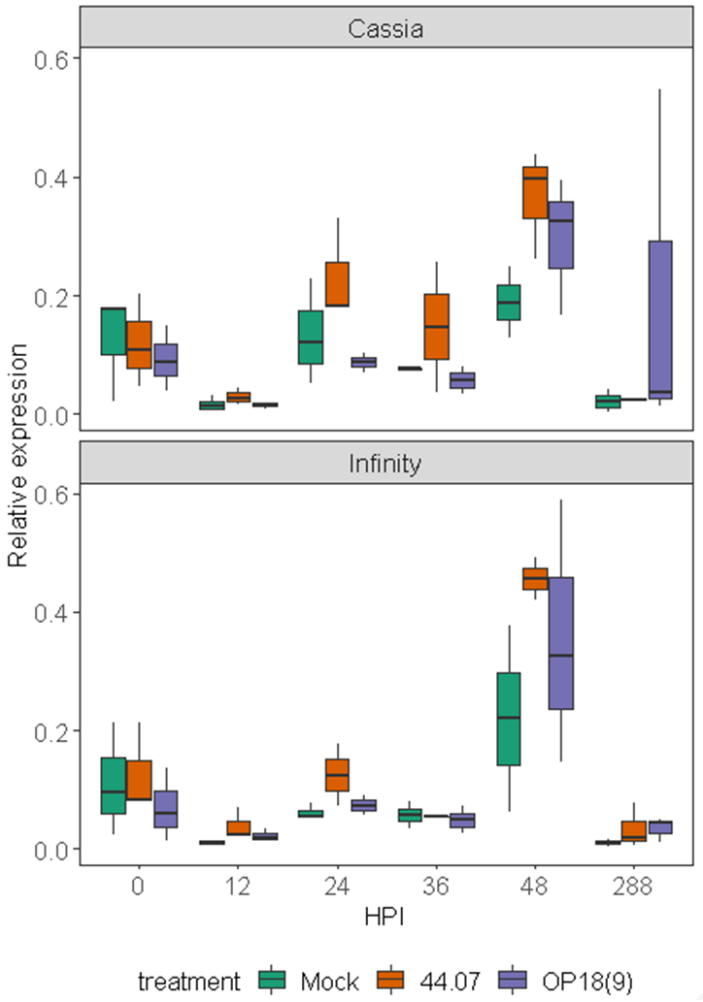


Figure 2.30: Expression of *WRKY50* relative to reference genes in Cassia and Infinity after treatment with *R. commune* isolates 44.07 and OP18(9) at a dose of 1 x 10^7 spores/ml. No significant changes between *R. commune* and mock treated samples (n = 3).

Relative quantification of *HB s*howed an increase in expression at 24 hpi in both Cassia and Infinity in response to both isolates (Figure 2.31). In Cassia, while a 3.4 fold and 7.6 fold increase compared to mock was observed at 24 hpi in response to OP18(9) and 44.07 respectively, values recorded remained

low (mean $2^{\Delta Cq}$ = 4.07e-3 in response to OP18(9) and 9.07e-3 in response to 44.07).

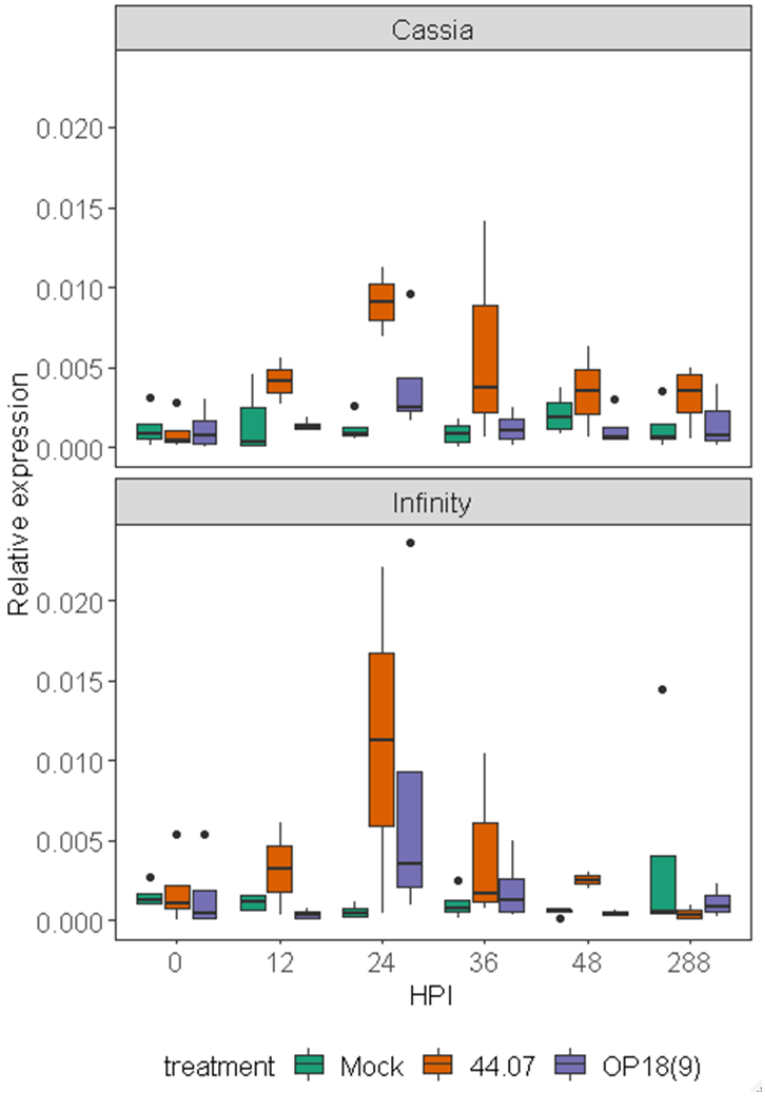


Figure 2.31: Expression of *HB* relative to reference genes in Cassia and Infinity after treatment with *R. commune* isolates 44.07 and OP18(9) at a dose of 1 x 10^7 spores/ml. No significant differences detected between *R. commune* and mock treated samples in either cv (n = 3)

This is similar to Infinity at 24 hpi, where an almost 20 fold increase is observed in response to 44.07 compared to mock, but value remain relatively low (mean $2^{\Delta Cq}$ = 1.1e-2) compared to other genes tested. However, in Infinity, this was inconsistent across replicates, with one replicate showing very low expression

relative to reference genes ($2^{\Delta Cq}$ = 4.6e-4). Values for *HB* relative expression in both cvs remain very low (> 0.025) across all treatments and timepoints (Figure 2.31). Previously, in Figure 2.15, in non-pooled samples, increased relative expression values of *HB* compared to mock treatment was only detected in response to the 1 x10^7 spores/ml dose of 44.07 in Cassia at 120 h (5 dpi).

2.5 Discussion

The first aim of this chapter was to identify a suitable protocol to study the interaction between different barley cvs against *R. commune*, to quantify the pathogenicity of Irish *R. commune* isolates and evaluate the response of barley cvs after *R. commune* inoculation. This was required to achieve the second aim of this chapter, which was to identify relevant timepoints in the barley x *R. commune* interaction.

Glasshouse pilot study

This was a preliminary screen to test the suitability of glasshouse use in studying the barley x *R. commune* interaction. This was also done to test the hypothesis that disease incidence of a given barley cv to *R. commune* is isolate dependant. To assess this, five barley cvs were inoculated with different *R. commune* isolates, and disease incidence was quantified as leaf area displaying scald lesions, and loss of green leaf area. Results displayed in Figures 2.2-2.5 indeed suggest that disease incidence on a given barley cv is *R. commune* isolate dependent. This was unsurprising and agrees with findings described by Arabi et. al., (2010) who showed that the disease incidence on 5 barley cvs varied dependent on *R. commune* isolate (of 49 *R. commune* isolates tested) (Arabi et. al., 2010). This is also observed in other crop pathosystems, for example, the disease incidence on wheat cultivars Gallant and Stigg varied significantly depending on which *Z. tritici* isolate they were exposed to (of 14 *Z. tritici* isolates tested) (Rahman et. al., 2020). In section 2.4.1, resistant cv Sanette showed a greater loss of green leaf area in response to OP16(16) compared to other *R. commune* treatments (Figures 2.4 and 2.6). However, these results were inconclusive because mock treated plants also showed disease symptoms. Although plants in the glasshouse were positioned in a random block design, they were placed in close proximity. It is possible that cross contamination between treatments occurred, so that mock treated plants may have been inadvertently exposed to a level of *R. commune* inoculum resulting in disease symptoms (Figures 2.3 and 2.5). If this was indeed the case, the lesions in *R. commune* treated plants cannot be accurately attributed to a single isolate. While the seed was not tested for *R. commune* infection, seed was collected from glasshouse plants with no

exposure to *R. commune* and sterilised prior to use to reduce potential for asymptomatic seed borne infection.

For an accurate quantitative assessment of pathogenicity, pre and post inoculation conditions must be strictly controlled as plants exposed to high temperature can affect both susceptibility and resistance to pathogen infection (Dhingra and Sinclair, 1995). For example, tomato hypersensitive response marker gene *HIN1* expression was detected at higher levels at 20°C compared to 33°C as early as 0.5 h after treatment with *Cladosporium fulvum* elicitor Avr9, as determined by northern blot analysis (Jong et. al., 2002). Conversely, increased temperature from 15°C to 35°C resulted in increased transcription of wheat stripe rust R gene *WKS1.1* both in the presence and the absence of *Puccinia striiformis f. sp. tritici* at 9 and 16 dpi (Fu et. al., 2009), suggesting that increased temperatures may also potentially improve resistance.

Due to weather conditions in Oak Park in 2018, during which time this work was completed, temperature increases (17-30°C) were recorded in the glasshouse despite cooling systems in place, resulting in unstable growth conditions. As the optimum growing temp for *R. commune* is ~ 18°C, increased temperature may have affected disease incidence in the cvs tested and the expression of defence related genes. In addition, heat stress could also contribute to loss of green leaf area, so the results documented may be unreliable. Therefore, it was necessary to investigate other approaches that would allow stricter control over experimental conditions.

Detached Leaf Assay

A detached leaf assay for the investigation of host pathogen interactions has advantages including reduced plant material needed, reduced inoculum and space requirements, and reduction of the risk of cross contamination. However, disadvantages include that this is a more artificial system compared to whole seedling/plant inoculation, it induces a wounding response in the plant and has temporal limitations due to leaf tissue senescence.

Despite these limitations, a detached leaf assay has been used in assessing the resistance of crop species to different pathogens. For example, Brown and

Cooke (2005) described the use of a detached leaf assay to assess resistance of barley, oat and wheat cvs to *Fusarium* head blight disease, specifically to determine changes in the length of latent period on different cvs. Similar to the work in section 2.4.2, Sayler and Yang (2007) used a detached leaf assay to quantify accumulation of the rice sheath blight pathogen *Rhizoctonia solani* in a susceptible and a resistant *Oryza sativa* subsp*. japonica* cvs. In that study, a standard curve was prepared similarly to that described in section 2.3.9, using serial dilution of pathogen DNA from 100 ng to 1 pg. However, dilutions of less than 1 pg were not tested. In section 2.3.9, gDNA in dilutions as low as 0.1 pg was successfully detected.

In section 2.4.2 it was hypothesised that, as colonisation of *R. commune* still occurs in the absence of visual symptoms in the field (Fountaine et. al., 2007), this may also occur under detached leaf conditions. If this was the case, this type of assay could be used to examine the pathogenicity of a given *R. commune* isolate in different barley cvs. As shown in section 2.4.2, fungal biomass accumulation was indeed greater in resistant Propino compared to susceptible Golden Promise, despite more severe symptoms observed in Golden Promise (Figure 2.7), however the timecourse of this work was limited to the timeframe during which cut leaf sections could survive on agar. It is possible that the wounding response induced by cutting the leaf may also affect susceptibility to the pathogen. For example, higher crown rust incidence was reported in detached leaves compared to non-detached leaves (Dhingra and Sinclair, 1995). An alternative proposed by Dhingra and Sinclair (1995) was to inoculate barley leaves of whole seedlings using *R. commune* inoculum in soft agar, which is then placed on selected spots along the leaf surface using a brush. This would allow for the application of more than two inoculation points on a single leaf or inoculation of multiple leaves followed by quantification of lesion formation induced by a single isolate, without the issue of cutting and wounding leaf sections.

In conclusion, while the detached leaf assay provided useful data in determining pathogenicity of the 44.07 isolate on different barley cvs and also demonstrated the efficacy of the qPCR protocol and standard curve generated, temporal and physiological limitations due to senescence would be

disadvantageous as this work aimed to study changes in host gene expression over time. Additionally, to achieve the aim of identifying host responses to *R. commune* that may contribute to susceptibility, a detached leaf assay was not appropriate, as results may not accurately correlate with seedling or plant resistance.

Controlled climate growth chamber seedling assay

The use of a controlled climate growth chamber overcame issues encountered in the glasshouse study and was identified as being the best method for the transcriptomic analysis presented in Chapter 3. This protocol allowed for the quantification of the *R. commune* symptoms without additional stress applied to the seedlings. However, senescence was still observed on small regions of mock treated seedlings. One possible explanation for this could be that seedlings in the growth chamber are in closer proximity to the light source compared to under glasshouse conditions. However, *R. commune* gDNA was not detected in mock treated samples, indicating the senescence observed is not a result of *R. commune* infection.

The colonisation of two *R. commune* isolates was examined in Cassia using the qPCR protocol described in section 2.3.9. A low level of *R. commune* gDNA was detected at the 0 dpi timepoint in *R. commune* treated samples, but not in mock treated samples (Figure 2.11). These leaf samples were harvested immediately after inoculation, and this detection is from the inoculum as opposed to inside the leaf tissue. The lack of *R. commune* gDNA increase prior to 14 dpi suggests that the pathogen might have remained dormant (i.e., in a latent phase), not undergoing significant replication during this first stage of the infection process.

A number of gene expression analyses were carried out in sections 2.4.3 – 2.4.5, to determine the relative expression of defence related genes at different timepoints and after different inoculation doses. An initial examination of relative gene expression at 0, 2, 7, 10, 14 and 21 dpi after inoculation with one of two *R. commune* isolates, 44.07 and OP18(9), at 10^6 spores/ml or a mock control was first carried out. At this inoculation dose, no statistically significant changes in gene expression were detected between *R. commune* and mock

treated samples for all genes examined, except *PR1,* which showed increased expression after OP18(9) treatment compared to mock at 21 dpi. This was largely due to variation between replicates. For example, there is an apparent increase in *HB* expression between *R. commune* and mock treated samples at 21 dpi, this was not found to be significant, as it was only detected in 2 of the 3 replicates. Additional replicates would therefore be required.

To ensure a strong pathogen load and potentially drive a more robust defence response, the inoculation dose was increased ten-fold from 10^6 spores/ml to 10^7 spores/ml. Changes in expression of *NPR1* (Figure 2.19)*, PR1* (Figure 2.20) and *HB* (Figure 2.25) were detected between *R. commune* and mock treated leaf sections after inoculation at the higher dose. This was observed in the earlier timepoints between 24 and 48 hpi. From this, it was concluded that using 10^7 spores/ml inoculation dose would be more suitable than a 10^6 spores/ml dose when determining appropriate timepoints in the host response. Following this, the relative expression of *PR1, NPR1, WRKY43, WRKY50* and *HB* in Infinity was confirmed to compare the response in Cassia to a more resistant cv (Section 2.4.5).

Interestingly, despite being classed as a resistant cultivar (Table 2.1), Infinity seedlings displayed scald like lesions after inoculation with the OP18(9) *R. commune* isolate at 17 dpi. This was not observed after inoculation with 44.07. This suggests that the OP18(9) isolate may be more virulent than the 44.07 isolate. As the OP18(9) - Infinity interaction displays a susceptible phenotype at 17 dpi, analysis of the changes in Infinity gene expression at this timepoint may yield further S gene candidates.

Genes involved in the SA signalling pathway were analysed. Expression of *NPR1* and *PR1* were found to be upregulated between 24 and 36 hpi in Cassia. However, in Figure 2.19, when testing the two different inoculation doses in Cassia alone, increased *NPR1* expression was observed in response to 44.07 at the 1 x 10^7 dose at 36 hpi, which was not recorded in Figure 2.28, which showed no significant changes in *NPR1* expression at 36 hpi after inoculation at the same dose. This may be as a result of biological variability between plants of the same cv undergoing the same treatment. Data in Figure

2.28 was obtained after pooling leaf sections from plants within a single replicate, which may have 'averaged' these differences. Increased *PR1* expression was also observed in Cassia at 21 dpi (Figure 2.13). As SA signalling is known to promote susceptibility to necrotrophic pathogens, it is possible that activation of SA response genes at the later necrotic stages of infection may be contributing to Cassia susceptibility to these *R. commune* isolates.

WRKY43 and *WRKY50* have been suggested as orthologues to *AtWRKY33* (Liu et. al., 2014), a key regulator of multiple defence related processes in *Arabidopsis* (Zheng et al., 2006). There is however no evidence in the literature that WRKY43 or WRKY50 activity is SA dependent in barley. While *WRKY43* was found to be upregulated in Cassia at 24 hpi in response to 44.07, this was not observed in Infinity. As SA response genes *PR1* and *NPR1* are upregulated in Cassia at 24 hpi when inoculated at 10^7 spores/ml with the 44.07 isolate only (Figures 2.27 and 2.28), along with *WRKY43* (Figure 2.29), it is possible that *WRKY43* is indeed an ortholog to *AtWRKY33* and may also play a role in the susceptible response downstream of SA signalling. If this is indeed the case, it is likely to act in an isolate dependent response, as increased *WRKY43* expression was not observed in response to OP18(9). No changes in *WRKY50* relative expression were detected in Cassia or Infinity in response to either *R. commune* isolate.

Interestingly, relative expression of *RAR1* and *SGT1* was not found to be upregulated after *R. commune* treatment compared to mock. This differs from findings by Al-Daoude et. al., (2014), in which inoculation with the Rs46 isolate led to increased *RAR1* expression at 24 hpi in susceptible barley. However, as previously outlined in section 2.1, isolate and cv selection can greatly affect disease incidence. As different isolates and cvs were used in this study, the activity of these genes are not likely to be identical as observed in response to the *R. commune* isolate Rs46. Activation of these R gene mediated response genes is likely to be part of early ETI response, more likely to be isolate specific.

HB expression was found to be upregulated in Cassia after treatment with 44.07 at the higher (1 x 10^7) inoculation dose at 120 hpi (5 dpi). This was not observed in response to OP18(9) (Figure 2.25). No other genes examined showed increased expression compared to mock at 5 dpi. Therefore, this timepoint was not further investigated in section 2.4.5. However, monitoring of the expression of other genes involved in NO signalling in barley in response to *R. commune* could be relevant, due to the central role of NO signalling in plant defences against pathogens (Shaprio, 2005; Groß et. al., 2013).

One of the overall goal of this thesis was to identify candidate S genes in barley through a global transcriptome analysis. Therefore, it was important to select the most appropriate timepoints to obtain meaningful data, based on the cost limitations associated with largescale RNA seq. Based on the results presented in this chapter, for the RNAseq work described in Chapter 3, timepoints were that encompass the earlier stage of infection to ensure important gene regulation events are captured (0, 12, 24, 36 and 48 hpi). The 288 hpi (12 dpi) timepoint was also included as it is at this point that the pathogen is transitioning to the necrotic phase of the infection cycle. Indeed, this timepoint may be particularly interesting to assist in the identification of specific host signals that trigger in the transition in the pathogenic stages of *R. commune.*

3 Identification of barley susceptibility genes involved in the *Rhynchosporium commune* x barley pathosystem.

3.1 Introduction

In order for a compatible host - pathogen interaction to occur, the pathogen must evade or supress the plant immune system. In addition to this, most pathogens also require host compatibility to support their pre- and post-penetration needs, which differ depending on the lifestyle of the pathogen. For example, some biotrophs such *as Bgh* require the establishment of feeding structures (Pliego et al., 2013), while many necrotrophs such as *Botrytis cinerea* induce host cell death (Tiedemann et. al., 1997).

Any host gene that facilitates the infection process can be considered an S gene. However, the primary function of these genes in the absence of stress is not necessarily linked to immunity or defence against pathogens. Disrupting the function of S genes can result in pathogen-specific resistance if the gene targeted is part of a specific pathway required by a given pathogen. Disruption of an S gene can also confer broad spectrum resistance, if the targeted gene is involved in immune signalling pathways. A large number of identified plant S genes were reviewed in van Schie and Takken (2014), with over 200 S genes described. While most of these S genes have been identified in *Arabidopsis*, over 60 are described in crop species, including 11 in barley. In barley, the role of S genes in susceptibility is primarily described in the biotrophic *Bgh* pathosystem (Shen et. al., 2007; Eichmann et. al., 2010). Of surprise, considering the importance of *R. commune* as a pest of economic importance, the involvement of S genes in the barley x *R. commune* pathosystem remains unknown.

Manipulation of S genes has been shown to confer disease resistance in a number of crop species. In rice, several *OsSWEET* genes have been identified as S genes (Streubel et. al., 2013). The bacterial blight pathogen *Rhizoctonia solani* was shown to induce the expression of *OsSWEET11* in rice after 48 h (Gao et. al., 2018). *OsSWEET11* knock out mutants were shown to be less susceptible compared to wild type (WT) in a detached leaf assay, while overexpression lines were more sensitive to *R. solani* (Gao et. al., 2018).

Notably, Oliva et. al., (2019) used CRISPR/Cas9 genome editing to manipulate *OsSWEET11, OsSWEET13* and *OsSWEET14* to confer broad spectrum resistance to 63 *Xoo* isolates in both lab and field trials (Oliva et. al., 2019).

Another example of an S gene whose mutation using CRISPR/Cas9 resulted in increased pathogen resistance is *DOWNY MILDEW RESISTANCE 6 (DMR6)* identified in *Arabidopsis.* This gene was shown to be required for downy mildew infection (van Damme et al., 2008). Using a phylogenetic analysis, two tomato *DMR6* orthologues *SlDMR6-1* and *SlDMR6-2* were identified by Thomazella et. al., (2021). CRISPR/Cas9 induced mutations in *SlDMR6-1* was shown to confer broad-spectrum disease resistance to biotrophic and hemi-biotrophic pathogenic bacteria, oomycetes and fungi. Indeed, impaired growth of *Pst, Xanthomonas gardneri, Phytopthera capsici, Pseudoidium neolycopersici* and *Xanthomonas perforans* was observed in mutant lines for *SlDMR6-1* compared to WT. Notably, RNA sequencing (RNAseq) has recorded the differential expression of 1,274 genes in *Sldmr6-1* mutants compared to WT in the absence of a pathogen. GO enrichment analysis of the upregulated genes in *Sldmr6-1* mutant revealed enrichment of biological processes associated with plant immunity including SA response (Thomazella et. al., 2021), indicating that *SlDMR6-1* functions as a negative regulator of these immune related genes, in an uninfected plant.

Changes in gene expression after pathogen infection may be the result of the onset of an immune response (Jones and Dangl, 2006) or may originate from the manipulation of the host's gene expression programme by the pathogen to increase infection or virulence (Lapin and Van den Ackerveken, 2013). S genes in particular are likely to be upregulated in a susceptible cultivar compared to a resistant cultivar. Comparison of *Arabidopsis* accessions with different levels of resistance to *Ralstonia solanacearum* showed that *INCOMPLETE ROOT HAIR ELONGATION 3* (*AtIRE3*) was expressed at higher levels at 7dpi after infection with *R. solanacearum* in susceptible *Arabidopsis* accessions Lm-2 and Nok-3, compared to resistant accessions Bu-0 and CIBC-5. *AtIRE3* was determined to function as an S gene as a T-DNA insertion mutation of this gene showed reduced disease index scores

compared to WT in the Col-0 accession (Demirjian et al., 2021). RNAseq can be used to examine genome wide changes in expression profiles of resistant and susceptible cultivars for the more systematic and genome wide identification of S genes. For example, RNAseq was carried out on one resistant and one susceptible tobacco cultivar at multiple timepoints after infection with *Phytophthora nicotianae* to screen for both R genes and S genes in *Nicotiana tabacum* (Meng et. al., 2021). This study identified the upregulation of seven S gene homologues compared to mock treatment in the susceptible cultivar only. These changes in expression occurred between 6 and 60 hpi, with the highest fold changes occurring at 24 hpi, hence at relatively early stages of the infection process.

The discovery of *SlDMR6* as an S gene highlighted that identification of orthologues to known S genes can also be used to identify candidate S genes. For example, the *POWDERY MILDEW RESISTANT 4 (AtPMR4)* and *DOWNEY MILDEW RESISTANT 1 (AtDMR1)* genes were previously identified to confer susceptibility to *Hyaloperonospora arabidopsidis* and *Hyaloperonospora parasitica* (downy mildew pathogens) in *Arabidopsis* (Vogel and Sommerville, 2000; Van Damme et. al., 2005). *AtPMR4* and *AtDMR1* were later shown to also be required for susceptibility to *Oidium neolycopersici* (tomato powdery mildew) in *Arabidopsis* (Huibers et. al., 2013). TBLASTN searches of AtPMR4 and AtDMR1 protein sequences were used to identify S gene orthologues in the tomato SOL Genomics Network database. Stable knockdown of the identified orthologues *SlPMR4* and *SlDMR1* showed reduced growth of *O. neolycopersici* in tomato (Huibers et. al., 2013). Similarly, BLASTp searches were carried out on the potato proteome sequence utilising 11 known *Arabidopsis* S protein sequences (Sun et. al., 2016). Sequences showing the highest degree of homology with each of the 11 the S genes in *Arabidopsis* were considered to be potential orthologues in potato. RNAi mediated knockdown of 5 of the 11 orthologues identified showed complete resistance to *P. infestans* (late blight) in transformed potato, and a sixth showed decreased susceptibility through reduced lesion size (Sun et. al., 2016). For a pathogen that requires ~11 fungicide sprays per season, this was

a highly significant result and emphasises the importance of S gene identification as a means to design robust genetic resistance.

A common method for identifying orthologous genes and constructing orthologous gene families employs the Markov Clustering (MCL) algorithm. Originally developed for graph clustering (van Dongen, 2008), this algorithm can be used to cluster groups of gene or protein sequences into families based on sequence similarities. This process has been particularly useful in clustering large sequence databases comprising sequences across multiple species (Enright et. al., 2002; Li et. al., 2003). OrthoMCL is an analysis pipeline that uses BLAST analysis to identify orthologous pairs of genes or proteins and then applies the MCL algorithm specifically to generate orthologous families from at least two species. In addition to identifying orthologous genes or gene families, analysing sequence conservation across multiple species can also be used to give insight into gene function and evolutionary history.

3.2 Chapter Aims

Research presented in this chapter aimed to use RNAseq and orthologue family clustering via the OrthoMCL pipeline to identify barley S gene candidates that are likely exploited by *R. commune* during infection. Based on the results of Chapter 2, an RNAseq analysis was completed across six time points post *R. commune* inoculation using barley cvs Cassia and Infinity, in response to two *R. commune* isolates (44.07 and OP18(9)).

To complement this transcriptomic approach, an *in silico* analysis was also conducted to identify barley orthologues to known S genes from other plant species using an extensive list of known dicot and monocot S genes published by Van Schie and Takken (2014). Of the plant species discussed in this publication, ten had a well annotated published proteome. These ten proteomes underwent an 'all against all' BLASTp search and results were put through the OrthoMCL process to generate orthologous protein families.

In order to identify S genes that may be specific to the *R. commune* - barley interaction, the lists of differentially expressed genes were compared to the set of barley S gene orthologues identified to generate a list of 'high confidence' S genes with a potential role in susceptibility to *R. commune*.

3.3 Methods

3.3.1 *In silico* identification of S protein families

The proteomes of ten plant species (Table 3.1) for which known S genes or S proteins had been listed in Van Schie and Takken (2014) were downloaded from publicly available online resources (Supplemental Table S3.1, Appendix 2) to create a plant protein database. Protein sequences were preferred over gene sequences in order to identify more efficiently those barley proteins that were closest to known S proteins, with the underlying assumption that protein sequences are typically more conserved than the corresponding DNA coding sequences. The protein database was then filtered to remove poor quality sequences (i.e., truncated sequences or sequences with >20% unknown amino acids). Additionally, all known protein isoforms arising from a single gene locus were removed, so that this database contained only the longest protein isoform. This was done to avoid 'pseudo-in paralogue' groups – isoforms that are classed incorrectly as paralogues, which in turn can skew the clustering analysis (Li et. al., 2003). From the comprehensive list of known S genes and proteins outlined in Van Schie and Takken (2014), the protein sequences for 203 of these were known. These 203 protein sequences were also included in the protein database.

An 'all-against-all' BLASTp was carried out on this protein database to identify homologous pairs of sequences. This involved both 'within species' BLASTp to identify potential paralogues, and 'between species' reciprocal BLASTp across any two proteomes to identify potential orthologous pairs of proteins. The threshold for the all-against-all BLASTp was set to an e-value ≤ 10^{-20}. This e-value was selected based on the size of the protein dataset and the level of stringency required. A lower e-value was not selected due to the diversity of the species in the database. The MCL algorithm was then applied to the BLASTp output to generate clusters of protein paralogues and orthologues. When using OrthoMCL, the inflation value parameter can be set to control cluster tightness. A higher inflation value increases the cluster tightness and will reduce the number of sequences clustered to the same family. In contrast, a lower inflation value will include more sequences into fewer families (Li et.

al., 2003). For this analysis, an inflation value of 1.5 was used as described in Li et. al., (2003).

Table 3.1: List of proteomes and known S proteins used in dataset.

Species	# of proteins	Reference	Version
Arabidopsis thaliana	27,382	TAIR 10	10
Hordeum vulgare	37,593	IBSC (2012)	IBSCv2*
Zea mays	39,161	Jiao et. al., (2017)	AGPv4*
Capsicum annum	35,336	Qin et. al., (2014)	V2
Oryza sativa	55,548	Kawahara et. al., (2013)	Version 7
Glycine max	56,044	Schmutz et al., (2010)	Wm82 2.1
Fragaria vesca	29,645	Li et. al., (2018)	V2.0. a2
Solanum lycopersicum	35,768	TCG (2012)	ITAGv3.2*
Triticum aesitivem (high confidence)**	110,789	IWGSC (2018)	V1.0*
Triticum aesitivem (low confidence)**	158,682	IWGSC (2018)	V1.0*
Medicago truncatula	55,344	Tang et. al. (2014)	Mt 4.0v2
Known S - genes	203	Van Schie and Takken (2014)	-

* A new version has been released since this work was completed
** Proteins assigned high confidence or low confidence classes based on sequence completeness (N's in DNA sequence corresponding to unknown bases), similarity to genes represented in protein and DNA databases and repeat content.
TAIR: The *Arabidopsis* Information Resource
IBSC: International Barley Sequencing Consortium
TGC: Tomato Genome Consortium
IWGSC: International Wheat Genome Sequencing Consortium

3.3.2 Transcriptome analysis for identification of candidate barley S genes

Barley cvs Cassia and Infinity were spray inoculated at a dose of 10^7 spores/ml at GS13 with either *R. commune* isolate OP18(9) (referred to as treatment A for remainder of chapter) or 44.07 (referred to as treatment B for remainder of chapter) and placed in a controlled atmosphere growth cabinet at 18˚C at a 16 h day photoperiod (Snijders Micro Clima MC1750) as described in Chapter 2 (section 2.3.2.3). A mock treatment (treatment M) of SDW with 0.1% (v/v) Tween 20 was used as a control. The second emerged leaf was collected at 0, 12, 24, 36, 48 and 288 (12 days) hpi, flash frozen in liquid nitrogen (LN_2) and stored at -80°C until RNA extraction. Therefore, the 0 hpi timepoint represents between 30 to 60 seconds post inoculation. Leaf samples from three individual plants per condition were pooled together to create one biological replicate. Therefore, a total of 36 samples were collected per biological replicate, totalling 144 samples over four independent experiments for RNAseq (Figure 3.1).

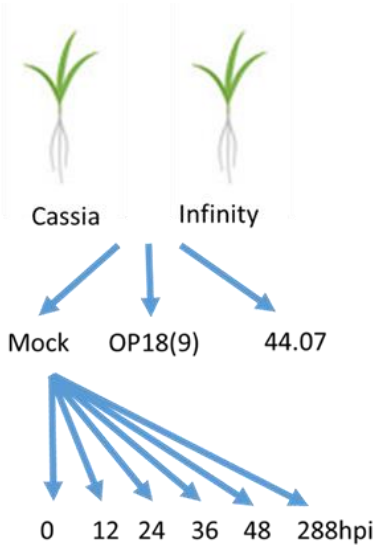


Figure 3.1: Schematic overview of RNAseq experiment. Two barley cultivars (Cassia and Infinity) were selected based on their resistance ratings to *R. commune* and inoculated with a spore suspension of OP18(9) (A), 44.07 (B) or a mock (M) control. Leaf tissue was collected from three individual plants and pooled for RNAseq at 0, 12, 24, 36, 48 and 288 hpi (12dpi) for each cultivar x treatment combination. Four biological replicates were completed for RNA-seq.

RNA extraction was carried out as described in Chapter 2 (section 2.3.8) using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich). Prior to sending the total RNA samples to Novogene, RNA quality was determined on a 1% (w/v) agarose gel and nanodrop spectrophotometer. Total RNA was sent to Novogene for library preparation and RNAseq. Quality control (QC) steps and analysis of raw data to identify DEGs were carried out by Novogene and are described below.

Prior to library construction, RNA quality was confirmed on an Agilent 2100 bio-analyser (Supplemental table S3.2, Appendix 2), a minimum RNA integrity value of 6.3 was recommended to pass QC. Once all samples passed QC, library construction was carried out by Novogene using a polyA enrichment protocol followed by reverse transcription resulting in ~300 bp cDNA fragments. The samples then underwent sequencing using the Illumina NovaSeq PE150 platform with at least 30 million paired reads per sample. Raw reads were filtered to remove reads containing adaptors, reads where N > 10% (where N corresponds to an undetermined base) and low quality reads. Error rate, GC content and percentage clean reads were determined for each sample.

Clean reads were mapped to the barley reference genome (IBSC_v2 cv. Morex, IBSC, 2002) using the HISAT2 software (Kim et. al., 2019). Gene expression levels were determined by the number of transcripts mapped to the reference genome, using fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) to normalise the effects of sequencing depth and gene length on read counts.

Differential gene expression analysis of mock treated versus *R. commune* infected tissue was carried out using the DESeq2 software (Love et. al., 2014) at each timepoint in both Cassia and Infinity. Differential gene expression analysis was also carried out comparing (i) the two *R. commune* treatments in each cultivar to determine potential isolate specific responses, and (ii) Cassia mock to Infinity mock samples to determine any intrinsic differences between the two cultivars in the absence of *R. commune*. In all comparisons,

differentially expressed genes (DEGs) were determined using the following thresholds: $|\log_2(\text{fold change})| > 1$ and adjusted p-value (padj) < 0.05.

To identify ‘core’ DEGs and isolate/cultivar specific DEGs, overlap analyses between the different datasets were carried out using the BioVenn software (Hulsen et al., 2008). Following this, a gene ontology (GO) analysis was carried out to identify the biological processes that were over-represented in each dataset using the g:GOSt function on g:Profiler (Raudvere et. al., 2019).

3.4 Results

3.4.1 *In silico* identification of barley S protein orthologues

To identify candidate barley orthologues to known S proteins, an *in silico* analysis was carried out using the OrthoMCL pipeline described in section 3.3.1. A total of 77,131 protein families comprising two or more proteins were identified by the OrthoMCL pipeline. Of these, only 142 families contained one or more known S protein(s). Within these 142 S protein families, 114 also contained at least one potential barley orthologue (Table 3.2).

Table 3.2: Overview of the protein families identified through the OrthoMCL pipeline.

	Count
Proteins in database	636,446
Protein families	77,131
Protein families containing a known S protein	142
Protein families containing a potential barley orthologue to a known S protein	114

The 142 protein families containing a known S protein revealed 10 families that contained proteins from the *Arabidopsis* proteome only. BLASTp of the S protein(s) in these families on the NCBI database into all species showed that two of these 10 families were in fact Brassicaceae specific. The remaining 8 of these 10 families were not *Arabidopsis*/Brassicaceae specific, as orthologous sequences were found in other dicot plant species, which were not included in the full protein dataset. Along with these 8 families, one additional family was also found to only contain orthologues in other dicot species (with orthologous proteins found in *Arabidopsis*, pepper, soybean and tomato from the dataset). From this, it was concluded that some S genes in this database may be dicot specific. Notably, no S protein family in this database was found to be monocot specific.

The number of members in an S protein family ranged from 2 to 955 proteins. In the 114 families that contained a potential barley orthologue to a known S

protein, the number of barley proteins in these families ranged from 1 to 63. In total, 682 potential barley S protein orthologues were assigned to these 114 protein families.

To determine if the expression of any of the genes coding for these 682 barley proteins was differentially regulated in response to *R. commune* in Cassia and Infinity, the expression pattern of these genes was subsequently analysed using the RNAseq datasets generated in section 3.4.2.

3.4.2 Transcriptional response of barley to *R. commune*

3.4.2.1 Quality control of RNAseq results

In parallel to the *in silico* identification of potential barley S protein orthologues, a transcriptomic approach to identify genes involved in the barley response to *R. commune* was carried out. In total, 144 RNA samples were sent for RNAseq. The quality of sequencing of each sample, as determined by percentage of clean mapped reads, error rate, quality score (Q20 and Q30) and GC content, are summarised in Supplementary table S3.3 (Appendix 2). Q20 represents an error rate of 1 in 100 (i.e. base call accuracy of 99%), while Q30 represents an error rate of 1 in 1000 (i.e. base call accuracy of 99.9%). Each sample had a Q30 of over 90%. An average of 92.18% of reads from each sample mapped to the barley reference genome (cultivar: Morex) and the read error rate was below 0.04% for every sample. GC content and distribution were found to be equal and stable across reads. Altogether, these QC results indicate accurate mapping and low probability of errors in base calling. In sum, the RNAseq data obtained was of good quality, so that it could be used for more in-depth analysis, including the identification of potential S genes that are relevant to a barley x *R. commune* interaction.

3.4.2.2 Analysis of gene expression changes in susceptible and resistant barley cultivars in response to two *R. commune* isolates.

Comparisons were carried out to determine significant differential gene expression between *R. commune* infected and mock treated barley in each cultivar and at each of the timepoints. As expected, there were no DEGs at 0 hpi in Cassia infected with A relative to mock. In contrast, some differential

expression was identified in Cassia x B, Infinity x A and Infinity x B at the 0 hpi timepoint with 74, 3 and 25 DEGs identified respectively (Figure 3.2). This was minor compared to the number of DEGs identified between 12 and 48 hpi.

The number of DEGs between 12 and 48 hpi relative to mock treatment was greater in response to the B isolate compared to A in Cassia (7 fold greater at 12 hpi, 6.4 fold at 24 hpi, 3.5 fold at 36 hpi and 2.5 fold at 48 hpi). This amplified response to B compared to A was also observed in Infinity between 12 and 48 hpi (8 fold greater at 12 hpi, 6.8 fold at 24 hpi, 3.3 fold at 36 hpi and 3.2 fold at 48 hpi) (Figure 3.2). The total number of DEGs was greatest between 12 and 48 hpi for each variety/treatment combination. Compared to the earlier timepoints, relatively few DEGs were detected at 288 hpi (12 dpi), with a total of 7 in Cassia in response to A, 52 in Cassia in response to B, 19 in Infinity in response to A, and 25 in response to B (Figure 3.2).

There was a higher proportion of upregulated genes compared to downregulated genes in all treatment x cv combinations. Also, a bi-modal expression pattern was observed in the number of upregulated genes independent of cv and treatment. A peak in upregulated genes was observed at 24 hpi in Cassia (973 and 4044 DEGs in response to A and B, respectively) and Infinity (666 and 3042 DEGs in response to A and B, respectively). This was followed by a second peak in upregulated genes observed at 48 hpi in Cassia (952 and 2283 DEGs in response to A and B) and Infinity (1070 and 2804 in response to A and B). This bi-modal expression pattern was also observed in downregulated genes in all treatment x combinations, except for Cassia inoculated with isolate A, in which there was no peak in down regulated genes at 24 hpi.

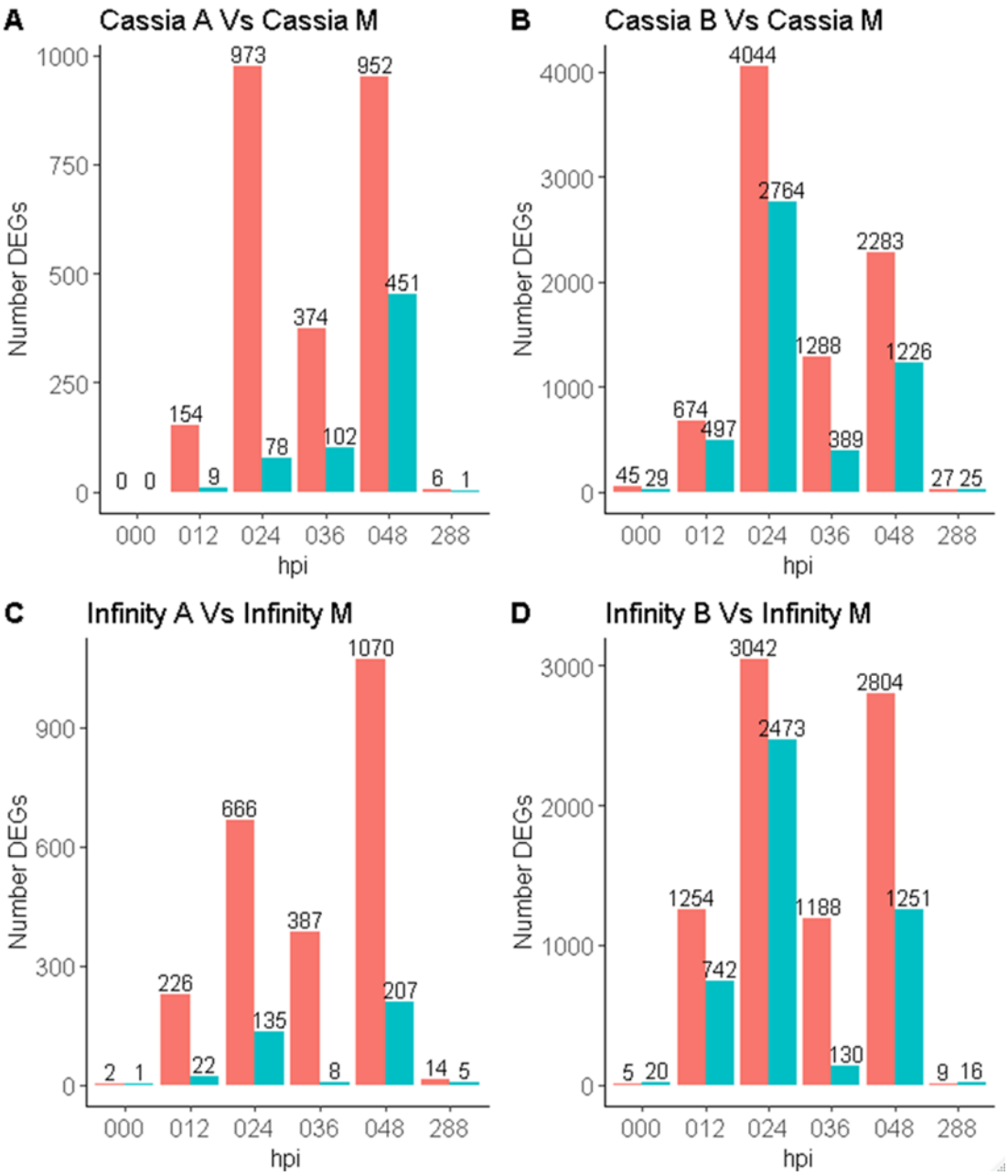


Figure 3.2: The number of differentially expressed genes in infected versus mock-treated samples, with the number of upregulated genes shown in red, and the number of downregulated genes shown in blue. A: OP18(9), B: 44.07, M: mock.

To further analyse the transcriptomic response of both cultivars to *R. commune,* pairwise comparisons of identified DEGs (relative to mock) were carried out between the two different isolate treatments in one given cultivar. This analysis has the potential to reveal isolate-specific differences in either

Cassia or Infinity. At the same time, this analysis may reveal DEGs that are common to both isolates. For this comparison, an overlap analysis between the sets of DEGs was carried out at each timepoint (except 0 hpi), comparing DEGs in response to A and B in Cassia, and then separately in Infinity (Figure 3.3). In Cassia, over half of the DEGs identified in response to A, were also identified as DEGs in response to B at every timepoint except 288 hpi. Specifically, at 12 hpi, 59.5 % of DEGs (97 DEGs) in response to A are also called as DEGs in response to B at the same timepoint, however these 97 genes only represent 8.2 % of the total DEGs in response to B (total 1171 DEGs at 12 hpi). At 24 hpi, the majority (96.4%) of DEGs in response to A are also responding to the B treatment. However, there were an additional 5795 genes differentially expressed in response to B at this time point. After 24 hpi, the proportion of DEGs in response to A also responding to B decreases, with 76% at 36 hpi (366 genes, representing 14.9% of B DEGs), 68% at 48 hpi (955 genes, representing 20% of B DEGs) and 28% at 288 hpi (2 genes, representing 4% of DEGs in response to B).

Similar to Cassia, the majority of DEGs in response to A are also differentially expressed in response to B in Infinity between 12 and 48 hpi. In Infinity, a total of 217 DEGs were determined to be common in response to both isolates at 12 hpi (representing 87% of total DEGs in response to A and 10.8% of total DEGs in response to B at 12 hpi), 774 DEGs shared by A and B response at 24 hpi (96.6% and 14% of total DEGs in response to A and B respectively), 376 DEGs shared at 36 hpi (95.1% and 28.5% of total DEGs in response to A and B respectively) and 1133 DEGs shared at 48 hpi (88.7% and 27.9% of total DEGs in response to A and B respectively).

This shows that while there is a conserved general response to the 2 isolates of *R. commune* shared across both cultivars at each timepoint, there are also isolate specific responses. Response to the B isolate induces a larger number of DEGs at each timepoint in both cultivars compared to the isolate A.

Figure 3.3: Overlap analysis of DEGs in response to different *R. commune* isolates in Cassia (susceptible) and Infinity (resistant) at 12, 24, 36, 48 and 288 hpi. Blue sets include DEGs in response to OP18(9). Yellow sets include DEGs in response to 44.07. (a: OP18(9), b: 44.07 e.g.: cas_a_012 represents the number of DEGs in response to A relative to mock identified in Cassia at 12 hpi)

A similar pairwise analysis was also carried out between the two cultivars in response to a single isolate in order to determine if there were common DEGs in response to both isolates between a resistant and a susceptible cultivar, indicating a conserved immune response regardless of the resistance rating of the cultivar. Additionally, this analysis will also identify any susceptible specific and resistant specific response to a given *R. commune* isolate at each timepoint. The overlap analysis of DEGs in Cassia and Infinity in response to A, and separately to B was completed (Figure 3.4). In response to the A isolate, at 12 hpi, the majority of DEGs were cv specific between Cassia (102 genes, 62.6% total DEGs at 12 hpi) and Infinity (187 genes, 75.4% total DEGs at 12hpi) with only 61 DEGs common to both cvs at 12 hpi. However, at 24, 36 and 48 hpi almost half of DEGs expressed in Cassia in response to A (42.7%, 40.1% and 40.2% respectively) are also differentially expressed in Infinity in response to A. At 288 hpi (12 dpi) there are 3 genes differentially expressed in both Cassia and Infinity in response to the A isolate.

In response to the B isolate, a similar trend is observed, in that almost half of the DEGs in Cassia are differentially expressed in Infinity at 24, 36 and 48 hpi (3643, 770 and 1738 genes representing 53.5%, 45.9% and 49.5% of total DEGs in Cassia at 24, 36 and 48 hpi in response to B respectively). At 288 hpi, only two DEGs are common to both cvs in response to the B isolate. These results suggest that while there was a conserved response between the two cvs detected in response to both isolates, there were also distinct groups of genes at each timepoint. These could be susceptible specific and resistant specific DEGs to each *R. commune* isolate, i.e., casB represents a susceptible interaction, while infB represents a resistant interaction, as determined in Chapter 2 (Figure 2.26). Barley S genes that are exploited by *R. commune* to promote susceptibility could potentially be found in the susceptible specific gene sets.

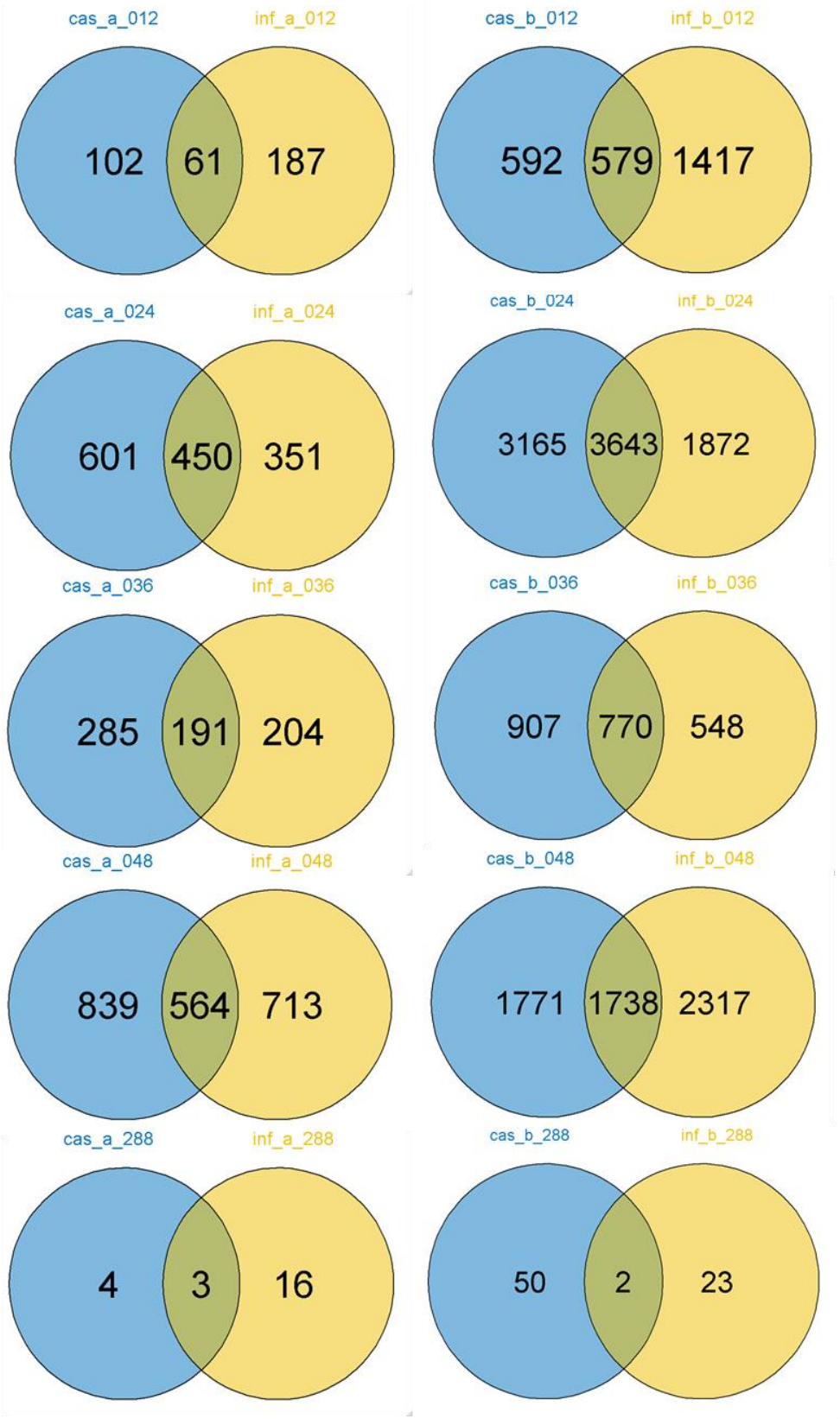


Figure 3.4: Overlap analysis of DEGs in response to a single *R. commune* isolate in Cassia (susceptible) and Infinity (resistant) at 12, 24, 36, 48 and 288 hpi. Blue sets include DEGs Cassia. Yellow sets include DEGs in response to Infinity. (a: OP18(9), b: 44.07)

For each cultivar x treatment condition, some genes were identified as differentially expressed at more than one timepoint. For example, in Cassia following treatment with A, 423 DEGs were identified at 2 timepoints, 188 DEGs were identified at three timepoints and 44 DEGs were identified at four timepoints. Similarly, in Cassia in response to B, there were a total of 2094 DEGs identified at 2 timepoints, 720 DEGs at three timepoints, 153 DEGs identified at four timepoints and 7 DEGs identified at all five timepoints. One such example is the HORVU7Hr1G113030 gene which was determined as significantly upregulated in Cassia at the 12 hpi and again at 48 hpi in response to A, but not at any other timepoint. This same gene was upregulated at 36 and 48 hpi in Cassia in response to B, but not at any other timepoint (Appendix 2, Supplemental Figure S3.1). This makes it difficult to identify clearly so called 'core' barley response genes to *R. commune* infection using pairwise comparisons at each timepoint because several genes will be accounted for more than once. In order to overcome this problem, 4 DEG groups were defined as follows:

- 2165 DEGs identified in ≥ 1 timepoint in Cassia after infection with OP18(9) (treatment A) compared to mock-treated [dataset 1, noted casA]

- 1831 DEGs identified in ≥ 1 timepoint in Infinity after infection with OP18(9) (treatment A) compared to mock-treated [dataset 2, noted infA]

- 9270 DEGs identified in ≥ 1 timepoint in Cassia after infection with 44.07 (treatment B) compared to mock-treated [dataset 3, noted casB]

- 8178 DEGs identified in ≥ 1 timepoint in Infinity after infection with 44.07 (treatment B) compared to mock-treated [dataset 4, noted infB]

These 4 datasets were used to identify 'core' response genes to *R. commune* independently of the timepoints at which samples had been collected (Figure 3.5). Within the overlap of the casA and casB datasets, there was a specific set of 370 genes (white circle in Figure 3.5) that were differentially expressed in Cassia in response to both *R. commune* treatments, but which were absent

from the infA and infB datasets. The expression of these genes was only affected in the susceptible cultivar and in response to both *R. commune* isolates, suggesting that they may be specifically required for the susceptibility phenotype. Genes in this set were considered as 'susceptible specific'. Within the overlap of the infA and infB datasets, there were 156 genes (red circle in Figure 3.5). These genes potentially make up a conserved response to *R. commune* within a resistant cultivar and were denoted 'resistant specific' response genes. However, as suggested in Chapter 2, the infA interaction may represent a more susceptible interaction compared to the infB interaction, due to the appearance of lesions on Infinity at 17 dpi after inoculation with the A isolate (Figure 2.26). No visible symptoms were observed in Infinity after inoculation with the B isolate. Therefore, the eight genes found in the overlap of the infA, casA and casB datasets (Figure 3.5) may also contain candidate genes required for a susceptible interaction.

Notably, 983 genes were differentially expressed in all 4 datasets (yellow circle in Figure 3.5), irrespective of the cultivar and of the *R. commune* isolate used in the experiments. These 983 genes are more likely to be part of a conserved or 'core' defence response to *R. commune.* This set of 'core' response genes is particularly interesting for the identification of S genes that may have a role in multiple cultivars and in response to a broader range of isolates.

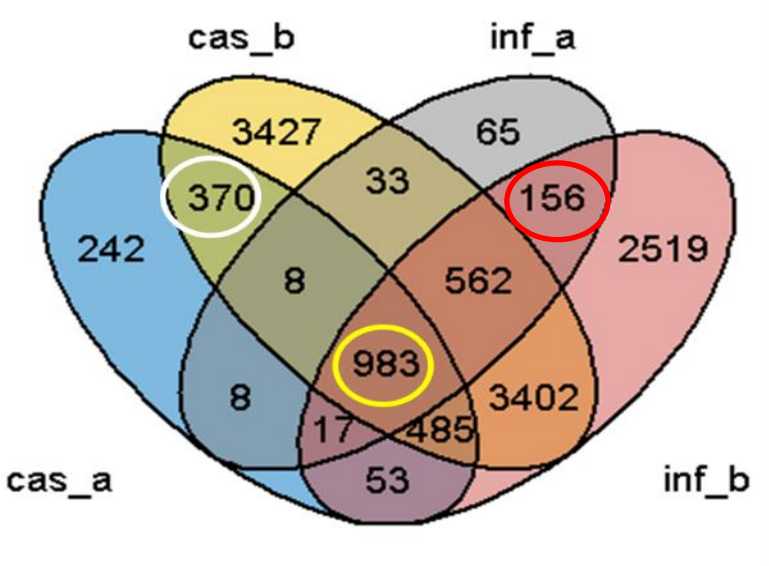


Figure 3.5: Overlap analysis of the different cultivar x treatment using datasets 1-4. The intersection representing the 'core' response to *R. commune* (i.e., DEGs present in all four datasets) is marked with a yellow circle (983 genes). The intersection marked in white represents putative 'susceptible specific' response genes (370 genes), which are only differentially expressed in Cassia but not in Infinity. Likewise, the intersection marked in red represents 'resistant specific' response genes (156 genes), which are only differentially expressed in Infinity. a: OP18(9), b: 44.07.

3.4.2.3 Functional analysis of DEGs

In order to identify the biological processes that were significantly overrepresented among DEGs in each treatment x cultivar combination, a GO analysis was carried out on each of the datasets (infA, casA, infB, casB) to reveal cultivar specific and isolate specific responses (Figure 3.6). As expected, GO terms associated with plant defences against pathogens were found to be enriched among the sets of DEGS. This contributes to the functional validation of the datasets obtained.

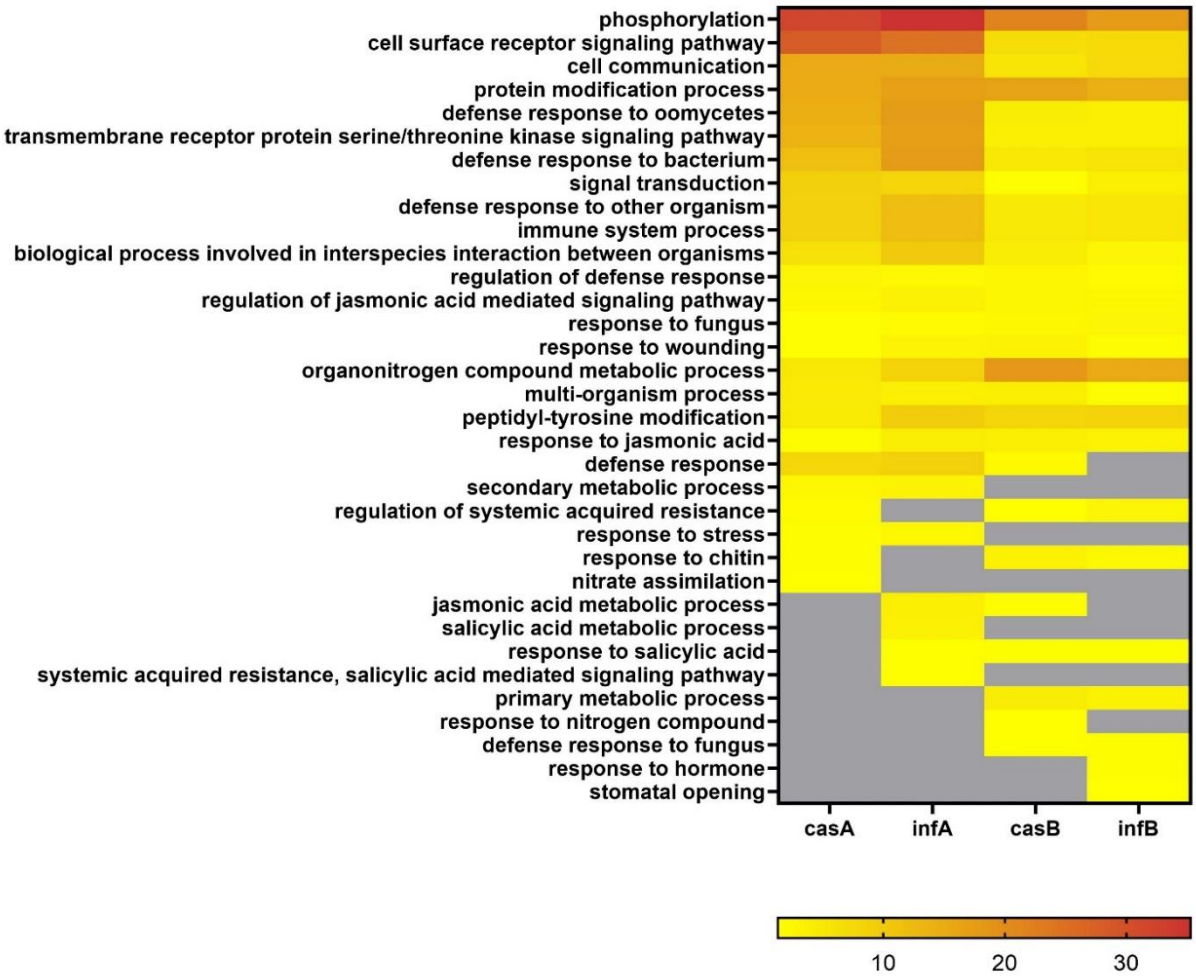


Figure 3.6: Heatmap of GO terms that are over-represented in the four pre-defined datasets. These biological processes are overrepresented in the set of genes found to be differentially expressed in each individual treatment x cultivar combination. Significance of over representation is shown as the -$\log_{10}$(p-adj) and is represented as a colour gradient from red (–$\log_{10}$(p-adj) > 35) to yellow (–$\log_{10}$(p-adj) < 5). Grey cells indicate no significant over representation. Heatmap generated using GraphPad Prism (version 9.1.0).

General terms such as ‘phosphorylation’, ‘cell surface receptor signalling pathway’ and ‘cell communication’ were the most significantly over-represented in each dataset (Figure 3.6). While these are broad functional GO terms, they relate to processes that can be associated with aspects of defence responses. For example, recognition of PAMPs induces PRR phosphorylation and the activation of signal transduction pathways, including MAPK signalling, eventually resulting in the phosphorylation of transcription factors that regulate defence gene expression (Park et. al., 2012). While GO terms such as ‘defence response to other organism’ and ‘immune system process’ were also over-represented in all four datasets, the more specific GO term ‘defence response to fungus’ was only over-represented in datasets describing the response to the B isolate (infB, casB). Other more specific GO terms including JA and SA processes and ‘systemic acquired resistance’ were also enriched in each of these datasets.

Other enriched GO categories represented in this analysis, which are not shown in Figure 3.6, included terms that would not typically be associated with the defence response. These include reproductive processes such as ‘recognition of pollen’, ‘pollination’ and ‘pollen-pistil interaction’. While these terms were not expected to be over-represented in these datasets, closer investigation of the genes assigned to these categories revealed a number of genes involved in protein phosphorylation and receptor protein signalling. Both of these processes are also required in immune signalling. It was also found that a number of genes found in these categories are also assigned to other general categories that can also be associated with the immune response, including ‘phosphorylation’ and ‘cell communication’. One possibility for this could be that GO annotation and categorisation is not as specific in barley as compared to other species such as *Arabidopsis*.

To further examine the DEGs in the core response (983 genes), susceptible specific (370 genes) and resistant specific (156 genes) subgroups identified in Figure 3.5, a GO analysis was also carried out on each subset of genes (Figure 3.7). The GO terms represented in this figure were selected based on their known role in defence response, but additional terms were overrepresented, as indicated below. There were 96 GO terms that were overrepresented in

both the core subgroup and the individual datasets (infA, casA, infB, casB) including ‘protein phosphorylation’, ‘kinase activity’ and ‘cell surface receptor signalling pathway’. More specific terms that also showed enrichment in this core group of DEGs include defence response and hormone signalling (Figure 3.7). From this, it appears that a large number of defence related processes are conserved across susceptible and resistant cultivars. Interestingly, the GO term ‘hydrogen peroxide metabolic process’ was found to be enriched for in the core subgroup of DEGs. This was not found to be enriched for in any of the DEG datasets when taken as a whole (infA, casA, infB, casB). Hydrogen peroxide (H_2O_2) is a ROS, which is known to be induced during PTI immediately after both compatible and incompatible host-pathogen interaction. A second, prolonged wave of ROS production has also been detected during ETI in compatible host-pathogen interactions and is involved in the hypersensitive response (Yuan et. al., 2021). This result suggests that ROS-mediated signalling may be particularly relevant in the context of *R. commune* infection.

A GO analysis on the sets of DEGs that were specific to the susceptible and resistant varieties (indicated in white and red, respectively, in Figure 3.5) was also conducted. This was carried out to examine the biological processes in response to *R. commune* that are unique to each cultivar. There was only one biological process enriched for in the resistant specific subgroup ‘rRNA base methylation’ and one biological process overrepresented in the susceptible specific subgroup ‘photosynthesis, light harvesting’. The relevance of these GO categories are unclear, although it is known that photosynthesis and chloroplast related processes are important in the context of plant/pathogen interaction (Bechtold et. al., 2005; Rojas et. al., 2014).

Due to the possibility that the infB dataset may also represent a susceptible interaction, the eight genes identified in the overlap of the infA, casA and infB datasets were also examined. However, there was no significant over representation of any GO terms. At the timepoints in this dataset (12, 24, 36 and 48 hpi), no visible symptoms were observed in Infinity. Therefore, these eight genes were not further investigated.

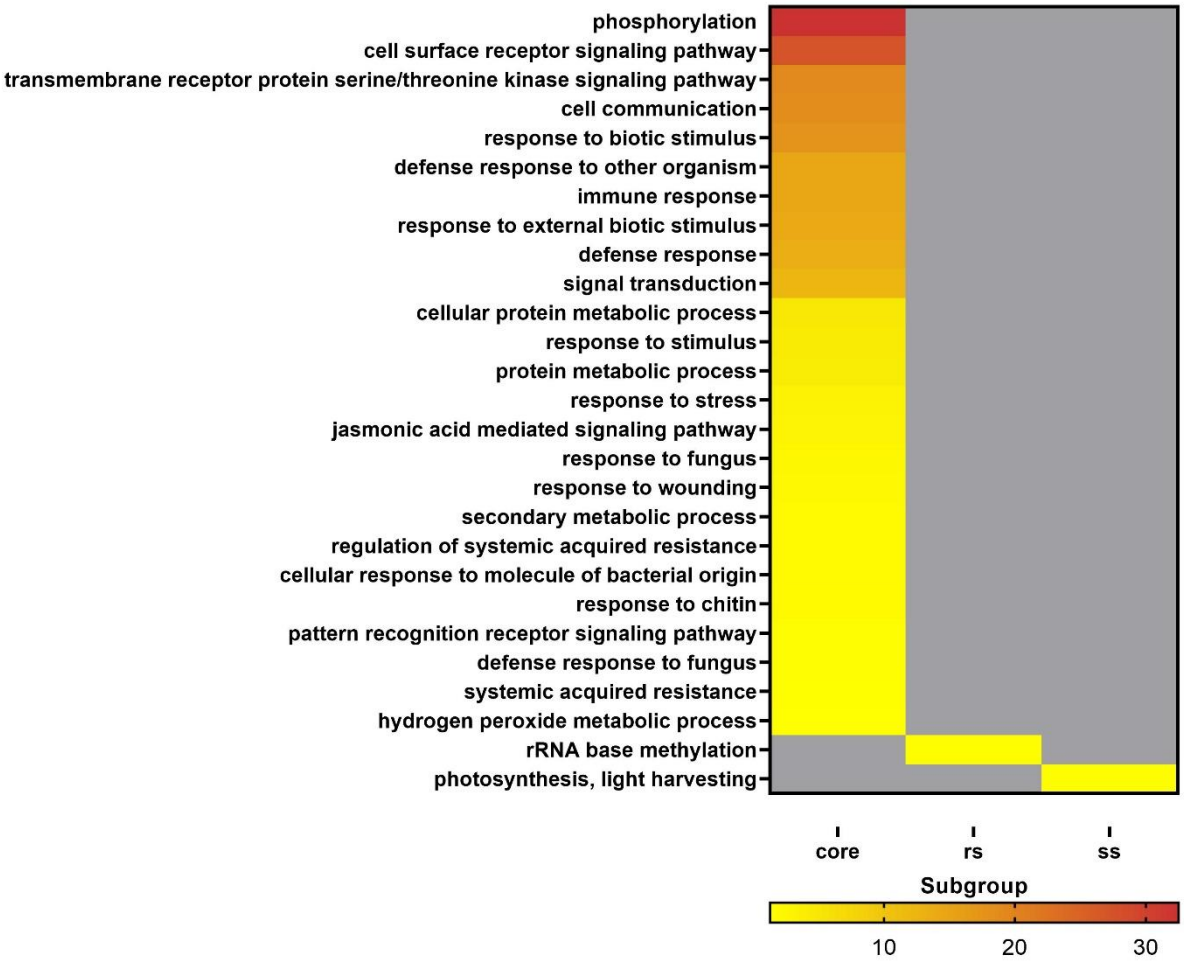


Figure 3.7: Heatmap of over-represented GO terms that are unique to the subgroups of DEGs identified in Figure 3.5 (core, resistant specific and susceptible specific). Significance of over representation is shown as the $-\log_{10}$(p-adj) and is represented as a colour gradient from red ($-\log_{10}$(p-adj) > 30) to yellow ($-\log_{10}$(p-adj) < 10). Grey cells indicate no significant over representation. Heatmap generated using GraphPad Prism (version 9.1.0). ss: susceptible specific; rs: resistant specific.

3.4.2.4 Validation of RNAseq and verification of DEGs by RT-qPCR

In order to further validate the RNAseq datasets, the FPKM values of four defence related genes (*NPR1, PR1, WRKY43, BI1*) were plotted. These genes were chosen because (i) of their potential role in the barley defence response; (ii) they are part of the 'core' response dataset identified in the previous

section; and (iii) they were shown (in section 2.4.5) to have changes in relative expression levels after treatment with the A and/or B isolates, except for *BI1* which showed no changes after *R. commune* treatment compared to mock. The FPKM values for the SA-related genes *PR1* (Figure 3.8) and *NPR1* (Figure 3.9) are in agreement with previously determined relative expresssion levels (Figures 2.27 and 2.28, Chapter 2). Specifically, *PR1* expression as determined by FPKM and by RT-qPCR increases compared to mock at 24 hpi and 36 hpi in response to both *R. commune* isolates in Cassia. In Infinity, the RT-qPCR results suggest increased expression in response to B compared to mock treatment only at 24 hpi, however this was not determined to be statistically significant. The FPKM values also show higher expression in response to B treated samples compared to mock treated samples at 36 hpi (Figure 3.8). However, FPKM vales also indicate increased expression of *PR1* in Infinity at 36 and 48 hpi in response to the A isolate. This was not detected by RT-qPCR.

FPKM plots representing *NPR1* expression in Cassia show that at 0, 12 and 288 hpi, FPKM values are similar between *R. commune* and mock treated samples (Figure 3.9 B). This was also observed in RT-qPCR plots (Figure 3.9 A). At 24 hpi, increased FPKM values are observed in *R. commune* treated Cassia (both isolates) compared to mock treatment. This apparent increase in expression is also seen in RT-qPCR plots for Cassia (Figure 3.9 A). However, expression determined by FPKM suggested an increase in expression in *R. commune* treated samples in Cassia at 36 and 48 hpi in response to both isolates, which was not detected by RT-qPCR. Furthermore, at 12, 24, 36 and 48 hpi FPKM values for *NPR1* expression in Infinity increase in response to both *R. commune* treated samples. These trends are only reflected in relative *NPR1* expression determined by RT-qPCR at 12 and 24 hpi, but not at 36 and 48 hpi. No changes in expression between *R. commune* and mock treated samples were observed in Infinity through FPKM or RT-qPCR data at any timepoint (Figure 3.9 A).

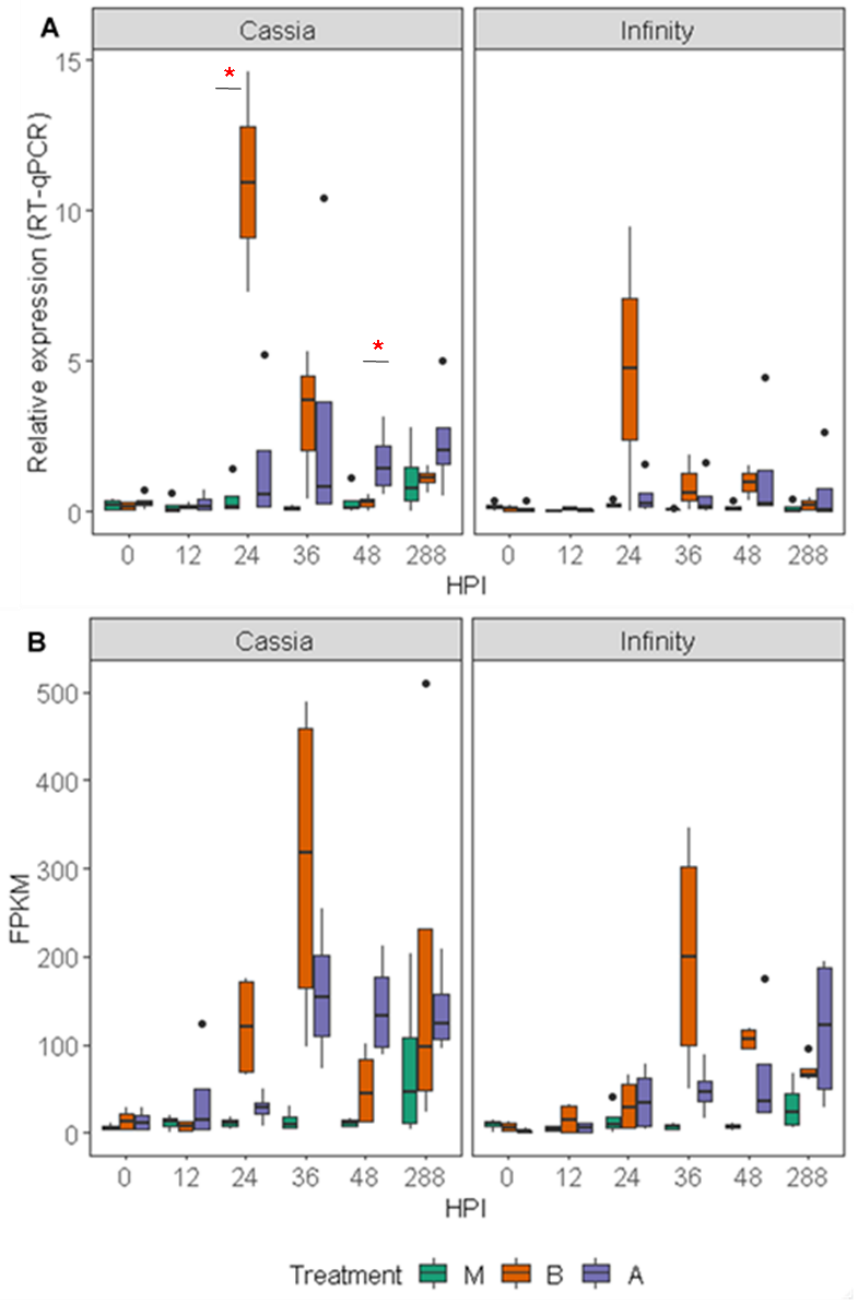


Figure 3.8: Validation of *PR1* gene expression changes. **A:** *PR1* expression relative to *ACTIN* as determined by RT-qPCR (n = 3) in Chapter 2. Significant differences between mock and 44.07 treatments at 24 hpi as determined by two-way ANOVA indicated by * (p-adj < 0.05) **B:** *PR1* expression as determined by FPKM values obtained from RNAseq datasets (n = 4). M: Mock, A: treatment A (OP18(9)), B: treatment B (44.07).

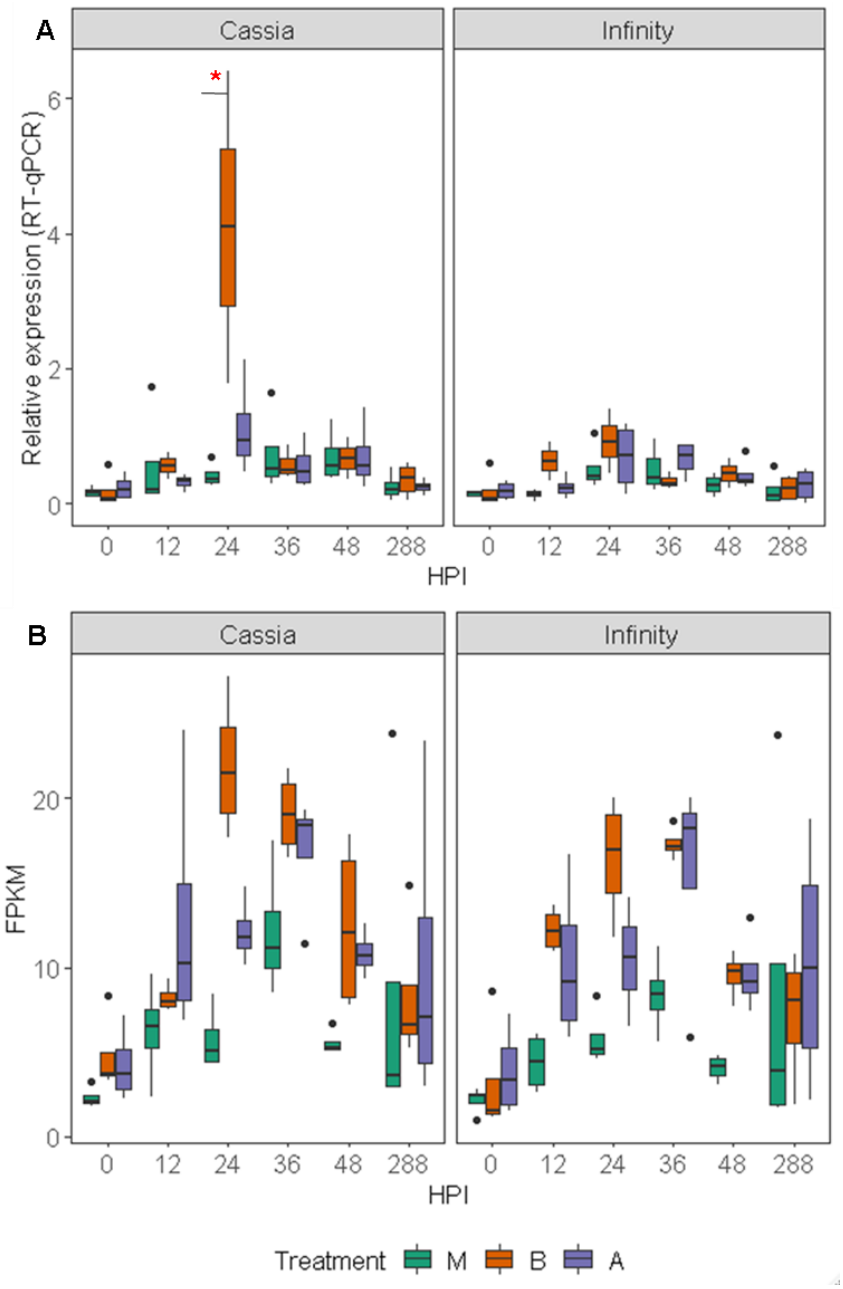


Figure 3.9: Validation of *NPR1* gene expression changes. **A:** *PR1* expression relative to *ACTIN* as determined by RT-qPCR (n = 3) in Chapter 2. Significant differences between mock and 44.07 treatments at 24 hpi as determined by two-way ANOVA indicated by * (p-adj < 0.05) **B:** *NPR1* expression as determined by FPKM values obtained from RNAseq datasets (n = 4). M: Mock, A: treatment A (OP18(9)), B: treatment B (44.07).

Expression of *WRKY43* in Cassia in response to B peaks between 12 and 48 hpi, and in response to A at 48 hpi, as determined FPKM. This increase is more pronounced in response to the B isolate. RT-qPCR data also shows increased *WRKY43* expression in Cassia in response to either isolate at the same timepoints, however this was only found to be statistically significant at 24 hpi in response to B (Figure 3.10). There is no difference in relative expression or FPKM values between *R. commune* and mock treated samples at 0 and 288 hpi in Cassia. An increase in *WRKY43* FPKM values was detected betwen 24 and 48 hpi in Infinity in response to both A and B. Corresponding RT-qPCR analysis did not detect any significant changes in *WRKY43* expression in Infinity between *R. commune* and mock treated samples. Expression of *WRKY43* as determined by FPKM and RT-qPCR in mock treated samples remained low across all timepoints in Cassia and Infinity (Figure 3.10).

Finally, *BI1,* a conserved cell death regulator known to be involved to susceptibility to *Bgh* (Eichmann et. al., 2010) was also chosen to validate the RNAseq data. RT-qPCR data indicated no significant changes in *BI1* relative expression between *R. commune* and mock treated samples in Cassia or Infinity (Figure 3.11 A). However, FPKM data suggests there was increased expression of *BI1* in Cassia between 24 and 48 hpi in response to B. The FPKM data detected increased *BI1* expression in response to B in Infinity at 36 and 48 hpi, but not 24 hpi.

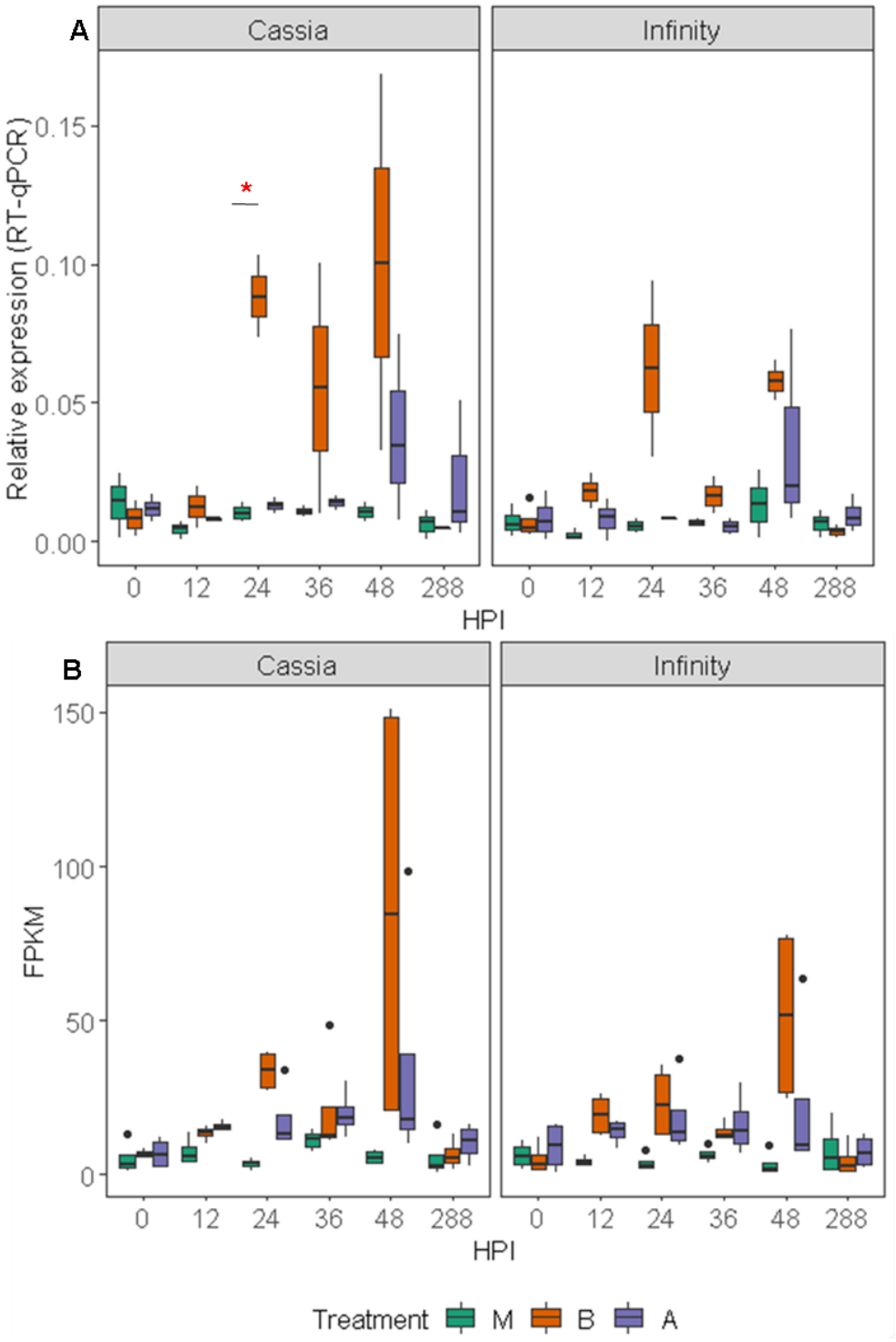


Figure 3.10: Validation of *WRKY43* gene expression changes. **A:** *WRKY43* expression relative to *ACTIN* as determined by RT-qPCR (n = 3) in Chapter 2. Significant differences between mock and 44.07 treatments at 24 hpi as determined by two-way ANOVA indicated by * (p-adj < 0.05) **B:** *WRKY43* expression as determined by FPKM values obtained from RNAseq datasets (n = 4). M: Mock, A: treatment A (OP18(9)), B: treatment B (44.07).

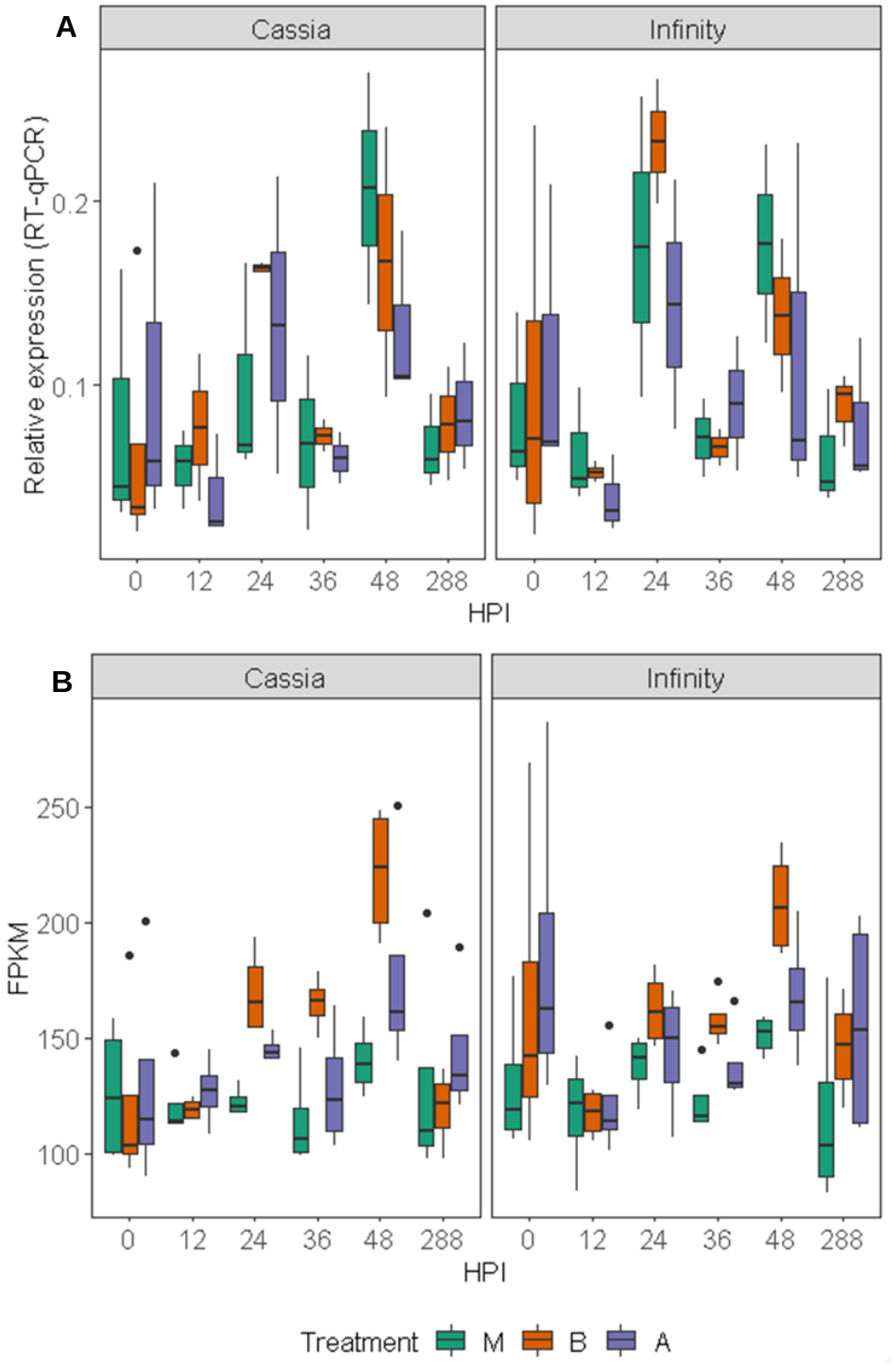


Figure 3.11: Validation of *BI1* gene expression changes. **A:** *BI1* expression relative to *ACTIN* as determined by RT-qPCR (n = 3). No significant changes in expression between different treatments at a given timepoint as determined by two-way ANOVA was detected **B:** *WRKY43* expression as determined by FPKM values obtained from RNAseq datasets (n = 4). M: Mock, A: treatment A (OP18(9)), B: treatment B (44.07).

3.4.3 Identification of candidate S genes in barley targeted by *R. commune.*

Using the RNAseq data, the expression profiles of the 682 potential barley orthologues that were identified through the *in silico* approach in Section 3.4.1 were next examined. Of these 682 genes, only 245 were found to be differentially expressed relative to mock treatment in at least one of the four previously defined datasets from section 3.4.2.2 (casA, casB, infA, infB). To track the changes in expression of these potential barley orthologues over time in response to each *R. commune* isolate, a heatmap of significant changes in expression of each of these 245 potential S gene orthologues relative to mock treatment, represented by the $\log_2$ of the fold change (FC), was generated (Figure 3.12). This analysis would allow for: (i) determination if the S gene orthologues are up or/and down regulated in each cultivar across the infection time-course; (ii) identification of any S gene orthologues that are differentially expressed in Cassia only or Infinity only in response to a given *R. commune* isolate; (iii) the reveal of isolate-specific differences within Cassia and Infinity; and (iv) identification of S gene orthologues that are responding to both isolates.

None of the 245 S gene orthologues were found to be differentially expressed at 0 hpi immediately following inoculation with isolate A, and only 2 genes (HORVU4Hr1G005920 and HORVU6Hr1G053090) showed differential expression in Cassia only at 0 hpi after inoculating with isolate B. This indicates that the expression of the majority of the S gene orthologues were not triggered by the inoculation method. Interestingly, none of the 245 S gene orthologues were found to be differentially expressed at 288 hpi (12 dpi). Because of the absence of differential expression, the 0 hpi and 288 hpi timepoints were not included on the heatmap (Figure 3.12).

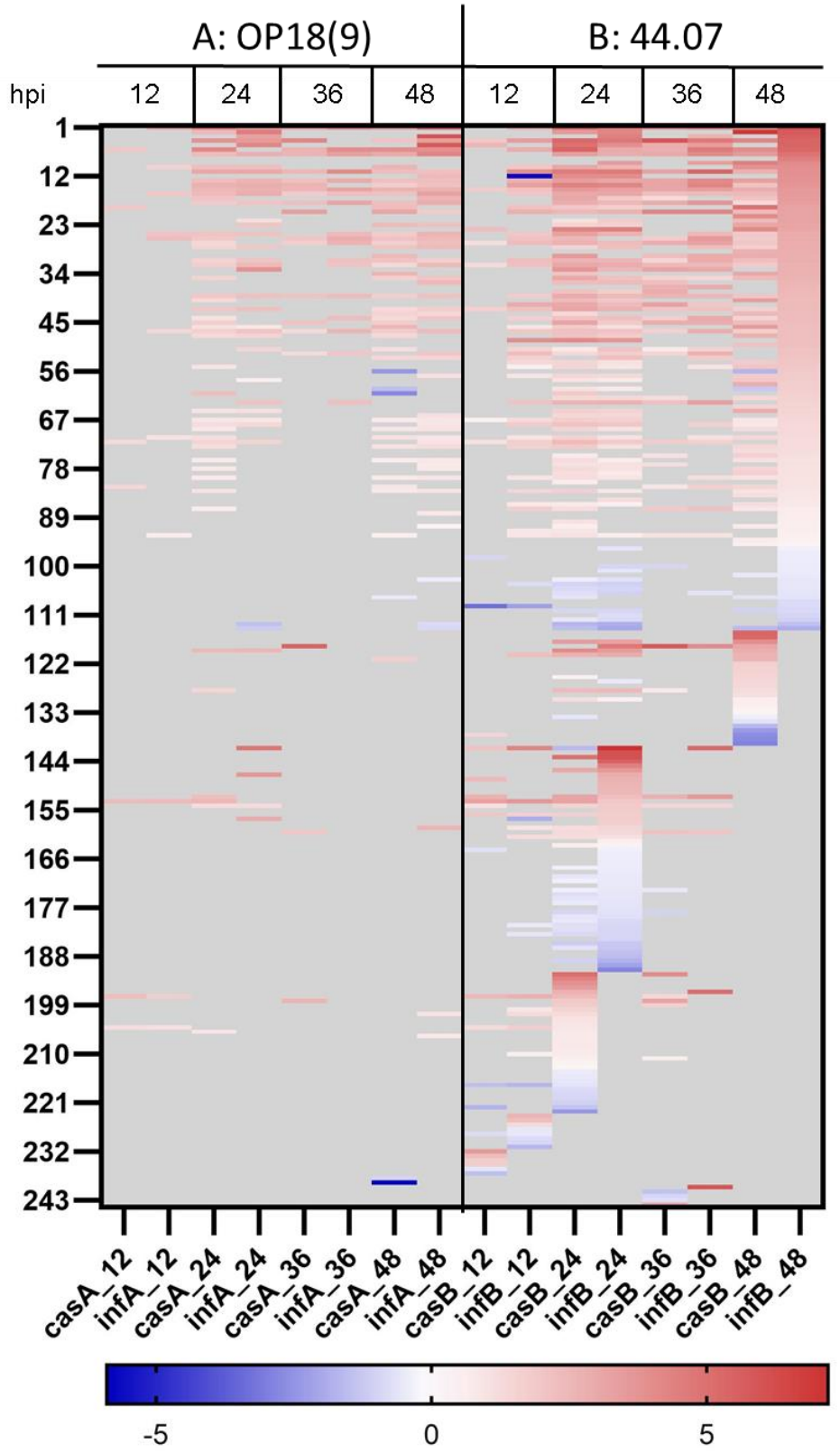


Figure 3.12: Heatmap of significant Log2(Fold change) of 245 identified potential barely S gene orthologues. Level of up or down regulation is represented as a colour gradient with upregulation shown in red and downregulation shown in blue. Grey indicates no significant change inexpression at this timepoint. Heatmap generated using GraphPad Prism (version 9.1.0).

143 of the 245 S gene orthologues showed significant differential expression at multiple timepoints. For example, HORVU5Hr1G060650 and HORVU7Hr1G019390 genes were differentially expressed in Infinity in response to both *R. commune* isolates at 12, 24, 36 and 48 hpi. These genes were also differentially expressed in Cassia in response to both isolates at 24, 36 and 48 hpi (Figure 3.13 and Figure 3.14).

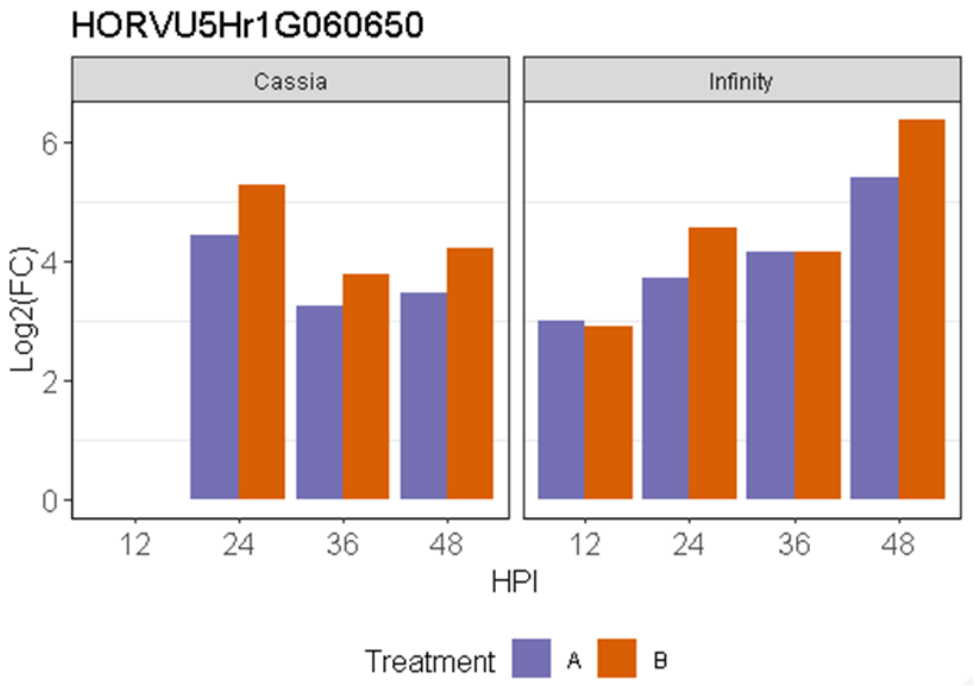


Figure 3.13: Log_2(FC) relative to mock treatment of DEG HORVU5Hr1G060650 in Cassia and Infinity after treatment with A or B isolate.

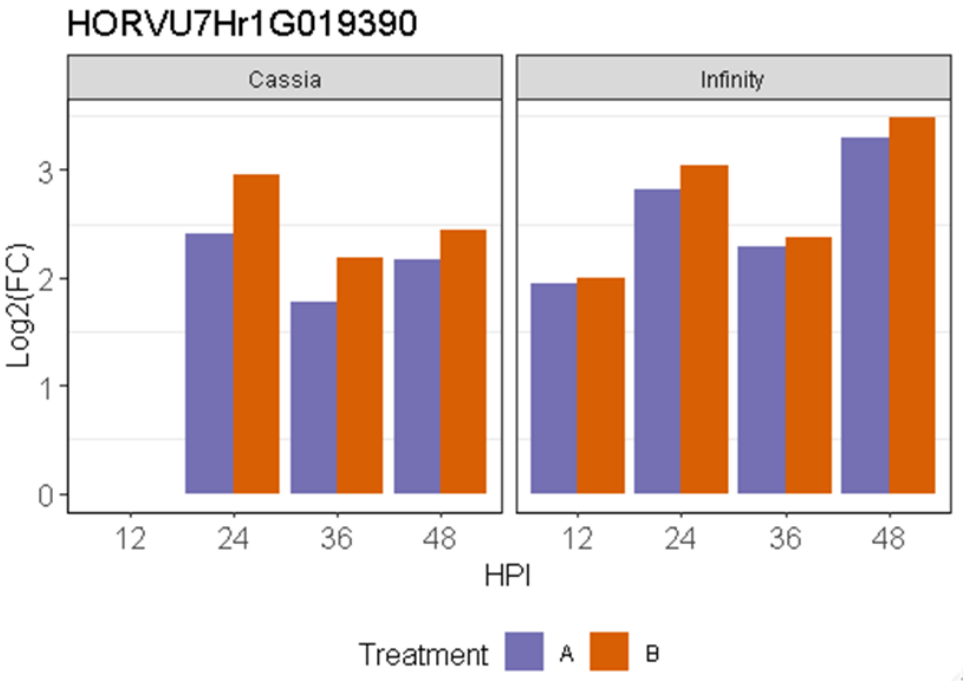


Figure 3.14: Log_2(Fold Change) relative to mock treatment of DEG HORVU7Hr1G019390 in Cassia and Infinity after treatment with A or B isolate.

Six of the 245 S gene orthologues show upregulation at one timepoint, and downregulation at another. For example, *ALCOHOL DEHYDROGENASE (ADH) (*HORVU5Hr1G057090) was upregulated in Cassia at 24 hpi in response to both A and B. It was also upregulated in Infinity in response to B at 48 hpi but downregulated in Cassia in response to B at 48 hpi (Figure 3.15). There were also 75 genes that are only down regulated in response to *R. commune* in Cassia or Infinity. For example, HORVU4Hr1G081990 is downregulated at 24 and 48 hpi in both Cassia and Infinity in response to B only (Figure 3.16).

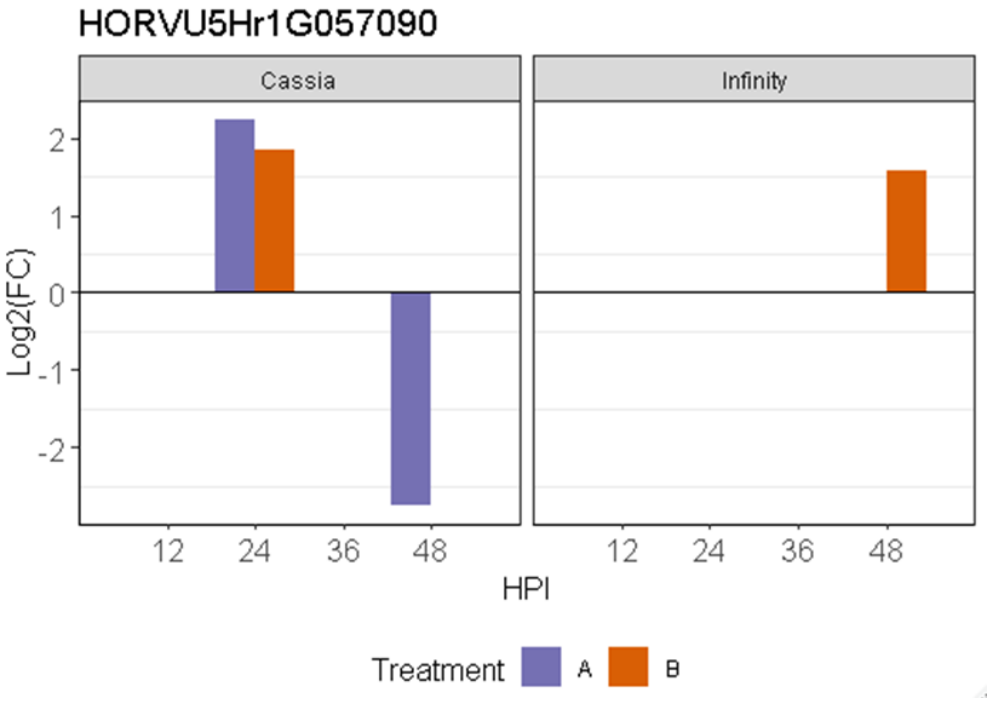


Figure 3.15: Log_2(Fold Change) relative to mock treatment of DEG *ADH* (HORVU5Hr1G057090) in Cassia and Infinity after treatment with A or B isolate

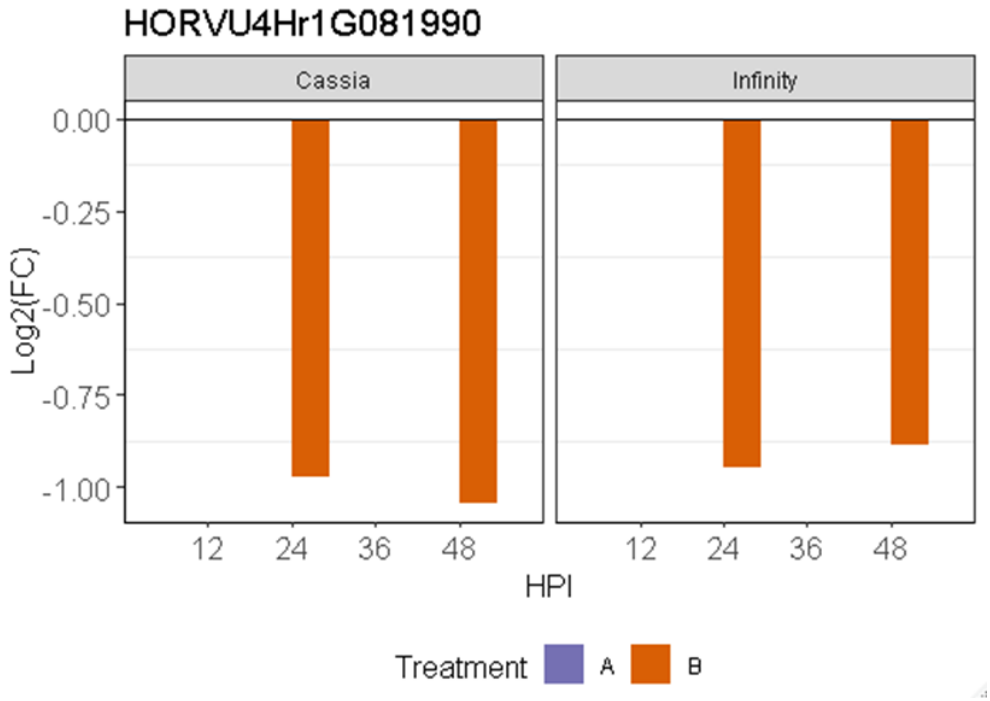


Figure 3.16: Log_2(Fold Change) relative to mock treatment of DEG HORVU4Hr1081990 in Cassia and Infinity after treatment with A or B isolate.

In order to narrow down the number of candidate S gene orthologues from the 245 genes shown in Figure 3.12, three different selection approaches were applied:

- Approach 1 - selection of S gene orthologues for which differential expression is observed in both Cassia and Infinity, in response to both *R. commune* isolates during at least one timepoint.
- Approach 2 - selection of S gene orthologues in which differential expression is observed in Cassia only in response to both *R. commune* isolates during at least one timepoint.
- Approach 3 - selection of S gene orthologues that show significantly higher endogenous expression in Cassia but not in Infinity in mock treated samples.

In order to identify DEGs that met criteria for approaches 1 and 2, an overlap analysis was carried out to examine which differentially expressed S gene orthologues were common to each cultivar x treatment combination, or unique to a specific dataset at 12, 24, 36 and 48 hpi (Figure 3.17). Two types of genes were of particular interest: (i) genes indicated in a blue circle in Figure 3.13 were differentially expressed in both Cassia and Infinity. Hence, they may correspond to candidate S genes involved in the susceptibility to *R. commune* more broadly (i.e., Approach 1) and (ii) genes indicated in a red circle in Figure 3.13 were potential S gene orthologues that were differentially expressed in response to both *R. commune* isolates in Cassia, but not differentially expressed in Infinity at that one particular timepoint (i.e. Approach 2). (Note that the same genes may be differentially expressed in Infinity at different timepoints).

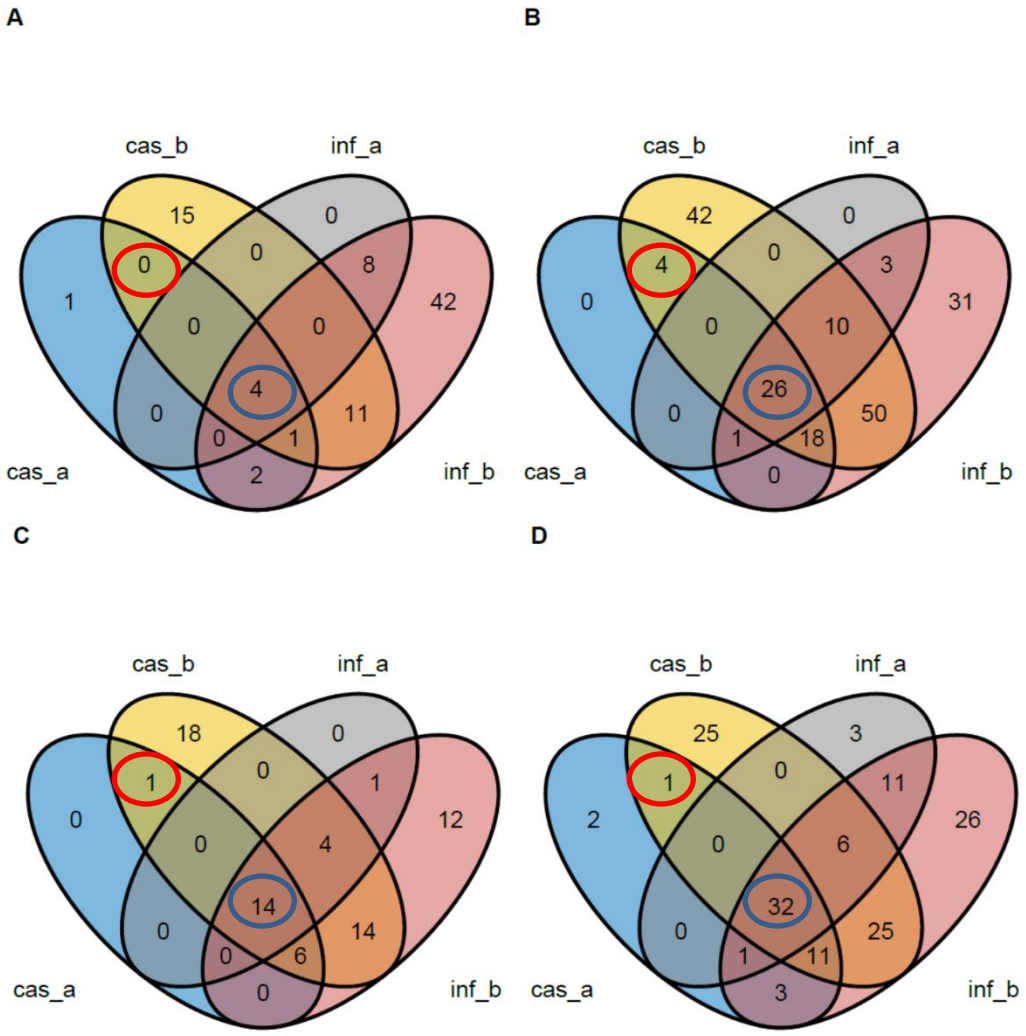


Figure 3.17: Overlap analysis of differentially expressed S gene orthologues A: 12 hpi, B: 24 hpi, C: 36 hpi, D: 48 hpi. Overlaps circled in blue represent the potential S genes that are differentially expressed in both cultivars in response to the two *R. commune* isolates. Overlaps circled in red represent the S gene orthologues that are specific to the Cassia response at a particular timepoint.

Results from Approach 1

The subset of potential S gene orthologues that were differentially expressed in both Cassia and Infinity in response to both isolates represent S gene orthologues involved in a conserved response. While Infinity is classified as resistant to *R. commune* (DAFM, 2022), it is not a fully resistant cultivar. As such, scald like symptoms still occur in this cv. A level of S gene activity is possible in this cultivar, therefore this set of genes were further examined so

as no to eliminate all potential S gene orthologues that may be differentially expressed in Infinity.

A total of 44 genes met this selection criterion (Table 3.3). A total of 4 genes were identified at 12 hpi, 26 at 24 hpi, 14 at 36 hpi and 32 at 48 hpi (Figure 3.17). 19 of these genes were identified as core response S gene orthologues at more than one timepoint. For example, HORVU0Hr1G030830 encodes a WRKY domain containing protein. It was differentially expressed in both Cassia and Infinity at 24 and 48 hpi in response to both *R. commune* isolates (Figure 3.18). In Cassia, it is also upregulated at 36 hpi in response to both isolates. In addition, in Infinity, it is upregulated in response to B at 12 and 36 hpi (Figure 3.18). This gene was identified as a proposed ortholog to the known rice S gene *OsWRKY45-1.*

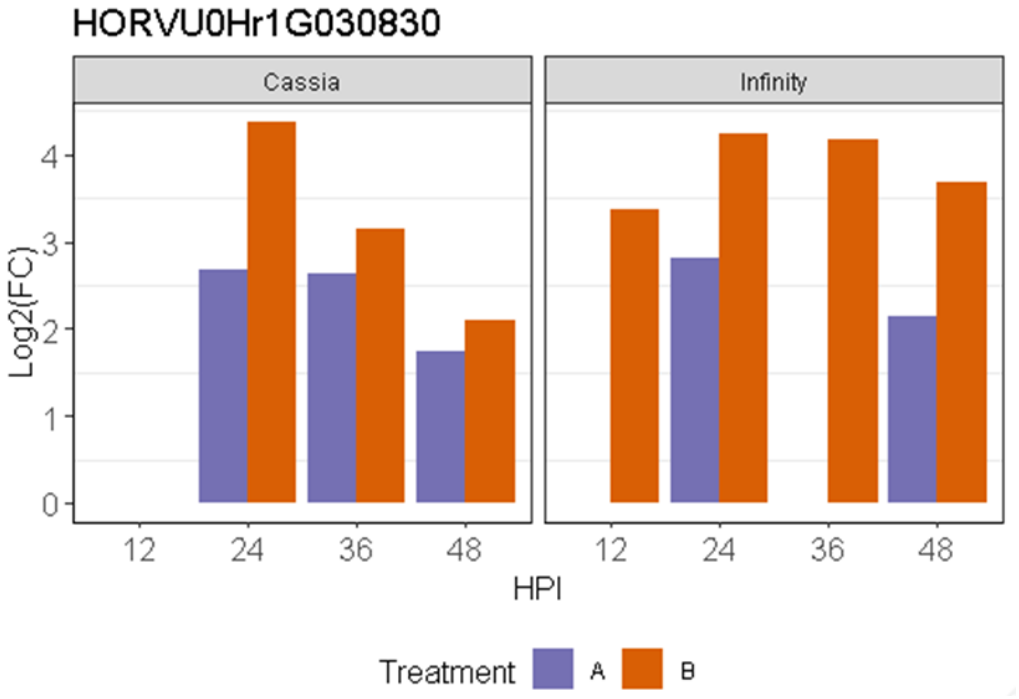


Figure 3.18: Log_2(Fold Change) relative to mock treatment of DEG HORVU0Hr030830 in Cassia and Infinity after treatment with A or B isolate.

Results of Approach 2:

Presuming that the differential expression of potential S gene orthologues in Cassia (but not in Infinity) might potentially contribute to the susceptible phenotype of Cassia compared to Infinity, this subset of S gene orthologues was of particular interest. A total of six genes met this selection criteria at the given timepoints. There were no potential S gene orthologues in this subset at 12 hpi, but 4 genes at 24 hpi, 1 at 36 hpi and 1 at 48 hpi (Figure 3.17) were identified. All six genes identified in this approach were only unique to the Cassia response at one timepoint. It is likely that candidate S genes specifically required for susceptibility to *R. commune* can be among these genes if their activity is required at a specific timepoint during infection. For example, *ADH* (HORVU5Hr1G057090) was selected through this approach as it is differentially expressed in Cassia in response to both *R. commune* isolates at 24 hpi, and is not differentially expressed in Infinity until later, at 48 hpi (Figure 3.15). It is possible that the earlier activation of *ADH* at 24 hpi in Cassia is required for the susceptible phenotype. This gene is involved in ethanol production and has been found to be upregulated in response to multiple stresses, including waterlogging and pathogen infection (Pathuri et. al., 2011). In barley, it has already been described as an S gene whose activity aids *Bgh* infection. Indeed, upregulation of *ADH* was recorded within 2 h after *Bgh* inoculation (Kasbauer et. al., 2018). It is possible that activation of this gene during early infection stages in Cassia may contribute to susceptibility to other fungal pathogens including *R. commune.*

Another gene identified through this approach, HORVU2Hr1G028470, was only differentially expressed in Cassia in response to both isolates at 12 hpi. It was not differentially expressed in Infinity at any timepoint (Figure 3.19). Although it codes for a myb-like DNA-binding protein, the function of this gene is unknown. The *in-silico* analysis in section 3.4.1 identified HORVU2Hr1G028470as a potential orthologue to *AtMYB46*. This *Arabidopsis* gene has been described as an S gene that contributes to susceptibility to *B. cinerea* (Ramirez et. al., 2011).

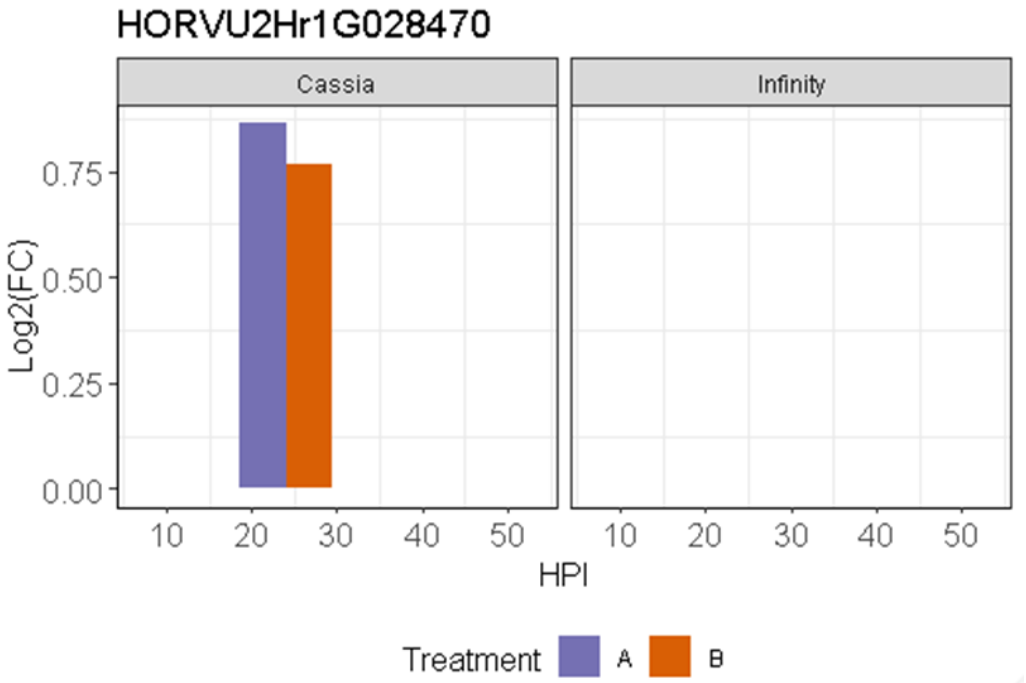


Figure 3.19: Log_2(Fold Change) relative to mock treatment of DEG HORVU2Hr028470 in Cassia and Infinity after treatment with A or B isolate.

Only one gene, HORVU5Hr1G122390, was identified through both Approach 1 and Approach 2. It was identified as unique to the Cassia response (Approach 2) at 12 dpi as it was found to be differentially expressed in Cassia in response to both isolates not differentially expressed in Infinity at this timepoint (Figure 3.20). In addition to this, it was also a potential S gene orthologue with a conserved response in both cvs (Approach 1), as it was found to be upregulated in both Cassia and Infinity in response to both isolates at 36 and 48 hpi. Interestingly, this gene was also differentially expressed in Infinity in response to both isolates at 24 hpi, and not differentially expressed in Cassia at this timepoint (Figure 3.20). It is possible that temporal regulation of this gene contributes to a susceptible or resistant phenotype. The function of this gene in barley is unknown, but here, it was identified as a potential orthologue to the known *Arabidopsis* S gene *AtIOS1.*

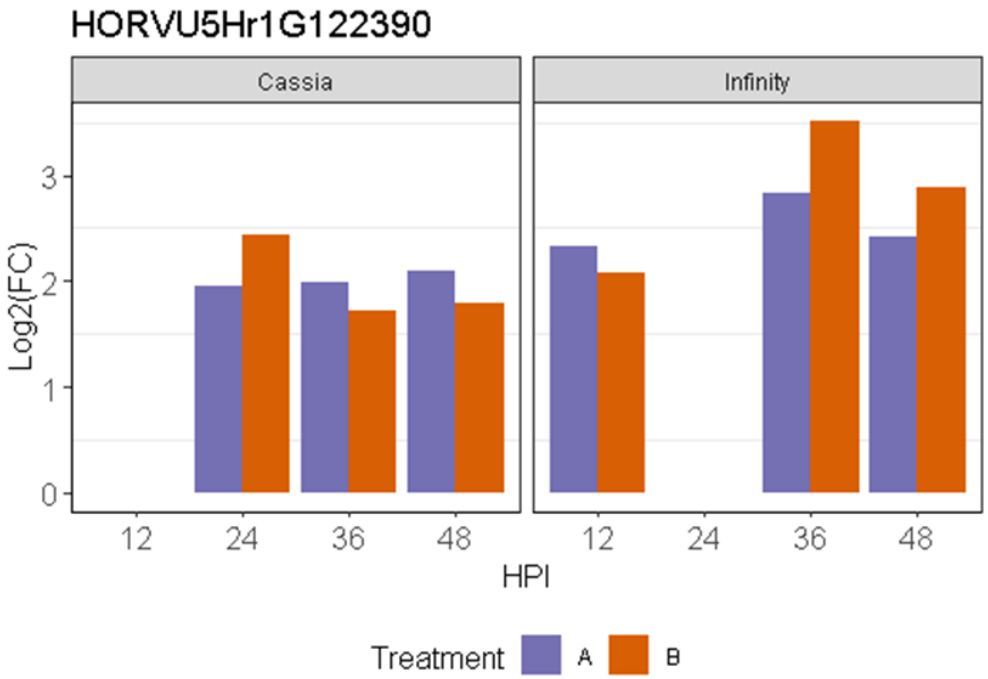


Figure 3.20: Log_2(Fold Change) relative to mock treatment of DEG HORVU5Hr1G122390 in Cassia and Infinity after treatment with A or B isolate

Results of Approach 3

Another approach taken to identify candidate S gene orthologues that could contribute to susceptibility to *R. commune* was to identify putative S gene orthologues that showed a significantly higher endogenous expression level in Cassia relative to Infinity. For example, type 1 S genes (i.e., genes whose activity benefits the pre-penetration needs of the pathogen) may have the ability to 'prime' susceptibility before infection. For this analysis, the FPKM values of all 682 potential S gene orthologues identified in section 3.4.1 in mock treated Cassia and Infinity at each time point examined in the RNAseq experiment were examined. This analysis detected 35 putative S gene orthologues that showed differential expression between Cassia relative to Infinity in the absence of pathogen infection at one timepoint only. A further 17 genes were differentially expressed at more than one timepoint. A two way ANOVA and pairwise comparison at each timepoint was carried out to determine any significant differences in corresponding FPKM values at individual time points. Six of these 17 genes were selected as they were defined as differentially expressed at more than 4 timepoints (Table 3.3).

Interestingly, one of these genes was significantly upregulated in Infinity, the remaining five were upregulated in Cassia (Figure 3.21).

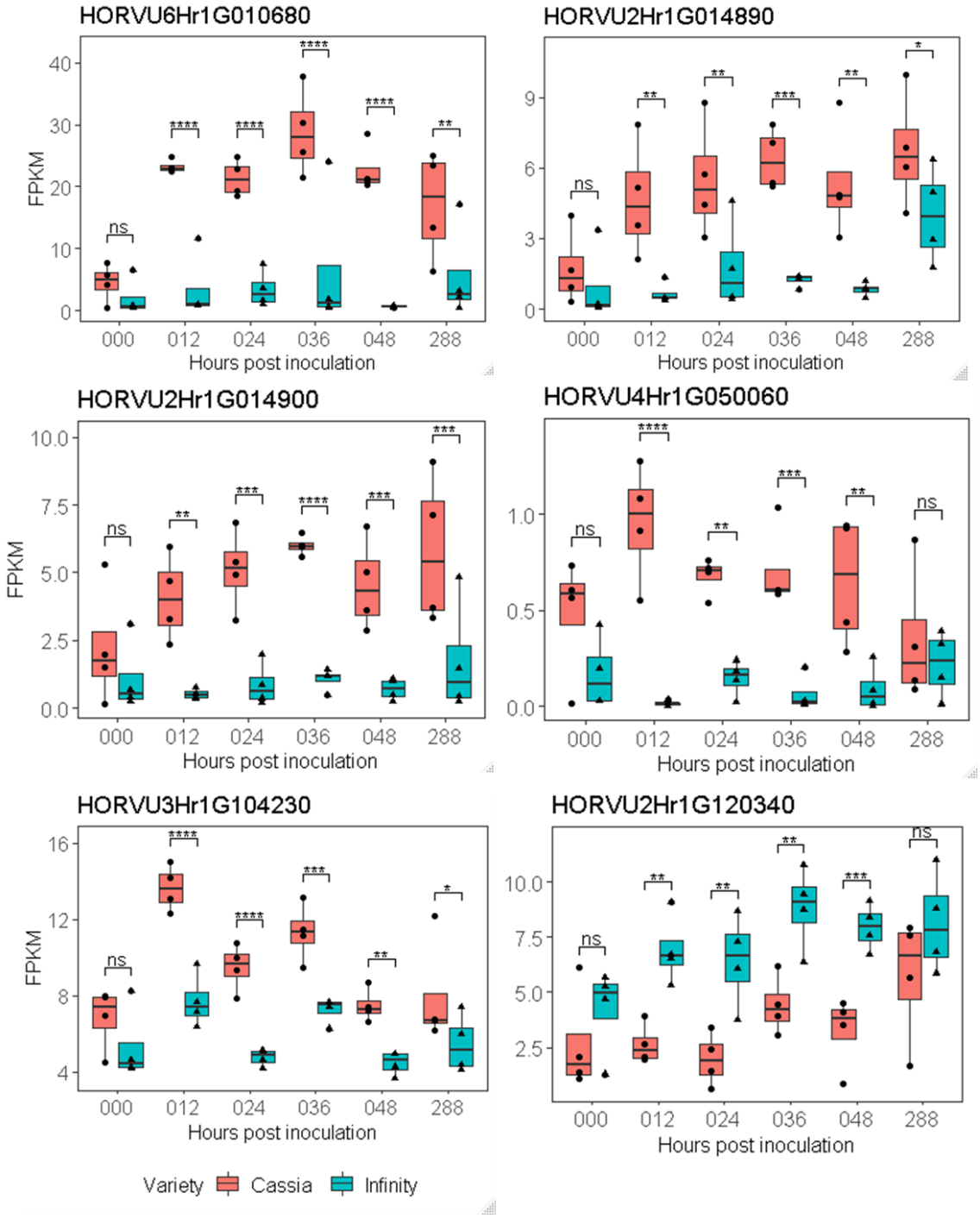


Figure 3.21: FPKM values of six candidate S gene orthologues with significant (p-adj < 0.05) differences between Cassia and Infinity mock treated samples, which also show differential gene expression at more than 4 timepoints (n = 4). * Indicates level of significance as determined by ANOVA using R (version 4.0.2). *: p-adj< 0.05, **: p-adj <0.01, ***: p-adj <0.005, ****: p-adj <0.001, ns: not significant.

A total of 53 ‘high confidence’ putative S gene orthologues were identified using the three approaches described in this chapter. The workflow for generating this list is outlined in Figure 3.22. The candidate genes are listed in Table 3.3, along with the approach used to identify them, as well as the proposed known S gene orthologue, and S gene type. Of these 53 candidates, only 1 had been previously identified as an S gene in barley: HORVU6Hr1G028790 (*WRKY1),* while a further two (HORVU7Hr1G113850 and HORVU5Hr1G057090) were identified as orthologues to previously identified barley S genes, *WRKY2* and *ADH* respectively.

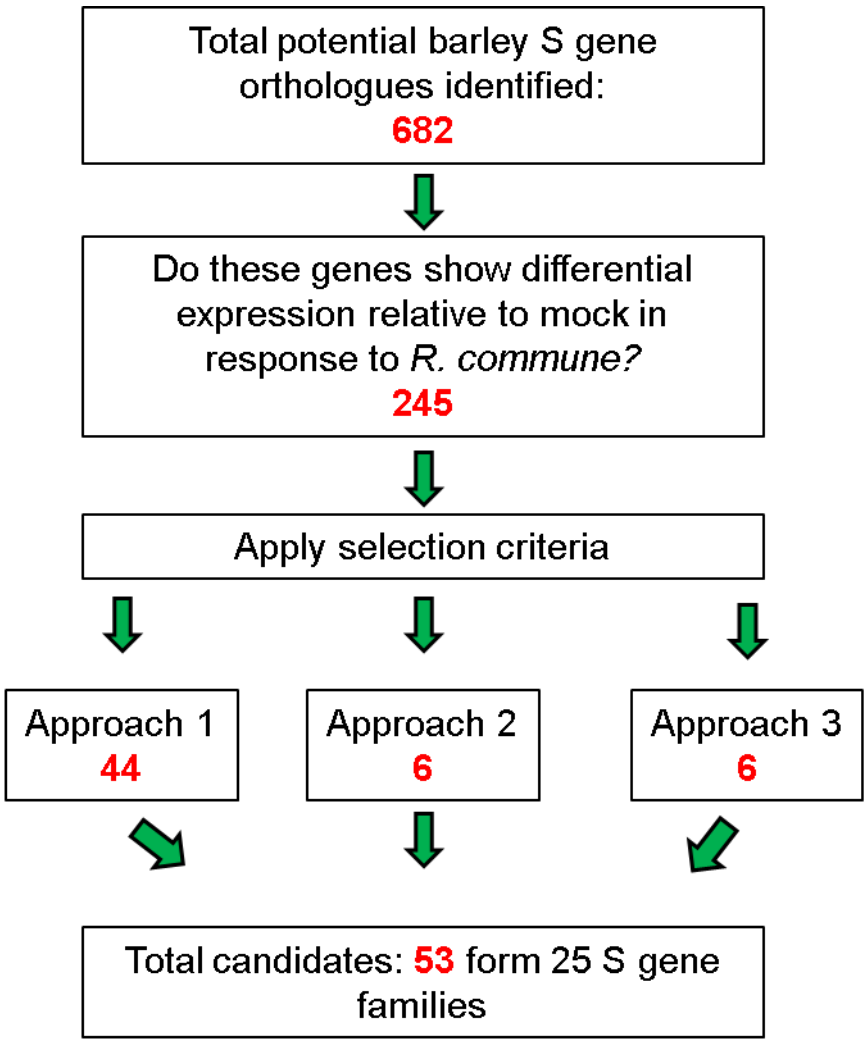


Figure 3.22: Overview of S gene candidate selection process. Number of candidate S genes after each selection stage shown in red. Approach 1: S gene orthologues for which differential expression is observed in both Cassia and Infinity. Approach 2: S gene orthologues in which differential expression is observed in Cassia only. Approach 3: S gene orthologues that show significantly different endogenous expression between uninfected cvs.

Table 3.3: 'High confidence' candidate S gene orthologues and their corresponding S gene family and known S gene orthologue.

Gene ID	Family ID	Selection Approach	Known S gene ortholog	S gene type
HORVU1Hr1G084170	Plant000003	1	MYB46_AT5G12870.1	2
HORVU2Hr1G028470	Plant000003	2	MYB46_AT5G12870.1	2
HORVU5Hr1G060650	Plant000003	1	MYB46_AT5G12870.1	2
HORVU2Hr1G120340	Plant000003	3	MYB46_AT5G12870.1	2
HORVU2Hr1G016720	Plant000020	1	BIK1_AT2G39660.1	2
HORVU2Hr1G095970	Plant000020	1	BIK1_AT2G39660.1	2
HORVU4Hr1G064260	Plant000020	1	BIK1_AT2G39660.1	2
HORVU6Hr1G010050	Plant000020	1	BIK1_AT2G39660.1	2
HORVU7Hr1G108150	Plant000020	1	BIK1_AT2G39660.1	2
HORVU2Hr1G006100	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU2Hr1G014890	Plant000040	1,3	LecRK_V.5_AT3G59700.1	1
HORVU2Hr1G014900	Plant000040	3	LecRK_V.5_AT3G59700.1	1
HORVU2Hr1G037200	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU2Hr1G037210	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU2Hr1G091360	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU5Hr1G020530	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU5Hr1G098640	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU6Hr1G025340	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU6Hr1G025350	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU7Hr1G000530	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU7Hr1G019390	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU7Hr1G019400	Plant000040	1	LecRK_V.5_AT3G59700.1	1
HORVU7Hr1G005270	Plant000059	1	CESA3_AT5G05170.1, CESA4_AT5G44030.1, CESA7_AT5G17420.1	2
HORVU7Hr1G084610	Plant000063	1	FDH_AT2G26250.1	1, 2
HORVU4Hr1G058220	Plant000069	2	FER_AT3G51550.1	1
HORVU5Hr1G084100	Plant000069	1	FER_AT3G51550.1	1
HORVU3Hr1G013380	Plant000076	1	IOS1_AT1G51800.1	1
HORVU5Hr1G122390	Plant000076	1,2	IOS1_AT1G51800.1	1
HORVU1Hr1G070250	Plant000088	1	Multiple WRKY genes	1, 2, 3
HORVU1Hr1G092130	Plant000088	1	Multiple WRKY genes	1, 2, 3
HORVU3Hr1G050590	Plant000088	1	Multiple WRKY genes	1, 2, 3
HORVU3Hr1G060500	Plant000088	1	Multiple WRKY genes	1, 2, 3
HORVU4Hr1G005920	Plant000115	1	LOX3_Zm00001d033623_P002	3
HORVU7Hr1G050680	Plant000115	2	LOX3_Zm00001d033623_P002	3
HORVU1Hr1G079150	Plant000161	1	AtPLDbeta1_AT2G42010.1 OsPLDbeta1_LOC_Os10g38060.1	2
HORVU5Hr1G084740	Plant000161	1	AtPLDbeta1_AT2G42010.1 OsPLDbeta1_LOC_Os10g38060.1	2
HORVU4Hr1G050060	Plant000385	3	PLP2_AT2G26560.1	2
HORVU7Hr1G113030	Plant000385	2	PLP2_AT2G26560.1	2

HORVU2Hr1G110110	Plant000592	1	Rhg4_SHMT_Glyma.08G108900.2	3
HORVU7Hr1G024350	Plant000648	1	LACS2_AT1G49430.1	1, 2
HORVU5Hr1G057090	Plant000975	2	ADH_HORVU4Hr1G016810.1	3
HORVU4Hr1G056470	Plant001126	1	FAD7_AT3G11170.1, FAD7_Solyc06g051400.3.1	2
HORVU3Hr1G099530	Plant001557	1	UGT76B1_AT3G11340.1, FAD8_AT5G05880.1	2
HORVU6Hr1G010680	Plant001599	3	BON1_AT5G61900.1	2
HORVU3Hr1G104230	Plant003135	3	AtPAM16L2_AT3G59280.1	2
HORVU0Hr1G005300	Plant003549	1	DMR6_AT5G24530.1	2
HORVU4Hr1G084810	Plant003549	1	DMR6_AT5G24530.1	2
HORVU4Hr1G018440	Plant003579	1	PTP1_AT1G71860.1	2
HORVU2Hr1G066100	Plant004299	1	bHLH27_AT4G29930.3	1
HORVU7Hr1G113850	Plant006394	1	HvWRKY2_HORVU7Hr1G113830.3 OsWRKY28_LOC_Os06g44010.1	2
HORVU5Hr1G065420	Plant014356	1	OsWRKY76_LOC_Os09g25060.1	2
HORVU0Hr1G030830	Plant014647	1	OsWRKY45.1_LOC_Os05g25770.1	2
HORVU2Hr1G109330	Plant014647	1	OsWRKY45.1_LOC_Os05g25770.1	2
HORVU6Hr1G028790	Plant018390	1	HvWRKY1_HORVU6Hr1G028790.1	2

3.5 Discussion

This chapter of the thesis aimed to identify potential barley S genes that are specifically targeted by *R. commune.* A number of S genes have been described in barley, the most well-known of which is *MLO,* whose activity promotes susceptibility to *Bgh.* Mutations in this gene confer broad spectrum resistance to multiple *Bgh* isolates (Jørgensen 1992). Since then, additional barley S genes whose mutations also confer resistance to *Bgh* have been described. For example, *WRKY1* and *WRKY2* expression increases within 3 h of *Bgh* infection, and virus induced gene silencing (VIGS) of *HvWRKY1* and *2* resulted in reduced fungal microcolonies by 48 hpi (Shen et. al., 2007). As such, *WRKY1* and *2* can also be classified as S genes. While a number of R genes such as *RRS1* and *RRS2* have been identified in barley to improve resistance to *R. commune*, there is a lack of information available on S genes in the barley x *R. commune* pathosystem. This chapter describes the combination of two complementary approaches, an *in silico* analysis and a transcriptomic approach, to identify potential barley S genes in the context of *R. commune* infection.

Identification of putative S gene orthologues in barley

An *in silico* approach was completed using the OrthoMCL pipeline to identify potential orthologues to known S genes and proteins from a comprehensive list reviewed by van Schie and Takken (2014). With this type of analyses, parameters must be adjusted as required. The e-value cut off for the BLASTp analysis was set at a low (stringent) e-value, to increase the similarity between pairs of proteins identified as 'orthologous'. If more plant species were included in the database, it might be necessary to increase this e-value cut-off to account for increased variability between species, due to potential sequence and evolutionary divergence. The inflation value set for the clustering analysis was selected based on recommendations in the literature (Li et. al., 2003). Increasing this inflation value would result in the construction of smaller, more homogenous protein families, but would increase the risk of potential orthologues being 'missed'. Lowering this value would increase protein family size, but also increase the risk of non-orthologous proteins being included in a given family. Similar types of *in silico* analyses aiming to identify orthologous

groups of proteins across different species have been described in the literature. For example, a clustering analysis was used to identify genes encoding evolutionary conserved proteins involved in response to oxygen deprivation in plants, animals, fungi and bacteria (Mustroph et. al., 2010). A total of 28,681 DEGs in response to oxygen deprivation were identified across 21 species from publicly available transcriptomic data. The OrthoMCL pipeline was used to compare the proteomes of 21 species. Over 40,000 clusters of related proteins were identified, 2,409 of which were found to contain two or more DEGs found to be differentially expressed during oxygen deprivation. Most responses were found to be conserved at a kingdom level in this analysis. The parameters used for the OrthoMCL analysis differed from parameters used in this study, in which they selected an e value cutoff of 10^{-5}, likely to compensate for the more diverse species used in this study (Mustroph et. al., 2010). This study highlights the ability of the OrthoMCL pipeline to construct orthologous gene groups across phylogenetic distance.

Clustering analysis has also been used in the identification of genetic markers of disease susceptibility. Specifically, over 200,000 protein sequences from 11 plant species were clustered into 27,222 families using the OrthoMCL pipeline with an e value cut off of 10^{-5}, and inflation value of 1.5 to identify putative *Fraxinus excelsior* (European ash) orthologues of gene expression markers for susceptibility to ash dieback (Sollars et. al., 2017). While orthology searches have been described for the identification of specific S genes, comparison of conserved protein families across different species has not been carried out for the identification of S genes in barley at the same scale as described in this chapter.

To confirm that the parameters selected are appropriate for this project, spot checks of known orthologous proteins can be carried out to determine the successfulness of the ortholog family clustering. For example, Mangleson et. al., (2008) identified barley *WRKY32* (NCBI accession: AK360029) as an orthologue to one of the known S genes of interest *OsWRKY45.* This was done through a phylogenetic analysis of 45 barley WRKY proteins and their putative orthologues in rice and *Arabidopsis* (Mangleson et. al., 2008). As discussed in section 3.4.1, the *in silico* analysis in this chapter identified

HORVU2Hr1G109330 as a potential orthologue to *OsWRKY45.* Alignment of *WRKY32* and HORVU2Hr1G109330 coding sequences showed that these were in fact the same gene, and that this analysis pipeline had correctly identified the same barley orthologue to *OsWRKY45* that had been described in the literature (Mangleson et. al., 2008).

A limitation to this analysis was that the list of known S genes included in the database were taken from an older review, published in 2014 (van Schie and Takken, 2014). Hence, more recently characterised S genes were not considered. For example, in rice, *RESISTANCE OF RICE TO DISEASES* 1 *(OsROD1)* has since been identified as an S gene (Gao et. al., 2021). This gene was found to be involved in Ca^{2+} sensing and regulation of H_2O_2, two key components of PTI. Specifically, *OsROD1* is required for degradation of H_2O_2. Loss of function resulted in increased JA and SA levels, downregulation of genes involved in Ca^{2+} signalling, and upregulation of genes involved in ROS homeostasis. Additionally, *rod1* mutants in rice showed increased resistance to *R. solani* and *M. oryzae* (Gao et. al., 2021).

A BLASTp analysis of OsROD1 into the barley proteome on Ensembl (cultivar: Morex) only found one similar protein: HORVU7Hr1G011230. The gene encoding for this barley protein was not found to be differentially expressed in either cultivar in the RNAseq dataset (section 3.4.2.2). The protein family OsROD1 was assigned to by the OrthoMCL clustering analysis also contained the HORVU7Hr1G011230 barley protein. However, this family was not one of the 142 S protein families identified as being differentially expressed in the presence of *R. commune,* indicating that HORVU7Hr1G011230 may not be required for defence against these specific *R. commune* isolates at the timepoints tested. Next steps would require a subsequent review of the literature to identify S gene candidates identified since 2014 to update this database.

In this analysis, 142 S protein families were identified in barley, containing at least 1 of 203 known S genes and proteins described in 2014. 682 putative barley orthologues to known S genes were identified within these families.

Transcriptomic analysis of susceptible and resistant barley to *R. commune*

A transcriptomic analysis of susceptible and resistant barley cultivars was completed to analyse global changes in gene expression after exposure to *R. commune* at 0, 12, 24, 36, 48 and 288 hpi. At 0 hpi, there were no DEGs (relative to mock treated plants) in Cassia after treatment with isolate A. This served as a control, indicating that there were no detectable changes in gene expression triggered by the inoculation process itself for Cassia x A. Indeed, as leaf tissue was collected immediately after inoculation for this timepoint, differential gene expression at the 0 hpi timepoint would not be expected. There was a very small number of DEGs detected in Cassia x B, Infinity x A and Infinity x B at the 0 hpi timepoint with 74, 3 and 25 DEGs identified respectively (Figure 3.2). Such a small number of DEGs corroborates the conclusion that the inoculation procedure itself did not trigger large genome-wide expression changes. In order to identify core response genes to *R. commune,* the expression of two barley cvs with different resistant phenotypes was examined. This work also aimed to identify changes in expression in response to different *R. commune* isolates, in order to identify potential isolate specific responses in a given cultivar, as well as genes or pathways involved in a conserved response. However, as this was limited to only two barley cvs and two *R. commune* isolates, it was not possible to predict true core response genes, or universal S genes. Further investigation into the transcriptomic response of additional barley cvs will validate the conservation of defence related process identified in this chapter.

During the first 48 hpi, the seedlings were placed into sealed plastic bags to aid fungal penetration into the leaf. It is during this time that the plant will detect the presence of the pathogen, so that the number of DEGs would be expected to increase during the first 48 hpi, correlating with the onset of the immune response. During that same period of time, the pathogen likely also releases effector proteins into the plant, further inducing changes in host gene expression. In chapter 2, it was determined that 12 dpi was the time at which scald like symptoms appeared in Cassia, suggesting that around that timepoint, the pathogen transitioned into the necrotic phase of its lifestyle. Because of this switch, it was expected that there would be large changes in

gene expression around 12 dpi (288 hpi) in Cassia, including genes involved in cell death. Surprisingly though, at this timepoint, few DEGs were identified compared to the timepoints between 12 and 48 hpi in Cassia (7 in response to A, 52 in response to B). In contrast to Cassia, at the 12 dpi timepoint, there were no visible symptoms in Infinity. As this is a more resistant cultivar, one possibility is that the latent phase is longer, and the onset of symptoms is delayed. Nevertheless, there were also very few DEGs in Infinity at 12 dpi (19 in response to A, 25 in response to B). One possibility is that the host response to *R. commune* has dampened at the later timepoint, and the plants are no longer responding to the presence of the pathogen.

Four datasets were described, infA, casA, infB and casB detailing the total DEGs detected in each cv x isolate combination. A GO enrichment analysis was carried out on these datasets, as well as on the overlapping core subset of genes. This served as a level of validation, as many defence related biological processes were over-represented in each dataset (Figures 3.6 and 3.7). However, there were also unexpected terms such as those involved in pollination. As indicated in section 3.4.2.3, a number of the genes in these categories represent genes involved in phosphorylation or receptor kinase signalling. It is also possible that the barley GO categories are not as well annotated as other model species such as *Arabidopsis* or rice. Despite the limitations of the GO enrichment analysis, the data shown in Figures 3.6 and 3.7 indicate that a general immune response is conserved across both the resistant and susceptible cultivars. However, as more specific GO terms are enriched for in the resistant specific subset of genes, there are a number of genes that are only differentially expressed in Infinity, and not in Cassia. These genes may contribute to the observed resistance phenotype of this cultivar.

Of the 682 potential S gene orthologues identified in section 3.4.1, 437 showed no differential gene expression in response to *R. commune* in these datasets. While these may be possible orthologues to genes with a potential susceptibility role in other pathosystems, they do not seem to be relevant in the context of *R. commune* isolates A and B in Cassia or Infinity. It remains possible that some of these genes might act as S genes in the presence of another isolate or in a different barley cv. Of the remaining 245 potential S

gene orthologues that were found to be differentially expressed in at least one cv in response to *R. commune,* 53 S will be further investigated in Chapter 4. These genes were identified through three different approaches to select genes with a higher probability of playing a role in the response to *R. commune.*

Approach 1 involved the selection of candidate S gene orthologues for which differential expression was observed in both Cassia and Infinity, in response to both *R. commune* isolates during at least one timepoint. Through this approach, 44 S gene candidates were selected. 12 of these candidates were clustered into the same protein family as *AtLecRK_V.5.* This was surprising, as this gene has a known role in COR induced stomatal reopening after *Pst* infection in *Arabisopsis* (Desclos-Theveniau et. al., 2012). *R. commune* does not enter through the stomata, and instead enters via the cuticle following appressoria formation. *AtLecRK_V.5* does not appear to be involved in PTI related gene expression. The 12 proposed barley *LecRK_V.5* orthologues have not been well annotated, and their role in the barley immune response remains unknown. As they were found to be differentially expressed in both Cassia and Infinity in response to both *R. commune* isolates, this suggests its role is not limited to a susceptible interaction. However, testing of additional cvs would be required to determine if they form part of a conserved immune response.

Approach 2 involved the selection of selection of S gene orthologues in which differential expression was observed in Cassia only in response to both *R. commune* isolates during at least one timepoint. Six S gene candidates were identified through this approach. One of these candidates was identified as a proposed orthologue to the *Arabidopsis FERONIA* gene (*AtFER). AtFER* is a receptor like kinase that has been identified as an S gene, as mutations in this gene confer improved resistance to the powdery mildew pathogen *Erysiphe orontii*. This may be associated with a hypersensitive response to ABA signalling also observed in *fer* mutants (Yu et. al., 2012).

Approach 3 involved the selection of S gene candidates that showed significantly higher endogenous expression in one cv, in the absence of a

pathogen. This approach was included as some S genes (e.g. type 1 S genes whose activity support the pre-penetration needs of the pathogen) may have the ability to 'prime' susceptibility. However, of the six barley gene candidates identified through this approach, none have a known role in either resistance or susceptibility priming.

Of these six genes, two are proposed orthologues to the *Arabidopsis* S gene *AtLecRK_V.5* (HORVU2Hr1G014890 and HORVU2Hr1G014900) which has been described as a negative regulator of coronatine (COR) induced stomatal reopening after Pst infection (Desclos-Theveniau et. al., 2012). HORVU4Hr1G050060 was identified as a proposed orthologue to *Arabidopsis* S gene *PATATIN-LIKE PROTEIN 2 (AtPLP2)* whose activity results in increased susceptibility to cucumber mosaic virus (La Camera et. al., 2009). HORVU6Hr1G010680 was identified as a candidate orthologue to *Arabidopsis BONZAI 1 (AtBON1)*, which is a known S gene in the *Arabidopsis* x *Pst* pathosystem. This gene encodes for a calcium-dependent, phospholipid binding protein and is likely involved in hormone defence signalling pathways and programmed cell death (Lee and McNeillis, 2008). An orthologue of *AtBON1* has also been identified in rice (*OsBON1*) and has been shown to confer susceptibility to *M. oryzae* and *R. solani* (Yin et. al., 2018). HORVU3Hr1G104230 is a candidate orthologue to *Arabidopsis AtPAM16L2*, which is a negative regulator of ROS production. Mutations in *AtPAM16L2* show increased ROS accumulation, as well as improved resistance to the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Huang et. al., 2013). The final gene identified through this approach, HORVU2Hr1G120340, is a potential orthologue to *AtMYB46*. This *Arabidopsis* gene is known to be involved in cell wall biosynthesis and lignin deposition. It has been described as an S gene as mutants show increased susceptibility to B. cinerea (Ramirez et. al., 2011). The barley genes identified as orthologous to these genes do not have a known role in the barley immune response.

By limiting the analysis to these three approaches, it is possible that potential candidates were missed. For example, the 53 candidates identified were limited to only examining the differential expression of proposed orthologues to known S genes identified in section 3.4.1. Further analysis into the full DEG

lists identified in overlaps in Figure 3.5 may lead to the identification of new S genes not previously described in other species. Additionally, a more in depth analysis in the timing and directionality of DEGs may also yield further insights into the early response of Cassia and Infinity to *R. commune.* The 53 candidates selected will be further discussed in Chapter 4.

4 Towards the characterisation of S genes in barley

4.1 Introduction

Genetic transformation of plant species is a key tool employed for functional analysis of candidate genes. Stable transformation of plants can be achieved through the use of both established (e.g., transgenesis) and more recent (e.g., cisgenics, site directed mutagenesis) breeding techniques. Site directed mutagenesis requires the use of restriction nucleases that locate and cleave specific gene sequences, down to single nucleotides. In stable transformation, the DNA insert is fully integrated into the host genome, and is heritable (Jones and Sparks, 2009). Disadvantages include difficulty in transformation in different plant species, the transgene coding for the different components also needs to be outcrossed, and mutations need to be screened for in a population of transformants. In contrast, transient based methods are advantageous over stable transformation in that they are rapid, have been reported to be relatively easy and less laborious than stable transformation once a protocol has been established (Lück et al., 2019). Notably, transient transformation allows for the temporary introduction or silencing of candidate genes and can also be used to study gene function in specific plant tissue or specific developmental stages.

Plant stable transformation or transient transfection often rely on *Agrobacterium* mediated transfer of DNA in plant cells based on a binary vector system consisting of a disarmed tumour inducing (Ti) plasmid containing virulence (*vir*) genes required for transfer DNA (T-DNA) mobilization and insertion, and a binary vector containing the T-DNA borders, in-between which scientists can clone DNA sequences of interest, including a selection cassette for transformants (Wang et. al., 1998).

Commonly used restriction nuclease approaches in stable and transient plant transformation include Transcription Activator-Like Effector-based Nucleases (TALENs) and Zinc Finger Nucleases (ZFNs) (Gaj et al., 2013). More recently the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems has also been described in plants (Shan et. al., 2013). This system employs the use of CRISPR associated protein (Cas) endonuclease

to induce double strand breaks in targeted DNA sequences, which then stimulates cellular DNA repair via non-homologous end joining (NHEJ) or the less common homologous recombination (Bortesi and Fischer, 2015). NHEJ mediated frameshift mutations typically result in knockout of gene function. Transgenes can be inserted via transfection of DNA with homologous overhangs prior to NHEJ repair (Ran et. al., 2013). CRISPR/Cas9 mediated gene editing is a robust tool that has been used in studying gene function for improvement of many crop traits such as pathogen resistance and abiotic stress tolerance. For example, CRISPR/Cas9 mediated knockout of the rice ethylene response transcription factor *OsERF922* resulted in enhanced resistance to *M. oryzae* (as determined by lesion size) in homozygous mutant lines compared to WT, without any effect on agronomic traits including number of grains per panicle and thousand grain weight (Wang et. al., 2016). CRISPR/Cas9 mediated disruption of the tomato coronatine (COR) co-receptor *SlJAZ2* resulted in increased resistance to coronatine-producing *Pst* (determined by lack of disease symptoms and bacterial titres), without compromising resistance to necrotrophic pathogens such as *B. cinerea* (determined by lesion size) (Ortigosa et. al., 2018).

Post transcriptional gene silencing (PTGS) can be achieved in plants via RNA interference (RNAi) (Smith et. al., 2000; Wesley et. al., 2001). This involves the introduction of a double stranded RNA (dsRNA) which is cleaved into short interfering RNAs (siRNAs) which target a complementary mRNA for degradation (Agrawal et. al., 2003). RNAi mediated gene silencing is a quicker and less laborious protocol compared to CRISPR/Cas9 mediated gene silencing. Disadvantages include incomplete knock down of target genes (which may also be considered an advantage if the target gene is required for survival of the plant) and increased risk of off-target effects (Senthil-Kumar and Mysore, 2011). PTGS by RNAi is a widely used tool to reduce gene expression (Hannon, 2002). Gene knockdown via RNAi has been achieved in barley using micro-projectile bombardment (Chowdhury et. al., 2016) and *Agrobacterium tumefaciens* mediated transformation (Lu et al., 2016). *Agrobacterium* mediated transformation is advantageous over biolistic mediated transformation in terms of efficiency (Travella et. al., 2005).

The pHANNIBAL vector (Wesley et. al., 2001) was designed as a generic intron spliced hairpin RNA vector to be used for RNAi knockdowns *in planta*. The presence of a functional intron between the arms of the hairpin RNA structure within an RNAi construct has been reported to improve silencing efficiency to almost 100% (Smith et. al., 2000, Wesley et. al., 2001). Using PCR and restriction digest, 400-800 bp DNA fragments from the target gene can be cloned as sense and anti-sense sequences separated by an intron into pHANNIBAL. Successful knockdown of barley *GLUCAN SYNTHASE LIKE-6* (*GSL6)* was achieved using the pHANNIBAL vector, and resulting plants identified a role for this gene in *Bgh* susceptibility (Chowdhury et. al., 2016).

The choice of promoter that drives the expression of the RNAi construct is also important. The Cauliflower mosaic virus (CaMV) 35S promoter is most commonly used to direct the constitutive expression of transgenes in dicot plants (Odell et. al., 1985). However, the CaMV 35S promoter is less suitable for transient expression in barley, as it is less active in monocots (Christensen et. al., 1992). Therefore, expression of transgenes in monocots is more often driven by promoters such as the maize *Ubiquitin 1* (Ubi) promoter (Christensen et. al., 1992). Non constitutive promoters such as tissue/organ specific, developmental stage specific and chemically inducible promoters have also been described in several plant species (Zuo et. al., 2000; Li et. al., 2011).

Another useful tool to monitor the efficiency of stable transformation or of transient expression experiments are reporter genes not endogenous to the species being studied. The *E. coli uidA* gene encodes for the commonly used reporter enzyme β-glucuronidase (GUS) whose activity can be visualised histochemically by cleavage of the β-glucuronide substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). X-gluc cleavage by the GUS enzyme results in the oxidation and dimerization of the released 5-bromo-4-chloro-3-indolyl group, which forms a blue precipitate (Jefferson et. al., 1987).

4.2 Chapter Aims

In Chapter 3, 53 candidate barley S gene orthologues that may play a role in barley susceptibility to *R. commune* were identified. In this Chapter, the expression of a selection of these genes in mock and *R. commune* treated Cassia and Infinity was validated. Following this, associated the literature on the corresponding known S gene with the respective RNAseq differential expression patterns after *R. commune* treatment relative to mock of the specific candidate gene was also investigated. From this, genes that were more likely to be involved in susceptibility to *R. commune* were prioritised and at least one gene identified through each approach defined in section 3.4.3 was included. This resulted in the selection of 17 candidate S gene orthologues whose differential expression after *R. commune* infection was validated using RT-qPCR. These 17 genes were then ranked based on (i) expression profile of candidate gene, (ii) expression profile of other barley genes in the corresponding S family, (iii) validation of expression after *R. commune* infection by RT-qPCR and (iv) known functions of orthologous S genes to determine the most suitable candidates to target for RNAi knockdown. The secondary aim of this chapter was to generate RNAi constructs for the top three ranked candidate genes using cloning methods to insert sense and antisense PCR fragments of the target genes into the pHANNIBAL vector. As the success of barley transformation is genotype dependent and Golden Promise is the most amenable variety to transformation (Tingay et. al., 1997), this aim was to design RNAi constructs suitable for knockdown of target S gene candidates in Golden Promise first and then in Cassia and Infinity.

Finally, with the aim to investigate gene function via transient *Agrobacterium* mediated RNAi knockdown of an S gene candidate, the efficiency of a transient expression protocol in barley through agroinfiltration of leaves with reporter constructs coding for the GUS reporter under the control of the CaMV 35S promoter of the maize Ubi promoter was investigated.

4.3 Methods

4.3.1 Validation of candidate barley S genes

The relative expression levels of S gene candidates identified in Section 3.4.3 was determined by RT-qPCR and compared to their corresponding FPKM values after *R. commune* infection. 1 µg total RNA (from an aliquot of the same RNA that was extracted for sequencing) was reverse transcribed using oligo-dT primers to produce cDNA using the Quantitect® Reverse Transcription Kit (Qiagen). RT-qPCR was carried out as described in section 2.2.3.2, with each sample represented by the gene of interest (Table 4.1) and *ACTIN* as a reference gene. Validation of HORVU1Hr1G070250 was previously carried out (Figure 3.10). Relative expression values were plotted and significant differences between mock and *R. commune* treated samples in each barley cultivar were determined using a two-way ANOVA ($p < 0.05$) in R (version 4.0.2).

Table 4.1: Barley target genes and primer sequences for validation of candidate S gene expression after *R. commune* infection

Primer name	Gene ID	Sequence (5' – 3')
Plant000115_1_F1	HORVU4Hr1G005920	CGACGAAGAGTACGAGCAGCT
Plant000115_1_R1	HORVU4Hr1G005920	ATCCGCTTCTCGATCTCCACC
Plant001557_1_F1	HORVU3Hr1G099530	AGGTACGTGGAGGAGGTGTG
Plant001557_1_R1	HORVU3Hr1G099530	CTACCAACTTGTCTACAGCCAGCT
Plant000161_1_F1	HORVU5Hr1G084740	GAGGAGCAGTTCTTCGTGCC
Plant000161_1_R1	HORVU5Hr1G084740	CTTGTAGGTCTTGCCGCTGG
Plant001599_1_F1	HORVU6Hr1G010680	GAGGACTCTGGATGCGGACTTG
Plant001599_1_R1	HORVU6Hr1G010680	GCGACGGAAGTTAGTCAGTCAGAC
Plant000076_1_F1	HORVU5Hr1G122390	ATGCAACTACAAGAGTGCCTCGATC
Plant000076_1_R1	HORVU5Hr1G122390	GTCTTCATCGTGCGGCTGCT
Plant000385_1_F1	HORVU7Hr1G113030	CCACATTCGACATCAAGCTCCTCC
Plant000385_1_R1	HORVU7Hr1G113030	CCTCGGTCGATTAGGTTGAAGGC
Plant014647_2_F1	HORVU2Hr1G109330	CCTCCATCTTCCTGAGCTG
Plant014647_2_R1	HORVU2Hr1G109330	CACAAGAGGTAGAGCGGAGCGC
Plant000040_1_F1	HORVU2Hr1G037210	AGCTAGGACACACCGGCAAG
Plant000040_1_R1	HORVU2Hr1G037210	GCTCGTTGATGAAGTGTATGGCATC
Plant000020_1_F1	HORVU7Hr1G108150	GCTTCCTGTTCTGTCGTGGTC
Plant000020_1_R1	HORVU7Hr1G108150	CTTGTCCACGGCTGCTCTTC
Plant000003_3_F1	HORVU5Hr1G060650	CTGTTCGATCACCAGGAGGC
Plant000003_3_R1	HORVU5Hr1G060650	TTGCCGCACCTACACCTACG
Plant000003_2_F1	HORVU2Hr1G028470	CTCGTGCTCGTCTCCTACGT
Plant000003_2_R1	HORVU2Hr1G028470	ATGAGCTTCTCCTCCTGGTCG
Plant003579_1_F1	HORVU4Hr1G018440	GCTACAGTCCTCAACAGGCAGTC
Plant003579_1_R1	HORVU4Hr1G018440	GTGGAGTGCTGGATGTGGAGA
Hv2OGO_F2	HORVU4Hr1G084810	TCCATTGCCTCGTTCCTCTGC
Hv2OGO_R2	HORVU4Hr1G084810	ACCTGACGAGTGGCTTATGTGTC
HvW2_1_F1	HORVU7Hr1G113850	ACGAGCCGTGCAACAGCAA
HvW2_1_R1	HORVU7Hr1G113850	TGTCCTTGGTCACCTTCTGCC
HvW1_1_F1	HORVU6Hr1G028790	CATTGCTTGCTGCCTGCCTC
HvW1_1_R1	HORVU6Hr1G028790	CGTCGTGTTCGCGGTCTATGTAC
HvADH_1_F1	HORVU5Hr1G057090	GGTGCAAGAGGTGATCGTGGAG
HvADH_1_R1	HORVU5Hr1G057090	TCTTGAACACCGCCTCCTTGTG

4.3.2 Seed sterilisation and growth conditions

Barley seeds were sterilised for 3 h by adding 3 ml HCl (37% v/v) to 100 ml bleach in a dessicator. Sterile seeds were stratified on moist filter paper at 4°C for 7 days then transferred to 9 cm pots containing John Innes #2 compost and grown under continuous light at 18°C until GS13. Tobacco seeds were planted in a 5:3:2 ratio of compost, perlite and vermiculite and grown at 18°C in continuous light conditions.

4.3.3 Preparation of chemically competent cells

4.3.3.1 Preparation of competent *A. tumefaciens* AGL1 cells

AGL1 cells were streaked onto Luria Bertani broth (LB) agar (Appendix 1) plates and incubated at 28°C for 3 days. A 5 ml LB containing carbenicillin (100 mg/l) and rifampicin (50 mg/l) starter culture was prepared from a single colony and grown overnight at 28°C. Following this, 250 µl of the starter culture was used to inoculate LB supplemented with 0.2 g/L $MgSO_4$ and grown overnight at 28°C shaking at 200 rpm until an OD_{600} > 1 was reached (approx. 12 h). The culture was then chilled on ice and centrifuged at 4500 x g for 10 min at 4°C.The pellet was resuspended in 10 ml ice-cold 10 mM sterile $CaCl_2$ then centrifuged again at 4500 x g for 10 min at 4°C. The supernatant was discarded, and the cells re-suspended in 2 ml ice-cold $CaCl_2$. 100 µl aliquots were prepared and frozen in LN_2. Competent AGL1 cells were stored at -80°C until needed.

4.3.3.2 Preparation of competent *E. coli* stbl2 cells

The protocol used to generate chemically competent *E. coli* stbl2 cells is described in Inoue et. al., (1990). Cells were streaked from a glycerol stock onto LB agar and incubated at 37°C overnight. A 2 ml LB starter culture was prepared from a single cell and grown overnight at 37°C and shaking at 200 rpm. Following this, 200 µl of the starter culture was used to inoculate 250 ml super optimal broth (Appendix 1), which was incubated at 18°C shaking at 200 rpm until OD_{600} = 0.6 (approx. 24 h). The culture was chilled over ice for 10 min then centrifuged at 4,000 x g for 10 min at 4°C to pellet the cells. The supernatant was discarded, and the cells were re-suspended in 80 ml ice cold

transformation buffer (TB) (Appendix 1). The cells were then centrifuged again at 4,000 x g for 10 min at 4°C. The pellet was resuspended in 20 ml ice cold TB and incubated kept on ice. 1.4 ml dimethyl sulfoxide (DMSO) was added (final concentration 7% v/v). The cells were incubated on ice for a further 10 min. Aliquots were made and frozen in LN_2. Competent stbl2 cells were stored at -80°C until needed.

4.3.4 Transformation of chemically competent cells

4.3.4.1 Transformation of competent *A. tumefaciens* cells

Competent AGL1 or pGV2260 cells were thawed on ice. 3 µl of plasmid DNA (~300 ng/µl) was added to the cells which were then transferred to LN_2 for 5 min. The cells were then thawed at room temperature (RT). Following the freeze thaw step, 1 ml LB medium was added to the suspension, and the cells were incubated at 28°C for 3 h. The cells were centrifuged at 5,000 rpm for 5 min and 800 µl of the supernatant was removed. The cells were then resuspended in the remaining supernatant and transferred to an LB plate with appropriate selection and incubated at 28°C for 3 days.

4.3.4.2 Transformation of competent *E. coli* stbl2 cells

Competent stbl2 cells (100 µl per transformation) were thawed on ice. 1 µl of plasmid DNA (5 ng/µl) or 10 µl ligation reaction was added to the cells and incubated on ice for 15 min. The cells were then transferred to a water bath and incubated at 42°C for 30 s then returned to ice for 5 min. Following the heat-shock step, 1 ml LB was added to the suspension, and the cells were incubated at 37°C for 45 min. The cells were centrifuged at 5,000 rpm for 5 min and 800 µl of the supernatant was removed. The cells were then resuspended in the remaining supernatant and transferred to an LB plate with appropriate selection.

4.3.5 Colony PCR

Colony PCR was used to confirm the presence of plasmid inserts into transformed cells. After incubation on LB agar plates with appropriate antibiotic selection, single colonies were transferred into 10 µl LB. One PCR reaction consisted of 1.5 µl cell suspension, 1x ThermoPol PCR buffer (Appendix 1),

0.2 µM primers, 2 µM dNTPs and Taq polymerase (home-made) in a 15 µl reaction. Volumes and component ratios were calculated and made up in master mixes to minimise the impact of pipetting. PCR amplification was carried out with 5 cycles [30 s at 95°C, 30 s at 50°C, 40 s at 72°C] followed by 20 cycles [30 s at 95°C, 30 s at 55°C, 40 s at 72°C].

4.3.6 Cloning methods

4.3.6.1 Genomic DNA extraction

Genomic DNA was extracted from barley seedlings using a protocol adapted from Edwards et. al., 1991. A 2 cm section of leaf tissue was collected into a 1.5 ml microcentrifuge tube to which 400 µl Edwards extraction buffer (Appendix 1) was added. The leaf tissue was ground in the extraction buffer using a miniature pestle and the mixture centrifuged at 14,000 rpm for 1 min to pellet debris. 300 µl of the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube, and 300 µl isopropanol was added. The sample was centrifuged again at 14,000 rpm for 1 min to pellet the DNA. The supernatant was removed, and the DNA pellet washed in 500 µl 70% ethanol (v/v). The sample was centrifuged, and the ethanol removed. The pellet was air dried then resuspended in 50 µl sterile water. Genomic DNA was stored at -20°C until needed.

4.3.6.2 RNA extraction and cDNA synthesis

RNA extraction was carried out on barley leaf tissue using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich) according to manufacturer's protocol as described in section 2.3.8 and a final elution volume of 50 µl. RNA quality and quantity were determined on a Nanodrop spectrophotometer. cDNA synthesis was carried out as described in section 2.3.8 using Quantitect® Reverse Transcription Kit (Qiagen). 1 µg total RNA was then reverse transcribed using oligo-dT primers to produce cDNA for each sample using the Quantitect® Reverse Transcription Kit (Qiagen) according to manufacturer's protocol. cDNA was stored at -20˚C until needed.

4.3.6.3 Plasmid DNA extraction

5 ml bacterial cultures were grown in LB with appropriate antibiotic selection overnight at 37°C with shaking at 200 rpm. Cultures were then centrifuged at 3,000 rpm for 5 min. The supernatant was discarded, and plasmid DNA was purified using the E.Z.N.A Plasmid Mini Kit (Omega) following manufacturer's instructions. Plasmid DNA was eluted into a final volume of 30 µl.

4.3.6.4 Generation of pUBI:GUSPlus T-DNA plasmid

A bacterial stab of *E. coli* DH5α carrying a plasmid containing a maize Ubiquitin (Ubi) promoter driven synthetic GUSPlus reporter gene (Vickers et. al., 2003) was obtained from Addgene (#64402). The pUbi:GUSPlus plasmid (Table 4.2) was purified from DH5α as described in section 4.3.6.3. The plasmid was digested with PvuII in a 30 µl reaction for 3 h at 37°C. The T-DNA vector pML_BART (Table 4.2) was NotI digested for 3 h at 37°C, blunted by adding 0.5 µl dNTP (100 µM) and 0.5 µl T4 polymerase and incubating at room temperature for 10 min then dephosphorylated for 15 min at 37°C using 1 µl alkaline phosphatase (AP).

Digestion was confirmed by gel electrophoresis on a 1 % agarose gel for pUbi:GUSPlus digestion and a 1.5 % agarose gel for pML_BART digestion. The products were extracted using the E.Z.N.A Gel Extraction kit (Omega) according to manufacturer's protocol. The Ubi:GUSPlus insert was ligated into the pML_BART backbone at 4°C overnight using 1 µl T4 ligase in a 10 µl reaction.

Competent *E. coli* stbl2 cells were transformed as described in section 4.3.4.2 with the full ligation reaction and grown overnight on LB agar with spectinomycin (100 mg/l). Plasmid DNA was purified from bacterial colonies as described in section 4.3.6.3 and the final pML_BART_Ubi:GUSPlus (noted pTOC1, Table 4.2) plasmid confirmed by digestion with XhoI.

Table 4.2: Plasmids used for generation of RNAi cloning vectors for S gene candidate and plant transformation with GUS reporter genes.

Plasmid name	Details	Selection	Reference
pJET1.2/blunt	Positive selection cloning vector	Amp	Thermo Fisher (Cat #: K1231)
pUBI:GUSPlus	Synthetic GUS gene under Ubi promoter	Amp	Addgene #64402 Vickers et. al., (2003)
pHANNIBAL	Cloning vector for RNAi in barley	Amp	Accession: AJ311872 Wesley et. al., (2001)
pML_BART	Plant transformation vector	Spec	Eshed et. al., (2001)
pEG356	pML-BART Ub-M-luc Plant transformation vector carrying GUS reporter gene under 35S promoter	Spec	Graciet et. al., (2010)
pTOC1	pML-BART_Ubi:GUSPlus Plant transformation vector carrying GUSPlus reporter gene under Ubi promoter	Spec	This study

4.3.6.5 RNAi construct design

RNAi constructs were designed using A Plasmid Editor (ApE) (RRID:SCR_014266) software, to insert target sites from barley S gene candidates into the pHANNIBAL RNAi cloning vector. The target site was cloned in both the sense and anti-sense direction (Figure 4.1). Barley target sites were amplified from cDNA or gDNA (barley cvs Golden Promise, Cassia and Infinity) by PCR using primers with added restriction sites (Table 4.3) and Phusion proofreading DNA polymerase for sense and antisense cloning. The restriction sites for XbaI and XhoI were added to the 5' end of all target sites, and restriction sites for Acc65I and ClaI to the 3' end of all target sites. One PCR reaction consisted of 5 µl cDNA or gDNA, 1x High Fidelity (HF) Phusion

PCR buffer, 0.2 µM primers, 2 µM dNTPs and Phusion DNA polymerase in a 50 µl reaction. Primers for *ACTIN* (Table 2.2) were used as a positive control. Volumes and component ratios were calculated and made up in master mixes to minimise the impact of pipetting. PCR amplification was carried out with 5 cycles [30 s at 95°C, 30 s at 50°C, 40 s at 72°C] followed by 25 cycles [30 s at 95°C, 30 s at 55°C, 40 s at 72°C].

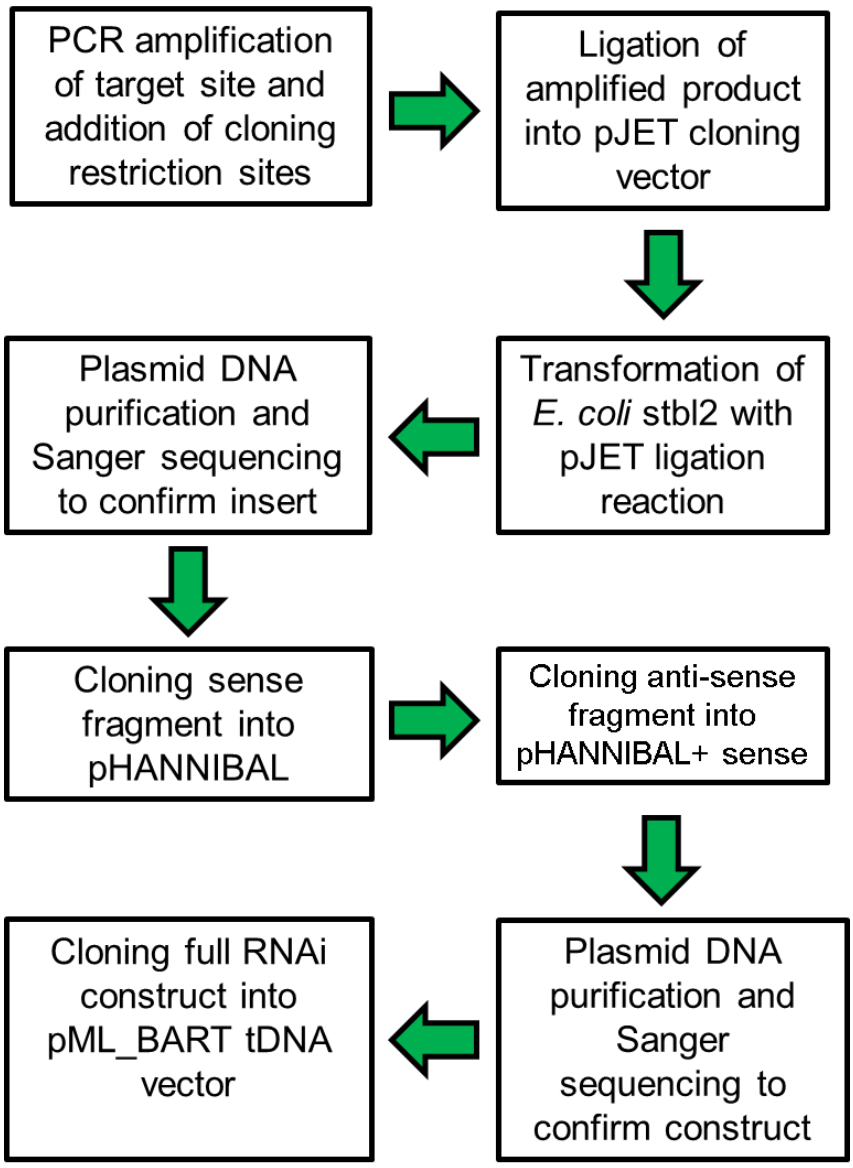


Figure 4.1: Workflow for the generation of RNAi constructs in pHANNIBAL for knockdown of candidate S genes.

Table 4.3: Primers used in this chapter for cloning and verification of plasmids. Bases in lowercase indicate added restriction sites.

Primer	Target	Sequence (5' → 3')
ToC1_for	HORVU6Hr1G010680	AAAAtctagactcgagGTCAGCGAAG TTCTGCAGT
ToC2_rev	HORVU6Hr1G010680	AAAAatcgatggtaccCTTGGACTTCC CTCATGGG
TOC3_for	HORVU5Hr1G122390	TTCCtctagactcgagGAGTTTTCCTA GTGACCTGCG
TOC4_rev	HORVU5Hr1G122390	AAAAatcgatggtaccCAAGCCATCC CAGTTGAGG
ToC5_for	HORVU2Hr1G109330	AAAAtctagactcgagACCGACGGCG CAAC
ToC6_rev	HORVU2Hr1G109330	AAAAatcgatggtaccTGGTCGACCA GGTTGGT
3'ocs_lo	OCS terminator	GGTAAGGATCTGAGCTACACAT GCTC
35S_up	35S promoter	GAAAAAGAAGACGTTCCAACCA C
TOC9_for	virD *A. tumefaciens*	TCAAGCGCGCCTGCTGGGAC
TOC10_rev	virD *A. tumefaciens*	CAGCTGGGGATGGCGCCTGG
ubi_for	Ubi promoter	TAACGGACACCAACCAGC
TOC11_for	GUSPlus	ATGGTAGATCTGAGGAACCGAC
TOC12_rev	GUSPlus	CTGGTAGAGATACGTGTTCAGT G
TOC13_for	HORVU2Hr1G109330	AAAAtctagactcgagACGCACAAGT ACGACCAG
TOC14_rev	HORVU2Hr1G109330	AAAAatcgatggtaccCAATGGTCGA GACCGTACG
TOC16_for	EF1alpha *N. benthamiana*	TGTGTATTGACTTGGAGGCTG
TOC17_rev	EF1alpha *N. benthamiana*	CCAGTCAAGGTTGGTTGATCTTT CGAT
qPCR13_up	GUS	GCCGATGTCACGCCGTATGTTA
qPCR14_lo	GUS	TTAACTATGCCGGAATCCATCGC AG

On successful amplification of the barley sense/antisense fragments, the PCR product was purified using the E.Z.N.A Cycle Pure kit (Omega) according to

manufacturer's protocol. The resulting purified PCR product was ligated into the pJET 1.2/blunt (Table 4.2) cloning vector using CloneJET PCR Cloning Kit (Thermo Fisher Scientific) in a 10 µl reaction for 30 min at room temperature. The full 10 µl ligation mixture was transformed into *E. coli* stbl2 as described in section 4.3.4.2 and grown on LB agar with ampicillin (100 mg/l). Colony PCR was carried out to confirm the target site insertion. The ligated pJET plasmid DNA was purified from positive colonies using E.Z.N.A Plasmid Mini Kit (Omega) as described in section 4.3.6.3. Plasmids were checked for the presence of the insert by PCR and Sanger sequencing (Eurofins).

Cloning sense fragment into pHANNIBAL

To clone the sense fragment into pHANNIBAL, 2 ng of the pJET plasmid containing sense/antisense sequence (noted pJET_insert) was first digested with the Acc65I restriction enzyme for 2 h at 37°C in a 30 µl reaction. The E.Z.N.A Cycle pure kit (Omega) was used to remove enzyme and buffer before a second digestion was carried out using XhoI for 2 h at 37°C in a 40 µl reaction. The same two step digestion was also carried out on the pHANNIBAL RNAi cloning vector (Table 4.2). Digestion was confirmed by gel electrophoresis on a 1 % agarose gel for pJET_insert and a 0.8 % agarose gel for pHANNIBAL. The products were extracted using the E.Z.N.A Gel Extraction kit (Omega) according to manufacturer's protocol. The RNAi insert was ligated into pHANNIBAL at 4°C overnight using 1 µl T4 ligase in a 10 µl reaction. The full 10 µl ligation reaction was transformed into *E. coli* stbl2 and the insertion of the sense fragment was confirmed by restriction digest with SacI after overnight culture of individual colonies and plasmid miniprep. This resulting plasmid is noted pHANNIBAL_sense.

Cloning antisense fragment into pHANNIBAL

To clone the antisense fragment into pHANNIBAL_sense, 2 ng of the same pJET_insert plasmid containing the target sequence was digested with the ClaI and XbaI restriction enzymes for 2 h at 37°C in a single 30 µl reaction. The same double digestion was also carried out on the pHANNIBAL_sense plasmid. Digestion was confirmed by gel electrophoresis on a 1 % agarose gel for pJET_insert and a 0.8 % agarose gel for pHANNIBAL_sense. The products

were extracted using the E.Z.N.A Gel Extraction kit (Omega) according to manufacturer's protocol. The RNAi antisense sequence was ligated into pHANNIBAL_sense at 4°C overnight using 1 µl T4 ligase in a 10 µl reaction. The full 10 µl ligation reaction was transformed into *E. coli* stbl2 and the insertion of the sense fragment was confirmed by restriction digest with XhoI.

Sanger sequencing (Eurofins) was carried out on the final RNAi construct to confirm correct sense and anti-sense fragment insertion. The workflow for generation of the RNAi construct is summarised in Figure 4.1.

4.3.7 Agroinfiltration of plant material

The *A. tumefaciens* strains AGL1 and C58 pGV2260 were used for the transient expression of GUS and GUSPlus genes in barley and tobacco. Protocols for agroinfiltration of barley and tobacco leaves were based on methods described by Mooney and Graciet (2020) and Lu et. al., (2016). The strains were transformed with either the pEG356 or the pTOC1 plasmids (Table 4.2) and grown on LB agar with carbenicillin (100 mg/l), rifampicin (50 mg/l) and spectinomycin (100 mg/l) selection for 3 days at 28°C. Colonies were then transferred to yeast extract broth (YEB) (Appendix 1) with carbenicillin (100 mg/l), rifampicin (50 mg/l) and spectinomycin (100 mg/l) and grown overnight at 28°C with shaking at 200 rpm. Cultures were centrifuged at 5,000 x g for 10 min at RT, and pellets resuspended in infiltration buffer (Appendix 1). The OD_{600} was checked and adjusted to the desired density. The suspension was infiltrated into the adaxial side of barley seedlings (cvs. Golden Promise, Cassia and Infinity) at GS13 or the abaxial side of 4-6 week old tobacco plants using a 1 ml blunt syringe. Infiltrated plants were returned to respective growth rooms (section 4.3.2) and two 1 cm leaf disks were collected using a cork borer from proximal, middle and distal sections of leaves 1 and 2 for GUS staining (Section 4.3.8) and gene expression analysis (Section 4.3.9) between 2–5 days post infiltration.

4.3.8 GUS staining

1 cm leaf sections from barley or 1 cm leaf disks from tobacco after agroinfiltration were collected into 1.5 ml microcentrifuge tubes containing 1 ml ice cold 90 % acetone (v/v). Leaf sections were incubated on ice for 20 min.

The acetone was completely removed, and leaf sections washed in GUS wash solution (Appendix 1). 500 µl of GUS staining solution (Appendix 1) was added and the samples were vacuum infiltrated for 2 to 30 mins. The leaf sections were incubated in the GUS staining solution at 37°C for 3 days.

Following the incubation period, the GUS staining solution was removed, and leaf sections rinsed in SDW. The leaf sections were placed in 70 % ethanol (v/v) for up to 5 days, then transferred to 100 % ethanol until all chlorophyll was removed.

4.3.9 Gene expression analysis

Tissue from agroinfiltrated leaves was collected and flash frozen in LN_2. The tissue was ground to a fine powder in LN_2 using a miniature pestle. RNA was extracted and cDNA synthesised as described in section 4.3.6.2. A PCR was carried out on the cDNA using primers to detect expression of either 35S:GUS (qPCR13_up and qPCR14_ lo primers, Table 4.3) or Ubi:GUSPlus (TOC11 and TOC12 primers). *ACTIN* was used as a control for barley samples (HvActin_AM45_F1 and HvActin_AM46_R1 primers, Table 2.2), and *NbEF1alpha* as a control in tobacco (TOC16 and TOC17 primers, Table 4.3). PCR amplification was carried out with 30 cycles [30 s at 95°C, 30 s at 56°C, 40 s at 72°C].

4.4 Results

4.4.1 Validation of barley S gene candidates

In Chapter 2, the identification of 53 candidate barley S genes through the combination of *in silico* and transcriptomic analyses was described. To visualise the expression of these genes in Cassia and Infinity after *R. commune* infection, a heatmap of Log_2(FC) relative to mock treatment as determined by RNAseq in Chapter 3 was generated (Figure 4.2). All candidates were upregulated in response to *R. commune* in Cassia and/or Infinity. Only one gene, HORVU5Hr1G057090, showed downregulation in its expression (log_2(FC) = -2.74) at 48 hpi in Cassia in response to A (Figure 4.2). Of note, five of the 53 candidate S genes are not reported on the heatmap, as they were identified through Approach 3 (i.e., differential expression detected between the two cvs in the absence of pathogen) and did not show any differential expression after *R. commune* treatment relative to mock treated plants.

This list of 53 high confidence S gene candidates was further narrowed down based on a comparative analysis that examined in detail the expression profiles versus the reported known function of orthologous S genes. For example, HORVU5Hr1G060650 is a proposed barley orthologue to *AtMYB46*, which has been described as an S gene in *Arabidopsis* (Ramirez et. al., 2011). Mutations in this gene in *Arabidopsis* resulted in increased resistance to *B. cinerea* but not to *Pst,* even though *AtMYB46* was not upregulated in response to *B. cinerea* or *Pst* infection. Additionally, separate experiments involving overexpression of *AtMYB46* did not result in increased susceptibility compared to WT (Ramirez et. al., 2011). Furthermore, microarray data comparing gene expression in an *atmyb46* mutant to the WT showed increased expression of peroxidase genes, suggesting a role in ROS scavenging (Ramirez et. al., 2011b). This gene also plays a role in cell wall biosynthesis and lignin deposition (Kim et. al., 2013). As *R. commune* penetrates the leaf cuticle rather than entering through the stomata, based on its known function and its upregulation after *R. commune* infection in this dataset, this candidate gene was selected for validation.

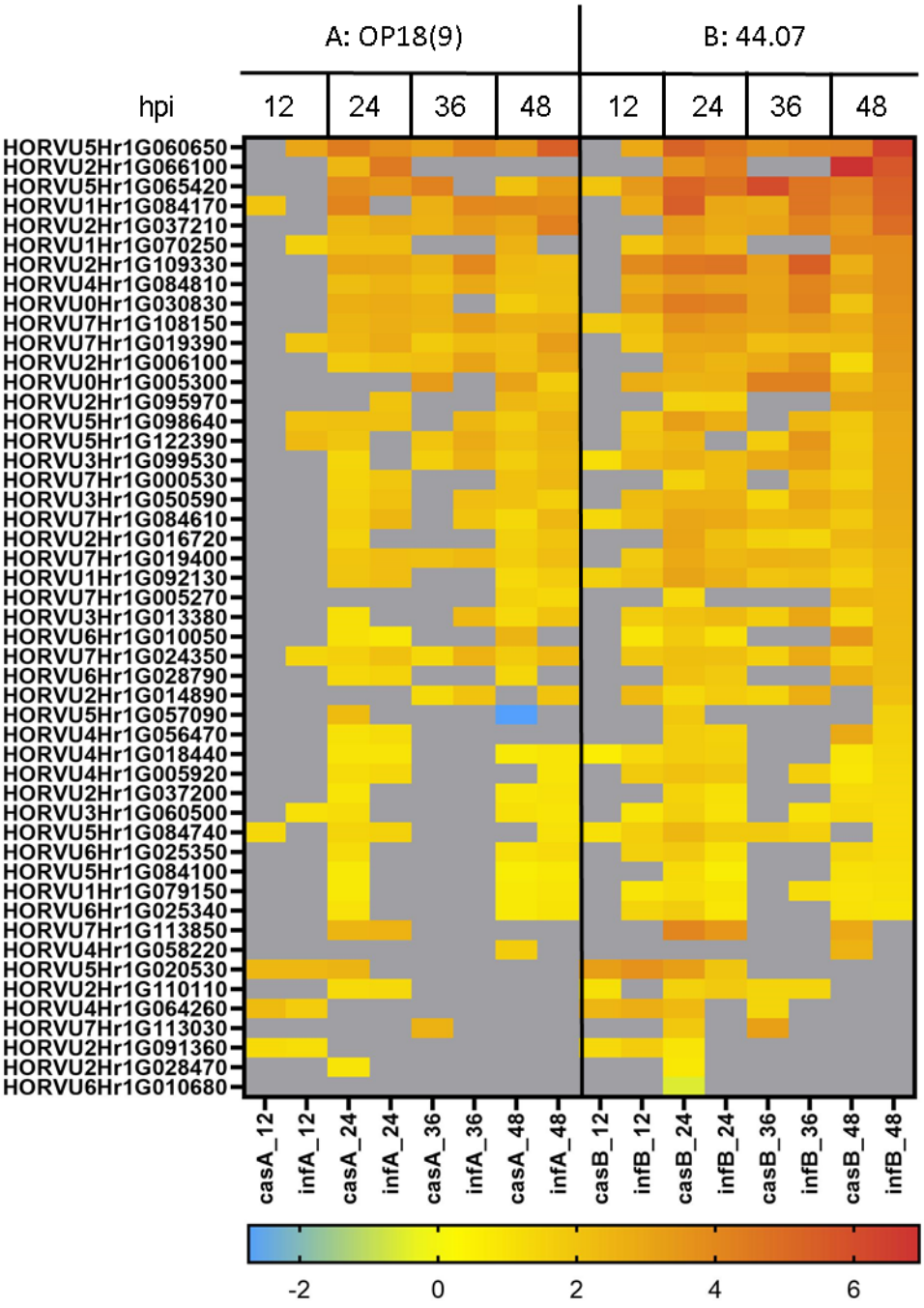


Figure 4.2: Heatmap of significant $\log_2$(FC) of high confidence S gene candidates. Level of up- or down- regulation is represented as a colour gradient. Grey indicates no differential expression relative to mock treatment detected at this timepoint. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. Heatmap generated using GraphPad Prism (version 9.1.0).

In contrast, HORVU2Hr1G066100 is a proposed orthologue to *AtBHLH27*, which has been described as an S gene that contributes to susceptibility to cyst nematode infection in *Arabidopsis* root tissue. Specifically, its activity is exploited by the nematode *Heterodera schachtii* to aid in the establishment of feeding structures in *Arabidopsis* root tissue (Puthoff et. al., 2003). As *R. commune* is a foliar disease that does not require the formation of feeding structures, HORVU2Hr1G066100 is less likely to be a suitable S gene, assuming a level of conserved function across orthologues. Hence, it was not considered further.

After analysis of the literature and of the gene expression differences, a total of 17 high confidence S gene candidates were selected for further analysis and validation by RT-qPCR (Table 4.4). The S gene family these candidates were assigned to were also investigated more in detail, and the fold changes after *R. commune* infection (relative to mock) of all genes in a given family were examined in order to assess if there were any other genes in the family following a similar expression pattern as the candidate orthologue (i.e. to identify potential functional redundancy). Following this, the 17 candidates were ranked based on their suitability for further investigation via RNAi mediated knockdown (Table 4.4). Criteria for this ranking was based on the following considerations (weighted in the following order):

(i) Expression profile of candidate gene
(ii) Expression profile of other barley genes in family
(iii) Validation of expression after *R. commune* infection by RT-qPCR
(iv) Known functions of orthologous S genes

Table 4.4: 17 candidate barley S genes selected for validation and their S gene families ranked by suitability for further investigation.

Rank	Gene ID	Identification approach	S gene orthologue	S gene type	S gene family	# barley genes in family
1	HORVU5Hr1G122390	1,2	*AtIOS1*	1	Plant000076	14
2	HORVU6Hr1G010680	3	*AtBON1*	2	Plant001599	2
3	HORVU2Hr1G109330	1	*OsWRKY45-1*	2	Plant014647	2
4	HORVU3Hr1G099530	1	*AtUTG7B1/ AtFAD8*	2	Plant001557	2
5	HORVU2Hr1G037210	1	*AtLecRK_V.5*	1	Plant000040	29
6	HORVU7Hr1G108150	1	*AtBIK1*	2	Plant000020	47
7	HORVU5Hr1G060650	1	*AtMYB64*	2	Plant000003	63
8	HORVU1Hr1G070250	1	Multiple WRKY genes	1,2,3	Plant000088	15
9	HORVU4Hr1G084810	1	*AtDMR6*	2	Plant003549	4
10	HORVU7Hr1G113850	1	*HvWRKY2/OsWRKY28*	2	Plant006394	2
11	HORVU6Hr1G028790	1	*HvWRKY1*	2	Plant018390	1
12	HORVU7Hr1G113030	2	*AtPLP2*	2	Plant000385	14
13	HORVU5Hr1G057090	2	*HvADH*	3	Plant000975	13
14	HORVU4Hr1G005920	1	*ZmLOX3*	3	Plant000115	14
15	HORVU4Hr1G018440	1	*AtPTP1*	2	Plant003579	2
16	HORVU5Hr1G084740	1	*AtPLDBeta1/OsPLDBeta1*	2	Plant000161	13
17	HORVU2Hr1G028470	1	*AtMYB46*	2	Plant000003	63

The top five candidates are discussed below in detail, the top three of which were selected for knockdown experiments. The RT-qPCR validation plots and heatmaps depicting significant fold changes after *R. commune* infection (relative to mock) for S gene families for the remaining 12 candidates are shown in Supplemental Figures S4.1 – S4.22 (Appendix 2).

The first candidate, HORVU5Hr1G122390 is a proposed orthologue to the known *Arabidopsis* S gene *IMPAIRED OOMYCETE SUSCEPTIBILITY1 (AtIOS1).* AtIOS1 is a putative LRR-RLK and has been shown to be involved in susceptibility to *Hyaloperonospora arabidopsidis (Hpa)* (Hok et. al, 2011). HORVU5Hr1G122390 was selected as a high confidence S gene candidate as it fulfilled selection criteria for Approach 1 (called as differentially expressed at the same timepoint in all cultivar x treatment combinations). It also fulfilled selection criteria for Approach 2 (differential expression observed in Cassia only during at least one timepoint). Essentially, it was part of the susceptible response at 24 hpi, and then later part of a core response at 36 and 48 hpi. Indeed, expression of this gene in Cassia is significantly upregulated 3 fold in response to the B isolate at 24 hpi compared to mock (Figure 4.3). While this gene also appears upregulated in Infinity in response to both isolates at 12 and 36 hpi, this was not found to be significant. Mean relative expression values determined by RT-qPCR for this gene remained low in mock treated samples compared to *R. commune* treated samples. The corresponding mean FPKM values from RNAseq show apparent increase in expression of HORVU5Hr1G122390 at 24 hpi in Infinity in response to A (3 fold) and B (2.7 fold) relative to mock, which is sustained until 48 hpi. This increase in expression in response to both isolates is also observed in Cassia and is more pronounced compared to Infinity (4 fold increase in response to A, 6 fold increase in response to B at 24 hpi) (Figure 4.3).

The S gene family that HORVU5Hr1G122390 and *AtIOS1* belong to is one of the larger families identified, containing 260 potential orthologues from all ten species in the database. Of these 260 potential orthologues, 14 were identified in barley. One of these genes, HORVU3Hr1G013380, was also upregulated at multiple timepoints in both Cassia and Infinity (Figure 4.4). Another barley

gene in this family, HORVU6Hr1G022360 was upregulated in Infinity only at 24 hpi, in response to A (log_2(FC) = 3.6) and B (log_2(FC) = 2.88). HORVU6Hr1G022310 was only upregulated in Cassia at 12 hpi (log_2(FC) = 2.3) in response to B. The remaining genes in this family were not detected as differentially expressed.

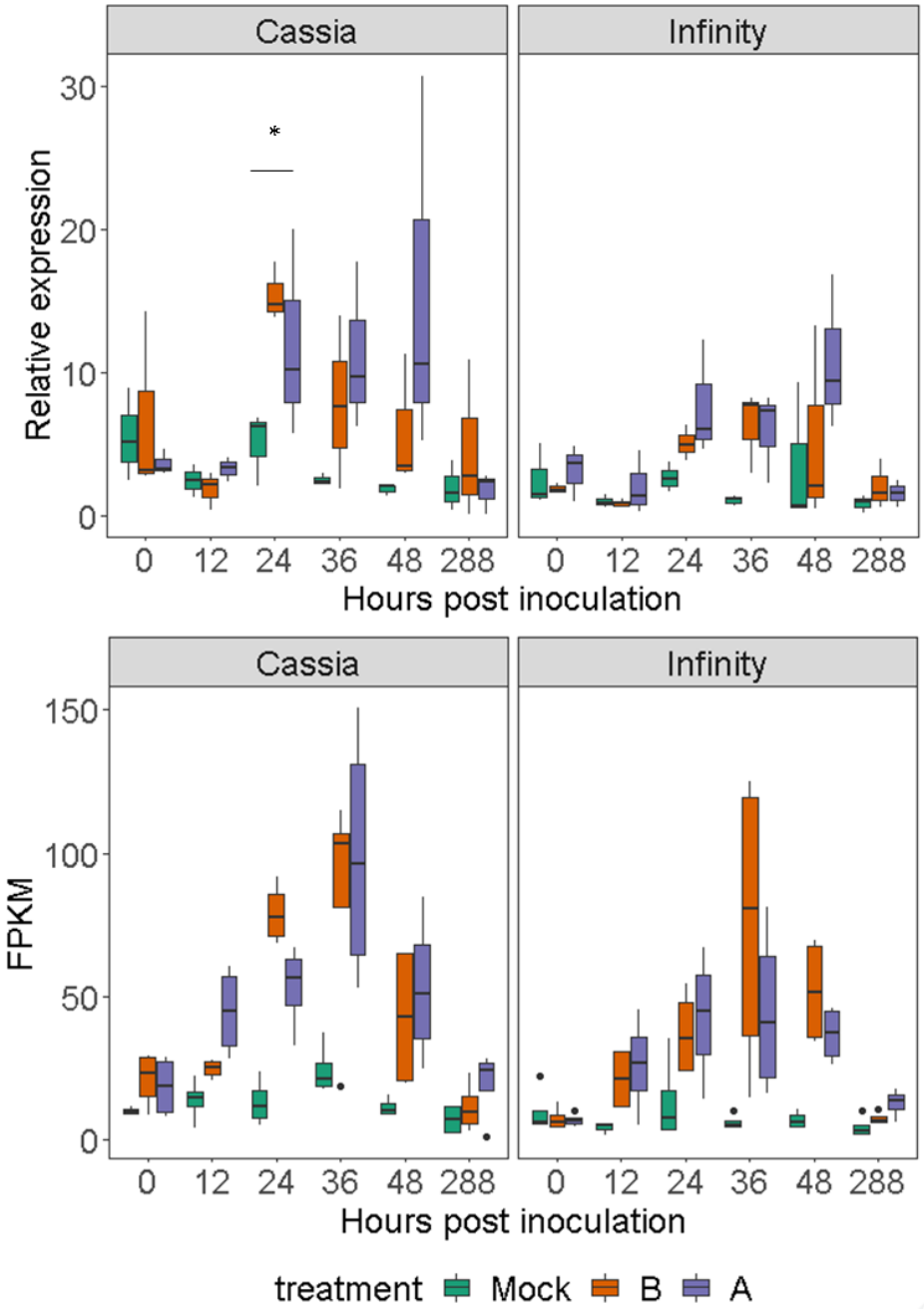


Figure 4.3: Relative expression level of HORVU5Hr1G122390, a proposed barley orthologue to *AtIOS1.* Expression levels of this S gene candidate relative to *ACTIN.* Significant (p-adj < 0.05, n = 3) differences in relative expression levels between A and mock treatment at a 24 hpi as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

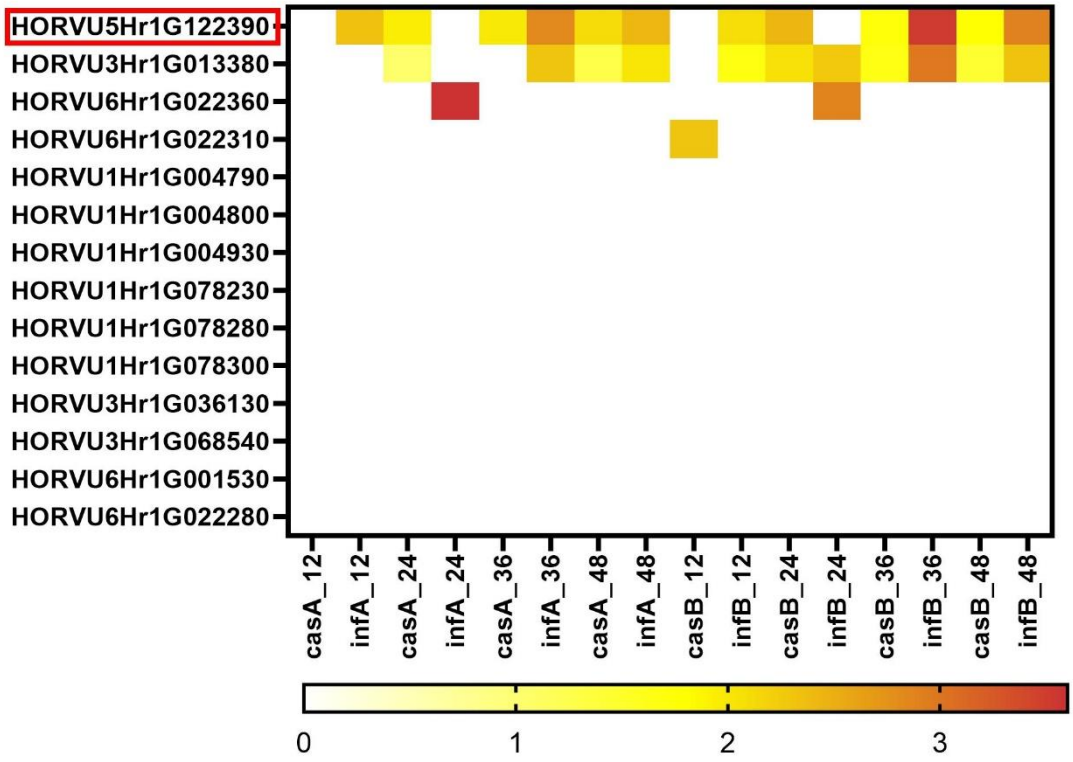


Figure 4.4: Heatmap of significant $\log_2$ (Fold change relative to mock) of all barley genes in S gene family Plant000076. Level of upregulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU5Hr1G122390 is indicated in red box.

The next barley gene selected for validation was HORVU6Hr1G010680. This gene was identified as a potential orthologue to the *Arabidopsis* S gene *BONZAI 1 (AtBON1)*. *AtBON1* encodes for a calcium-dependent, phospholipid binding protein, and has been shown to confer *Arabidopsis* susceptibility *Pst,* likely as part of the hormone signalling defence pathways (Lee and McNeillis, 2008). The gene family containing this S gene contained 40 genes from all species in the database, including two barley genes. This gene was selected as an S gene candidate as endogenous expression in mock treated samples was significantly increased in Cassia compared to Infinity at all timepoints except 0 hpi (Approach 3, Figure 3.21). Indeed, as determined by RT-qPCR, relative expression of this gene is higher in Cassia than in Infinity. Interestingly, the expression of this gene did not change after infection with either *R. commune* isolate, except in Cassia at 288 hpi, where it was in fact significantly

downregulated almost 2 fold in response to A relative to mock (Figure 4.5). This gene also appears downregulated relative to mock treatment in Cassia in response to B at 24 hpi, however, this was not found to be statistically significant. These trends observed in the expression patterns of this gene were mirrored in FPKM values extracted from the RNAseq datasets (Figure 4.5).

The S gene family that HORVU6Hr1G010680 and *AtBON1* were clustered into (Plant001599) contained one other barley gene: HORVU1Hr1G058000. This gene was also not called as being differentially expressed in response to *R. commune*, and this gene was expressed at similar levels in both Cassia and Infinity mock treated samples (Figure 4.6).

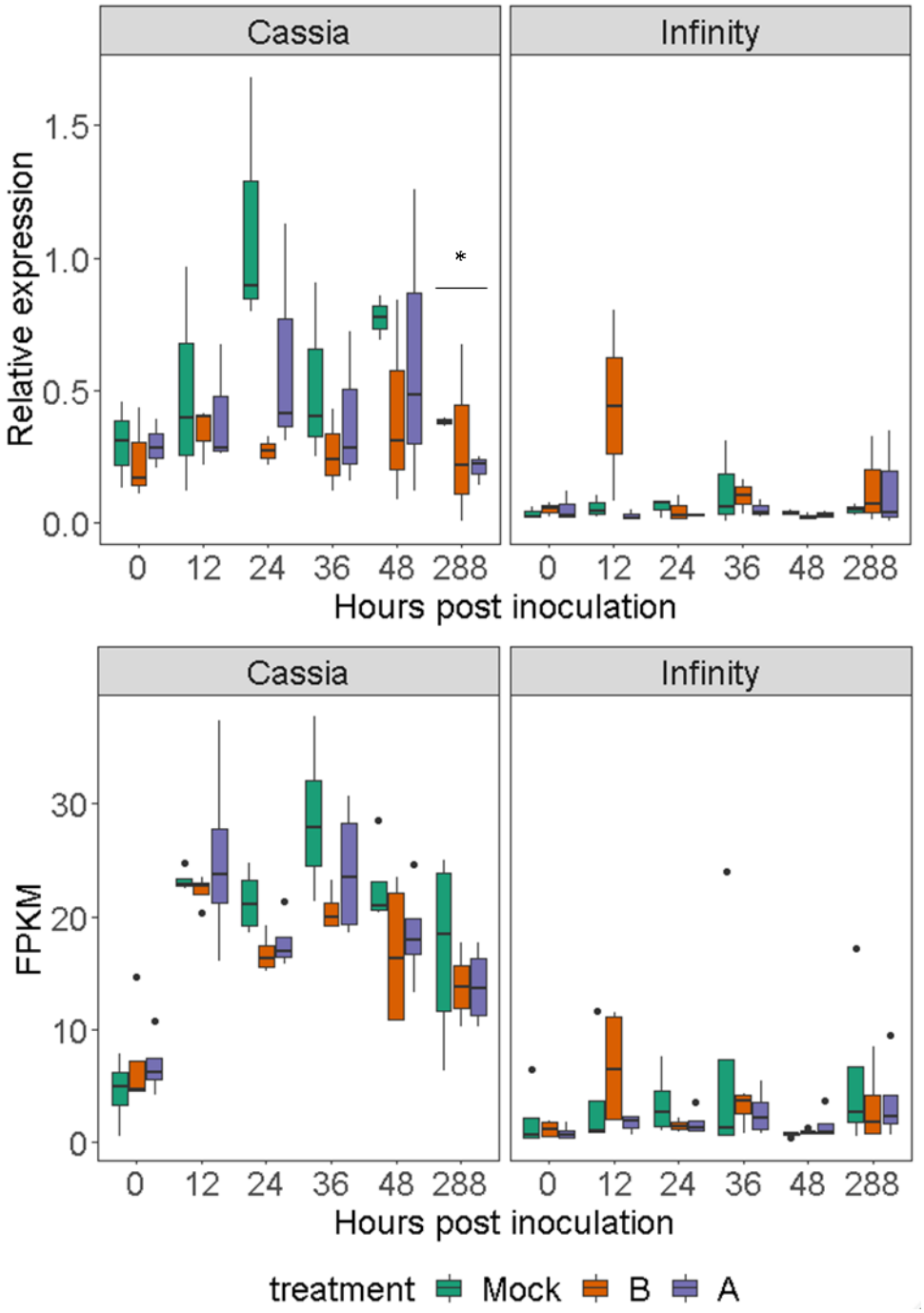


Figure 4.5: Relative expression level of HORVU6Hr1G010680, a proposed barley orthologue to *AtBON1*. Expression levels of this S gene candidate relative to *ACTIN*. Significant (p-adj < 0.05, n = 3) differences in relative expression levels between different A and mock treatment at a 288 hpi as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

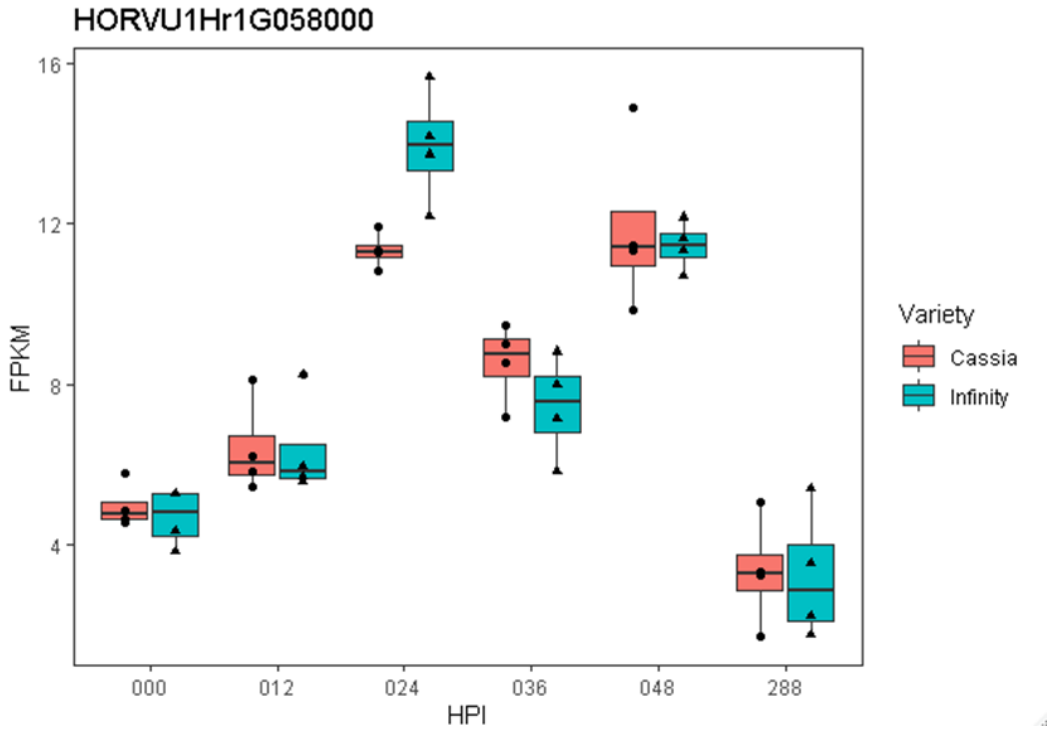


Figure 4.6: FPKM values of HORVU1Hr1G058000 in Cassia and Infinity Mock treated samples (n = 4). No significant differences between Cassia and Infinity endogenous expression of HORVU1Hr1G058000.

HORVU2Hr1G109330 was identified as a potential barley orthologue to rice *OsWRKY45-1.* This gene has been shown to confer susceptibility to *Xoo* in *O. sativa* subsp. *Japonica* (Tao et. al., 2009). RT-qPCR analysis (Figure 4.7) shows that, in Cassia, expression of HORVU2Hr1G109330 after treatment with isolate B peaks at 24 hpi and 36 hpi (17 and 8.6 fold increase relative to mock respectively). Expression levels in *R. commune* treated samples are similar to mock treatment at 288 hpi. This peak of expression at 24 and 36 hpi in Cassia was found to be significant in response to the B isolate. An apparent increase is also observed in response to the A isolate in Cassia at these timepoints, however there is more variation between replicates, and this was not found to be statistically significant. This pattern of expression observed in Cassia between 24 and 48 hpi is also observed in corresponding FPKM from RNAseq, with mean FPKM values in Cassia peaking at 24 and 36 hpi in Cassia B treated samples (60.92 and 49.47 respectively). The expression of this gene in Infinity in response to both *R. commune* isolates is similar to that of Cassia, in that it peaks at 24 hpi (9.5 fold increase relative to mock), however in Infinity,

expression levels remain upregulated until 48 hpi. This is more pronounced in response to the B isolate compared to isolate A (Figure 4.7); however, this was only statistically significant at 24 hpi.

HORVU2Hr1G109330 was clustered into the S gene family Plant014647 along with *OsWRKY45-1.* Within this S gene family, there were a total of 9 potential orthologues, found in rice, maize, barley and wheat, however subsequent BLASTp of the corresponding *OsWRKY45-1* protein sequence into other species on NCBI suggested this gene was not monocot specific. One other barley gene clustered to this S gene family and was also found to be differentially expressed in response to *R. commune* (relative to mock). Indeed, the two barley genes in this family were found to have similar expression patterns in both Cassia and Infinity (Figure 4.8).

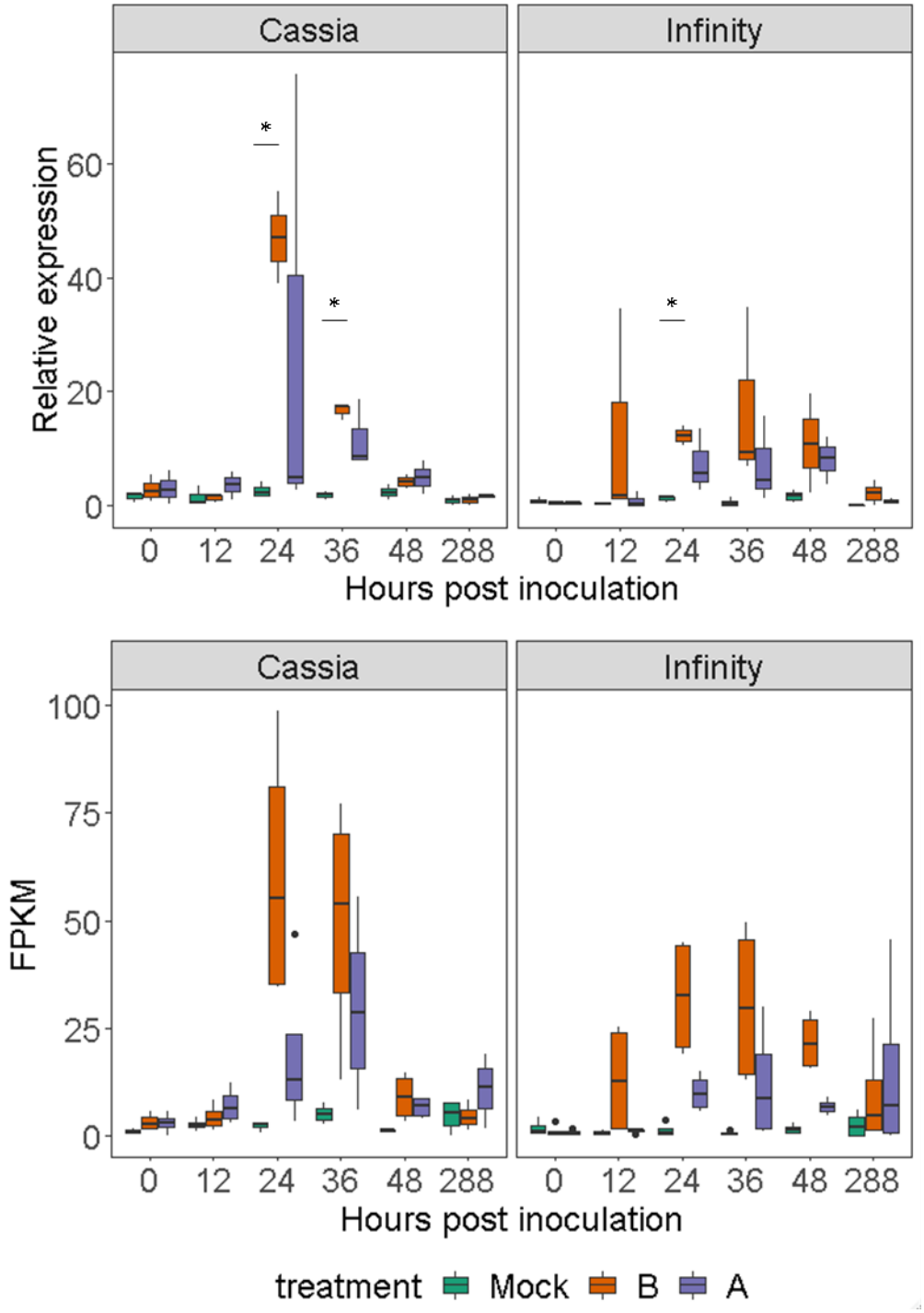


Figure 4.7: Relative expression level of HORVU2Hr1G109330, a proposed barley orthologue to *OsWRKY45-1*. Expression levels of this S gene candidate relative to *ACTIN*. Significant (p-adj < 0.05, n = 3) differences in relative expression levels between B abd mock trearments at a 24 and 36 hpi as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

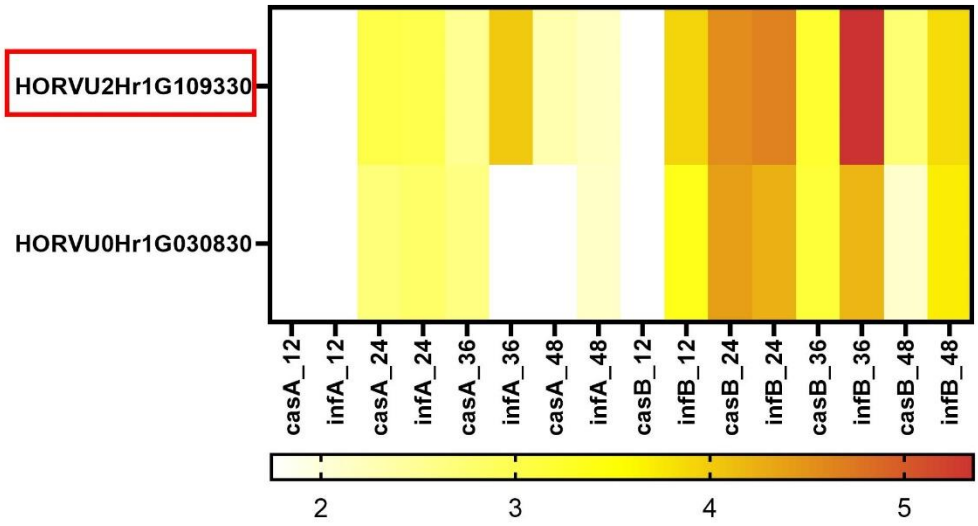


Figure 4.8: Heatmap of significant $\log_2$(FC) of all barley genes in S gene family Plant014647. Level of upregulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU2Hr1G109330 is indicated in red box.

HORVU3Hr1G099530 was identified as a potential barley orthologue to the *Arabidopsis* S gene *UDP-DEPENDENT GLYCOSYLTRANSFERASE 76B1 (AtUGT76B*) by the OrthoMCL clustering analysis. This gene is involved in the modulation of N-hydroxypipecolic acid (NHP) and SA during pathogen invasion. Loss of function results in an SAR like, NHP dependant immune response to *Pst* (Ge et. al., 2021). HORVU3Hr1G099530 was included for validation as it met the selection criteria for Approach 1 (called as differentially expressed at the same timepoint in all cultivar x treatment combinations). Expression of this gene in Cassia begins to increase at 24 hpi, peaking at 36 hpi before decreasing again at 48 hpi in response to B. Interestingly, the expression of this gene remains elevated between 24 and 48 hpi in Cassia in response to A. In Infinity, a similar expression profile is observed in response to B, however expression begins to increase earlier, at 12 hpi. This continues to increase until 36 hpi. The response of Infinity to A shows a similar dynamic (Figure 4.9). The relative expression of this gene in response to both *R. commune* strains is more pronounced in Cassia compared to Infinity, however changes in expression relative to mock treatment were only significant in

Infinity in response B at 24 hpi. FPKM plots for both Cassia and Infinity follow similar trends as those determined by RT-qPCR (Figure 4.9).

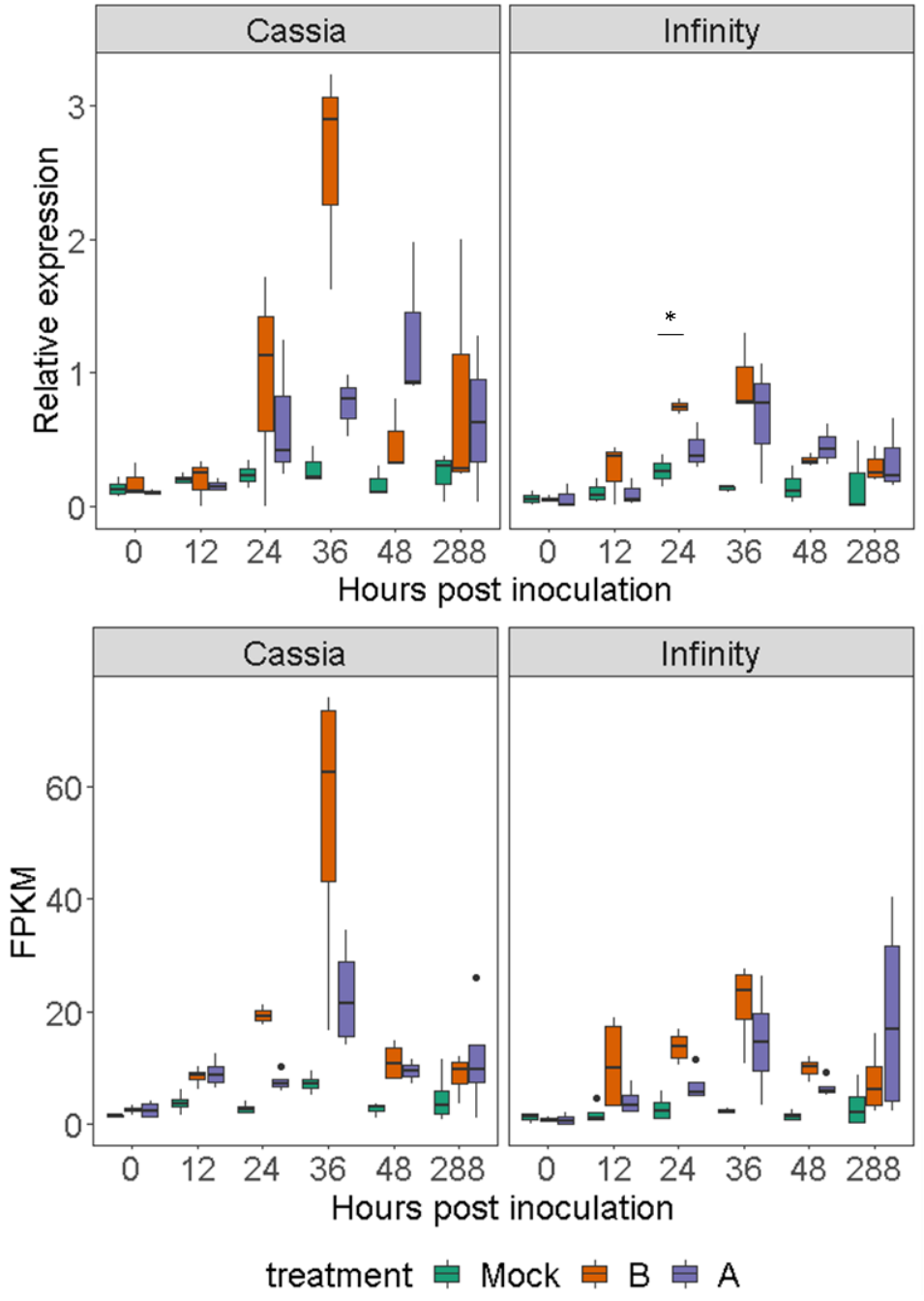


Figure 4.9: Relative expression level of HORVU3Hr1G099530, a proposed barley orthologue to *AtUGT76B*. Expression levels of this S gene candidate relative to *ACTIN*. Significant (p-adj < 0.05, n = 3) differences in relative expression levels between B and Mock at a 24 hpi in Infinity as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

This gene was grouped into a family containing 41 other genes from all of the species in the database except pepper. This family (Plant001557) only contained one other barley gene, HORVU4Hr1G011760. The latter was found to be strongly upregulated (log_2FC = 5.8), but only in Infinity at 36 hpi in response to B (relative to mock) (Figure 4.10).

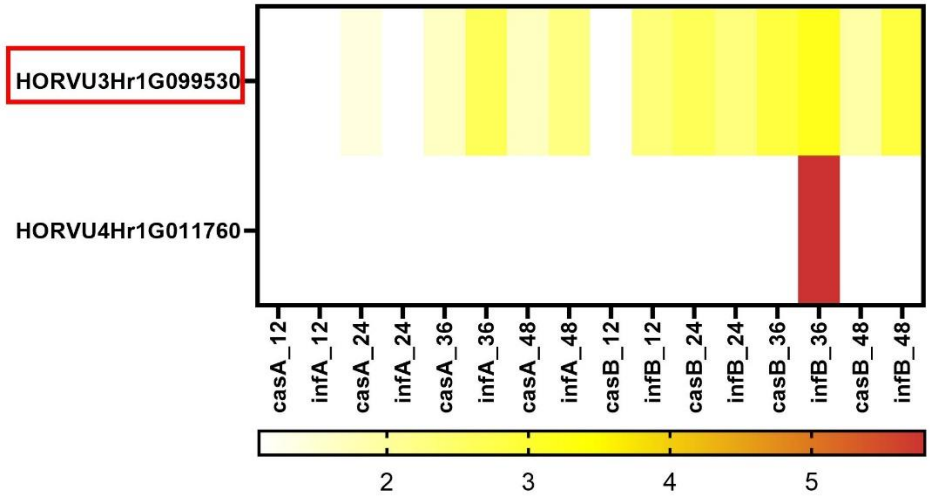


Figure 4.10: Heatmap of significant log_2 (FC) of all barley genes in S gene family Plant001557. Level of upregulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU3Hr1G099530 is indicated in red box.

The HORVU2Hr1G037210 gene was identified as a potential barley orthologue to the known *Arabidopsis* S gene *L-TYPE LECTIN RECEPTOR KINASE V.5* (*AtLecRK-V.5*). *AtLECRK-V.5* has been shown to play a role in negative regulation of PAMP induced stomatal closure, conferring increased susceptibly to *Pst* (Desclos-Theveniau et. al., 2012). The corresponding S gene family (Plant000040) was the fourth largest family constructed using OrthoMCL, with a total of 334 genes assigned to this family. Genes from all 10 species in the database were present in this family. RT-qPCR analysis HORVU2Hr1G037210 in both cultivars indicates that the expression of this gene remains relatively low in mock treated samples compared to *R. commune* treated samples in both Cassia and Infinity (Figure 4.11). Expression of this gene appears to increase 3 fold in Cassia in response to A relative to mock at 12 hpi and remains upregulated until 48 hpi. This activation

is delayed in Cassia in response to B, and upregulation (3.1 fold relative to mock) is not observed until 24 hpi. However, this apparent increase in expression was not found to be significant. In Infinity a significant upregulation compared to mock can be seen in response to A at 36 hpi and 48 hpi (4.8 fold increase at both timepoints). There was no difference in expression between treatments at 288 hpi (12 dpi) in both Cassia and Infinity (Figure 4.11).

The S gene family Plant000040 containing HORVU2Hr1G037210 and *AtLecRK-V.5* also contained 28 other barley genes. Of these, differential gene expression relative to mock was detected in 20 (Figure 4.12). Two of these genes (HORVU5Hr1G020530 and HORVU2Hr1G091360) were only found to be differentially expressed in Cassia and Infinity between 12 and 24 hpi. HORVU5Hr1G104850 was down regulated at 24 hpi in Cassia and Infinity in response to the B isolate only (log_2FC = -0.756 and -0.95 relative to mock in Cassia and Infinity respectively). One gene, HORVU6Hr1G093300, was strongly downregulated (log_2FC = -2.73) relative to mock in Cassia at 48 hpi in response to B only. Differential expression of this gene was not detected at any other timepoint (Figure 4.12).

Based on the data presented, it was hypothesised that these five candidates may play a role in barley susceptibility to *R. commune* and would also be suitable candidates for knockdown analysis. Due to time constrains, only the top three ranked candidates: HORVU5Hr1G122390, HORVU6Hr1G010680 and HORVU2Hr1G109330 (Table 4.4) were brought forward for knockdown analysis. While these genes are not well annotated in barley beyond identification of functional domains, for ease of reporting these genes will be referred to as *HvIOS1, HvBON1* and *HvWRKY45-1*, respectively.

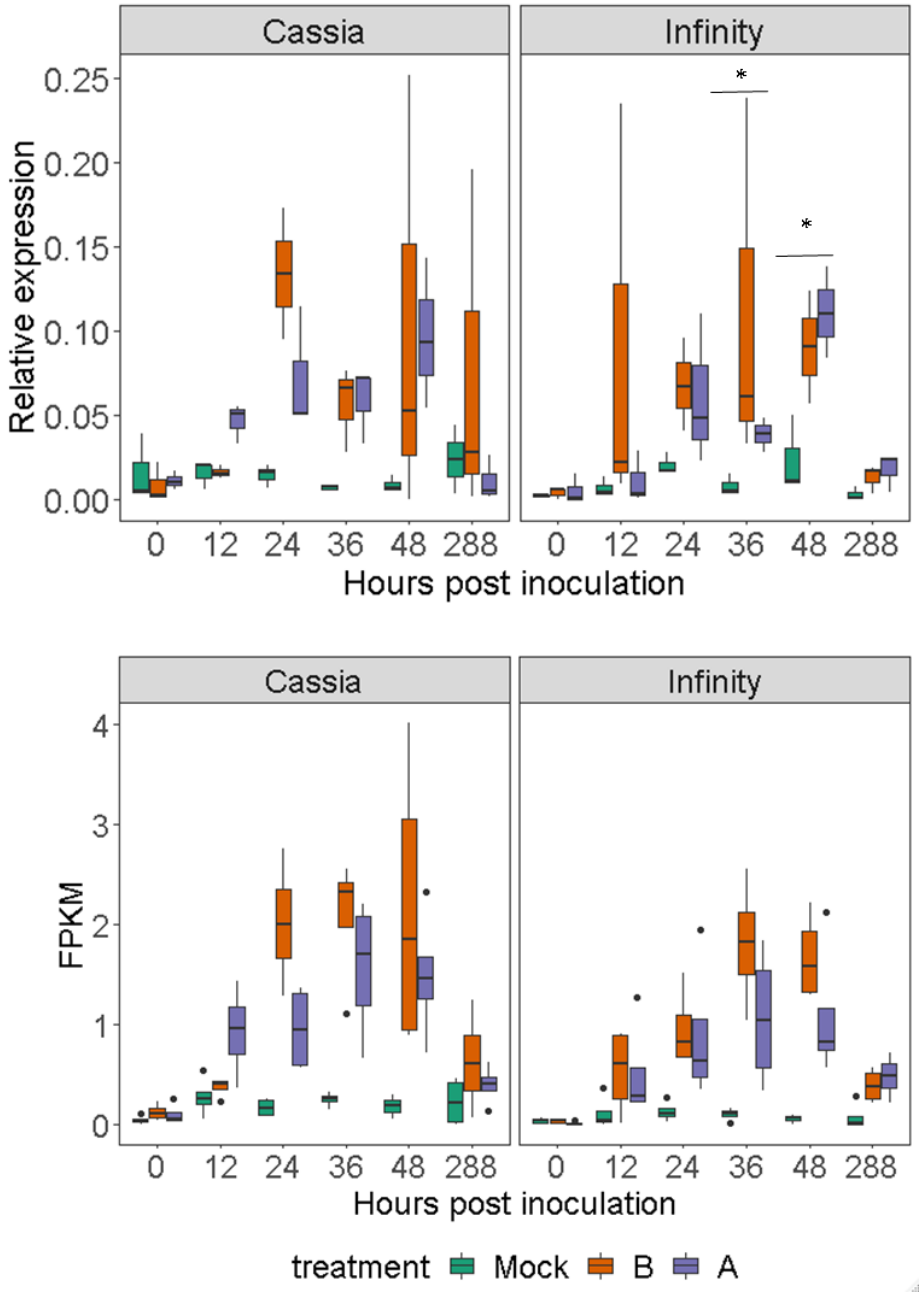


Figure 4.11: Relative expression level of HORVU2Hr1G037210, a proposed barley orthologue to *AtLecRK-V.5*. Expression levels of this S gene candidate relative to *ACTIN*. Significant (p-adj < 0.05, n = 3) differences in relative expression levels between A and mock treatments at a 36 and 48 hpi in Infinity as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

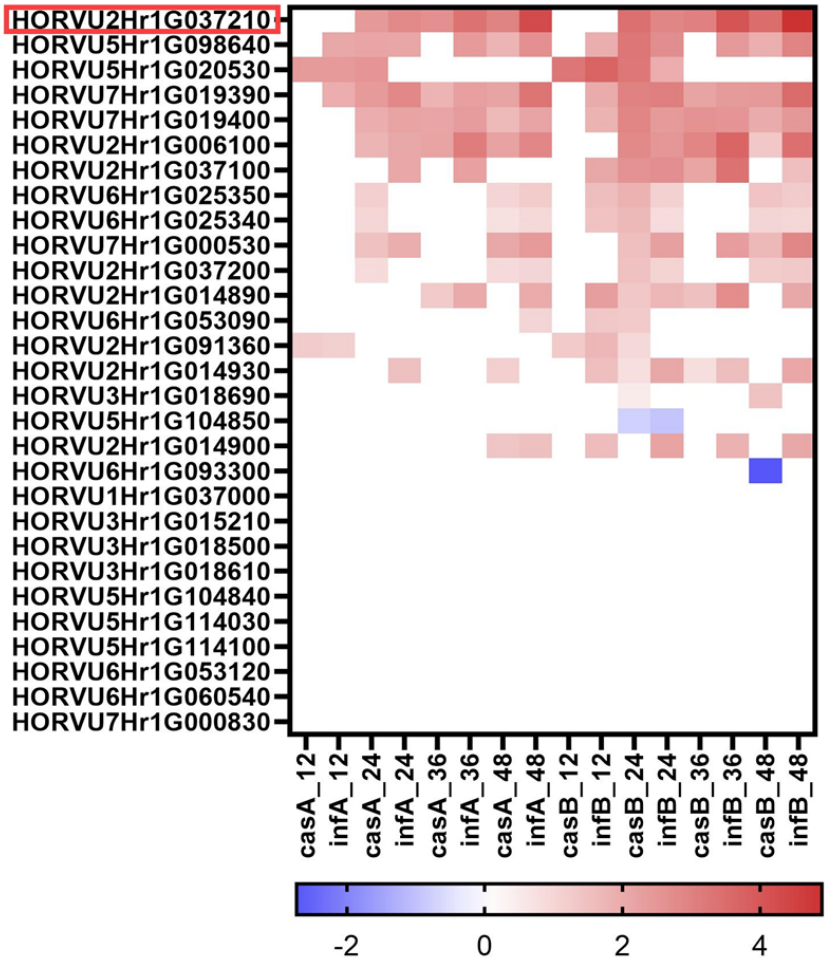


Figure 4.12: Heatmap of significant $\log_2$ (FC relative to mock) of all barley genes in S gene family Plant000040. Level of upregulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU2Hr1G037210 is indicated in red box. White squares indicate no significant FC relative to mock detected.

4.4.2 Generation of RNAi knockdown constructs

In order to further elucidate the role of *HvIOS1, HvBON1* and *HvWRKY45-1* in the defence response to *R. commune*, this section aimed to generate intron containing hairpin RNAi constructs in the pHANNIBAL cloning vector to knockdown each of these genes using *Agrobacterium* mediated transient expression in leaf tissue. In order to identify any potential off-targets, a BLASTn search against cDNAs/transcripts/splice variants cDNA sequence of each of the genes of interest was performed. While it was expected to find most or all of the other barley genes that clustered in the respective gene

families, this approach was taken to identify more carefully genes that might have lower similarities with the genes of interest (i.e., resulting in higher e-values).

HvIOS1

BLASTn analysis identified three additional cDNA sequences showing similarity to the *HvIOS1* target region. These cDNA sequences correspond to HORVU3Hr1G013380, HORVU1Hr1G004790 and HORVU1Hr1G078280, all of which had also been identified as being part of the Plant000076 family. HORVU3Hr1G013380 showed a similar expression pattern to *HvIOS1* after *R. commune* treatment, while the remaining two genes were not determined to be differentially expressed in either cv (Figure 4.4). The sequences of these genes were aligned to identify a ~450 bp region with the least similarity (Figure 4.13) with the aim of using this region to generate the sense and antisense fragments.

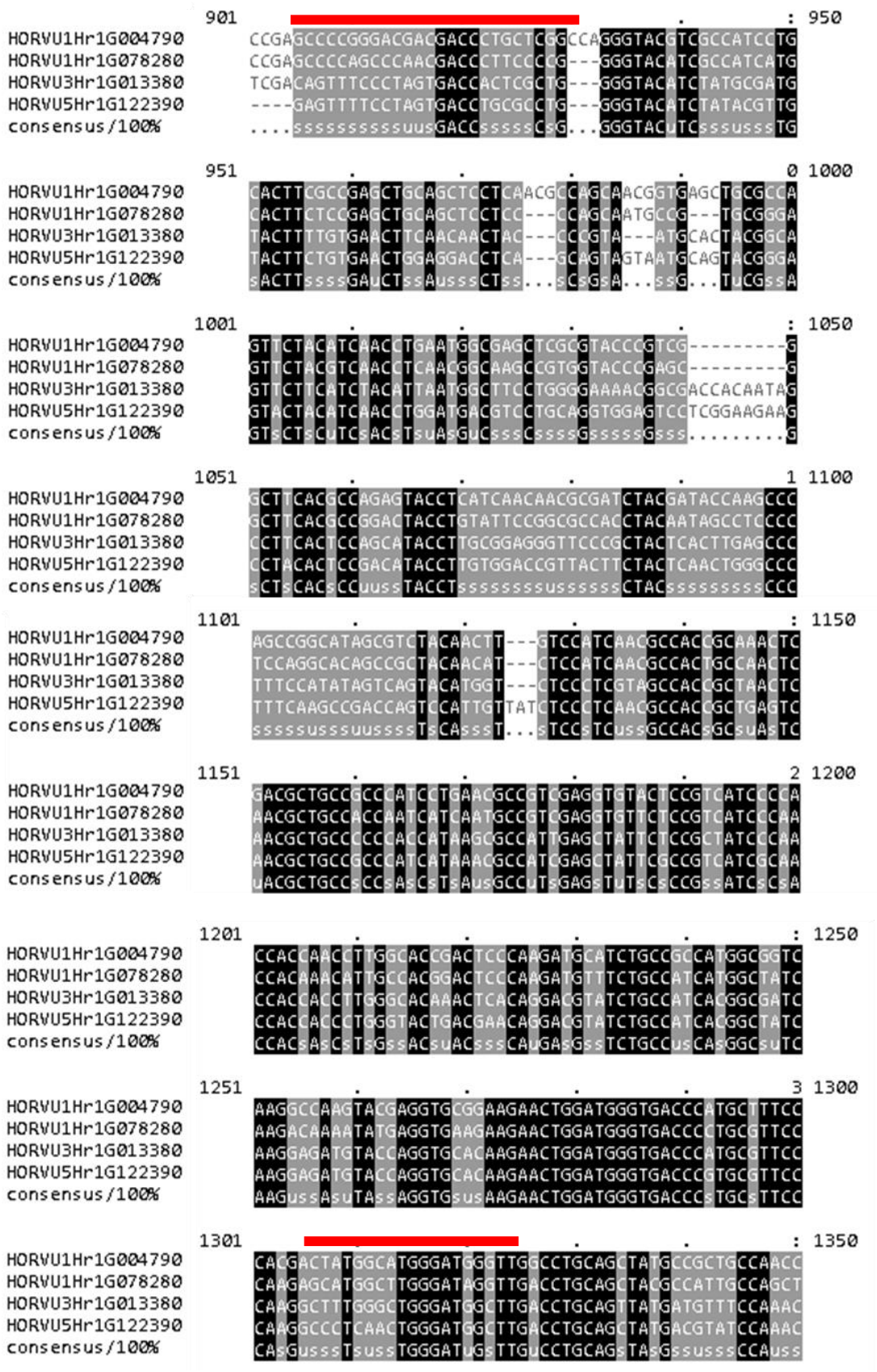


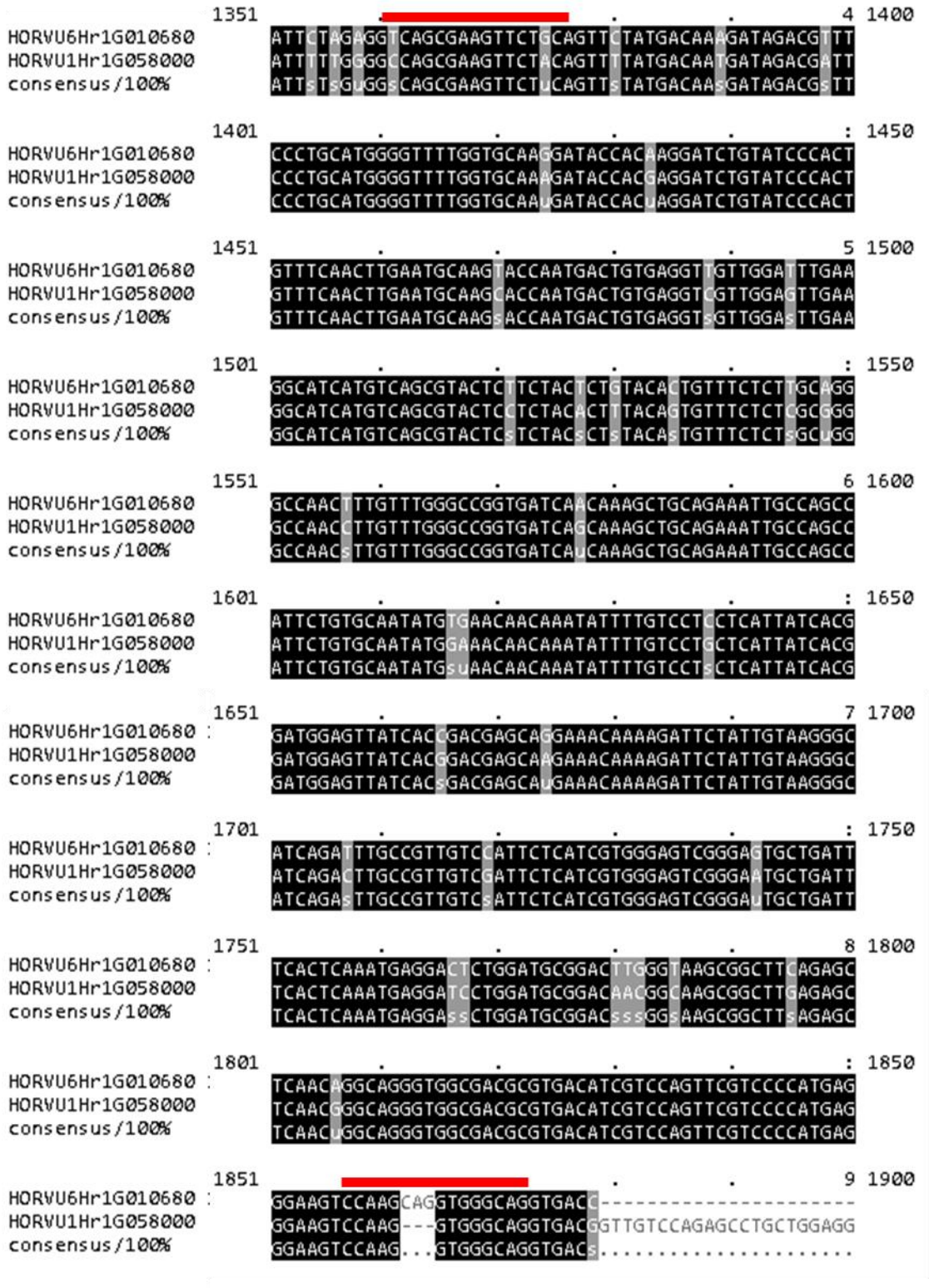
Figure 4.13: ~450bp region of *HvIOS1* (HORVU2Hr1G109330) and alignment with similar cDNA sequences identified by BLAST analysis. Location of primers marked in red.

HvBON1

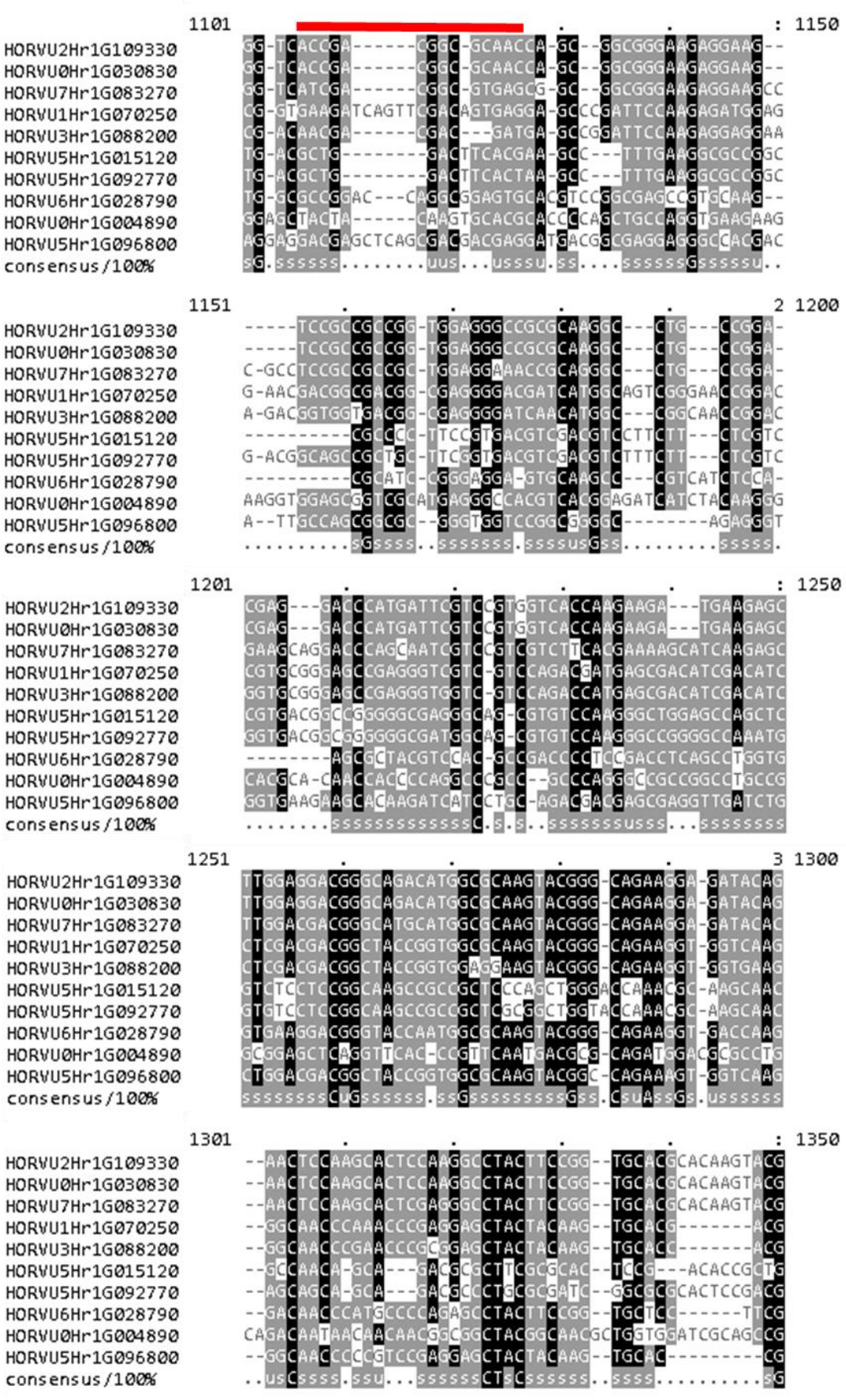
BLASTn of *HvBON1* CDS identified only one additional cDNA sequence showing similarity to the *HvBON1* target sequence: HORVU1Hr1G058000. This was the other barley gene in the Plant001599 family alongside *HvBON1.* The sequences of these two genes were aligned and showed high conservation. A 502 bp region (Figure 4.14) was selected to generate the RNAi construct but given the remaining level of similarity between the 2 coding sequences in this region, it was concluded that HORVU1Hr1G058000 would be a likely off target.

HvWRKY45-1

BLASTn of *HvWRKY45-1* identified 9 other genes showing sequence similarity. One of the genes identified was the other barley gene in the S gene family Plant014647. This gene was shown to have a similar expression pattern in Infinity and Cassia after *R. commune* infection, relative to mock (Figure 4.8). Alignment of these 10 genes showed some areas of strong conservation, however there were regions with sufficient differences to place primers and clone a region that would likely be specific to target the gene of interest (Figure 4.15).



4.14: ~510bp region of *HvBON1* (HORVU6Hr1G010680) and alignment with similar cDNA sequences identified by BLASTn. Location of primers marked in red.


1101 : 1150
HORVU2Hr1G109330
HORVU0Hr1G030830
HORVU7Hr1G083270
HORVU1Hr1G070250
HORVU3Hr1G088200
HORVU5Hr1G015120
HORVU5Hr1G092770
HORVU6Hr1G028790
HORVU0Hr1G004890
HORVU5Hr1G096800
consensus/100%
1151 2 1200
HORVU2Hr1G109330
HORVU0Hr1G030830
HORVU7Hr1G083270
HORVU1Hr1G070250
HORVU3Hr1G088200
HORVU5Hr1G015120
HORVU5Hr1G092770
HORVU6Hr1G028790
HORVU0Hr1G004890
HORVU5Hr1G096800
consensus/100%
1201 : 1250
HORVU2Hr1G109330
HORVU0Hr1G030830
HORVU7Hr1G083270
HORVU1Hr1G070250
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HORVU5Hr1G092770
HORVU6Hr1G028790
HORVU0Hr1G004890
HORVU5Hr1G096800
consensus/100%
1251 3 1300
HORVU2Hr1G109330
HORVU0Hr1G030830
HORVU7Hr1G083270
HORVU1Hr1G070250
HORVU3Hr1G088200
HORVU5Hr1G015120
HORVU5Hr1G092770
HORVU6Hr1G028790
HORVU0Hr1G004890
HORVU5Hr1G096800
consensus/100%
1301 : 1350
HORVU2Hr1G109330
HORVU0Hr1G030830
HORVU7Hr1G083270
HORVU1Hr1G070250
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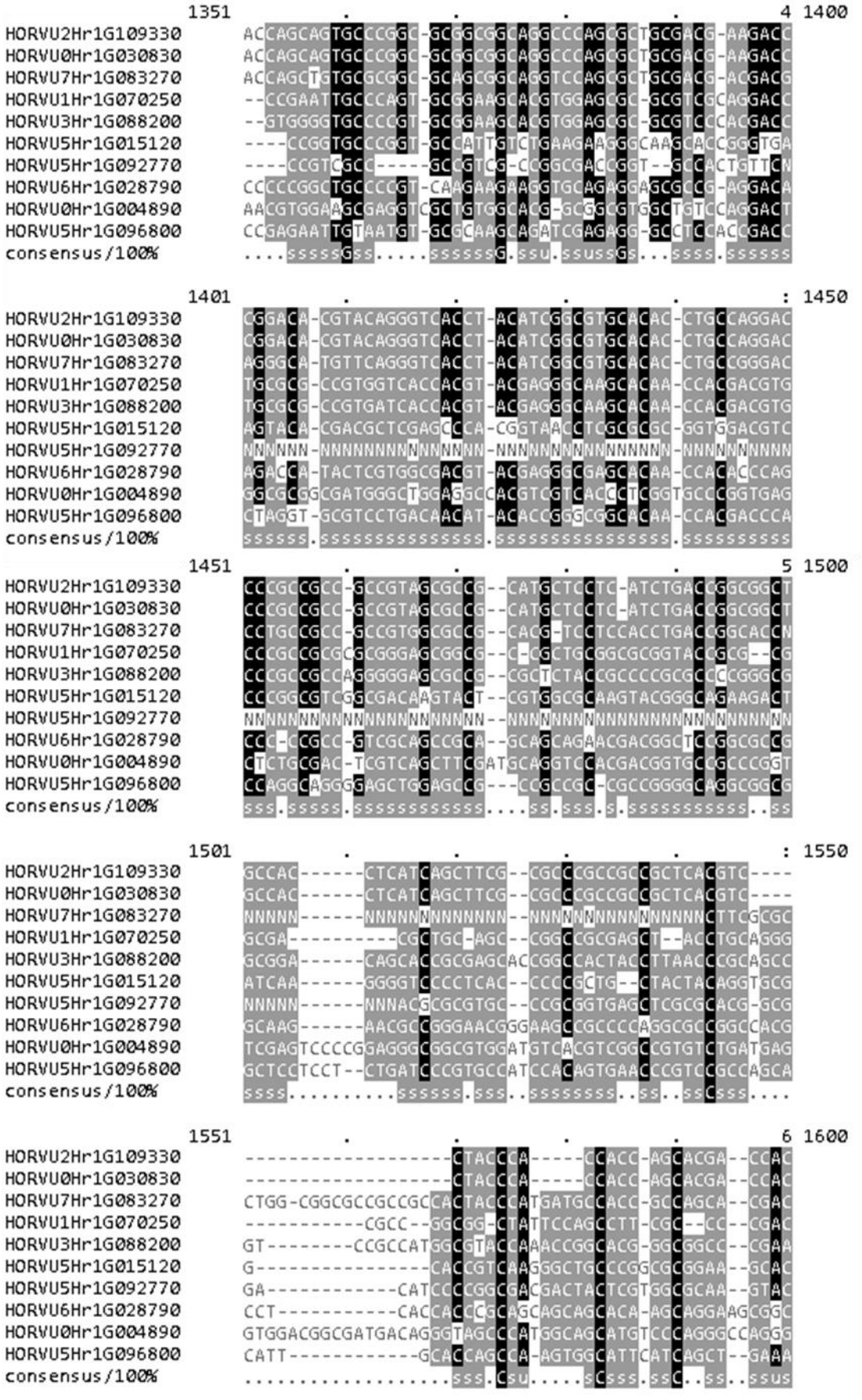
consensus/100%


1351 . . . . 4 1400
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HORVU7Hr1G083270 ACCAGCTGTGCGCGGC-GCAGCGGCAGGTCCAGCGCTGCGACG-ACGACG
HORVU1Hr1G070250 --CCGAATTGCCCAGT-GCGGAAGCACGTGGAGCGC-GCGTCGCAGGACC
HORVU3Hr1G088200 --GTGGGGTGCCCCGT-GCGGAAGCACGTGGAGCGC-GCGTCCCACGACC
HORVU5Hr1G015120 ----CCGGTGCCCGGT-GCCATTGTCTGAAGAAGGGCAAGCACCGGGTGA
HORVU5Hr1G092770 ----CCGTCGCC-----GCCGTCG-CCGGCGACCGGT--GCCACTGTTCN
HORVU6Hr1G028790 CCCCCGGCTGCCCCGT-CAAGAAGAAGGTGCAGAGGAGCGCCG-AGGACA
HORVU0Hr1G004890 AACGTGGAAGCGAGGTCGCTGTGGCACG-GCGGCGTGGCTGTCCAGGACT
HORVU5Hr1G096800 CCGAGAATTGTAATGT-GCGCAAGCAGATCGAGAGG-GCCTCCACCGACC
consensus/100% ....sssssGss.....ssssssG.ssu.ssussGs...ssss.ssssss
1401 . . . . : 1450
HORVU2Hr1G109330 CGGACA-CGTACAGGGTCACCT-ACATCGGCGTGCACAC-CTGCCAGGAC
HORVU0Hr1G030830 CGGACA-CGTACAGGGTCACCT-ACATCGGCGTGCACAC-CTGCCAGGAC
HORVU7Hr1G083270 AGGGCA-TGTTCAGGGTCACCT-ACATCGGCGTGCACAC-CTGCCGGGAC
HORVU1Hr1G070250 TGCGCG-CCGTGGTCACCACGT-ACGAGGGCAAGCACAA-CCACGACGTG
HORVU3Hr1G088200 TGCGCG-CCGTGATCACCACGT-ACGAGGGCAAGCACAA-CCACGACGTG
HORVU5Hr1G015120 AGTACA-CGACGCTCGAGCCCA-CGGTAACCTCGCGCGC-GGTGGACGTC
HORVU5Hr1G092770 NNNNNN-NNNNNNNNNNNNNN-NNNNNNNNNNNNNNN-NNNNNNNNNN
HORVU6Hr1G028790 AGACCA-TACTCGTGGCGACGT-ACGAGGGCGAGCACAA-CCACACCCAG
HORVU0Hr1G004890 GGCGCGGCGATGGGCTGGAGGCCACGTCGTCACCCTCGGTGCCCGGTGAG
HORVU5Hr1G096800 CTAGGT-GCGTCCTGACAACAT-ACACCGGGCGGCACAA-CCACGACCCA
consensus/100% ssssss.ssssssssssssss.sssssssssssssss.ssssssssss
1451 . . . . 5 1500
HORVU2Hr1G109330 CCCGCCGCC-GCCGTAGCGCCG--CATGCTCCTC-ATCTGACCGGCGGCCT
HORVU0Hr1G030830 CCCGCCGCC-GCCGTAGCGCCG--CATGCTCCTC-ATCTGACCGGCGGCCT
HORVU7Hr1G083270 CCTGCCGCC-GCCGTGGCGCCG--CACG-TCCTCCACCTGACCGGCACCN
HORVU1Hr1G070250 CCCGCCGCGCGCGGGAGCGGCG--C-CGCTGCGGCGCGGTACCGCG--CG
HORVU3Hr1G088200 CCCGCCGCCAGGGGGAGCGCCG--CGCTCTACCGCCCCGCGCCCCGGGCG
HORVU5Hr1G015120 CCCGGCGTCGGCGACAAGTACT--CGTGGCGCAAGTACGGGCAGAAGACT
HORVU5Hr1G092770 NNNNNNNNNNNNNNNNNNNNN--NNNNNNNNNNNNNNNNNNNNNNNNN
HORVU6Hr1G028790 CCC-CCGCC-GTCGCAGCCGCA--GCAGCAGAACGACGGCTCCGGCGCCG
HORVU0Hr1G004890 CTCTGCGAC-TCGTCAGCTTCGATGCAGGTCCACGACGGTGCCGCCCGGT
HORVU5Hr1G096800 CCAGGCAGGGGAGCTGGAGCCG---CCGCCGC-CGCCGGGGCAGGCGGCG
consensus/100% sss.sssss.sssssssssss....ss.sss.s.sssssssssss..ss
1501 . . . . : 1550
HORVU2Hr1G109330 GCCAC------CTCATCAGCTTCG--CGCCCGCCGCCGCTCACGTC----
HORVU0Hr1G030830 GCCAC------CTCATCAGCTTCG--CGCCCGCCGCCGCTCACGTC----
HORVU7Hr1G083270 NNNNN------NNNNNNNNNNNNN--NNNNNNNNNNNNNNNNCTTCGCGC
HORVU1Hr1G070250 GCGA----------CGCTGC-AGC--CGGCCGCGAGCT--ACCTGCAGGG
HORVU3Hr1G088200 GCGGA------CAGCACCGCGAGCACCGGCCACTACCTTAACCCGCAGCC
HORVU5Hr1G015120 ATCAA------GGGGTCCCCTCAC--CCCCGCTG--CTACTACAGGTGCG
HORVU5Hr1G092770 NNNNN------NNNACGCGCGTGC--CCGCGGTGAGCTCGCGCACG-GCG
HORVU6Hr1G028790 GCAAG------AACGCCGGGAACGGGAAGCCGCCCCAGGCGCCGGCCACG
HORVU0Hr1G004890 TCGAGTCCCCGGAGGGCGGCGTGGATGTCACGTCGGCCGTGTCTGATGAG
HORVU5Hr1G096800 GCTCCTCCT--CTGATCCCGTGCCATCCACAGTGAACCCGTCCGCCAGCA
consensus/100% ssss.........ssssss.sss..sssssssss..ss..ssCsss....
1551 . . . . 6 1600
HORVU2Hr1G109330 ----------CTACCCA-----CCACC-AGCACGA--CCAC
HORVU0Hr1G030830 ----------CTACCCA-----CCACC-AGCACGA--CCAC
HORVU7Hr1G083270 CTGG-CGGCGCCGCCGCCACTACCCATGATGCCACCC-GCCAGCA--CGAC
HORVU1Hr1G070250 ----------CGCC--GGCGG-CTATTCCAGCCTT-CGC--CC--CGAC
HORVU3Hr1G088200 GT--------CCGCCATGGCGTACCAAACCGGCACG-GGCGGCC--CGAA
HORVU5Hr1G015120 G----------CACCGTCAAGGGCTGCCCGGCGCGGAA--GCAC
HORVU5Hr1G092770 GA----------CATCCCCGGCGACGACTACTCGTGGCGCAA--GTAC
HORVU6Hr1G028790 CCT----------CACCACCCGCAGCAGCAGCACA-AGCAGGAAGCGGC
HORVU0Hr1G004890 GTGGACGGCGATGACAGGGTAGCCCATGGCAGCATGTCCCAGGGCCAGGG
HORVU5Hr1G096800 CATT----------GCACCAGCCA-AGTGGCATTCATCAGCT--GAAA
consensus/100% ....sss.Csu.....sCsss.ssC..ss..ssus

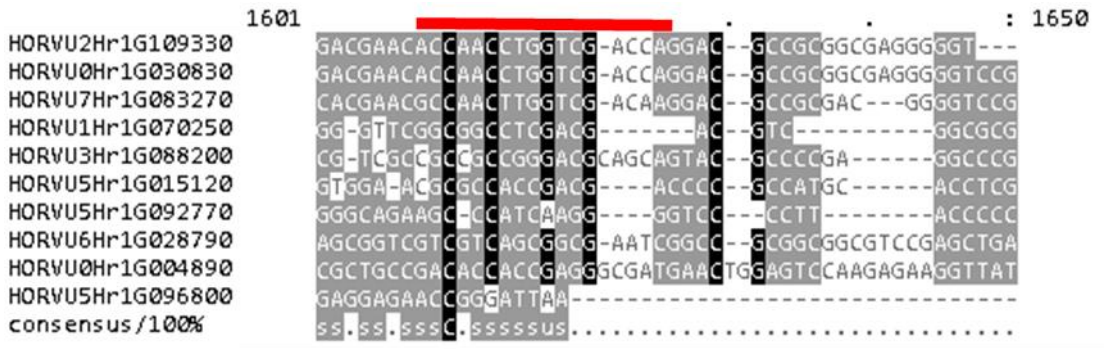


Figure 4.15: ~550bp region of *HvWRKY45-1* (HORVU2Hr1G109330) and alignment with similar cDNA sequences identified by BLASTn. Location of primers marked in red.

PCR was carried out to amplify the target region of *HvBON1* (using primers TOC1 and TOC2, Table 4.3), *HvIOS1* (primers used: TOC3 and TOC4), and *HvWRKY45-1* (primers used: TOC5 and TOC6) from cDNA from barley that was not inoculated with *R. commune*, as described in section 4.3.6.5. Bands were expected for *HvBON1* at 501 bp, for *HvIOS1* at 401 bp and for *HvWRKY45-1* at 434 bp. Faint bands were observed at 500 bp following PCR amplification of *HvBON1* in Cassia and Golden Promise only (Figure 4.16).

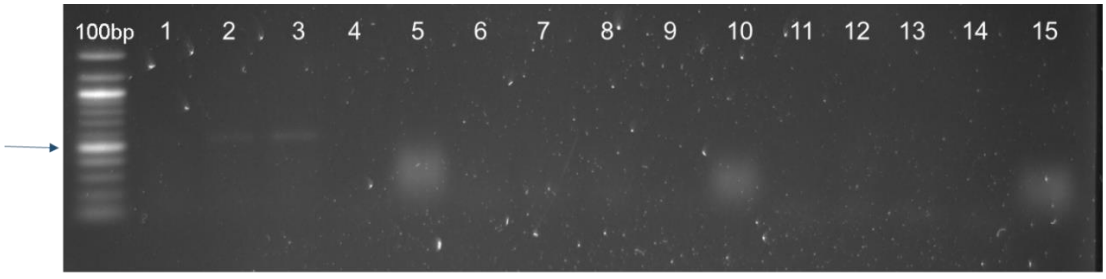


Figure 4.16: PCR amplification of *HvBON1, HvIOS1* and *HvWRKY45-1* cDNA fragments in Infinity, Cassia and Golden Promise. No template control with water in the place of template DNA; nonRT control with total RNA instead of cDNA to monitor for the presence of residual gDNA contamination. Blue arrow indicates 500 bp marker on Gene Ruler 100bp ladder (Thermo). Faint bands in lanes 2 and 3 correspond to the *HvBON1* amplicon in Cassia and Golden Promise respectively.

As no amplicons were detected for *HvIOS1* and *HvWRKY45-1,* to determine if the issue was related to the PCR conditions or the lack of target transcript in cDNA samples, PCRs using the same sets of primers using gDNA as a template were carried out. The primers AM103 and AM104 were used as a positive control for the amplification of the *ATE1* gene (1.2 kb) (Miricescu, 2019). Due to the presence of introns, the expected PCR amplicon size for *HvBON1* was 1.6 kb (3 introns present), *HvIOS1* was 501 bp (0 introns present) and *HvWRKY* was 727 bp (1 intron present). Faint bands were visible for *HvIOS1* amplicons in Golden Promise and Cassia at the 500 bp marker. Bands of the appropriate size were also visualised for *HvWRKY45-1* and the *ATE1* control in all three cvs. No amplicon was detected for *HvBON1* in any cv (Figure 4.17). As the same gDNA was used for each primer pair, it was concluded this is likely a result of issues in primer annealing. As amplicons ~1.2 kb were detected for the positive *ATE1* control it was concluded that there was no issues with the PCR protocol.

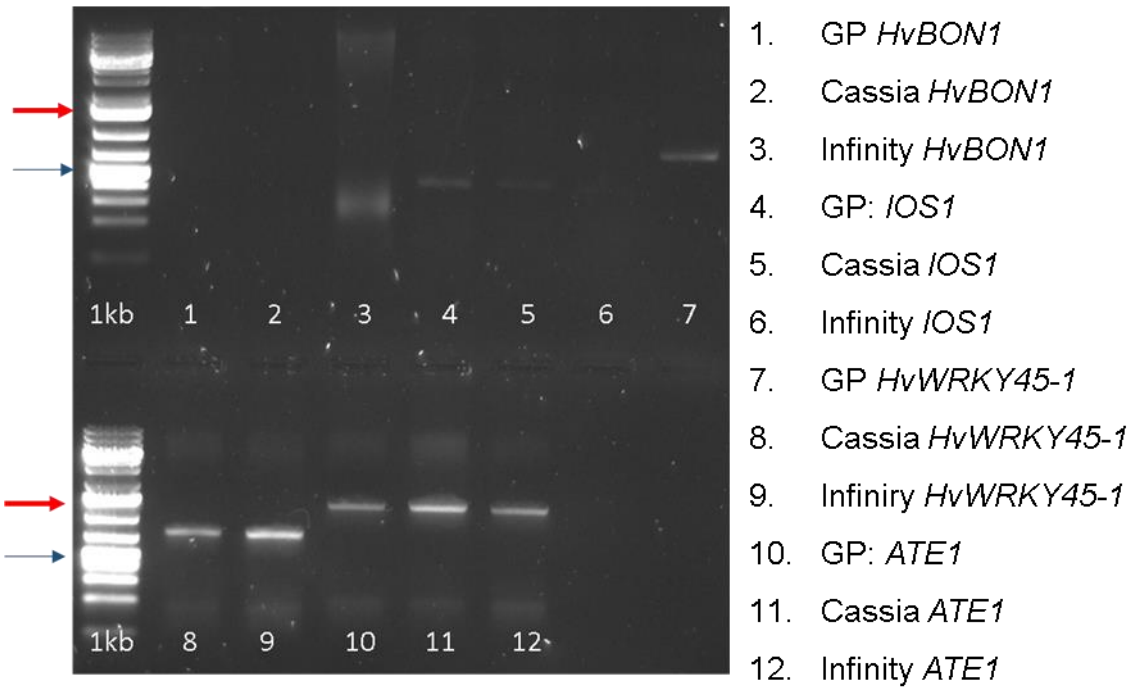


Figure 4.17: PCR amplification of *HvBON1, HvIOS1* and *HvWRKY45-1* using Infinity, Cassia and Golden Promise (GP) gDNA. Positive control with primers for *ATE1*. Blue arrow indicates 500 bp marker on Gene Ruler 1 kb+ ladder. Red arrow indicates 1 kb marker.

As cDNA for Cassia and Infinity was previously synthesised from RNA aliquots from the RNAseq experiment, the FPKM for the target gene in each sample was checked and the sample with the highest FPKM values in Cassia and Infinity were specifically selected to ensure sufficient transcript concentration. The volume of template DNA was increased from 5 µl to 9 µl in a 50 µl reaction. *ACTIN* was used as a positive control. No bands were visible for *HvIOS1* or *HvWRKY45-1* in any cv (Supplemental Figure S4.23, Appendix 2). A PCR product of ~500 bp for *HvBON1* was successfully amplified in Cassia and Infinity, as well as ~120bp amplicons representing *ACTIN* amplification. Multiple bands were detected using *HvBON1* primers, and PCR product of incorrect size using *ACTIN* primers were visualised for Golden Promise (Figure 4.18).

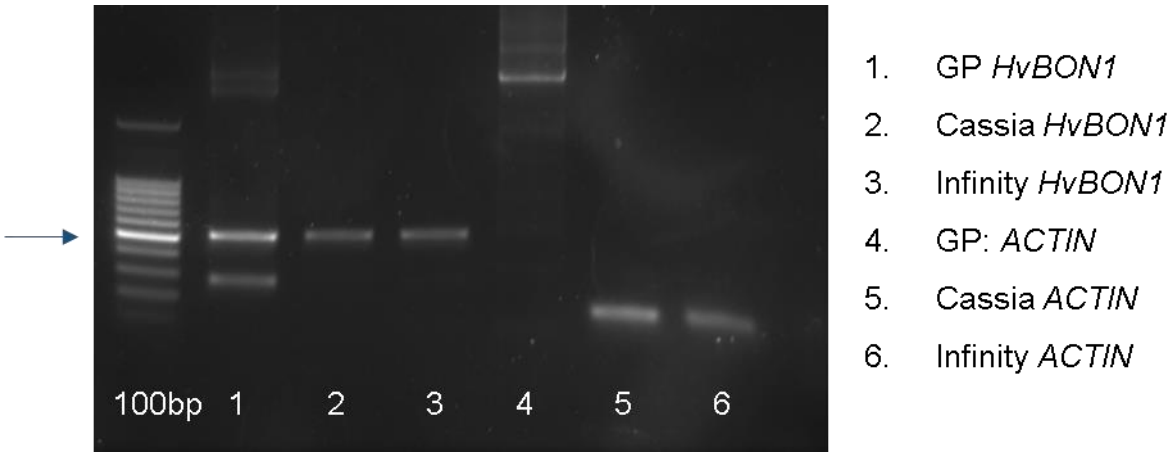


Figure 4.18: PCR amplification of *HvBON1* in Infinity, Cassia and Golden Promise (GP) cDNA. Positive control with primers for *ACTIN*. Blue arrow indicates 500bp marker on Gene Ruler 1 kb+ ladder.

All primers were originally designed based on the Morex genome sequence. However, as deletions were observed in the *HvWRKY45-1* sequence in Golden Promise compared to Morex, primers were redesigned for amplification of *HvWRKY45-1* using information from the Golden Promise genome sequence (TOC13 and TOC14, Table 4.3). However, this was done towards the end of this PhD, and therefore there was no opportunity to test

these primers. As the primers for *HvIOS1* target sequence were not intron spanning, cloning of the target sequence into pHANNIBAL using PCR fragment amplified from gDNA would also be suitable. As a result, the PCR for *HvIOS1* was repeated on gDNA of each cv but was only successful in Cassia (data not shown).

PCR fragments for *HvBON1* from Cassia, Golden Promise (Figure 4.16), Infinity (Figure 4.18) and *HvIOS1* from Cassia (data not shown) were cloned into pJET (Table 4.2) as described in section 4.3.6.5. After transformation of *E. coli* stbl2 competent cells, the sequence of each of the inserts in pJET was checked by Sanger sequencing. Sequences for *HvBON1* in Cassia, Infinity and Golden Promise were aligned to the Morex reference sequence to determine the presence of SNPs between the cultivars (Figure 4.19). Cassia and Infinity showed 100% alignment with the Morex reference sequence. Seven SNPs were detected between Golden Promise and Morex. Therefore, it was concluded that the same RNAi construct could be used for the knockdown of *HvBON1* in all three cvs. The sequence obtained for Cassia *HvIOS1* did not align to the Morex or the Golden Promise reference sequence. This would suggest that, unexpectedly, an incorrect gene was amplified during the PCR process.

The *HvBON1* fragment was cloned into the pHANNIBAL cloning vector in the sense orientation as described in section 4.3.6.5. The digestion of pJET_BON1 and pHANNIBAL was confirmed by agarose gel electrophoresis (Supplemental Figure S4.24, Appendix 2). The appropriate digestion products were extracted from the gel as described in section 4.3.6.5. After stbl2 transformation with the ligated pHANNIBAL_BON1sense plasmid, one colony was observed after 48 h incubation at 37 °C. The presence of the insert was confirmed with colony PCR (Figure 4.20 A) and SacI digest (Figure 4.20 B). The BON1 fragment was cloned again into the resulting pHANNIBAL_BON1sense plasmid, this time in the anti-sense orientation as described in section 4.3.6.5. The digestion of pJET_BON1 and pHANNIBAL_BON1sense was confirmed on an agarose gel (Supplemental Figure S4.25). Following ligation and *E. coli* transformation, the presence of both sense and antisense fragments was confirmed with XhoI digestion

(Figure 4.20 C) and Sanger sequencing, and final vector visualised in Figure 4.20 D.

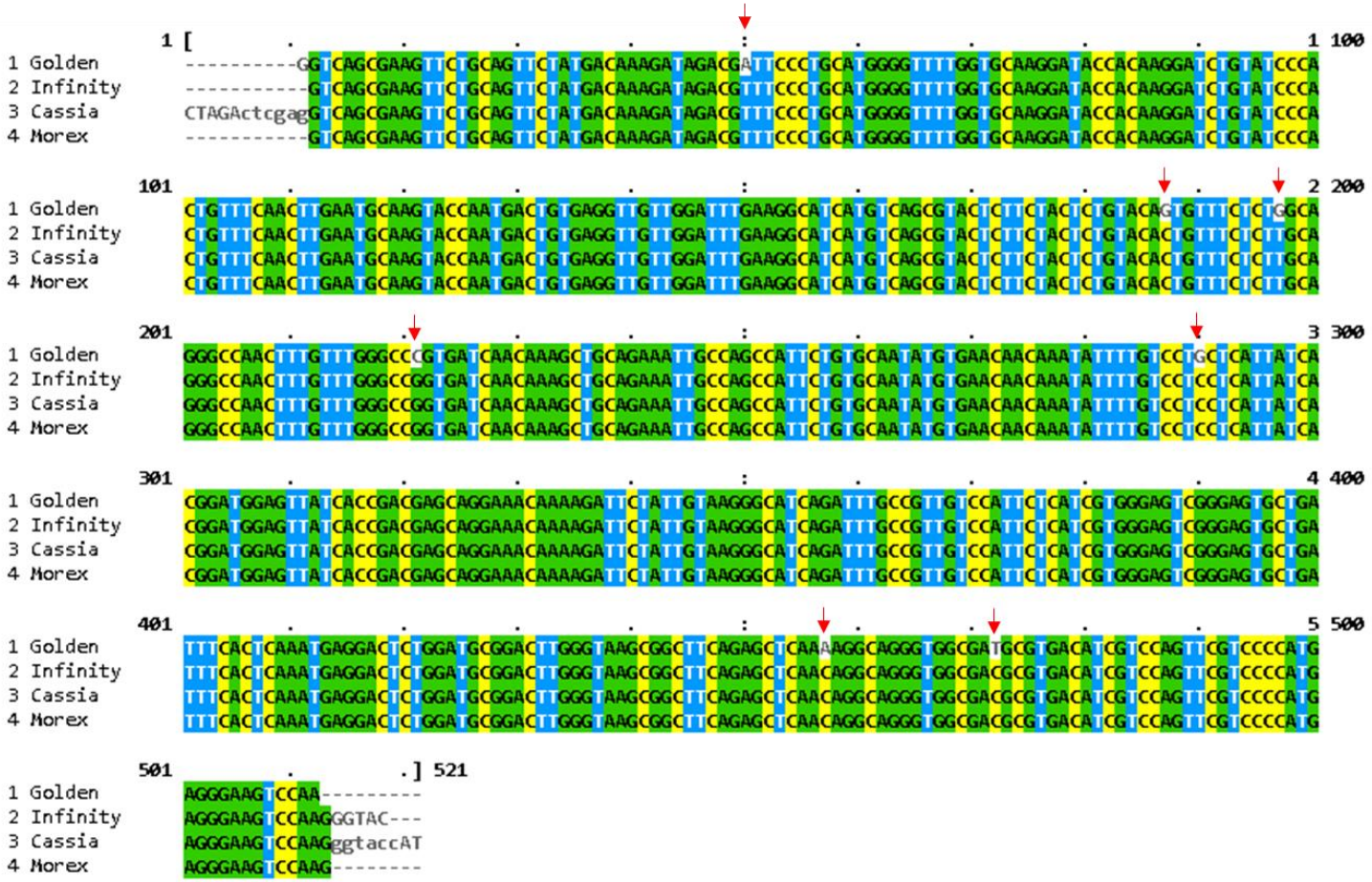
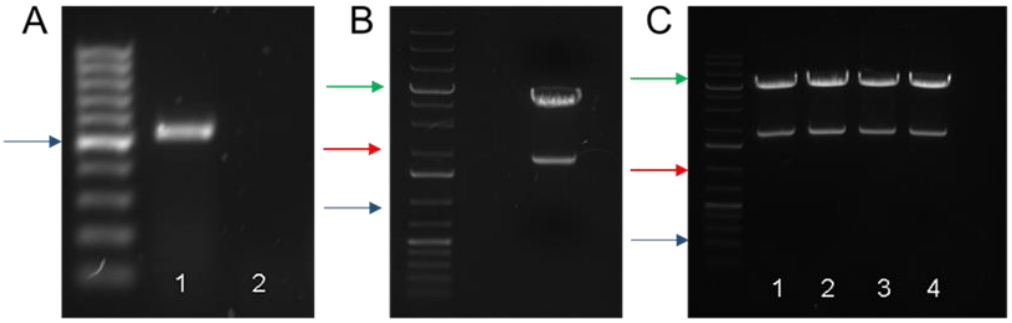


Figure 4.19: Nucleotide sequence of *HvBON1* fragment in barley cvs Golden Promise, Infinity, Cassia and Morex. SNPs indicated with a red arrow.



1. Colony from stbl2 transformation
2. Empty agar control

1-4. pHANNIBAL_BON1 plasmid DNA after XhoI digest

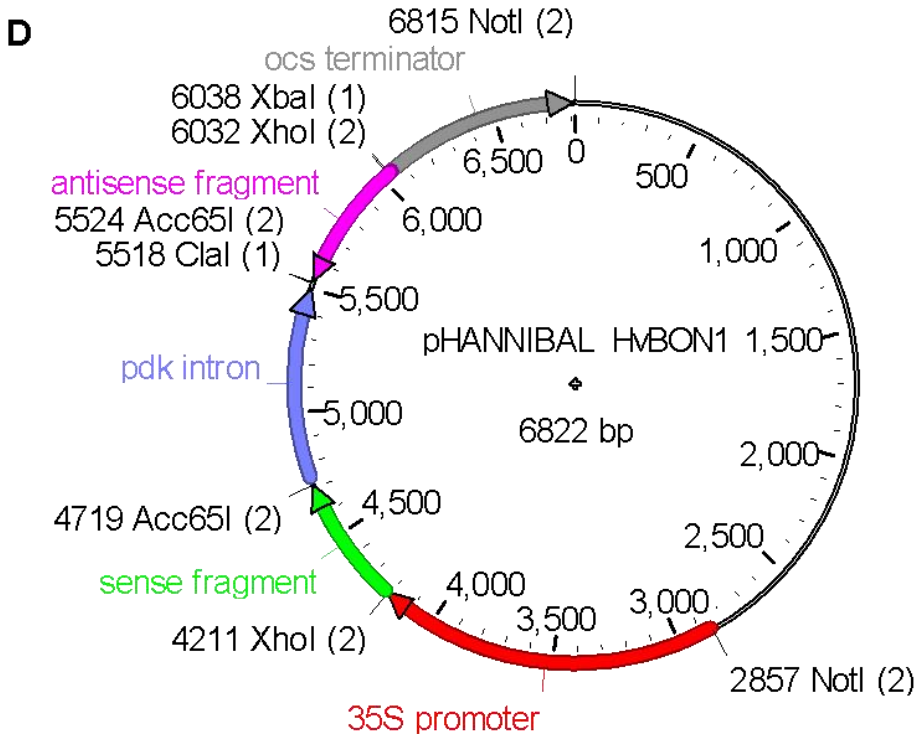


Figure 4.20: Cloning sense/anti sense *HvBON1* fragments into pHANNIBAL cloning vector. A: Colony PCR to confirm the presence of the *HvBON1* insert in sense orientation using the TOC1 and TOC2 primers. Blue arrow indicates 500 bp marker on Gene Ruler 100 bp ladder. B: Restriction digest to confirm insertion of BON1 in sense orientation using SacI to yield ~4.5 kb and ~1.8 kb bands. Blue, red and green arrows indicate 500 bp, 1 kb and 5 kb markers on Gene Ruler 1 kb+ ladder respectively. C: Restriction digest to confirm insertion of BON1 in both sense and anti-sense orientations using XhoI to yield ~5 kb and ~1.8 kb bands. Blue, red and green arrows indicate 500 bp, 1 kb and 5 kb markers on Gene Ruler 1 kb+ ladder respectively. Plasmids from four colonies tested. D: Visualisation of final pHANNIBAL_BON1 vector with both sense and anti-sense fragment successfully inserted. Figure generated using ApE software.

The next steps would have been to clone the relevant portion of the construct (in-between the 2 NotI sites) into the pML-BART plasmid, which codes for the T-DNA. However, due to time constraints this task was not completed.

4.4.3 Establishing a transient expression system in barley

One of the goals of this project was to validate, if time permitted, the function of the top candidate S genes. Considering the time needed to generate stable transformants in barley (~12 months from initiative to T_1 line confirmation), a transient approach to deliver and express the RNAi constructs was preferred. To do so, a series of preliminary experiments were completed to establish a reliable transient expression system using agroinfiltration on cvs Cassia, Infinity and Golden Promise with an *Agrobacterium* strain (C58 pGV2260) carrying the pEG356 plasmid (strain noted pGV2260_pEG356). This plasmid codes for a GUS reporter gene under the control of the CaMV 35S promoter. In a first experiment, leaves 1 and 2 of barley seedlings at GS13 were agroinfiltrated with pGV2260_pEG356 plated from glycerol stock before being transferred to liquid culture in YEB. Leaves were infiltrated at an OD_{600} = 2. The agro infiltrated leaf tissue was collected at 3, 4 and 5 dpi and each leaf was divided into 3 sections (proximal, middle and distal, corresponding to base, middle and tip of the leaves). As a positive control, leaves 1 and 2 of 4-6 week tobacco were also agroinfiltrated with the same suspension. GUS staining was observed at 2 dpi in leaf 2 of Infinity (Figure 4.21). This was localised to the mid-vein and leaf edge in middle and distal sections of the leaf. A small region of Infinity leaf 1 also showed localised staining. GUS staining was not observed in Cassia or Golden Promise. GUS staining was also not observed in any of the barley cvs at 4 and 5 dpi (Supplemental Figure S4.26, Appendix 2). Strong GUS staining was observed in tobacco leaf sections at 3 and 4 dpi, and was most intense at the cut edge. No staining was observed in tobacco at 5 dpi (Figure 4.22).

The transformation efficiency of the AGL1 *Agrobacterium* strain carrying the pEG356 reporter plasmid (strain noted AGL1_pEG356) was also tested. Similarly, seedlings of Golden Promise, Cassia and Infinity were agroinfiltrated with a bacterial suspension at an OD_{600} = 2, and tissue was collected at 3 and

4 dpi. Only Cassia and Golden Promise tissue was collected at 5 dpi, due to poor germination of Infinity resulting in insufficient plant material. In parallel, additional seedlings of Cassia and Golden Promise were agroinfiltrated with pGV2260_pEG356 and tissue collected at 3 and 4 dpi only. pGV2260_pEG356 was streaked from previously cultured plates. No GUS staining was observed in barley at any timepoint after infiltration with either strain (Supplemental Figures S4.27 and S4.28, Appendix 2). Unfortunately, no GUS staining was observed in tobacco after infiltration with either strain (Supplemental Figure 4.29, Appendix 2), suggesting a technical problem with the experiment, perhaps due to the use of a previous plate to restart the cultures for this experiment. Therefore, in the remaining agroinfiltration experiments, *Agrobacterium* strains were always plated straight from glycerol stocks. As a low level of GUS staining was observed in barley after the first agroinfiltration experiments (Figure 4.21), the C58 pGV2260 strain was used for all remaining experiments.

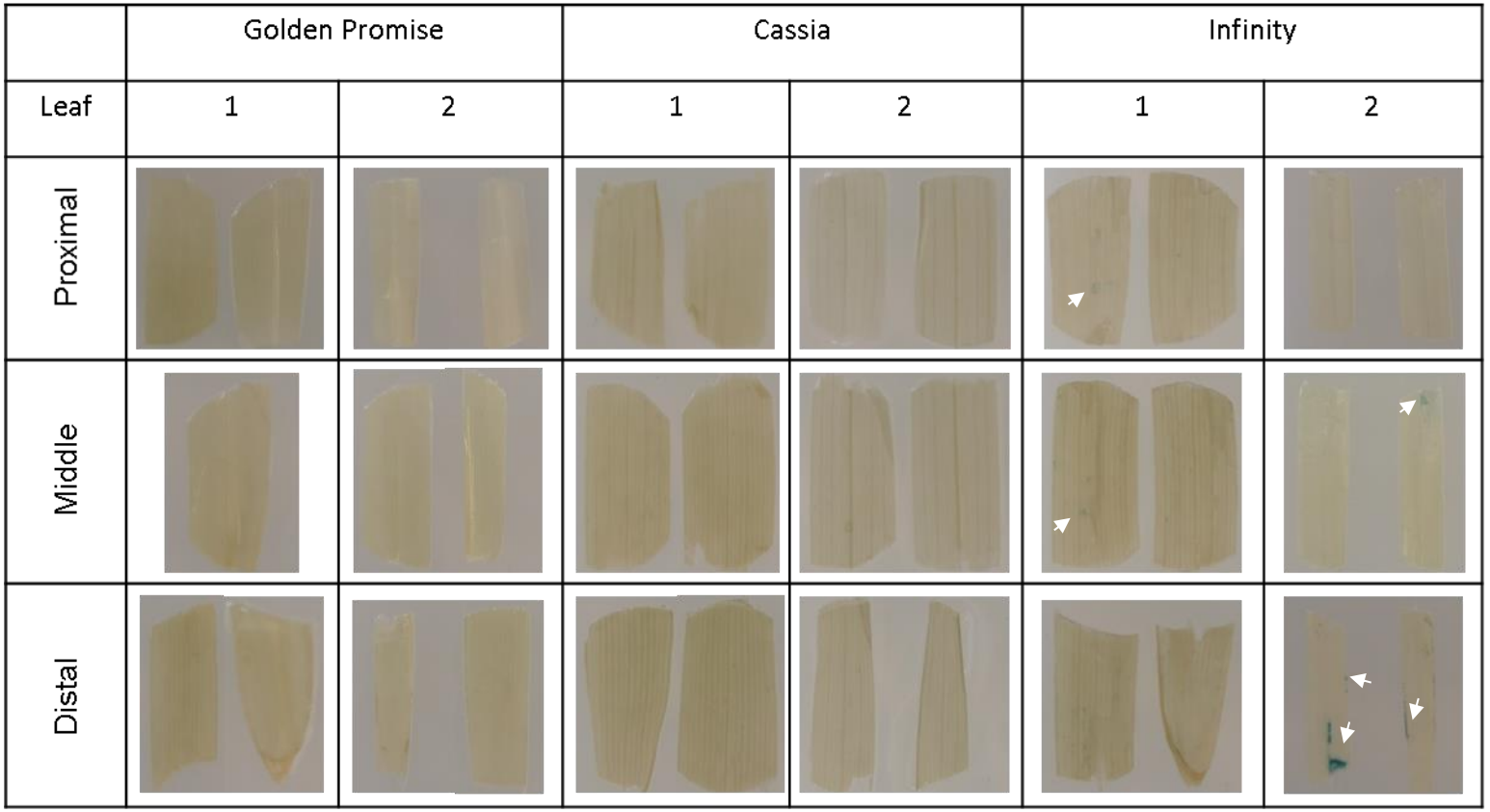


Figure 4.21: Barley leaf sections from proximal, middle and distal sections of leaf 1 and 2 of cvs Golden Promise, Cassia and Infinity 2 dpi after agroinfiltration with pGV2260_35S:GUS suspension of OD_{600} = 2. Localised staining was observed in Infinity in the distal and middle sections of leaf 2. Staining indicated with white arrows.

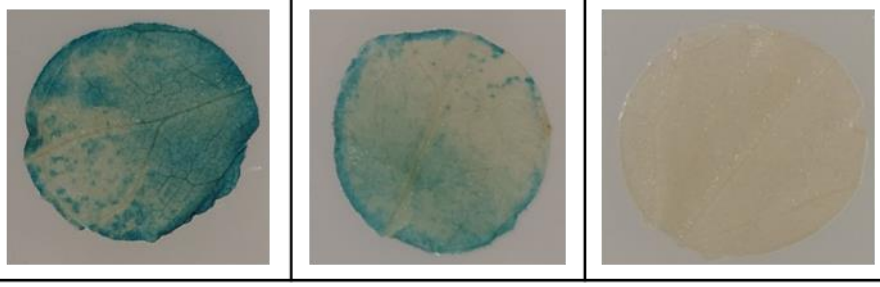
	Tobacco		
DPI	3	4	5
			

Figure 4.22: GUS staining of tobacco leaf disks Agroinfiltrated with pGV2260 with the 35S: GUS construct at a suspension of OD_{600} = 2. Leaf disks collected at 3, 4 and 5 dpi.

As GUS staining in barely was only detected at 3 dpi, and not observed at 4 or 5 dpi, it was hypothesised that the transient expression of the *iudA* gene may be occurring earlier than the timepoints tested. Therefore, the agroinfiltration process was repeated with pGV2260_pEG356 into Cassia, Infinity and Golden Promise, but collected leaf disks at 2, 3 and 4 dpi instead. The OD_{600} was also increased to 3 in order to have a denser suspension and potentially increase the efficiency of the transient transfection. As previously, leaves 1 and 2 of tobacco plants were used as a positive control. At 2 dpi, faint localised staining was observed in leaf 2 of Cassia and Golden Promise, but this was not observed in all leaf sections. No staining was observed in Infinity at 2 dpi. At 3 dpi, only a small portion (< 2%) of Cassia leaf 1, and leaf 2 of Golden Promise and Infinity showed any GUS staining (Figure 2.23). At 4 dpi, <1 % of Infinity leaf 1 showed GUS staining (Figure 4.23). GUS staining in tobacco at all three timepoints was almost completely abolished with staining only observed at the periphery of the leaf disk at 3 and 4 dpi (Figure 4.25) compared to infiltration of tobacco at OD_{600} = 2 (Figure 4.23). It was concluded that increasing the OD_{600} of the infiltration suspension from 2 to 3 did not increase transfection efficiency.

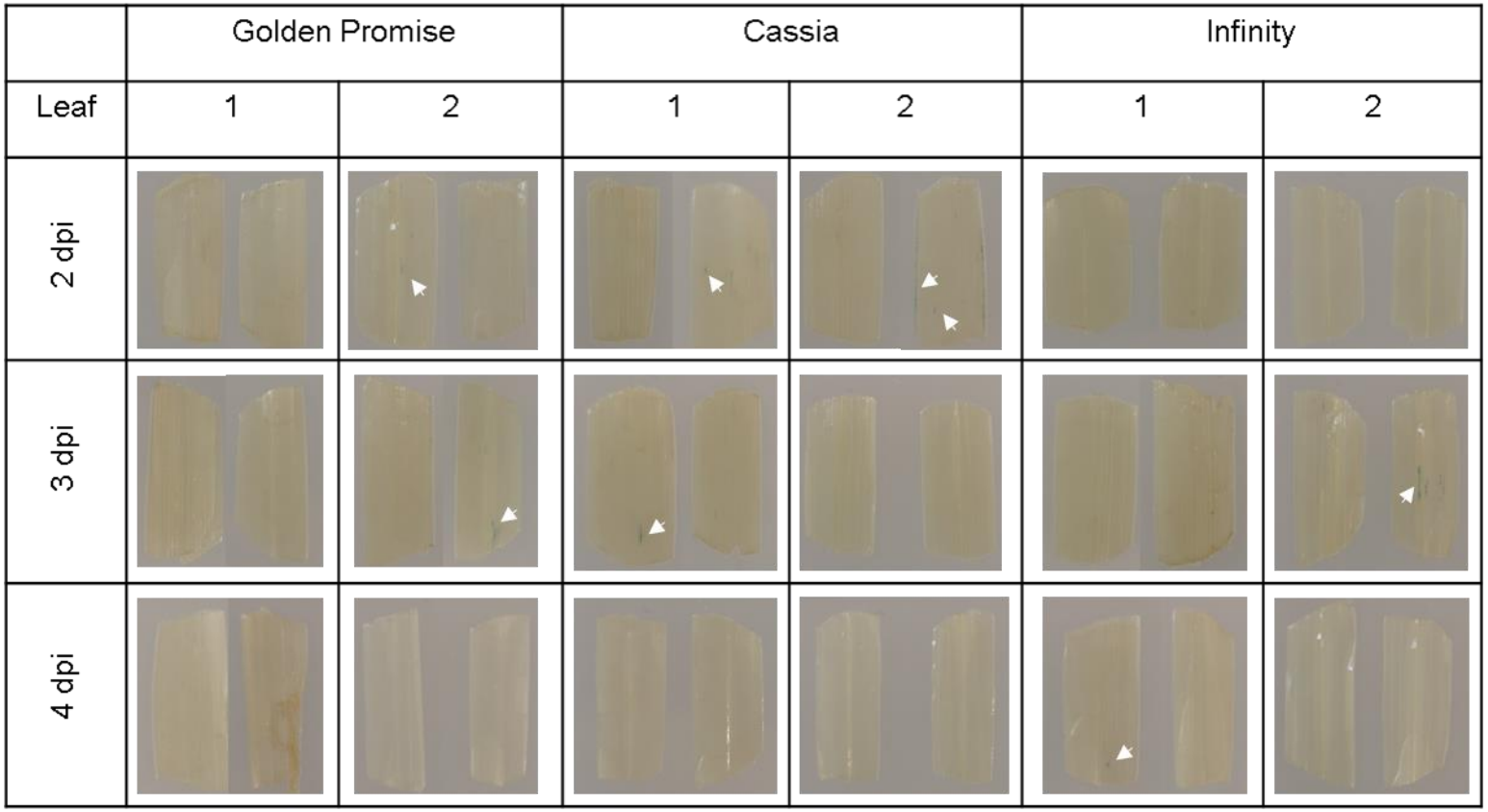


Figure 4.23: GUS staining of barley leaf sections agroinfiltrated with pGV2260 with the 35S:GUS construct at a suspension of OD_{600} = 3. Leaf disks collected at 2, 3 and 4 dpi are representative of the whole leaf blade. Staining indicated with white arrows.

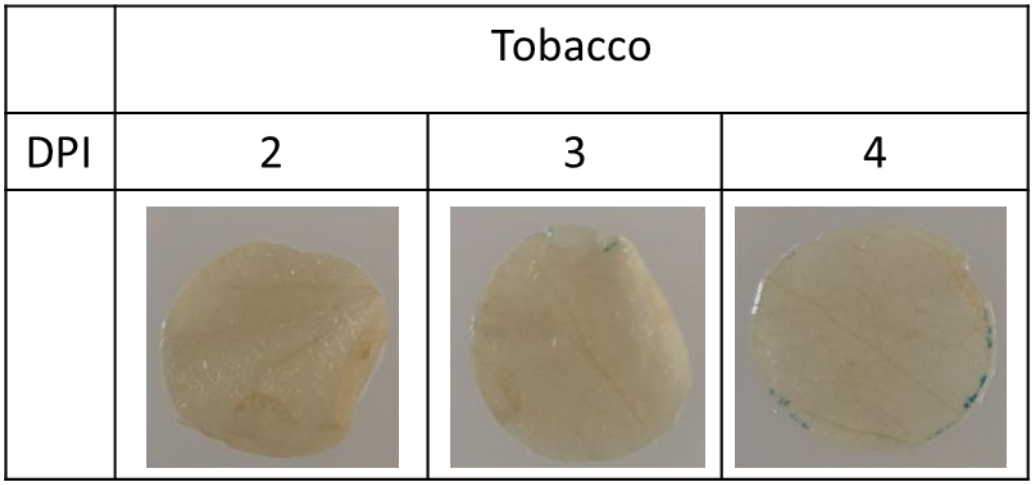


Figure 4.24: GUS staining of tobacco leaf disks Agroinfiltrated with pGV2260 with the 35S:GUS construct at a suspension of OD_{600} = 3. Leaf disks collected at 2, 3 and 4 dpi.

The CaMV 35S promoter is less active in monocots than in dicots. Therefore, the expression of a synthetic GUSPlus gene (this GUS gene is interrupted by an intron, to ensure that any signal obtained originated from *in planta* expression) driven by the maize *Ubi1* promoter (Vickers et. al., 2003) was also tested. The Ubi1:GUSPlus fragment, including the transcription terminator, was transferred from pUbiGUSPlus into the pML-BART plant transformation vector as described in section 4.3.6.4. The resulting plasmid (termed pTOC1) was confirmed by XhoI digest. Successful insertion of the Ubi1:GUSPlus region resulted in fragments of 5 kb and 10 kb after XhoI digest, as expected (Figure 4.25). Leaves 1 and 2 of Cassia, Golden Promise and Infinity (at GS13) were agroinfiltrated with the C58 pGV2260 *Agrobacterium* strain carrying the pTOC1 plasmid (noted pGV2260_pTOC1) at an OD_{600} of 2. Tissue from proximal, middle and distal sections of the leaves were collected for GUS staining at 2, 3 and 4 dpi. Leaves 1 and 2 of 4-6 week old tobacco plants were also agroinfiltrated with pGV2260_pTOC1 at an OD_{600} of 2 and at an OD_{600} of 0.75, as is typically done for tobacco agroinfiltrations. A positive control of tobacco infiltrated with pGV2260_pEG356 was also carried out, with tissue collection at the same timepoints. Also, as previous results showed more intense GUS staining along the cut edge of tobacco leaf disks (Figures 4.22

and 4.24), It was also possible that there may be an issue with the X-gluc substrate entering the cells. Therefore, the vacuum infiltration time was increased from 2 to 30 mins. In order to confirm expression of the GUS gene, RT-PCR was also carried out on leaf tissue after agroinfiltration. In order to ensure sufficient tissue volume for RNA extraction, barley leaf sections from same area (proximal, middle or distal) of leaves 1 and 2 were pooled. Remaining leaf disks from the proximal, middle and distal leaf sections of leaves 1 and 2 were kept for histochemical staining.

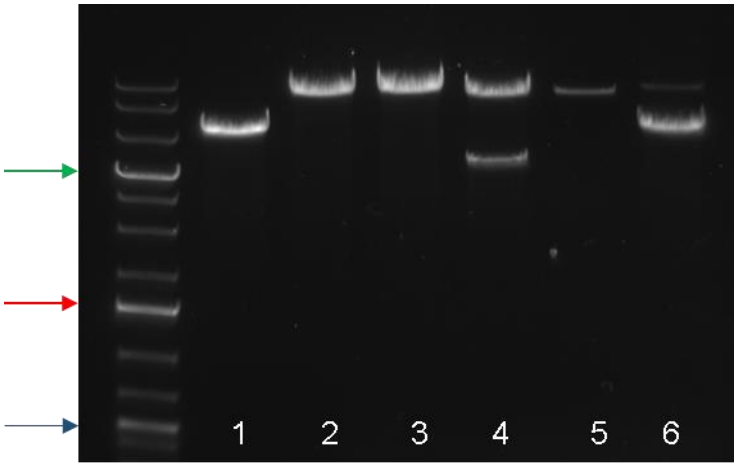


Figure 4.25: XhoI digest of plasmid DNA after cloning UBI:GUSPlus insert into pML_BART. Lanes 1-4: plasmid DNA from 4 colonies after stbl2 transformation and XhoI digest. Lane 4 shows presence of insert and bands of predicted size at 10 kb and 5 kb. Lane 5 represents pML_BART with no insert. Lane 6 represents pUBI:GUSPlus plasmid. Blue, red and green arrows indicate 500 bp, 1 kb and 5 kb markers on Gene Ruler 1 kb+ ladder respectively.

There was no GUS staining observed in barley after agroinfiltration with the pTOC1 plasmid (Figure 4.26). In addition, no expression of the GUSPlus gene was detected by RT-PCR (Figure 4.27). GUS staining in tobacco controls was only observed when driven by the 35S promoter at 3 and 4 dpi (Figure 4.28), which was also confirmed by RT-PCR (Figure 4.29). Interestingly, GUS expression was detected by RT-PCR in tobacco agroinfiltrated with pGV2260_pEG356 at 2 dpi, despite no GUS staining observed.

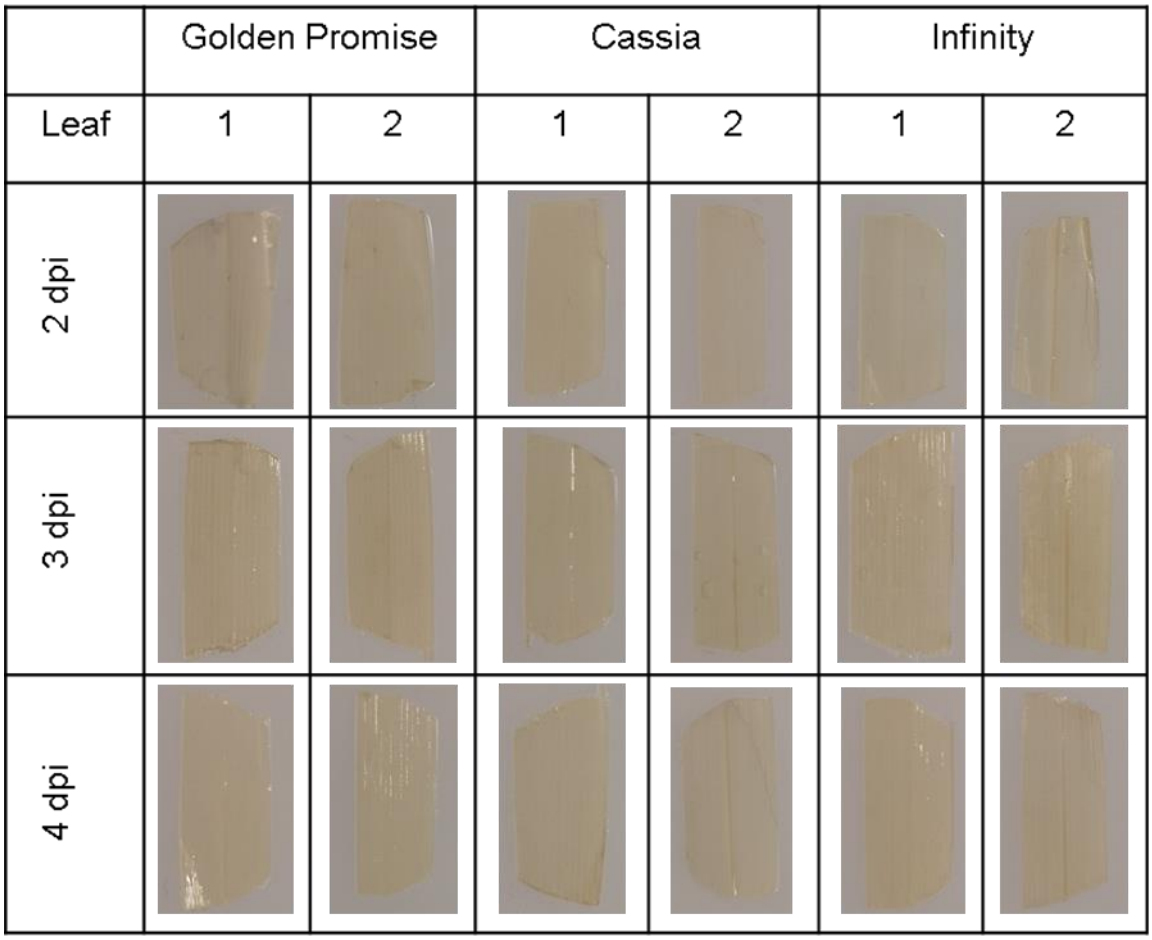


Figure 4.26: GUS staining of barley leaf sections agroinfiltrated with pGV2260_pTOC1 at a suspension of OD600 of 2. Leaf disks collected at 2, 3 and 4 dpi are representative of the whole leaf blade.

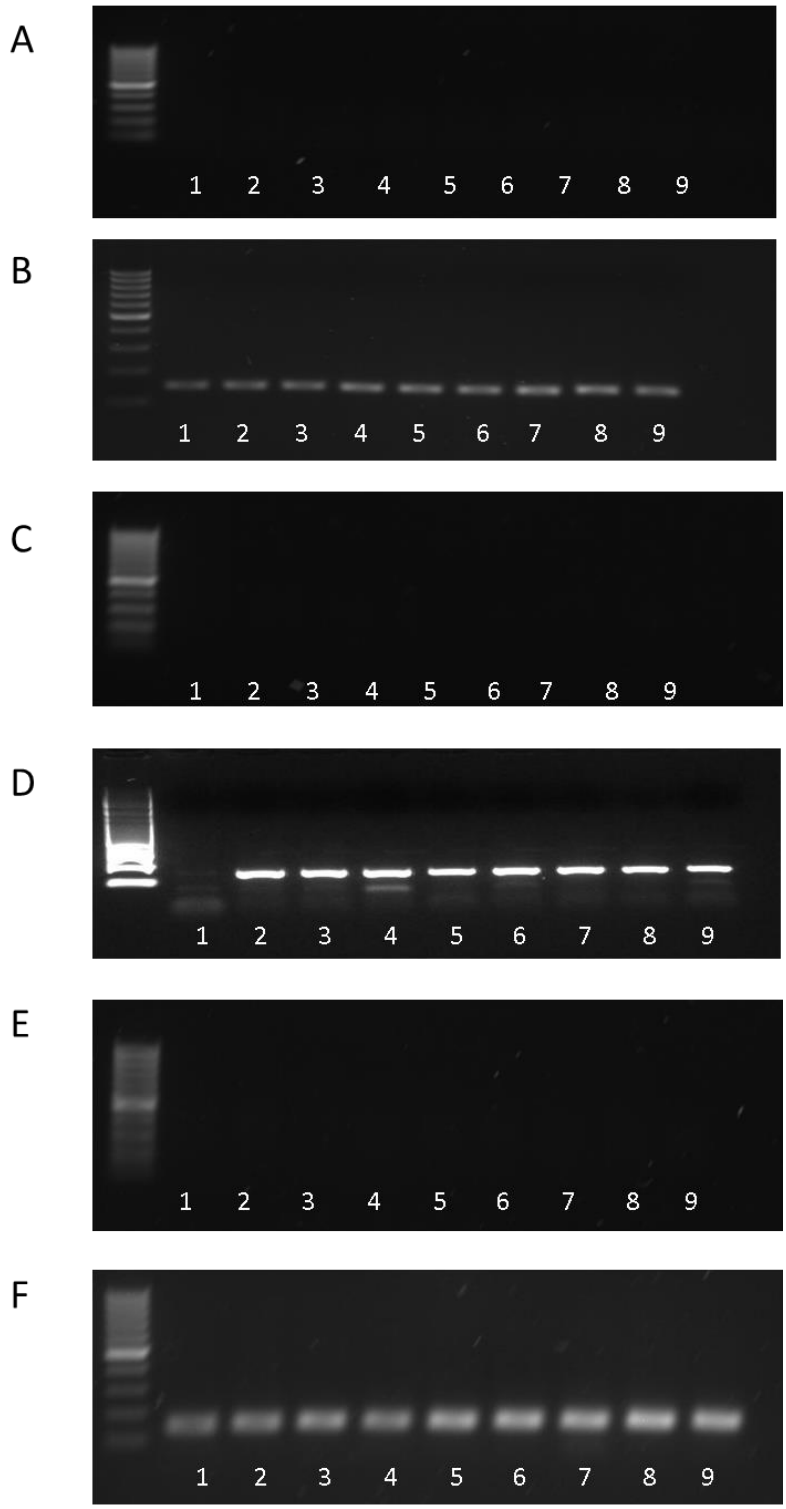


Figure 4.27: RT-PCR detection of GUS expression after agroinfiltration with pGV2260_pTOC1 at OD_{600} = 2 at A: 2 dpi, C: 3 dpi and E: 4 dpi. Detection of *ACTIN* after agroinfiltration with pGV2260_pTOC1 plasmid at OD_{600} = 2 at B: 2 dpi, D: 3 dpi and F: 4 dpi. Lanes 1-3: Golden Promise (proximal, middle and distal sections respectively). Lanes 4-6: Cassia (proximal, middle and distal sections respectively). Lanes 7-9: Infinity (proximal, middle, distal sections respectively). 100 bp Gene Ruler ladder was used in each panel.

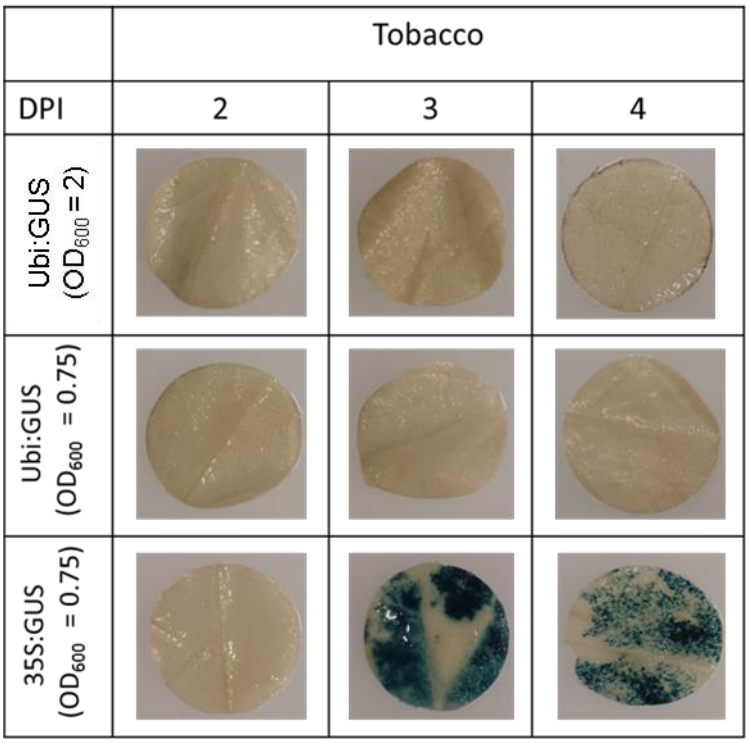


Figure 4.28: GUS staining of tobacco leaf disks agroinfiltrated with pGV2260_pTOC1 pGV2260_pEG356 at an OD_{600} of 2 or OD_{600} of 0.75. Leaf disks collected at 2, 3 and 4 dpi.

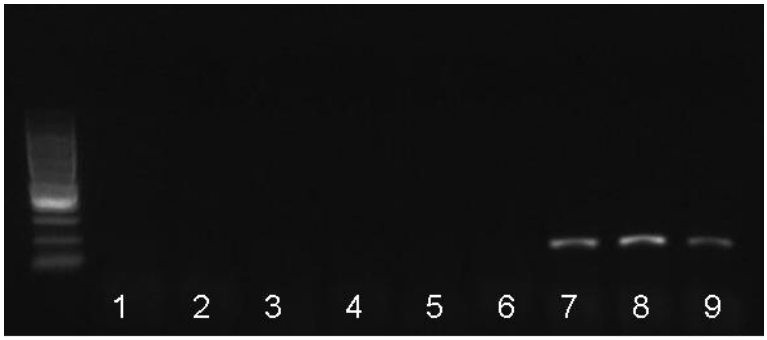


Figure 4.29: RT-qPCR detection of GUS gene expression in tobacco. Lanes 1-3: Tobacco agroinfiltrated with pGV2260_pTOC1 at an OD_{600} of 2, at 2 3 and 4 dpi respectively. Lanes 4 -6: Tobacco agroinfiltrated with pGV2260_pTOC1 at an OD_{600} of 0.75, at 2 3 and 4 dpi respectively. Lanes 7-9: Tobacco agroinfiltrated with pGV2260_pEG356 at an OD_{600} of 0.75, at 2 3 and 4 dpi respectively. 100 bp gene ruler ladder.

Agroinfiltration of barley seedlings with pGV2260_pEG356 and increased vacuum infiltration time resulted in stronger GUS staining observed in Golden Promise at the proximal end of leaf 2 at 2 dpi. However, this was not consistent

across the leaf blade (Figure 4.30). GUS staining was also observed at 2 dpi on the proximal end of Cassia leaf 2, and Infinity leaf 1. At 3 dpi, weak localised GUS staining was observed in Golden Promise leaf 1 and 2, but not in Cassia or Infinity. At 4 dpi, weak localised staining was recorded in leaf 1 and 2 in Cassia, but not in Golden Promise or Infinity (Figure 4.30). This was not consistent across the leaf blade, as separate portions of the same leaf did not show expression of GUS when confirmed with RT-PCR (Figure 4.31). Strong GUS staining was observed at 2, 3 and 4 dpi after agroinfiltration at OD_{600} = 2 and at OD_{600} = 0.75 (Figure 4.32). As previously observed (Figures 4.22 and 4.24). This was most intense at the cut edge of the leaf disk. GUS expression in tobacco leaves was confirmed by RT-PCR (Figure 4.33).

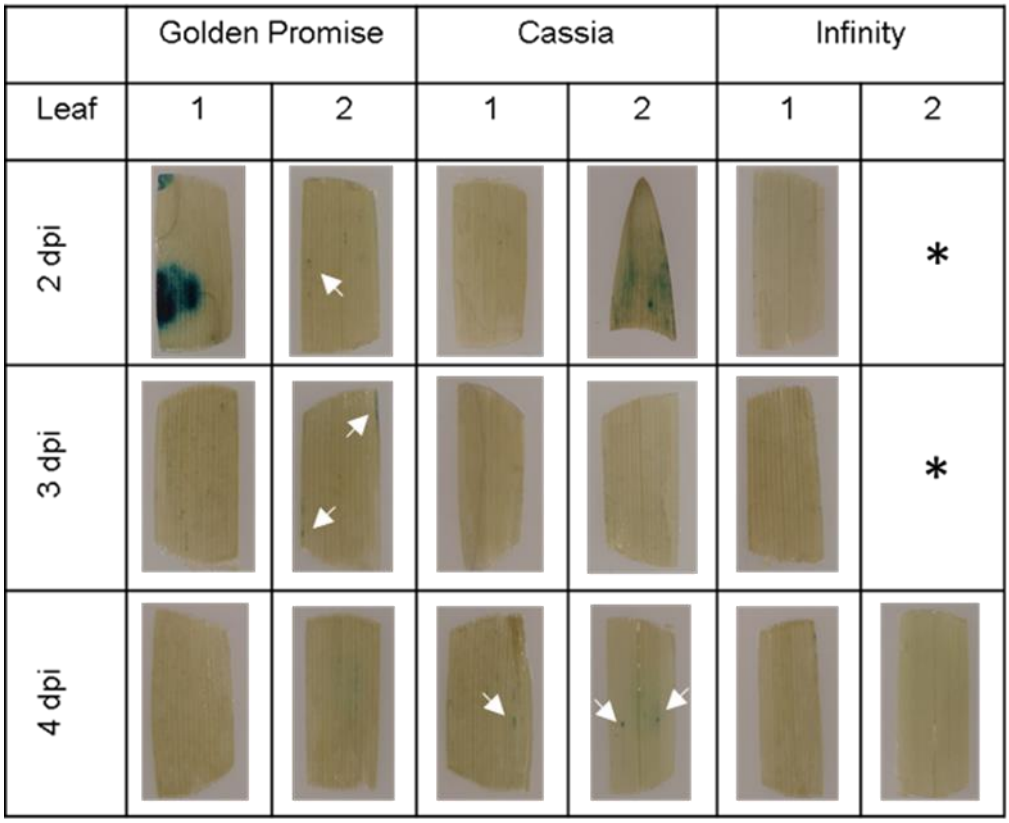


Figure 4.30: GUS staining of barley leaf sections Agroinfiltrated with pGV2260_pEG356 construct at a suspension OD_{600} of 2. Leaf disks collected at 2, 3 and 4 dpi. * Indicates insufficient leaf material available to complete histochemical analysis of GUS expression. Faint staining indicated with a white arrow.

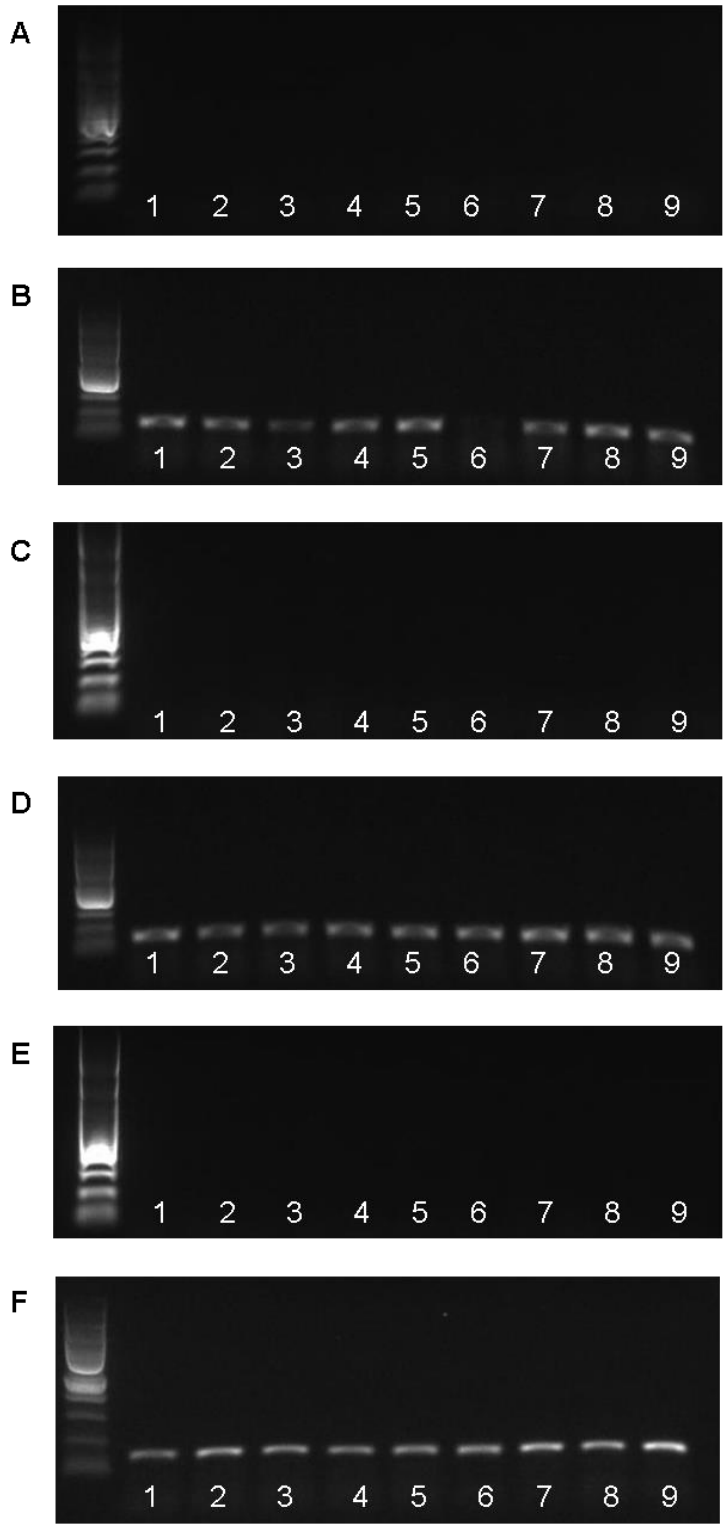


Figure 4.31: RT-PCR detection of GUS expression after agroinfiltration with pGV2260_pEG356 at an OD_{600} of 2 at A: 2 dpi, C: 3 dpi and E: 4 dpi. Detection of *ACTIN* after agroinfiltration with pGV2260_pEG356 plasmid at OD_{600} = 2 at B: 2 dpi, D: 3 dpi and F: 4 dpi. Lanes 1-3: Golden Promise (proximal, middle and distal sections respectively). Lanes 4-6: Cassia (proximal, middle and distal sections respectively). Lanes 7-9: Infinity (proximal, middle, distal sections respectively). 100 bp Gene Ruler ladder was used in each panel.

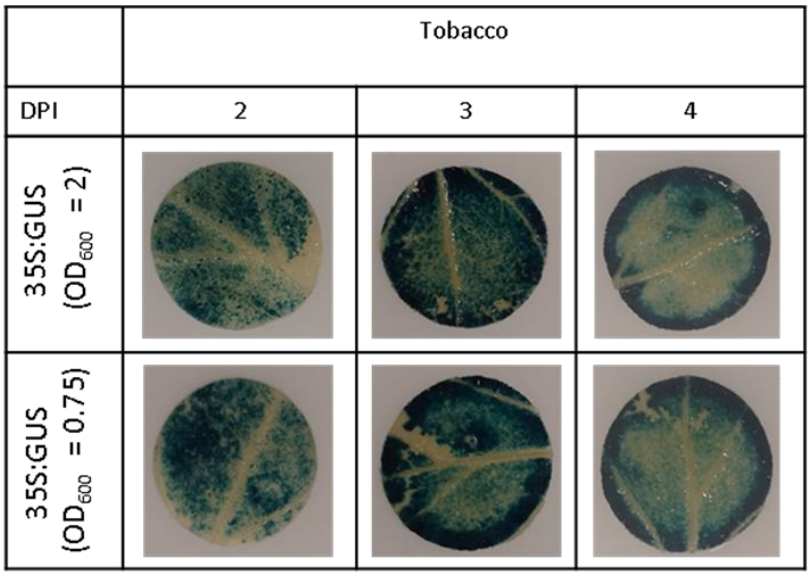


Figure 4.32: GUS staining of tobacco leaf disks Agroinfiltrated with pGV2260_pEG356 at a suspension of OD600 = 2 or OD600 = 0.75. Leaf disks collected at 2, 3 and 4 dpi.

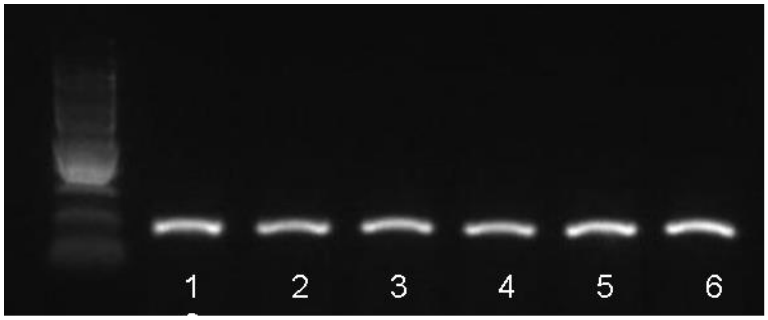


Figure 4.33: RT-qPCR detection of GUS gene expression in tobacco. Lanes 1-3: Tobacco agroinfiltrated with pGV2260_pEG356 at an OD_{600} of 2, at 2 3 and 3 dpi respectively. Lanes 4-6: Tobacco agroinfiltrated with pGV2260_pEG356 at an OD_{600} of 0.75, at 2 3 and 3 dpi respectively100 bp gene ruler ladder.

4.5 Discussion

On conclusion of Chapter 3, 53 candidate S gene orthologues had been identified from transcriptomic and *in silico* analyses. Based on (i) the expression profile of candidate genes determined by log_2(FC) after *R. commune* treatment relative to mock and (ii) the known functions of orthologous S genes, 17 genes were selected for validation by RT-qPCR and a deeper analysis of additional barley genes that belonged to the same family.

S gene selection and validation

In order to narrow down the list of 53 S gene candidates, the expression profile of each gene after *R. commune* treatment was examined. Genes that were strongly upregulated (as indicated by a higher log_2(FC) relative to mock) in either cv, or genes that were more strongly upregulated in Cassia compared to Infinity were selected. At least one gene that was selected through each approach described in section 3.4.3 (Figure 3.22) was included. Additionally, the role of the identified known S gene orthologue (Table 4.4) was considered and at least one gene corresponding to the different types of S gene (Type 1: aid the pre-penetration needs of the pathogen, Type 2: role in modulation of host defences, Type 3: facilitate post penetration needs of invading pathogen) was included. A total of 17 genes were selected for RT-qPCR validation, 5 of which are presented in section 4.4.1. The remaining validation plots can be found in Appendix 2. The expression profiles of each of these genes was examined, and how well RT-qPCR data correlated with FPKM values determined by RNAseq. The expression profiles of all genes within the same orthologous family were also examined. This was done to identify any similarly expressed genes that might suggest a level of functional redundancy between homologues in the same family. Genes were then ranked based on these criteria (Table 4.4). While only a small selection of genes were considered, of course if cannot be ruled out that within the remaining cohort of genes that were not selected for validation, some could still play an important role in susceptibly to *R. commune.* While their characterisation and validation as potential S genes will require follow on research, the datasets generated in

this project will provide the requisite foundation to support any future investigations.

Of the potential S genes prioritised, HORVU6Hr1G010680 was identified as a potential orthologue to *AtBON1* and selected because it was expressed at higher levels in Cassia compared to Infinity even in the absence of *R. commune* (Approach 3). In *Arabidopsis*, *AtBON1* has two known homologues (*AtBON2* and *AtBON3).* In *Arabidopsis*, the promoter region of *AtBON1* has SA, ABA and Ca^{2+} responsive elements, indicating it is likely to have a role in response to hormone signalling and during early defence signalling events (Lee and McNeillis, 2008). *AtBON1* expression has been shown to be upregulated by avirulent *Pst,* and the effect was more pronounced after inoculation with virulent *Pst* strains (Lee and McNeillis, 2008). Mutation of *AtBON1* resulted in increased programmed cell death and higher *PR1* expression, as well as reduced *Pst* growth within 3 dpi (Li et. al., 2009). In rice, there are two *BON* homologs: *OsBON1* (closely related to *AtBON1)* and *OsBON3* (closely related to *AtBON3)* (Yin et. al., 2018). RNAi mediated knockdown of *OsBON1* resulted in increased resistance to *Xoo* as determined by lesion size and colony forming units (c.f.u.). Knockdown lines also showed reduced hyphal growth of *M. oryzae* and increased field resistance to necrotropic *R. solani* (Yin et. al., 2018). This gene therefore appears to be an S gene that confers susceptibility to a wide range of pathogens with different lifestyles. Given the role of *BON1* homologues in rice and *Arabidopsis*, it is possible that orthologous barley genes also play a similar role. Therefore, disruption of this gene in a susceptible barley variety such as Cassia may confer an 'Infinity like' resistant phenotype, if expression was reduced to levels observed in Infinity.

Also within the top ranked genes was HORVU5Hr1G122390, a proposed orthologue to *AtIOS1.* The function of this gene in barley is not characterised. In *Arabidopsis*, this gene has been shown to be upregulated after infection with the downy mildew pathogen *Hpa* during both early (8-12 hpi) and late defence responses (4-6 dpi). *Agrobacterium* mediated mutations of *AtIOS1* by floral dip showed between 40-60% reduced sporulation of virulent *Hpa* and reduced hyphal growth, yet no change in haustoria formation (Hok et. al.,

2011). Identification of *AtIOS1* orthologues (such as HORVU5Hr1G122390 whose mutation reduce sporulation and hyphal growth in barley would be beneficial for improving resistance to *R. commune.*

However, *AtIOS1* does not appear to act as an S gene in other pathosystems. Indeed, *Arabidopsis ios1* T-DNA mutant lines also show weaker association of FLS2 with BAK1, an essential step in the activation of downstream signalling pathways (Chinchilla et. al., 2007), and increased susceptibility to *Pst* (Yeh et. al., 2016). *AtIOS1* also appears to be involved in the ABA-dependent regulation of stomata opening (Hok et. al., 2014). After infection with *R. solanacearum, PR1* and *PR4* expression was reduced in *Arabidopsis ios1* mutant lines compared to WT (Hok et. al., 2014). While mutations in *Arabidopsis ios1* confer resistance to *Hpa,* dampened PTI responses in mutant lines after *Pst* and *R. solanacearum* indicate that this actually may not be a suitable candidate for *R. commune* S gene mediated resistance. Further functional analysis of the proposed *AtIOS1* orthologue, HORVU5Hr1G122390, is required to determine its role in the barley x *R. commune* pathosystem.

HORVU2Hr1G109330 codes for a potential orthologue to *OsWRKY45-1.* In barley, it has been shown to play a role in the general stress response. Specifically, it is upregulated in 7 day old seedlings in response to drought and cold stress (Blake et. al., 2014). There are two *OsWRKY45* alleles described in rice, *OsWRKY45-1* (described in *O. sativa* subsp. *japonica*) and *OsWRKY45-2* (described in *O. sativa* subsp. *indica)* (Tao et. al., 2009). The role of *OsWRKY45-1* in rice susceptibility appears to be pathogen dependent. For example, overexpression of *OsWRKY45-1* has been shown to have increased resistance to *M. grisea* at the pre-invasive stage through blocking of appressorium formation, but also at the post-invasive stage through increased H_2O_2 production at the site of penetration, resulting in HR and restricted fungal growth in a detached leaf assay (Shimono et. al., 2007). During early stages of infection, *R. commune* also forms appressoria. Assuming a similar role of the barley orthologue HORVU2Hr1G109330, knockdown of this gene may not reduce *R. commune* colonisation of barley tissue during early stages of infection.

OsWRKY45-1 knockout lines have also been shown to have resistance to *Xoo* with increased expression of defence related genes such *PAL1, PAD4, PR1a, NH1, LOX,* and *PR1b*, many of which act downstream of SA and JA signalling. Interestingly, SA production was reduced in *Oswrky45-1* lines (Tao et. al., 2009). Therefore, this may pose problems if the HORVU2Hr1G109330 orthologue is also involved in SA signalling. Further functional analysis of the proposed orthologue, HORVU2Hr1G109330 is required to determine its suitability as a target for *R. commune* resistance breeding.

Generation of RNAi knockdown constructs

Transient RNAi knockdown of candidate S gene orthologues was preferred over CRISPR/Cas9 mediated editing, as it has been reported to be a relatively quick method compared to stable systems (Lück et al., 2019). CRISPR/Cas9 mediated knockout of candidate S genes would be a lengthier procedure requiring the co-cultivation of immature barley embryos with *Agrobacterium* carrying relevant constructs followed by regeneration and selection of transformants (Hardwood et. al., 2009). As this work was completed towards the end of the PhD, it was decided that transient RNAi knockdown would be a more suitable strategy, with which to attempt to obtain seedlings with reduced candidate S gene activity. All three of these S gene candidates had additional barley genes in their respective gene families identified by OrthoMCL. Additionally, *HvIOS1* and *HvWRKY45-1* both had additional genes in their respective S gene families that followed similar expression patterns in Cassia and Infinity after *R. commune* infection (Figures 4.4 and 4.8). When designing RNAi constructs for these genes, primer sequences were placed in regions unique to the target gene of interest to clone fragments with more sequence variation between the genes of the same family. However, for *HvBON1,* it is likely not possible to avoid targeting HORVU1Hr1G058000 along with *HvBON1* (Figure 4.14)

Several issues were encountered when generating RNAi constructs for these genes. The most significant relating to the fact that a reference genome is not available for Cassia and Infinity; so it was not possible to check primer sequences for SNPs between Cassia, Infinity and Morex. However, it was

possible to check the sequences against the published genome for Golden Promise (Schreiber et. al., 2020). Surprisingly, there were deletions in the Golden Promise Hv*WRKY45*-1 sequence compared to Morex, including a deletion at the location of the reverse primer (TOC6). While there was a ~700 bp amplicon detected after PCR amplification on gDNA (Figure 4.17), amplification of the target sequence was not achieved on cDNA (Supplemental Figure 4.23, Appendix 2). Sequencing of the gDNA fragment is required to confirm if it was indeed *HvWRKY45-1,* and if so, accurate primers could be redesigned that are appropriate for PCR amplification on cDNA. While bands of the predicted size were observed for *HvIOS1* amplification, Sanger sequencing showed that the fragment cloned did not correspond to *HvIOS1* in Morex or Golden Promise. This would suggest that the primers used were not specific and had amplified a different gene region. Despite these limitations, the RNAi construct for *HvBON1* was successfully generated*,* however, this was achieved towards the end of the PhD timeline, and therefore the construct has not yet been validated.

The next steps in this work would be to validate the *HvBON1* RNAi construct via agroinfiltration followed by RT-qPCR to confirm downregulation of *HvBON1* in Cassia, Infinity and Golden Promise. *HvBON1* is expressed at low levels in Infinity in the absence of *R. commune* compared to Cassia (Figure 4.3), was not determined to be differentially expressed after *R. commune* inoculation. RT-qPCR could be used to determine if RNAi knockdown in Cassia reaches similar expression levels as in Infinity, and if this confers a resistant phenotype in Cassia similar to that observed in Infinity. Next steps would also include the generation of RNAi constructs to test additional S gene candidates described in Table 4.4. Further functional characterisation of S gene candidates could be achieved via CRISPR/Cas9 mediated stable knockout. This could be used to validate the role of these genes in the barley response to *R. commune* as well as to determine any pleiotropic effects on other important agronomic traits such as yield that may arise from S gene manipulation.

Virus-induced gene silencing (VIGS) would be another approach that could be used to validate the function of the S gene candidates discussed in this

chapter. This involves the use of a viral vector to trigger transient systemic silencing of a gene of interest (Burch-Smith et. al., 2004). Indeed, the barley stripe mosaic virus (BSMV) has been successfully used to achieve significant silencing of *RAR1, SGT1* and *HSP90* in barley to determine the role of these genes in *Mla* mediated resistance to *Bgh* (Hein et. al., 2005). As VIGS is dependent on the plant's ability to tolerate the presence of the virus, barley cultivars must first be tested to determine their ability to tolerate BSMV accumulation without resulting in infection symptoms. VIGS has also been successfully achieved in wheat with the BSMV vector (Tai et. al., 2005), and in barley, rice and maize with a Brome mosaic virus (BMV) vector (Ding et. al., 2006).

This chapter also aimed to establish an *Agrobacterium* mediated transient expression system in barley. The agroinfiltration protocol is advantageous because it does not require specialised equipment as micro projectile bombardment, or the use of plant protoplasts. Agroinfiltration has also been successfully described in a number of crop species including barley (Lu et al., 2016), wheat, maize (Zhang et. al., 2017), potato (Bhaskar et. al., 2009), and soybean (King et. al., 2015). While the AGL1 strain has been reported to efficiently transform barley plants (Lu et. al., 2016), this strain did not grow well in culture over the course of the work described in this chapter, and also did not successfully transform Cassia, Golden Promise or Infinity. The transformation efficiency of the C58 pGV2260 strain which has been reported to efficiently transform tobacco, *Brassica rapa* and *Brassica napus* (Mooney and Graciet, 2020) was also tested. However, despite attempts with different $OD_{600,}$ expression of the GUS gene was inconsistent in barley. When GUS staining was observed, it was weak and localised, and not to the level that would be considered robust enough for use in this work. Further work to optimise this protocol is required. For example, increasing the $OD_{600} > 2$ is an option but $OD_{600} = 2$ was selected based on previous success with AGL1 infiltration of barley tissue (Lu et. al., 2016) and increased OD_{600} may also induce post transcriptional gene silencing (PTGS) (Faizal and Geelen, 2012). The timepoints sampled were selected based on success in other plant species, however, additional timepoints should be sampled to determine

optimum *Agrobacterium* duration in barley. Co-infiltration with the p19 protein has been shown to increase *Agrobacterium* mediated transgene expression through suppression of PTGS (Norkunas et. al., 2018). Additionally, the use of 5-azacytidine, ascorbate acid and Tween 20 were shown to improve transformation of tobacco six fold (Zhao et. al., 2017). The agroinfiltration protocol was only tested on barley plants at GS13, as this was the age of seedlings that were used to describe the infection process of the two *R. commune* isolates OP18(9) and 44.07 (Chapters 2 and 3). Transformation efficiency should also be tested at an additional growth stage, as plant age can also affect transient transformation (Kaur et. al., 2021). Indeed, while a set of gene candidates have been identified, their validation must be complemented via both transient and stable transformation, with a view to disrupting and over-expressing the target sequence. Based on the latter results of this chapter, the transformation-based validation protocol will require significant optimisation in order to analyse the function of the putative candidate S genes.

5 Conclusions

The main aim of this PhD was to identify novel sources of resistance to *R. commune* in barley. This is of particular importance, as genetic host resistance to pathogens is a cornerstone of maintaining sustainable agricultural practices via Integrated Pest Management (IPM). A key concept of IPM is in prioritising biological, physical and other non-chemical methods over chemical control to reduce reliance on conventional pesticides and combat issues such as pesticide resistance, decline in insect populations and environmental contamination (FAO, 2020). This, combined with the EU's 'Farm to Fork' strategy, which aims to reduce pesticide usage by up to 50% by 2030 (European Commission, 2020), highlights the importance of research into host-pathogen interactions with the ultimate goal of expanding our knowledge base and identifying novel sources of genetic resistance.

However, chemical control still remains the basis of many plant protection programmes, for example, control of *P. infestans* requires fungicide application on a regular and preventive basis, with high pressure conditions resulting in treatment intervals that are as low as 5 days (Cooke et. al., 2011). Control of winter wheat diseases requires four fungicide applications (both single site and multi-site) during a single growing season (Collins and Phelan, 2020), however, *Zymoseptoria tritici* establishment is still problematic on fungicide treated wheat (Kildea et. al., 2021). Control of *R. commune* still currently requires 2-3 fungicide treatments in a single growing season, usually depending on the developmental stage of the plant as opposed to the presence/absence of the disease. While fungicide application is effective in controlling *R. commune*, it does not contribute to long term, sustainable disease management and ultimately erodes farmer's margins.

Qualitative R gene mediated resistance is well documented in the literature (reviewed by McDowell et. al., 2003; Gururani et. al., 2012; van Wersch et. al., 2020). However, there are also numerous reports of R gene breakdown in different pathosystems. For example, a number of R genes that confer resistance to the wheat leaf rust fungus *Puccinia triticina* have been described (Long and Kolmer, 1989), however it has been found that 22 stem rust R genes

are ineffective against *P. graminis* races belonging to the Ug99 lineage (Singh et. al., 2011). In rice, the cv IRBLta2-Re containing the R gene *PITA2* was ranked as resistant to the rice blast pathogen *M. oryzae* in the Philippines and Burundi between 2014 and 2016 but was later found to be susceptible in 2017 and 2018 as a result of the breakdown *PITA2* mediated resistance (Meng et. al., 2020). In wheat, the winter cv Cougar was consistently ranked as resistant to *Z. tritici,* however recently, high levels of disease were recorded on Cougar and other cvs derived from Cougar as a result of R gene breakdown (Kildea et. al., 2021). Virulent *Z. tritici* has also been detected on resistant cv Cellule carrying the *Stb16q* R gene (Kildea et. al., 2020). The impact of both is significant, as the Cougar source of resistance has been bred into several elite lines set for commercialisation in Ireland and the UK.

As with over reliance on pesticide use, single gene mediated resistance applies selection pressure on the pathogen, resulting in evolution of new pathotypes lacking corresponding avirulence genes (Stukenbrock and McDonald, 2008). The issue is also compounded by the population dynamics of the pathogen. In the case of *Septoria*, a single field can account for 92% of the global genetic diversity, due to the promiscuous nature of the pathogen, which drives the rapid evolution of novel strains (McDonald and Mundt, 2016). Similarly, with *R. commune,* 76% of the global genetic diversity can be attributed to within a single barley field (Linde et. al., 2003). As a result of its high genetic diversity, *R. commune* has the ability to adapt at a field level, within a single growing period. Taken together, it is therefore critical to re-evaluate resistance breeding strategies to rely less on sole R gene resistance and to focus more on the potential overlapping roles of more durable approaches such as S gene mediated resistance as part of quantitative 'partial' resistance and disease tolerance approaches.

The potential of S gene mediated resistance has emerged in recent years as an alternative strategy to promote IPM-based strategies. However, the primary difficulty is in the identification of candidate S genes, as the primary role of these genes may not necessarily be related to defence responses. For example, *Arabidopsis DIHYDRODIPICOLINATE SYNTHASE 2* (*DHDPS2)* is involved in lysine production during seed development (Galili et. al., 2001),

however this gene was later shown to contribute to susceptibility to *Hpa.* Stable knockdown of this gene showed reduced colonisation of *Hpa,* although the mechanism behind this remains unclear (Stuttmann et. al., 2011).

'Disease tolerance' was first described in susceptible wheat that did not show any loss in yield or quality after severe *Puccinia recondite* infection (Caldwell et. al., 1934; Caldwell et. al., 1958). 'Disease tolerance' is now described as the ability of a host to minimise the effects of infection, regardless of pathogen load (Pagán and García-Arenal, 2018). Disease tolerance allows harmful pathogens to exist at acceptable levels (i.e., levels that do not trigger disease symptoms in the host). While tolerance would lead to increased prevalence of the pathogen, this would potentially confer broad spectrum 'resistance' without exerting selection pressures on pathogen populations. A number of traits contributing to wheat tolerance of *Z. tritici* have been described, including traits for thousand grain weight (TGW), increased grains per ear and radiation interception (Appelgren 2017). Disease tolerance needs to be further investigated in respect to specific pathosystems to determine the threshold at which the level of infection becomes an economic problem. However, tolerance related traits in barley to *R. commune* have not yet been investigated.

One of the aims of this thesis was to characterise host responses of susceptible and resistant barley cvs to two Irish *R. commune* isolates. A primary question in the barley x *R. commune* pathosystem is the fact that the temporal response of the host has yet to be fully characterized. Therefore, one of the challenges faced during this project was to identify timepoints in the barley *R. commune* interaction that would yield meaningful information on host responses. To address this, in chapter 2, the expression of several barley genes with a potential role in defence against *R. commune* was first investigated. The work described in chapter 2 and 3 focused on early infection stages and transition to necrotic stages, as initial phases of plant-pathogen interactions are often decisive for the successful infection. This work suggested a role of SA signalling during the early stages of infection, as evident by the upregulation of SA response gene *PR1* which was subsequently confirmed by RNAseq in chapter 3. From this work, five timepoints in the

infection process during which defence related pathways and signalling were likely to be active were identified. RNAseq at these five timepoints was carried out, and at 0 hpi to evaluate global transcriptional response to two *R. commune* isolates in Cassia and Infinity, with an aim to identify genes potentially conferring susceptibility to *R. commune.*

According to Thirugnanasambandam et. al., (2011), extensive hyphal growth within the apoplastic space occurs during the early asymptomatic stages of infection as determined through the use of a GFP tagged *R. commune* isolate. As shown in Figure 2.7, in a detached leaf assay, *R. commune* DNA was detectable at 10 dpi in cvs Cassia, Propino and Golden promise. A timepoint evaluation would be useful to determine the extent of fungal proliferation in the early stages. However, in Figure 2.11, fungal biomass accumulation of the 44.07 isolate was determined in Cassia and was not shown to significantly increase between 0 – 14 dpi, suggesting that extensive hyphal growth is not likely occurring during the asymptomatic stage. An alternative approach would use trypan blue staining to assess *R. commune* proliferation at different timepoints. Indeed Griffe (2017), describes the use of trypan blue staining to visualise the increase in conidia and hyphae at 4, 7 and 9 dpi in the susceptible cultivar Optic, after inoculation with *R. commune* isolate L2A at a spore concentration of 10^7 spores/ml.

In chapter 3, two S gene identification approaches were described, an *in silico* identification of putative barley orthologues to known S genes and a transcriptomic approach to determine up/down regulation of these putative orthologues in the course of *R. commune* infection. A similar approach was described by Meng et. al., (2021), in which a transcriptomic analysis of susceptible and resistant cvs of tobacco (Xiaohuangjin 1025 and Beinhart 1000–1 respectively) after *P. nicotianae* infection was completed. The authors complemented this analysis by determining the expression profiles of 28 S gene orthologues (Meng et. al., 2021). The role of these genes in the tobacco x *P. nicotianae* pathosystem has yet to be validated. However, this work was limited, in that only orthologues to S genes reported to be involved in susceptibility to fungi or oomycetes were considered. This equated to only 56 orthologues in tobacco being analysed, despite the hundreds of known S

genes recorded in the literature. It is unclear why the initial 28 S genes were selected. In contrast, in chapter 3, the *in silico* analysis was completed to identify orthologues to over 200 known S genes, regardless of the pathosystem they were identified in. This broad approach was used because a number of these known S genes are associated with key signalling pathways in the defence response, therefore manipulation of these genes may confer broad spectrum resistance, if the S gene candidate was part of a conserved response. Additionally, the role of putative S gene orthologues identified through this approach in other barley pathosystems can be validated in future studies. From this *in silico* analysis, 682 putative S gene orthologues were identified, 245 of which were differentially expressed in at least one barley cv after *R. commune* infection. This list of candidates was narrowed down to three genes for functional analysis in chapter 4, however, further work could also be carried out on the 245 putative S gene orthologues, or indeed the full list of 682 genes, not only in the *R. commune* response, but also to elucidate their role in response to other barley pathogens. Unfortunately, large-scale screening for the role of these potential S-genes would require a significant amount of time and would ideally involve the use of transient approaches to overcome difficulties associated with the generation of stable mutant barley lines. A problem that may remain is that of functional redundancy, whereby high-order mutants may need to be generated in order to assess the role of these genes as susceptibility factors. This limitation is particularly relevant, because S gene mediated resistance is typically recessive (Eckardt, 2002).

One aspect of the barley x *R. commune* pathosystem that is largely understudied is that of the latent phase. While it is known that increasing temperatures decreases the latent period in controlled conditions (Davis and Fitt, 1994), the mechanisms that trigger the transition to the necrotic phase are yet to be described. Identification of cvs that show increased resistance during the early stages and the latent phase could be implemented into disease management strategies. An analysis of different barley cvs to characterise the importance *R. commune* latent phase on successful colonisation would be beneficial. For example, the resistance of wheat cv Stigg to *Z. tritici* is largely attributed to lengthening of the latent period (36.5 days) compared to

susceptible cv Gallant (21.3 days) (Hehir et. al., 2018). Under glasshouse conditions, during this latent phase, Stigg showed less fungal biomass accumulation compared to Gallant (Rahman et. al., 2020). This differs from reports that during *R. commune* colonisation of barley, cvs showing field resistance showed comparable fungal biomass accumulation to susceptible cvs, despite the lack of external symptoms (Fountaine et. al., 2007), which suggests that the resistant cv is able to tolerate higher levels of pathogen load. In chapter 2, the efficacy of a similar qPCR protocol as the one used in (Fountaine et. al., 2007) was tested. This was done on three barley cvs using a detached leaf assay, and showed that despite fewer lesions being observed, cv Propino showed higher levels of *R. commune* DNA compared to susceptible Golden Promise, suggesting that some cvs are able to tolerate a certain level of disease. Although these experiments were carried out on detached leaves, as opposed to *in planta*, the results throw into question the current definition of resistance, which currently represents absence of symptoms, but not necessarily inhibition of pathogen growth and disease tolerance.

When new varieties are sent from breeders and seed agents to DAFM for evaluation of disease resistance, varieties are tested at a range of sites across Ireland to account for different environments such as climate and soil type. The varieties are assessed for a minimum of two years and evaluated for disease resistance. Only varieties resulting in a positive Value for Cultivation and Use (VCU) are allowed onto the National Catalogue of Agricultural Plant Varieties (NCAPV) and are then allowed to be marketed in Ireland. A further three years of more comprehensive trials are required to qualify to be listed on the recommended growing list (DAFM, 2020). At no point in this evaluation is the accumulation of fungal biomass measured to distinguish between disease resistance and disease tolerance. Indeed, the Innovations in Plant Variety Testing in Europe (INVITE) initiative aims to improve testing of variety performance under abiotic and biotic stress including the identification of characteristics and bio indicators associated with resilience (https://www.h2020-invite.eu/, Date accessed: 30/03/2022), which will aid in the identification of tolerant varieties. Increased understanding of the *R.*

commune latent phase to identify mechanisms involved in prolonging the latent phase will contribute to the identification of tolerance traits in barley.

R. commune virulence is primarily a quantitative trait, therefore disease management strategies should also focus on quantitative resistance and combining multiple approaches. To my knowledge, the work described in chapter 3 is the first instance of a transcriptomic analysis of the early infection stages of *R. commune* isolates. While the scope of this project was limited to the identification of S genes, the data from this chapter serves as a substantial knowledge base on how barley responds at a transcriptomic level to *R. commune,* for example the involvement of hormone and ROS signalling pathways within the first 48 h of infection, which could potentially be used for further investigation of traits in susceptible and resistant cvs, or conserved defence related pathways. However, this work was limited to two barley cvs, therefore it is not possible to predict true conserved responses. In addition, the inclusion of two isolates in this analysis can provide insights into isolate specific and potentially broad spectrum host responses, however screens against additional isolates is required to validate this.

The work described in this thesis also aimed to establish a transient expression system in barley, with the goal of completing a functional analysis of candidate S genes. While low levels of GUS expression was detected via histochemical staining of leaf sections, it was not at the level required to represent efficient transformation. As this work was completed towards the end of the PhD, further work is required for the optimisation of the agroinfiltration protocol. Other approaches for the RNAi mediated knockdown of candidate S genes were not considered in chapter 4. These include microprojectile bombardment or virus induced gene silencing (VIGS), both of which have been described to work in barley (Hein et. al., 2005; Douchkov et. al., 2005; Chowdhury et. al., 2016). A problem that may remain is that of functional redundancy, as S gene mediated resistance is usually recessive. This may affect the resistant phenotype of S gene mutants. Additionally, the role of candidate S genes in growth and development may also be problematic, as trade-offs associated with induced resistance are often

associated with a fitness penalty (van Schie and Takken, 2014), for example, lesion mimic phenotype observed in barley *mlo* mutants (Jørgensen, 1992).

As stated in Chapter 4, the functional analysis of the candidate S genes is key to identifying those S genes which could be manipulated to delay progression of *R. commune* and exploited for resistance to *R. commune* and indeed possibly other important barley pathogens such as ramularia (*R. collo-cygni*), net blotch (*P. teres*), and powdery mildew (*Bgh*). While it is possible the silencing of S gene candidates may confer broad range resistance against many different pathogens, this is largely dependent on the pathogen lifestyle. For example, a number of S genes have been identified which are involved in the regulation of plant defence hormones JA and SA. Due to the antagonistic interaction between JA and SA (Turner et. Al., 2002), silencing gene candidates involved in the negative regulation of SA signalling pathway to increase resistance to biotrophs can also increase susceptibility to necrotrophs and insects due to reduced JA signalling. Indeed, any genes coding for proteins involved in the regulation of JA and SA signalling pathways could be considered candidate S genes, however their manipulation to improve resistance would be entirely dependent on pathogen lifestyle.

Overall though, the work described in this thesis has generated valuable datasets that has identified genes with potential as S genes. In light of the legislative and environmental challenges that the Irish arable sector will face in the years ahead, it is clear that such a strategy would be highly relevant to underpin the economic and environmental sustainability of the Irish tillage sector.

6 Reference List

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7 Appendix 1

List of reagents and buffers used in this thesis.

Name	**Composition**
CTAB buffer	100 mM Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, 2% (w/v) CTAB, 1% (w/v) PVP
Czapek Dox agar (CDA)	4.5% (w/v) Czapek Dox agar (Duchefa), 0.6% (w/v) peptone
Edward's extraction buffer	200 mM Tris-HCl (pH 8), 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS
GUS wash solution	0.2% (v/v) Triton, 50 mM NaPi (pH 7.2)
GUS staining solution	0.2% (v/v) Triton, 50 mM NaPi (pH 7.2), 2 mM X-gluc
Infiltration buffer	10 mM MES (pH 5.5), 10 mM $MgCl_2$, 400 µM acetosyringone
Luria Bertani (LB) broth	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl
LB agar	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 4.5% (w/v) agar
Seed germination media (SGM)	5% (w/v) Murashige and Skoog basal medium (Duchefa), 5% (w/v) agar
Super optimal broth (SOB)	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$
Transformation buffer (TB)	10 mM PIPES/KOH (pH 6.7), 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$

10x ThermoPol Buffer	200 mM Tris-HCl pH8.8 100 mM $(NH_4)2SO_4$ 100 mM KCl 20 mM MgSO4 1% Triton X100
Yeast extract broth (YEB)	0.5% (w/v) beef extract, 1% (w/v) yeast extract, 5% (w/v) peptone, 5% (w/v) sucrose, 5 mM $MgCl_2$

8 Appendix 2

8.1 Supplemental Figures:

Chapter 2:

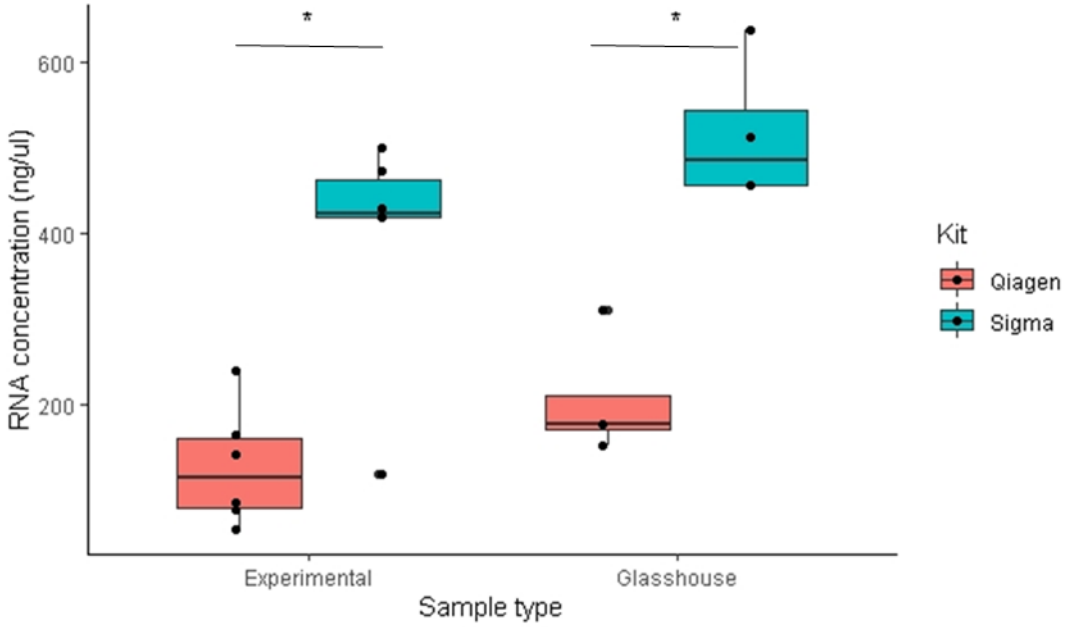


Figure S2.1: Comparison of RNA concentration (ng/µl) from Spectrum™ Plant Total RNA kit (Sigma-Aldrich) (blue) or the RNeasy® Plant Mini Kit (Qiagen) (red) extraction kits using untreated barley tissue grown in the glasshouse, or experimental samples inoculated with *R. commune*. Significant differences in concentration obtained from each kit is indicated with a * (t-test, $p < 0.05$). Sigma-Aldrich kit produces a significantly higher RNA yield in both experimental ($p = 0.005$, $n = 6$) and glasshouse ($p = 0.019$, $n = 4$) samples. Presence of fungal RNA does not affect yield for either extraction kits ($p > 0.05$).

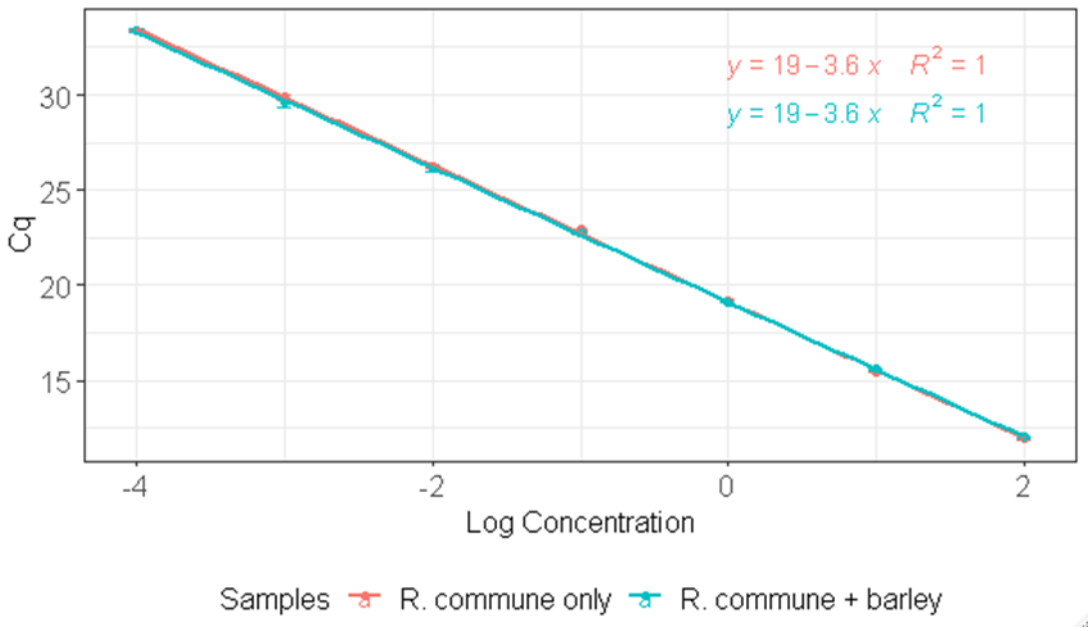


Figure S2.2: Standard curve for *R. commune* gDNA quantification. Blue line shows Cq values for *R. commune* DNA over seven 10-fold dilutions (from 100 ng/µl to 100 fg/µl). Red line represents standard curve for same *R. commune* DNA dilutions spiked with 100 ng barley DNA. There is no difference in the regression equations, which shows the presence of barley DNA has no effect on *R. commune* quantification. Data is for two biological replicates, each run in triplicate, error bars represent standard error

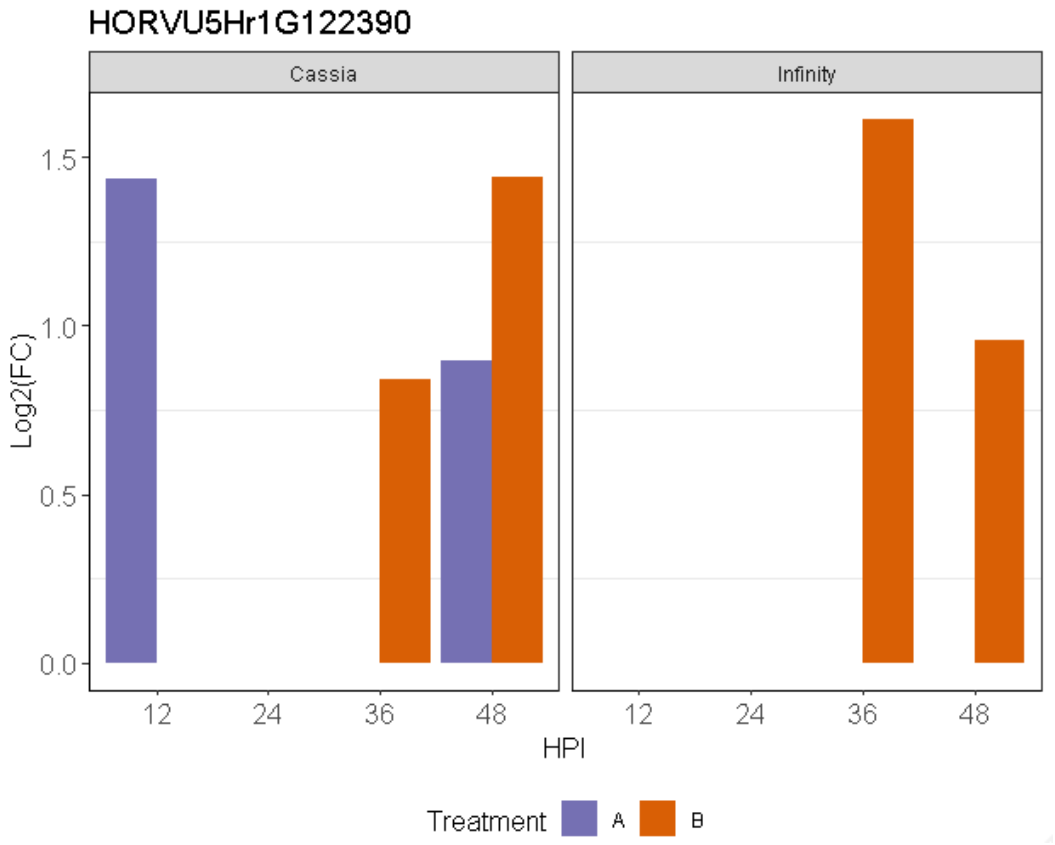


Figure S3.1: Log_2(Fold Change) relative to mock treatment of DEG HORVU5Hr1G122390 in Cassia and Infinity after treatment with A or B isolate.

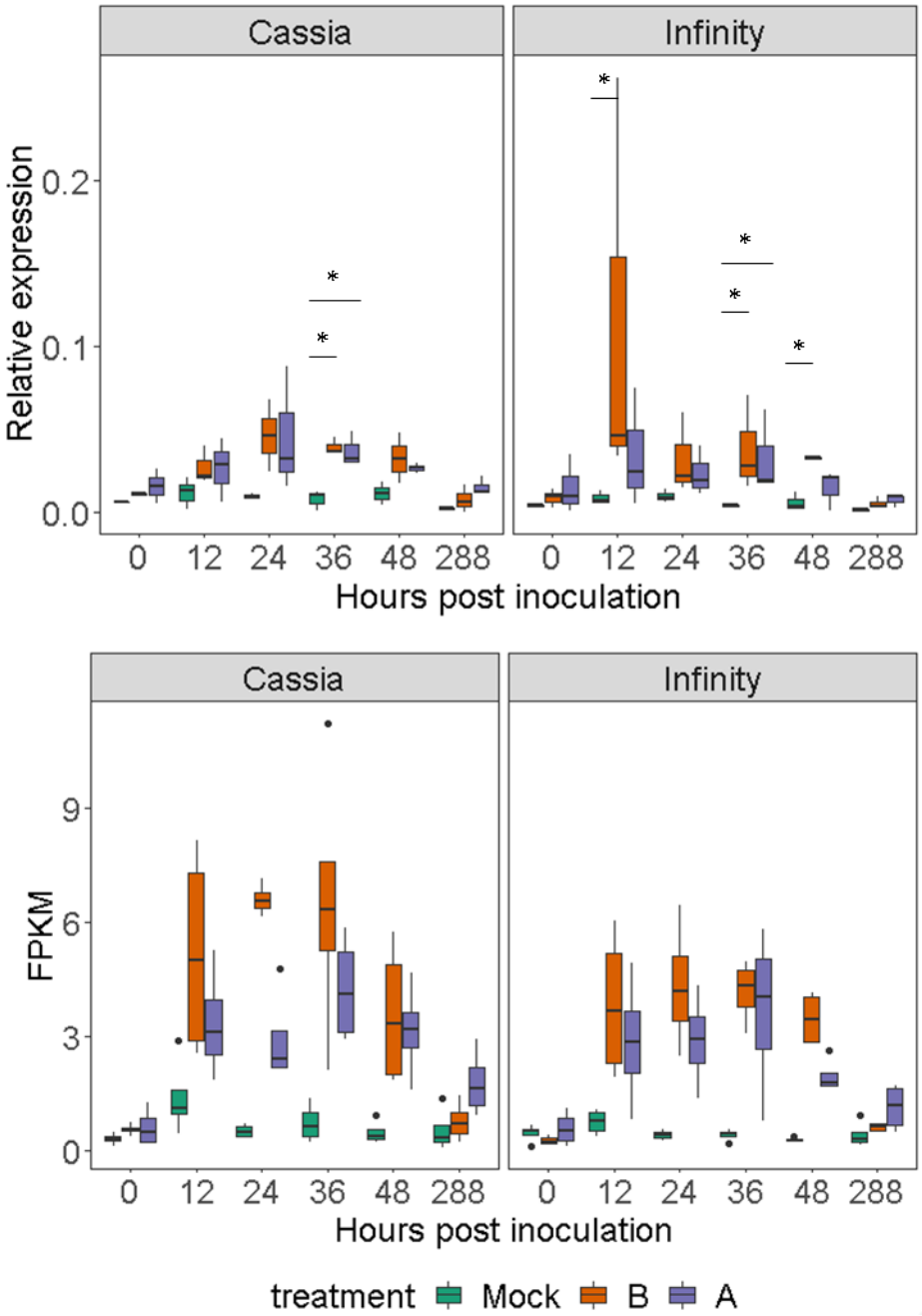


Figure S4.1: Relative expression level of HORVU7Hr1G108150, a proposed barley orthologue to *AtBIK1.* Expression levels of this S gene candidate relative to *ACTIN.* Significant differences (p-adj < 0.05, n = 3) in relative expression levels between different treatments at a given timepoint as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene (n = 4).

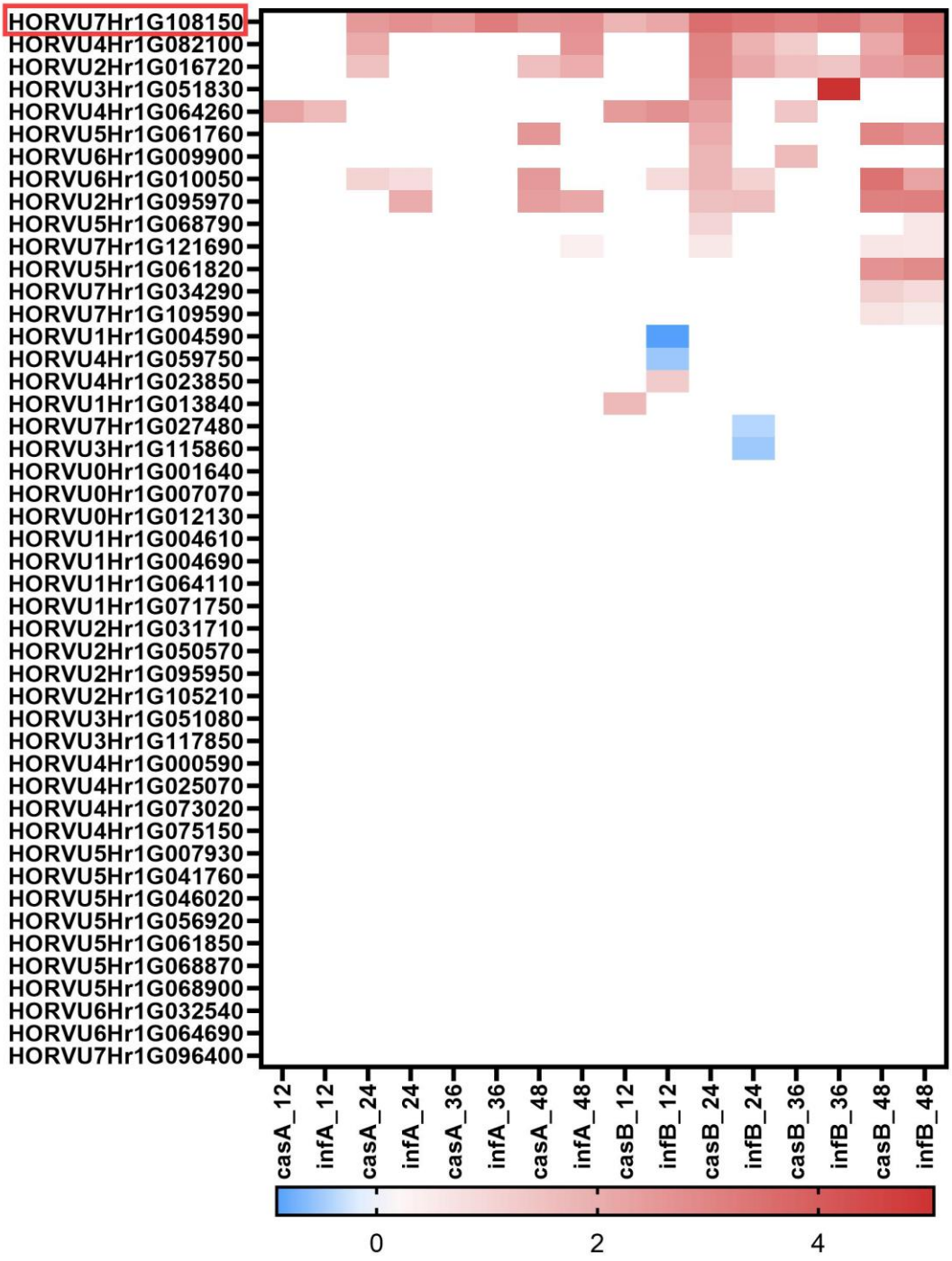


Figure S4.2: Heatmap of significant Log_2(Fold change) of all barley genes in S gene family Plant000020. Level of up/down regulation is represented as a colour gradient. Significance determined as p-adj < 0.05, as outlined in section 3.3.2. S gene candidate HORVU7Hr1G108150 is indicated in red box.

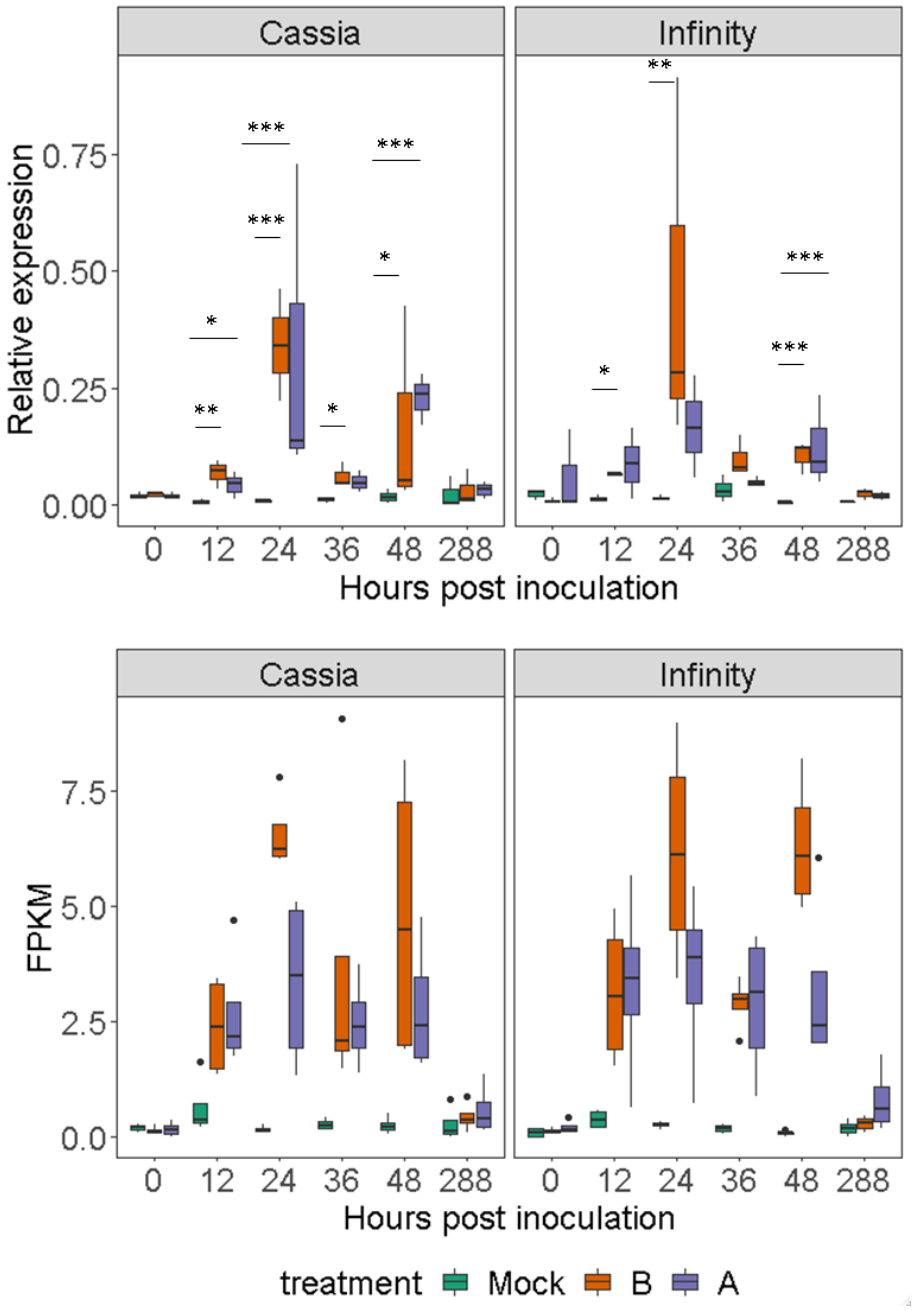


Figure S4.3: Relative expression level of HORVU5Hr1G060650, a proposed barley orthologue to *AtMYB46.* Expression levels of this S gene candidate relative to *ACTIN* are presented. Significant differences (p-adj < 0.05, n = 3) in relative expression levels between different treatments at a given timepoint as determined by two way ANOVA and Tukey post-hoc analysis is indicated. *: p-adj < 0.05, **: p-adj < 0.01, *** p-adj < 0.001. FPKM values of same gene (n = 4).

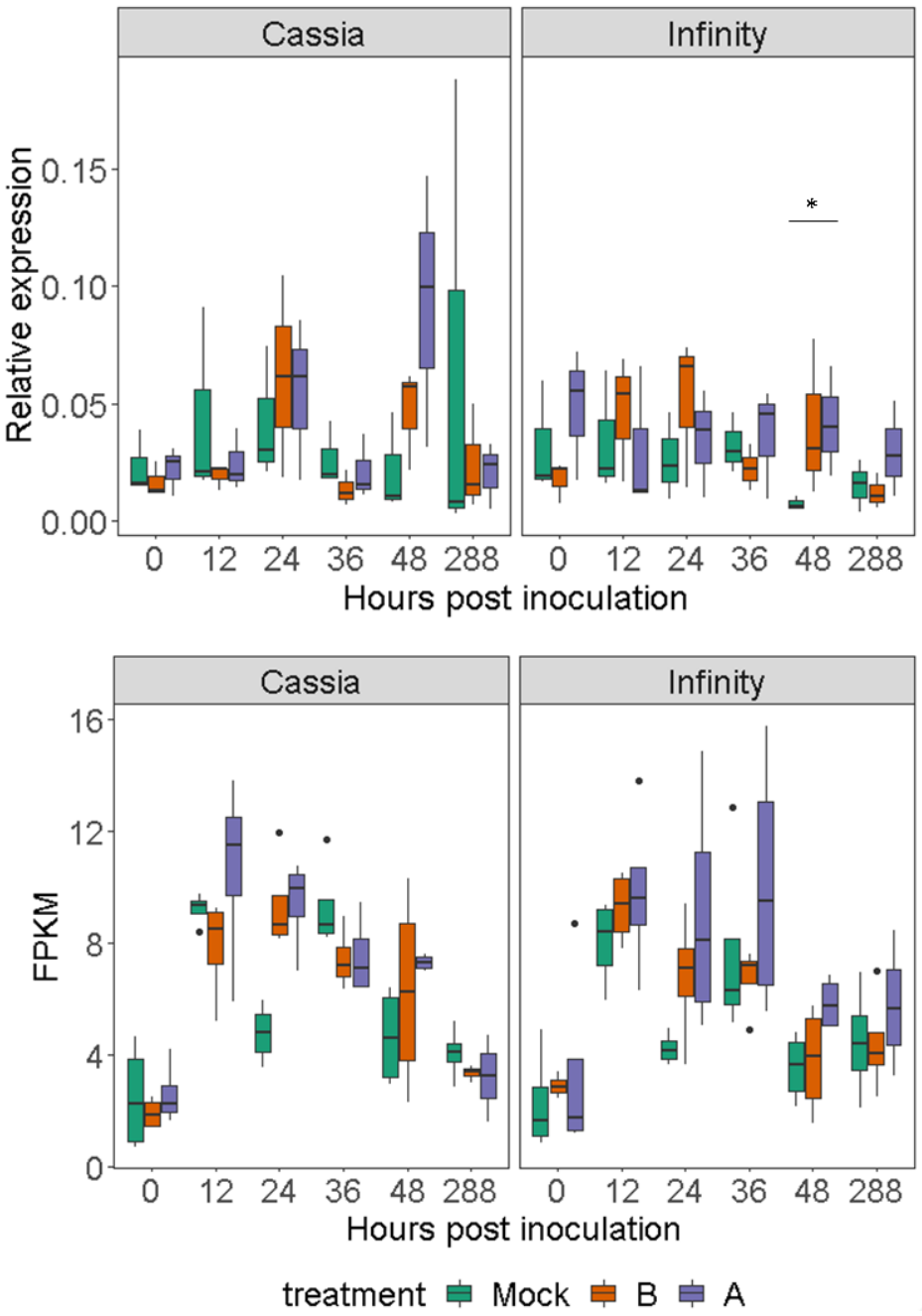


Figure S4.4: Relative expression level of HORVU2Hr1G028470, a proposed barley orthologue to *AtMYB46.* Expression levels of this S gene candidate relative to *ACTIN.* Significant differences (p-adj < 0.05, n = 3) in relative expression levels between different treatments at a given timepoint within a single cv as determined by two way ANOVA and Tukey post-hoc analysis. FPKM values of same gene (n = 4).

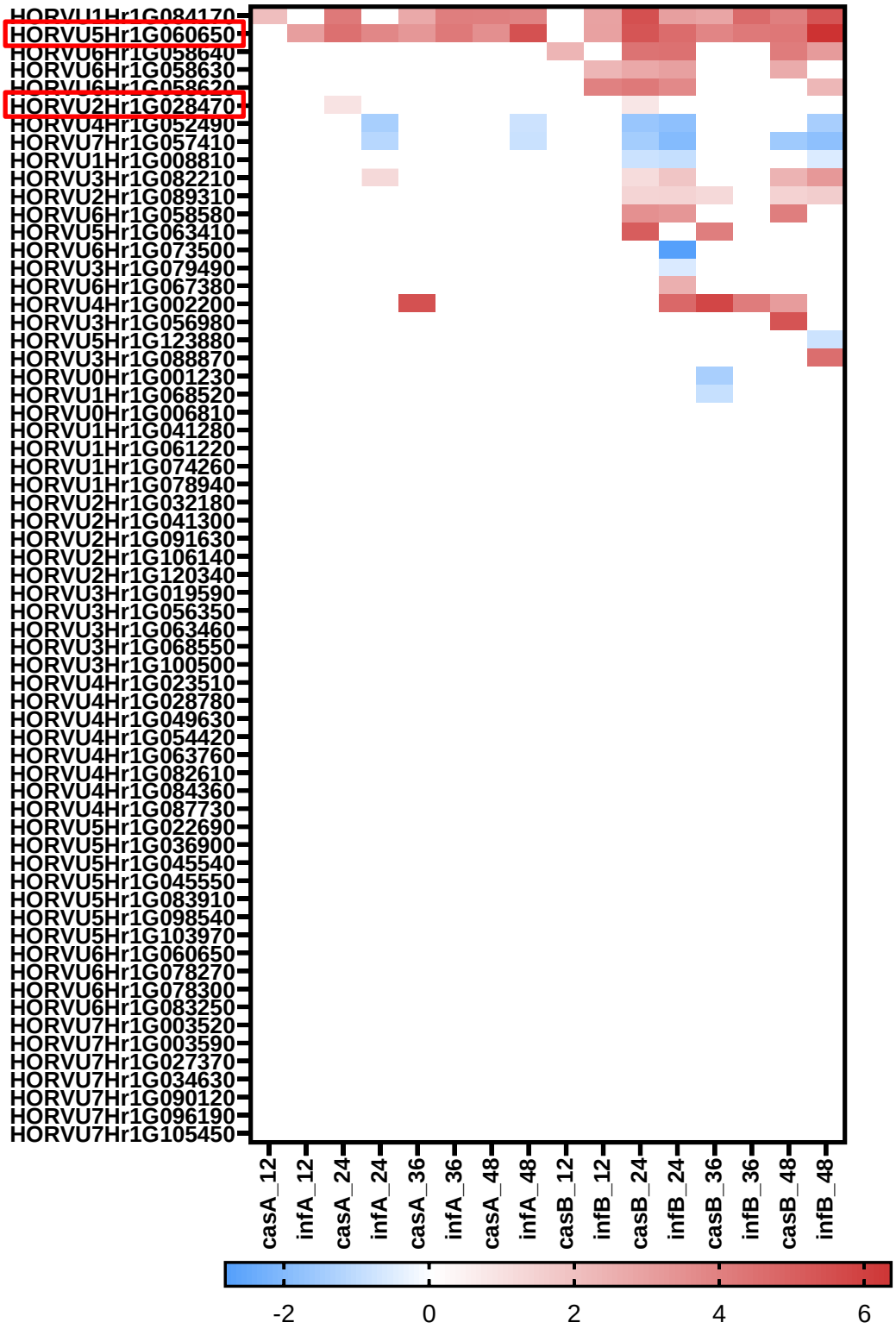


Figure S4.5: Heatmap of significant Log_2(Fold change) of all barley genes in S gene family Plant000003. Level of up/down regulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidates HORVU2Hr1G028470 and HORVU5Hr1G060650 are both present in this family and are indicated in red boxes.

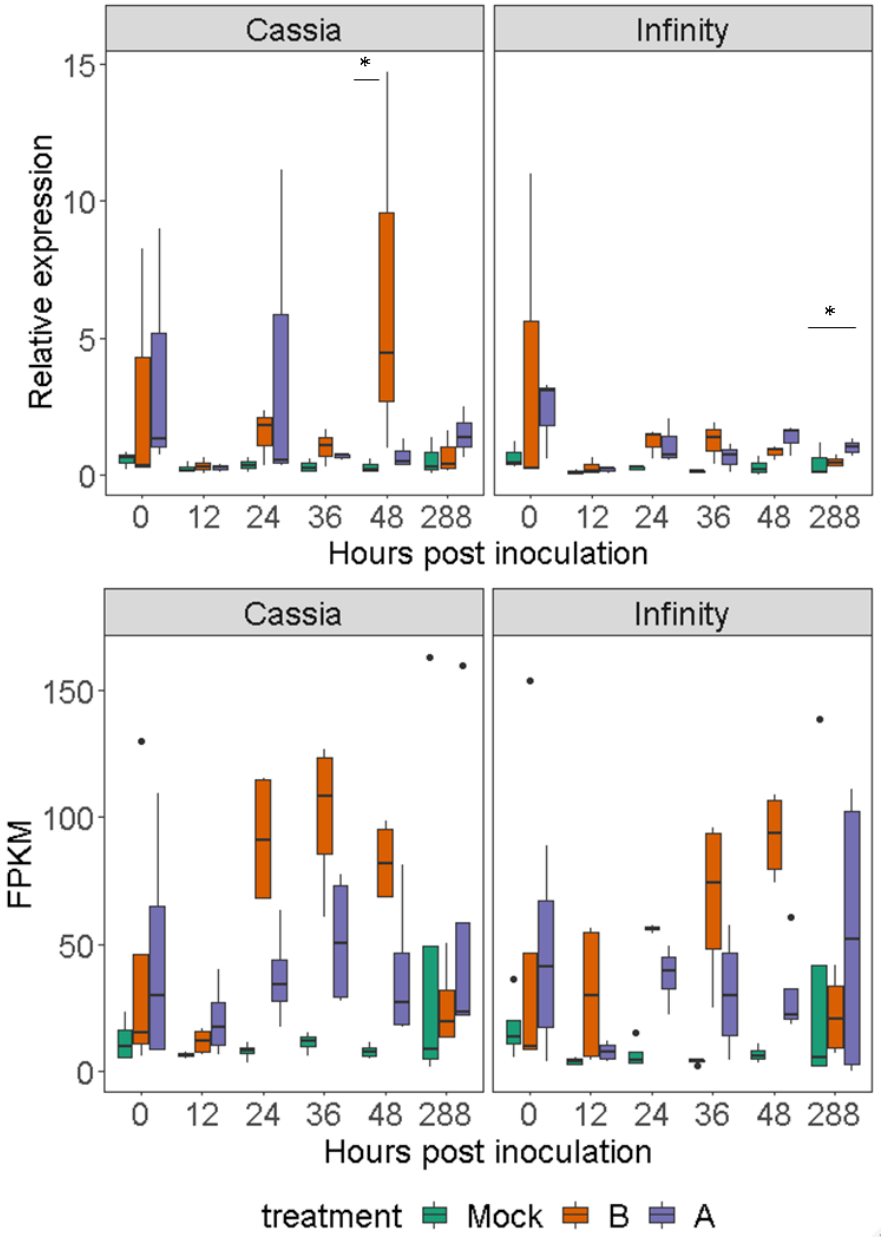


Figure S4.6: Relative expression level of HORVU4Hr1G084810, a proposed barley orthologue to *AtDMR6.* Expression level of this S gene candidate relative to *ACTIN* is presented. Significant differences (p-adj < 0.05, n = 3) in relative expression levels between different treatments at a given timepoint in a single cv as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

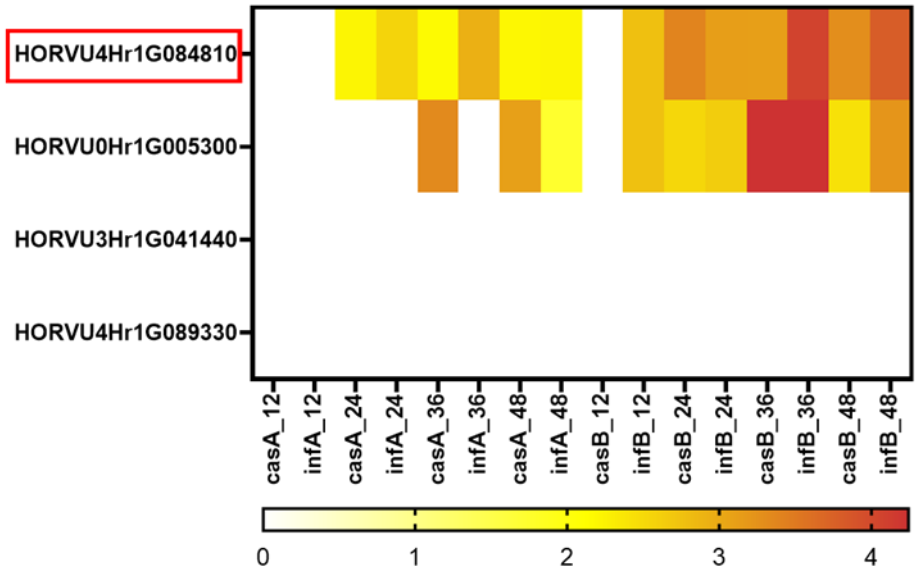


Figure S4.7: Heatmap of significant Log_2(Fold change) of all barley genes in S gene family Plant003549. Level of upregulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU4Hr1G084810 is indicated in red box.

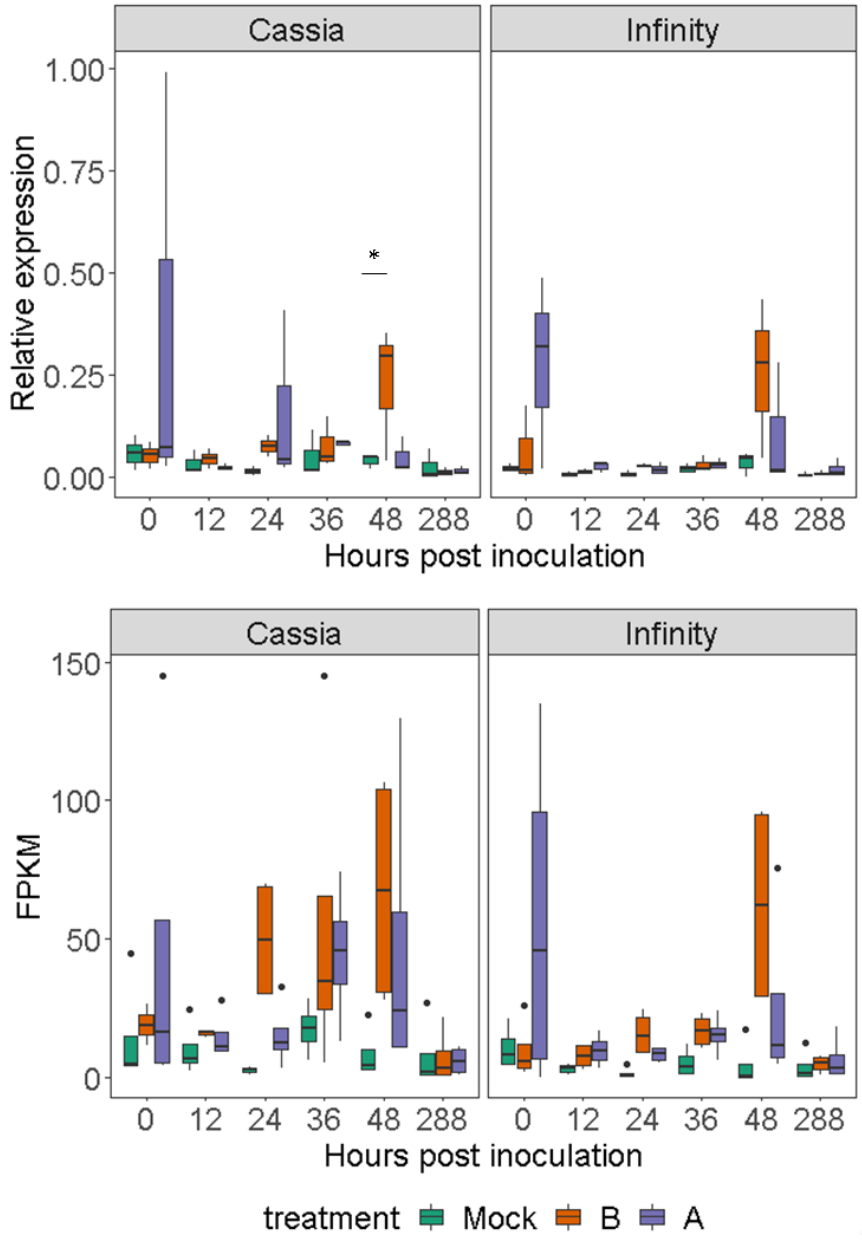


Figure S4.8: Relative expression level of HORVU7Hr1G113850, a proposed barley paralogue to *HvWRKY2.* Expression levels of this S gene candidate relative to *ACTIN.* Significant differences (p-adj < 0.05, n = 3) in relative expression levels between different treatments at a given timepoint in a single cv as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

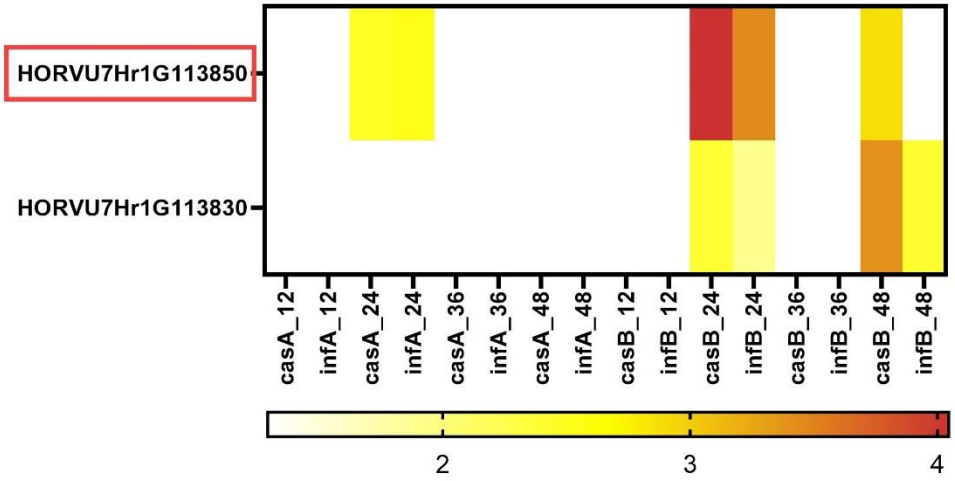


Figure S4.9: Heatmap of significant Log_2(Fold change) of all barley genes in S gene family Plant006394. Level of upregulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU7Hr1G113850 is indicated in red box. The other gene in this family (HORVU7Hr1G113830) corresponds to the known S gene *HVWRKY2.*

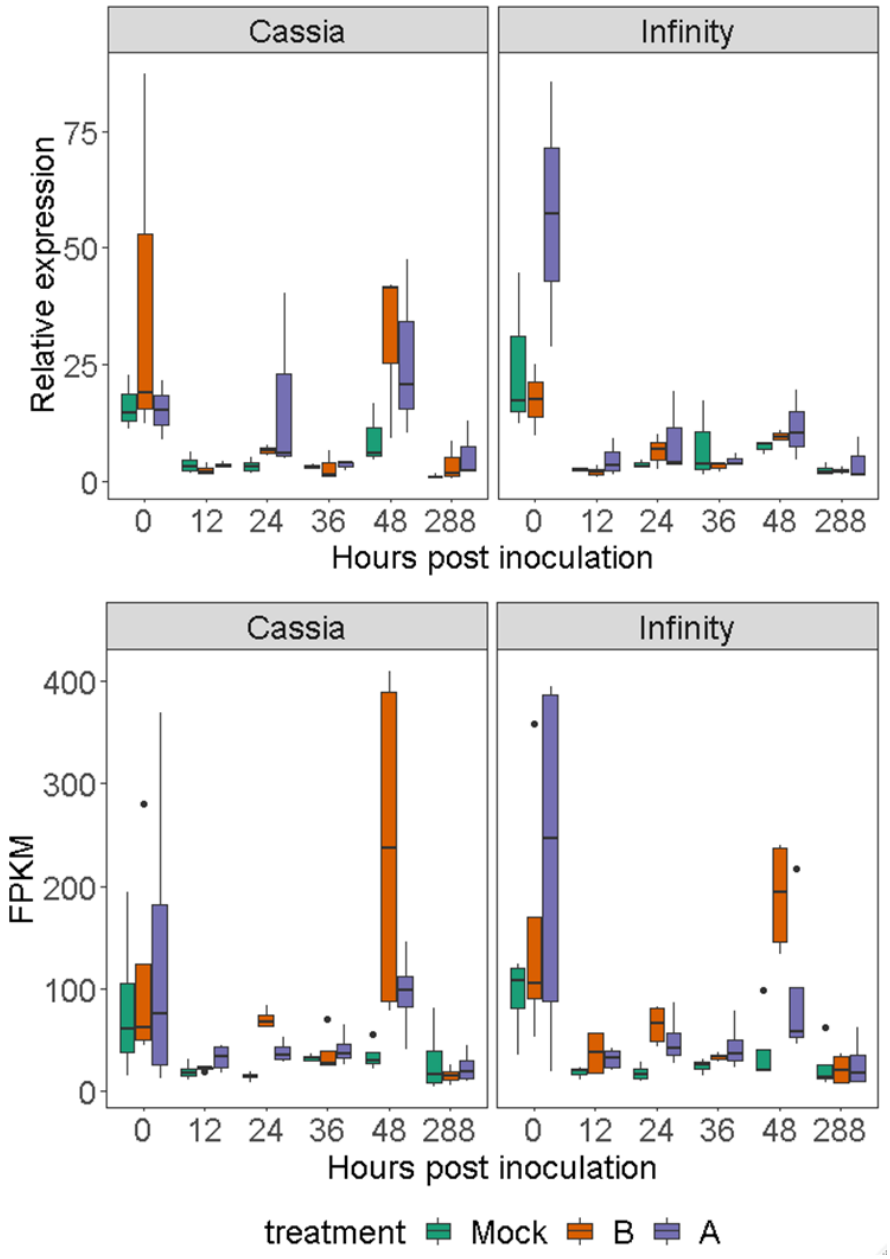


Figure S4.10: Relative expression level of *HvWRKY1 (*HORVU2Hr1G028470), a previously identified barley S gene. Expression levels of this gene relative to ACTIN. No significant differences (p-adj < 0.05, n = 3) in relative expression levels between different treatments at a given timepoint within a single cv as determined by two way ANOVA and Tukey post-hoc analysis. FPKM values of same gene (n = 4).

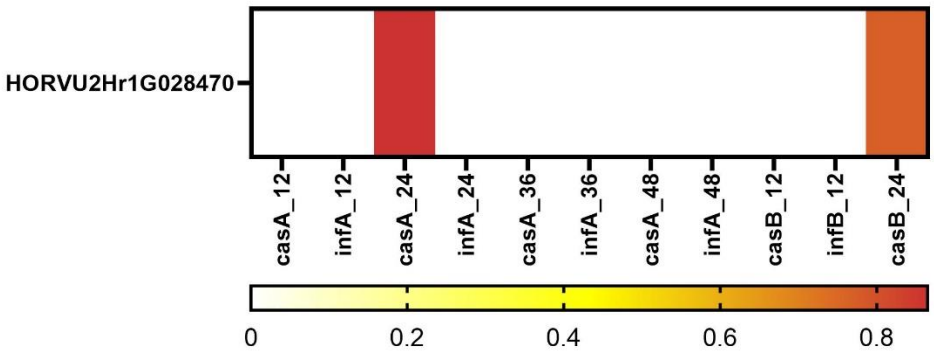


Figure S4.11: Heatmap of significant $\log_2$ (Fold change relative to mock) of *HvWRKY1 (*HORVU2Hr1G028470). No other barley genes were clustered to this gene family (Plant018390) by the OrthoMCL pipeline. Level of upregulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2.

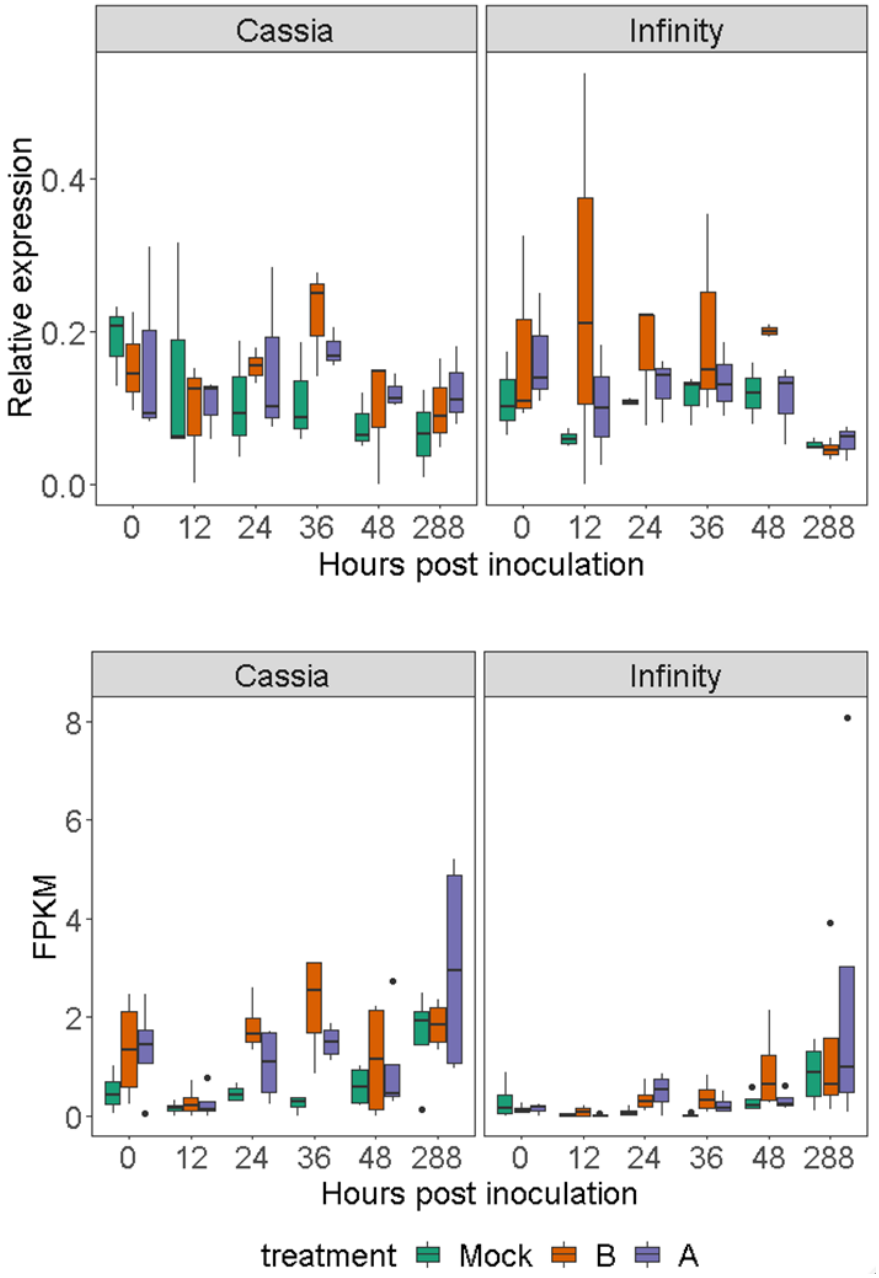


Figure S4.12: Relative expression level of HORVU7Hr1G113030, a proposed orthologue to the known S gene *AtPLP2*. Expression levels of this gene relative to *ACTIN*. No significant differences (p-adj < 0.05, n = 3) in relative expression levels between different treatments at a given timepoint within a single cv as determined by two way ANOVA and Tukey post-hoc analysis. FPKM values of same gene (n = 4).

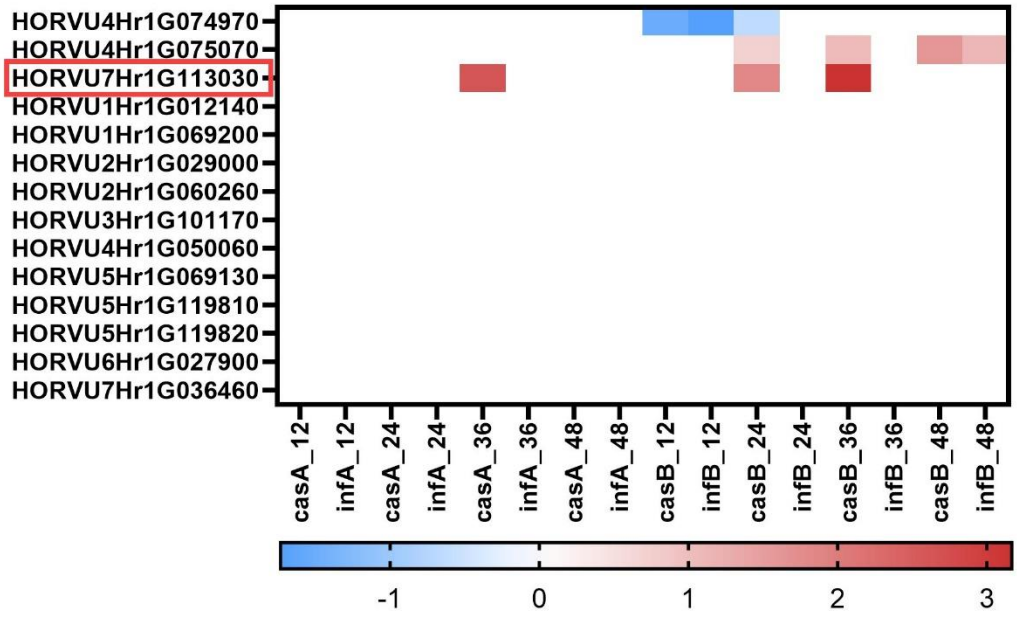


Figure S4.13: Heatmap of significant log_2 (Fold change relative to mock) of all barley genes in S gene family Plant000385. Level of up/down regulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU7Hr1G113030 is indicated in red box.

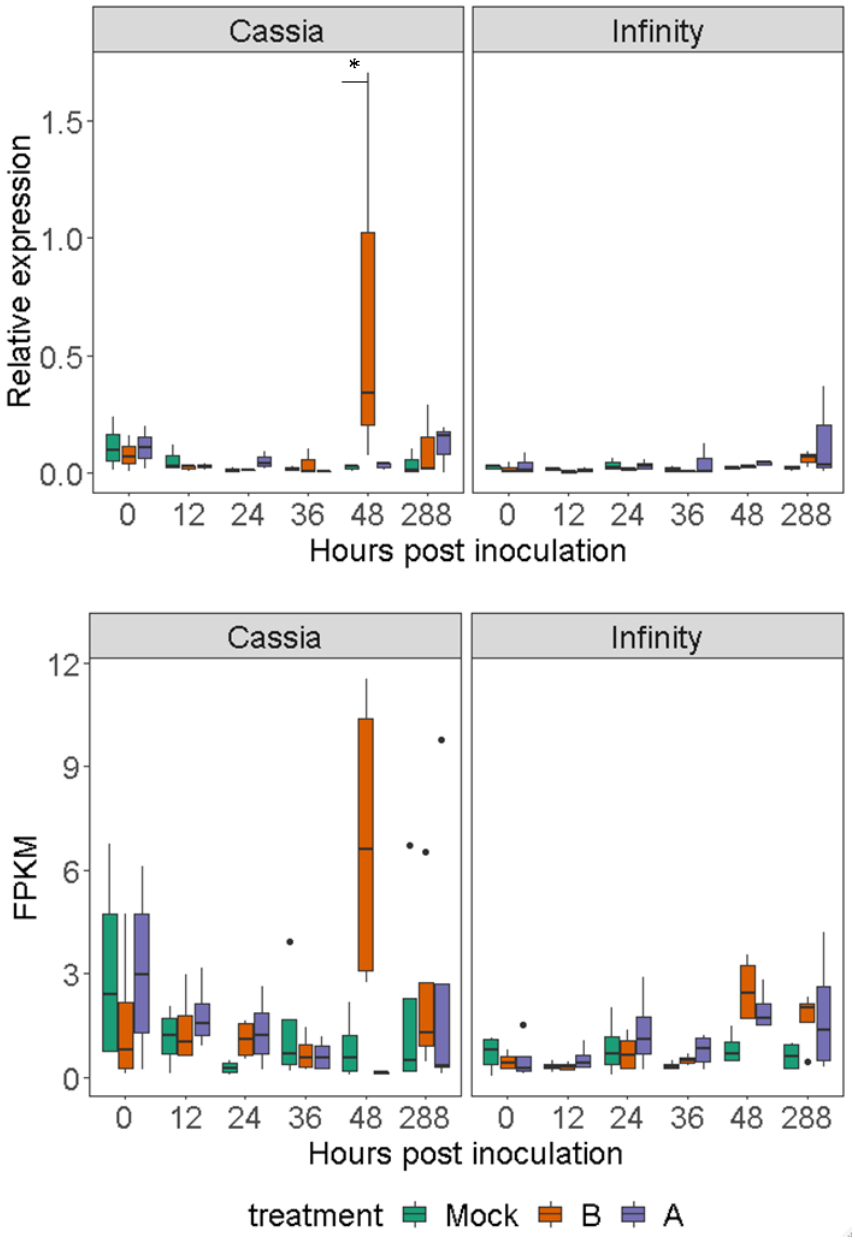


Figure S4.14: Relative expression level of HORVU5Hr1G057090, a proposed barley paralogue to *HvADH*. Expression levels of this S gene candidate relative to *ACTIN*. Significant differences (p-adj < 0.05, n = 3) in relative expression levels between different treatments at a given timepoint in a single cv as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

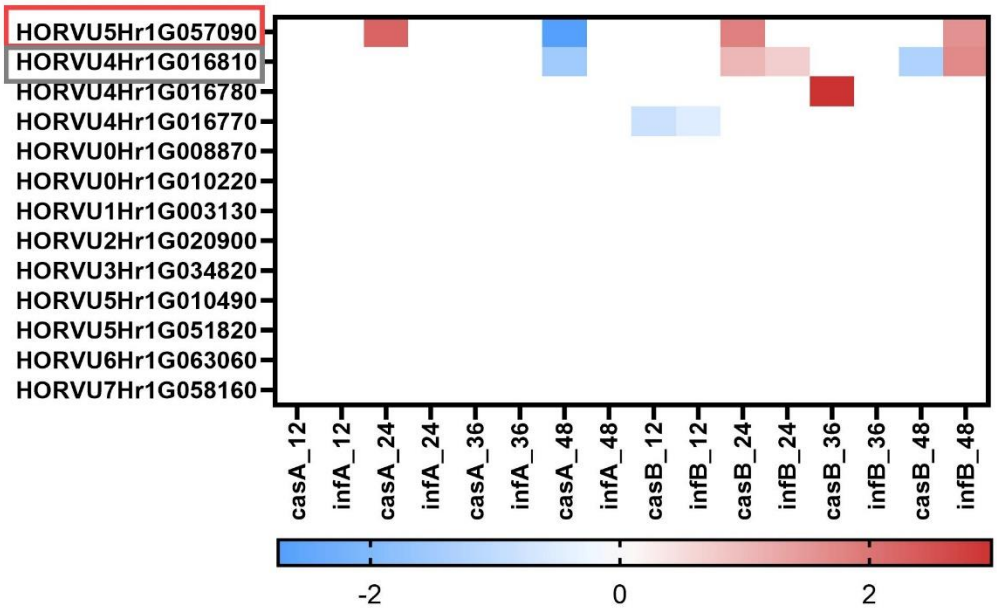


Figure S4.15: Heatmap of significant $\log_2$ (Fold change relative to mock) of all barley genes in S gene family Plant000975. Level of up/down regulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU5Hr1G057090 is indicated in red box. Previously identified barley S gene paralog *HvADH* (HORVU4Hr1G016810) is indicated with grey box.

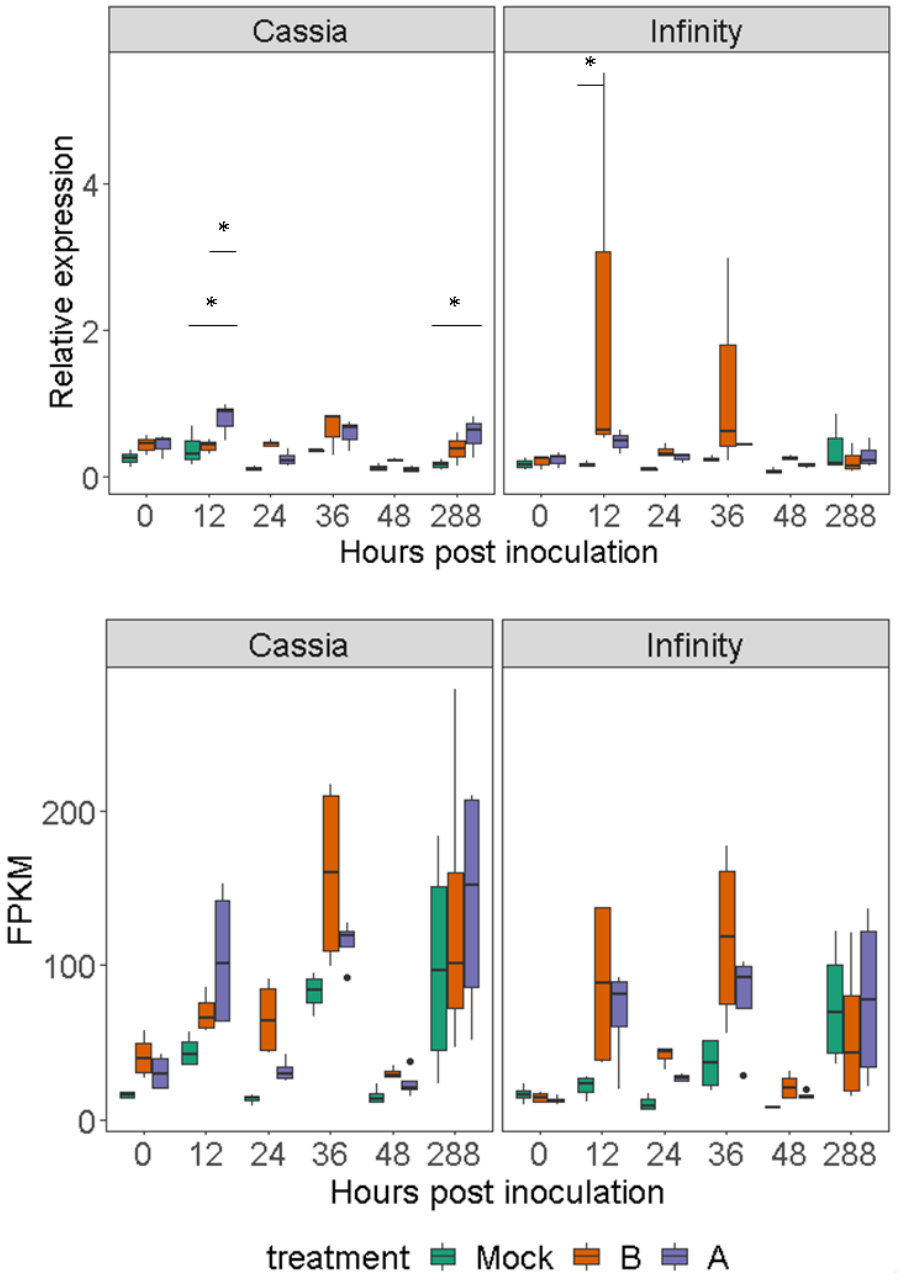


Figure S4.16: Relative expression level of HORVU4Hr1G005920, a proposed barley orthologue to *ZmLOX3*. Expression levels of this S gene candidate relative to *ACTIN*. Significant (p-adj < 0.05, n = 3) differences in relative expression levels between different treatments at a given timepoint in a single cv as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

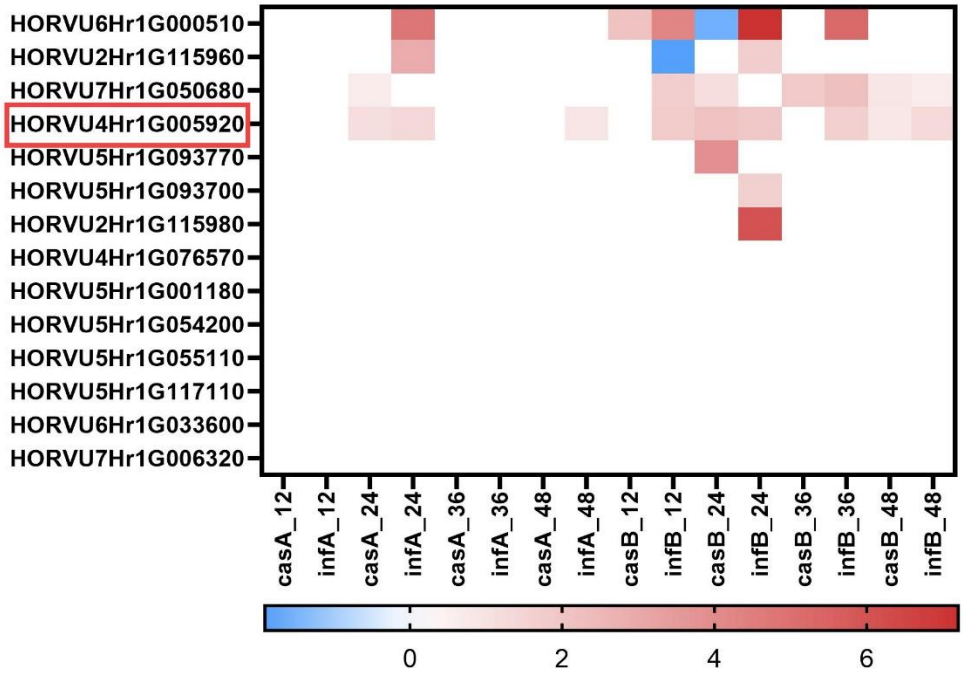


Figure S4.17: Heatmap of significant $\log_2$ (Fold change relative to mock) of all barley genes in S gene family Plant000115. Level of up/down regulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU4Hr1G005920 is indicated in red box.

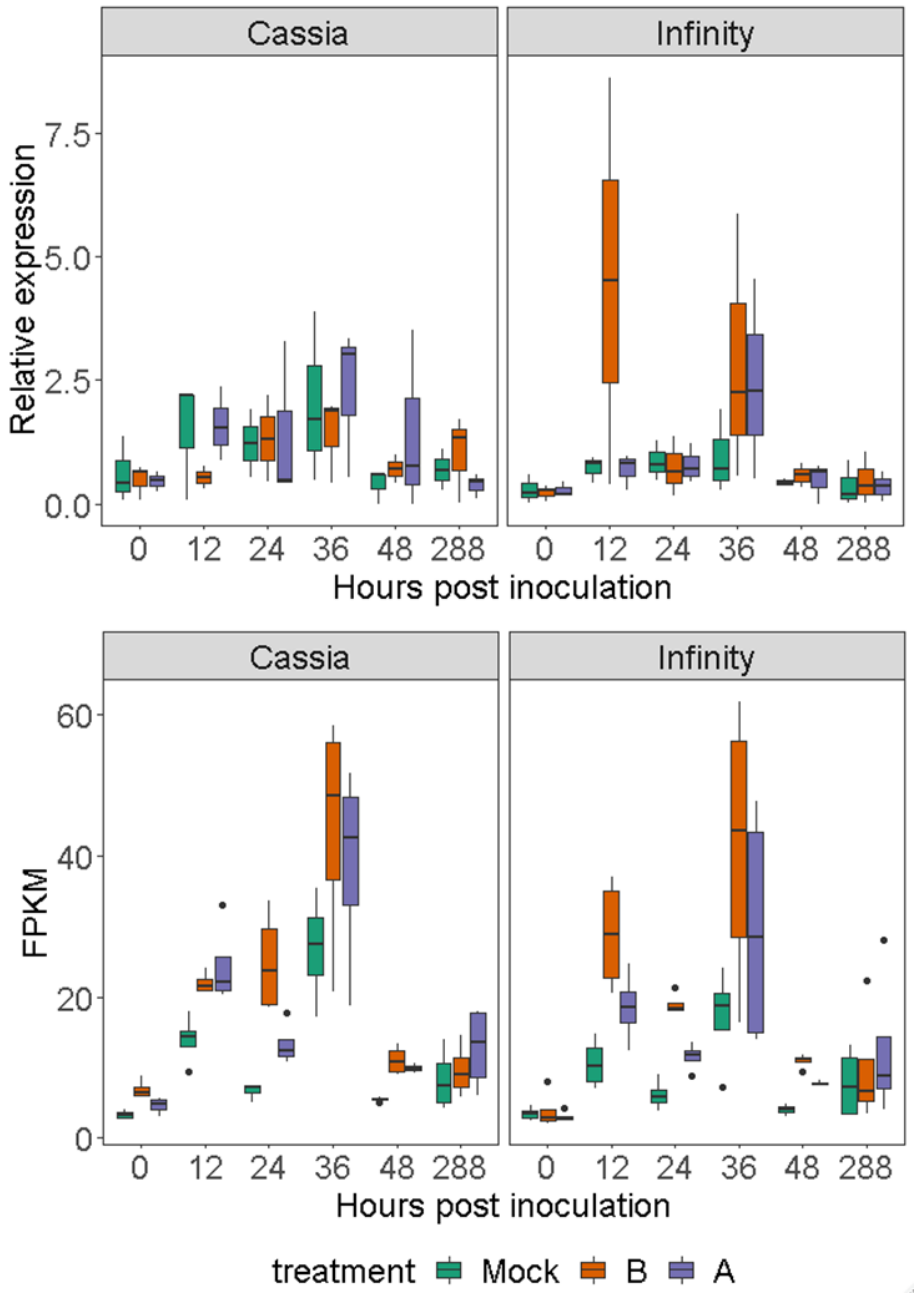


Figure S4.18: Relative expression level of HORVU4Hr1G018440, a proposed orthologue to the known S gene *AtPTP2*. Expression levels of this gene relative to *ACTIN*. No significant (p-adj < 0.05, n = 3) differences in relative expression levels between different treatments at a given timepoint within a single cv as determined by two way ANOVA and Tukey post-hoc analysis. FPKM values of same gene (n = 4).

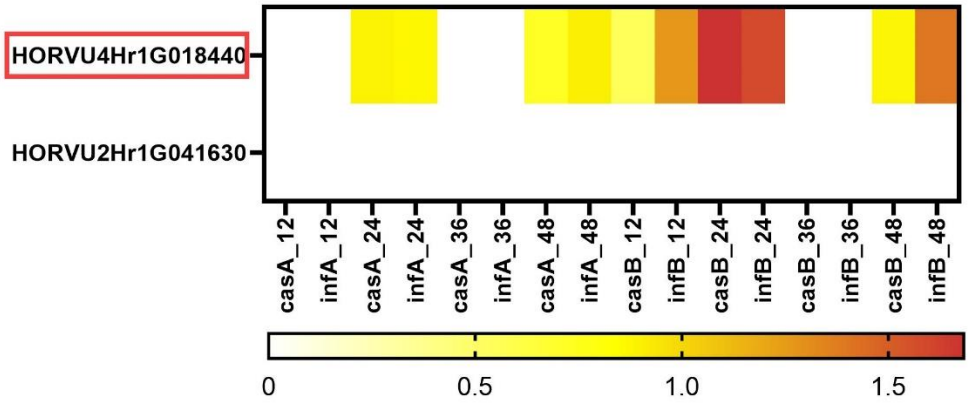


Figure S4.19: Heatmap of significant $\log_2$ (Fold change relative to mock) of all barley genes in S gene family Plant003579. Level of upregulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU4Hr1G018440 is indicated in red box.

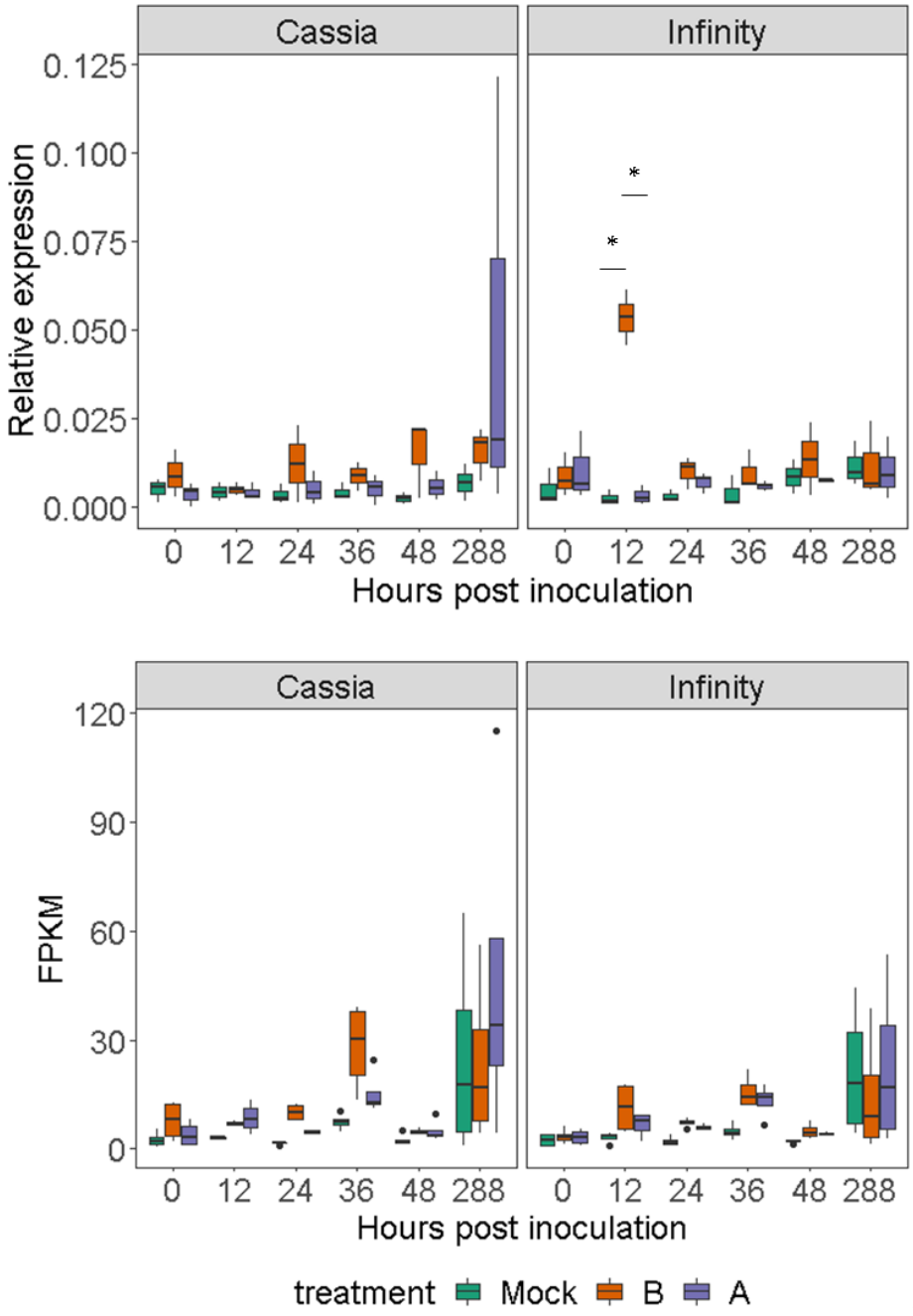


Figure S4.20: Relative expression level of HORVU5Hr1G084740, a proposed barley orthologue to *AtPLDβ1* and *OsPLDβ1*. Expression levels of this S gene candidate relative to *ACTIN*. Significant (p-adj < 0.05, n = 3) differences in relative expression levels between different treatments at a given timepoint in a single cv as determined by two way ANOVA and Tukey post-hoc analysis is indicated with * (p-adj < 0.05). FPKM values of same gene FPKM (n = 4).

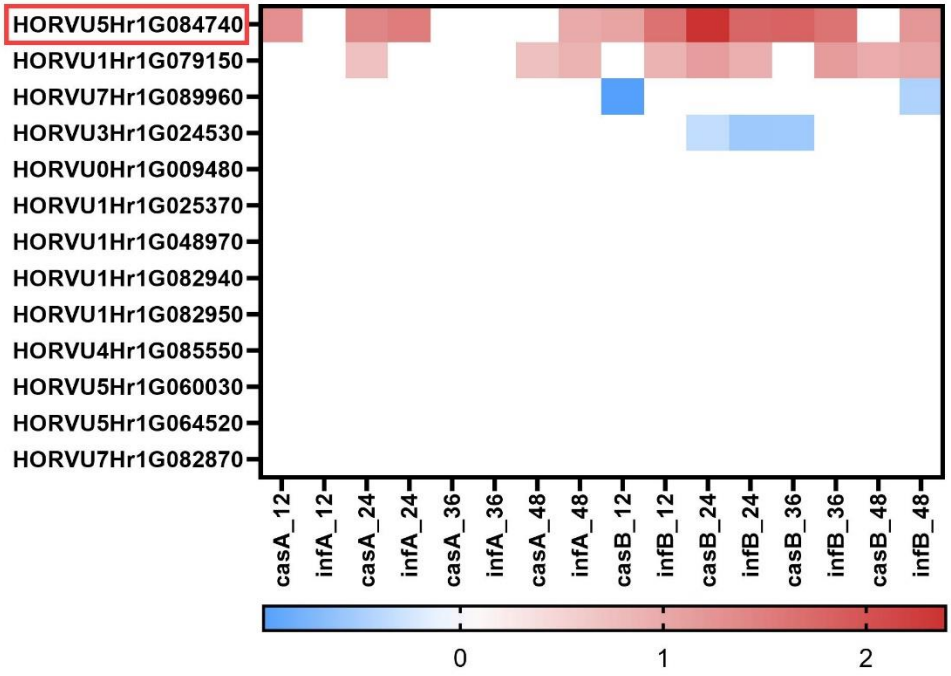


Figure S4.21: Heatmap of significant log_2 (Fold change relative to mock) of all barley genes in S gene family Plant000161. Level of up/down regulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU5Hr1G084740 is indicated in red box.

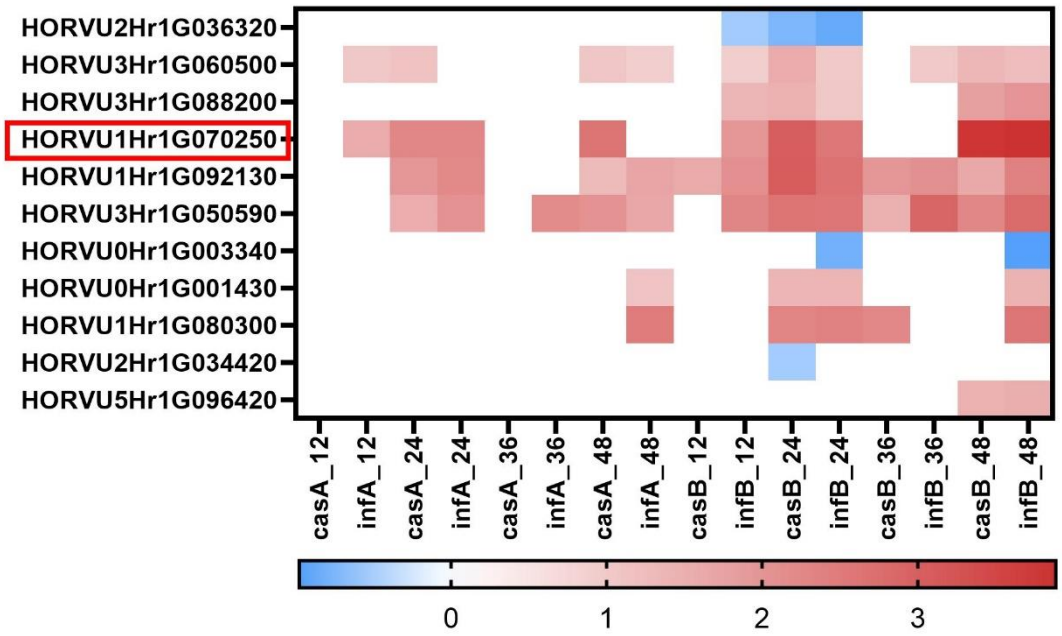
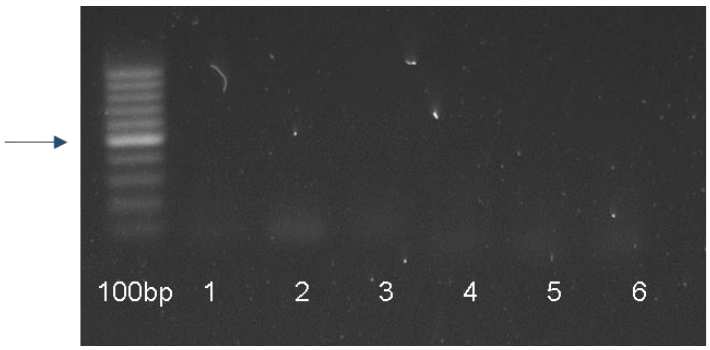


Figure S4.22: Heatmap of significant $\log_2$ (Fold change relative to mock) of all barley genes in S gene family Plant000088. Level of up/down regulation is represented as a colour gradient. Significance determined as p-adj < 0.05 as outlined in section 3.3.2. S gene candidate HORVU1Hr1G070250 is indicated in red box. RT-qPCR plots for this gene are displayed in chapter 3 (Figure 3.10)



1. Infinity *IOS1*
2. Cassia *IOS1*
3. GP IOS1
4. Infinity *WRKY45-1*
5. Cassia *WRKY45-1*
6. GP *WRKY45-1*

Figure S4.23: Phusion PCR amplification of *HvIOS1* (401bp) and *HvWRKY45-1* (434bp) in Infinity, Cassia and Golden Promise (GP) cDNA. Blue arrow indicates 500bp marker on Gene Ruler 1 kb+ ladder.

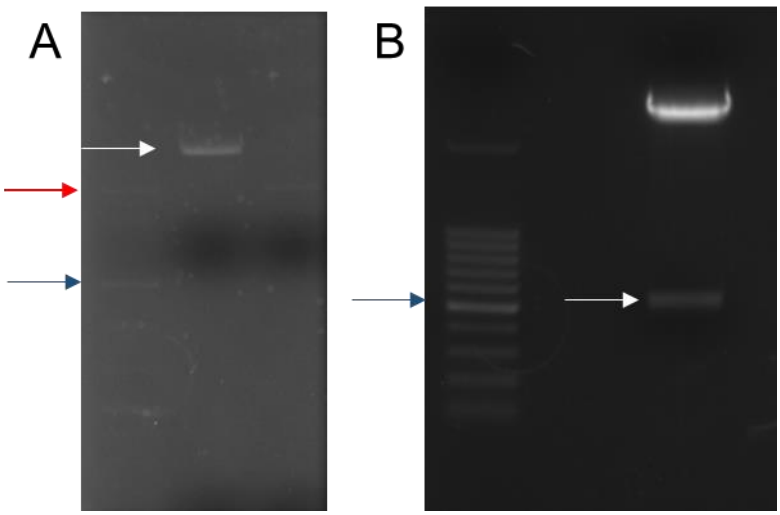


Figure S4.24: Cloning Hv*BON1* sense fragment into pHANNIBAL. A: pHANNIBAL after restriction digest with XhoI and Acc65I. Blue and red arrows indicate 500 bp, 1 kb markers on Gene Ruler 1 kb+ ladder respectively. B: pJET_BON1 after restriction digest with the same enzymes. Blue arrow indicates 500 bp marker on Gene Ruler 100 bp ladder. White arrows indicate fragments extracted using E.Z.N.A Gel Extraction Kit (Omega).

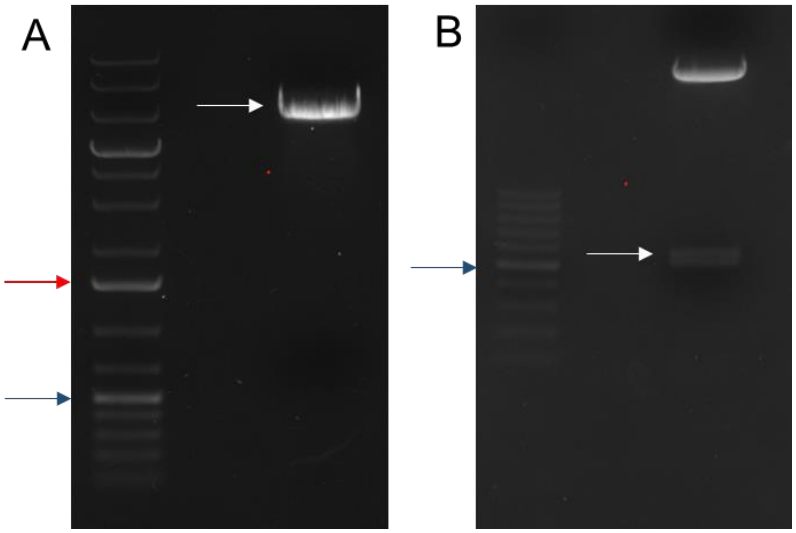


Figure S4.25: Cloning *HvBON1* anti-sense fragment into pHANNIBAL. A: pHANNIBAL_sense after restriction digest with XbaI and ClaI. Blue and red arrows indicate 500 bp, 1 kb markers on Gene Ruler 1 kb+ ladder respectively. B: pJET_BON1 after restriction digest with same. Blue arrow indicates 500 bp marker on Gene Ruler 100 bp ladder. White arrows indicate fragments extracted using E.Z.N.A Gel Extraction Kit (Omega)

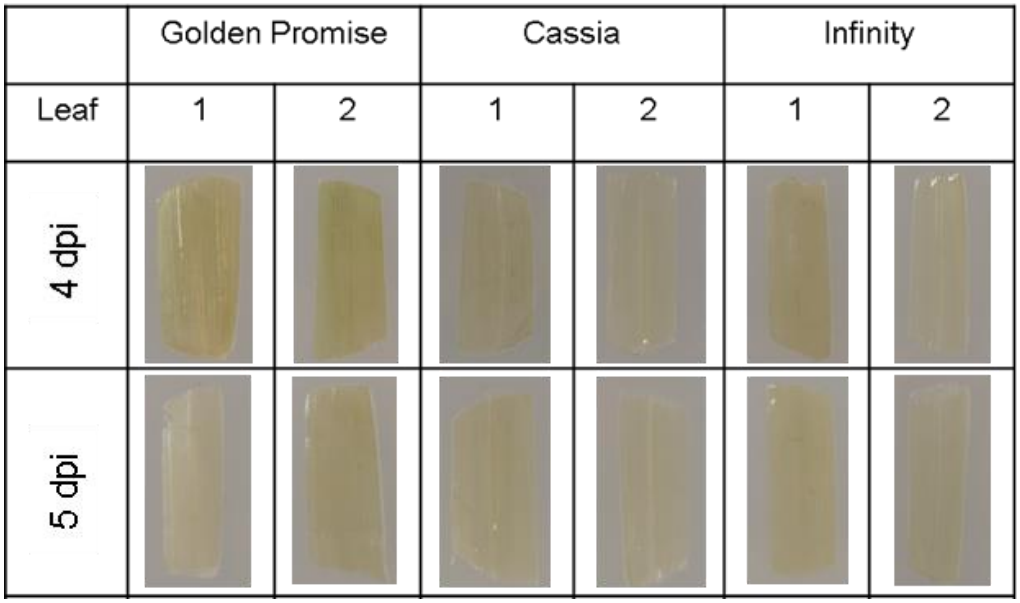


Figure S4.26: Barley leaf sections from proximal, middle and distal sections of leaf 1 and 2 of cvs Golden Promise, Cassia and Infinity 4 and 5 dpi after agroinfiltration with pGV2260_35S:GUS suspension of OD_{600} = 2. No GUS staining was observed in any cv. Leaf disks displayed are representative of the whole leaf blade.

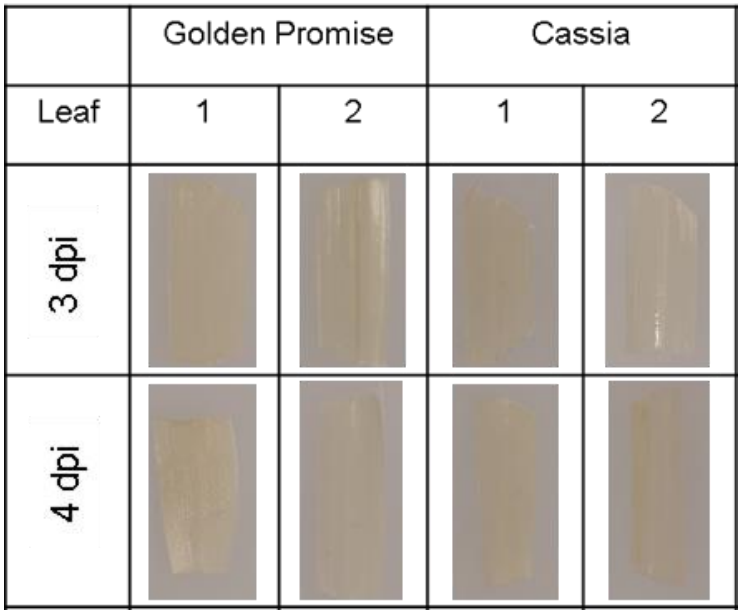


Figure S4.27: Barley leaf sections from proximal, middle and distal sections of leaf 1 and 2 of cvs Golden Promise and Cassia at 3 and 4 dpi after agroinfiltration with pGV2260_35S:GUS suspension of OD_{600} = 2. No GUS staining observed in any cv. Leaf disks displayed are representative of the whole leaf blade.

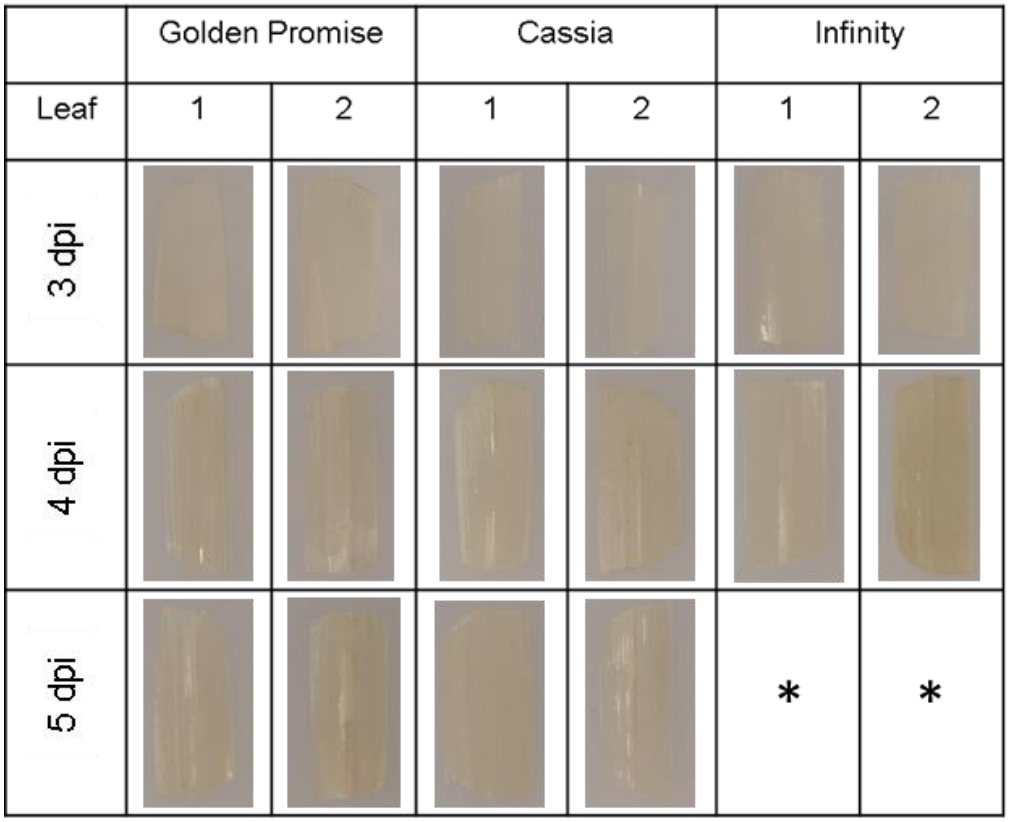


Figure S4.28: Barley leaf sections from proximal, middle and distal sections of leaf 1 and 2 of cvs Golden Promise, Cassia and Infinity at 3, 4 and 5 dpi after agroinfiltration with AGL1_35S:GUS suspension of OD_{600} = 2. No GUS staining observed in any cv. Leaf disks displayed are representative of the whole leaf blade. * Infinity not tested at 5 dpi due to poor germination rate.

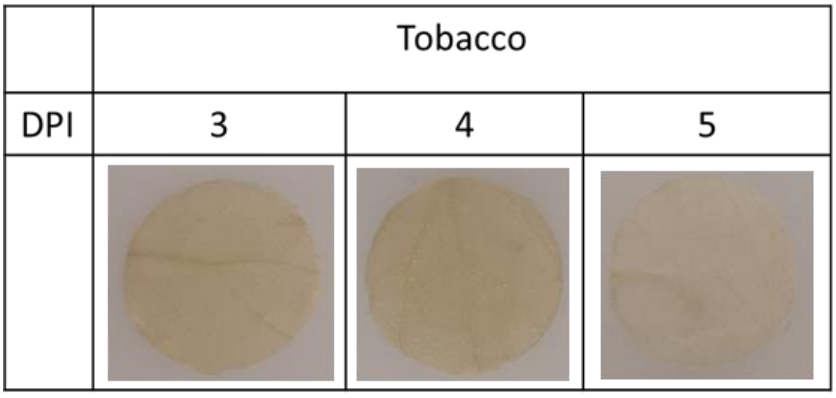


Figure S4.29: Leaf disks from leaf 1 and 2 of 4-6 week old tobacco at 3, 4 and 5 dpi after agroinfiltration with AGL1_35S:GUS suspension of OD_{600} = 2. No GUS staining observed at any timepoint.

8.2 Supplemental Tables

Chapter 3

Table S3.1: Online resources for proteome sequences and date accessed.

Species	Source	Date Accessed	# Proteins
Arabidopsis thaliana	https://www.arabidopsis.org/download/index-auto.jsp?dir=%2Fdownload_files%2FSequences%2FTAIR10_blastsets	19/10/2017	27416
Medicago truncatula	http://www.medicagogenome.org/downloads	25/10/2017	55344
Zea mays	http://ensembl.gramene.org/Zea_mays/Info/Index	24/10/2017	39499
Solanum lycopersicum	ftp://ftp.solgenomics.net/tomato_genome	24/10/2017	35768
Hordeum vulgare	http://plants.ensembl.org/Hordeum_vulgare/Info/Index	19/10/2017	37674
Glycine max	https://soybase.org/GlycineBlastPages/blast_descriptions.php	25/10/2017	56246
Oryza sativa	http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/	26/10/2017	55985
Fragaria vesca	https://www.rosaceae.org/organism/Fragaria/vesca	24/10/2017	33538
Triticum aestivum	https://wheat-urgi.versailles.inra.fr/Seq-Repository/Annotations	20/11/2017	110790
Capsicum annuum	http://peppersequence.genomics.cn/page/species/download.jsp#2	24/10/2017	35336

Table S3.2: RNA concentration and sample integrity for Cassia and Infinity post-treatment with *R. commune* strains A and B, or mock as determined on Agilent2100 by Novogene, prior to RNA sequencing.

Sample Name*	Concentration(ng/μl)	Volume(μl)	Total amount(μg)	Integrity value
cas_a_000_r1	399	14	5.586	7.2
cas_a_000_r2	441	13	5.733	7.6
cas_a_000_r3	177	10	1.77	7.8
cas_a_000_r4	294	14	4.116	7.1
cas_a_012_r1	405	14	5.67	7.3
cas_a_012_r2	244	14	3.416	6.9
cas_a_012_r3	318	15	4.77	7.2
cas_a_012_r4	82	15	1.23	7.7
cas_a_024_r1	153	10	1.53	7.8
cas_a_024_r2	796	13	10.348	7
cas_a_024_r3	92	13	1.196	8
cas_a_024_r4	125	13	1.625	7.1
cas_a_036_r1	240	15	3.6	7.2
cas_a_036_r2	152	14	2.128	7.4
cas_a_036_r3	182	13	2.366	7.2
cas_a_036_r4	240	14	3.36	7.2
cas_a_048_r1	190	13	2.47	7.2
cas_a_048_r2	240	13	3.12	7.1
cas_a_048_r3	144	13	1.872	7.2
cas_a_048_r4	130	16	2.08	7
cas_a_288_r1	192	16	3.072	7.3
cas_a_288_r2	46	14	0.644	7.5
cas_a_288_r3	113	15	1.695	7.3
cas_a_288_r4	164	10	1.64	7.5
cas_b_000_r1	168	12	2.016	7.5
cas_b_000_r2	152	14	2.128	6.7
cas_b_000_r3	93	10	0.93	7.4
cas_b_000_r4	258	12	3.096	7.7
cas_b_012_r1	256	13	3.328	7.3
cas_b_012_r2	399	14	5.586	7
cas_b_012_r3	176	17	2.992	7.6
cas_b_012_r4	345	16	5.52	7
cas_b_024_r1	88	14	1.232	7.8
cas_b_024_r2	252	12	3.024	7.2
cas_b_024_r3	152	21	3.192	8.1
cas_b_024_r4	381	16	6.096	7.2
cas_b_036_r1	96	14	1.344	7.6
cas_b_036_r2	369	9	3.321	6.6
cas_b_036_r3	174	14	2.436	7.3
cas_b_036_r4	462	20	9.24	7
cas_b_048_r1	99	20	1.98	6.9

cas_b_048_r2	158	15	2.37	7
cas_b_048_r3	152	14	2.128	7
cas_b_048_r4	267	16	4.272	6.9
cas_b_288_r1	51	15	0.765	7.4
cas_b_288_r2	124	16	1.984	7.6
cas_b_288_r3	248	14	3.472	7.7
cas_b_288_r4	162	17	2.754	7.9
cas_m_000_r1	497	13	6.461	7.4
cas_m_000_r2	98	16	1.568	7
cas_m_000_r3	255	11	2.805	7.2
cas_m_000_r4	186	10	1.86	7
cas_m_012_r1	150	11	1.65	7.3
cas_m_012_r2	196	13	2.548	7
cas_m_012_r3	236	13	3.068	7
cas_m_012_r4	141	14	1.974	7.5
cas_m_024_r1	104	11	1.144	7.6
cas_m_024_r2	171	12	2.052	7.2
cas_m_024_r3	148	13	1.924	7.8
cas_m_024_r4	142	13	1.846	7.4
cas_m_036_r1	273	15	4.095	7.3
cas_m_036_r2	296	14	4.144	7
cas_m_036_r3	158	14	2.212	7
cas_m_036_r4	530	13	6.89	7.1
cas_m_048_r1	152	14	2.128	6.8
cas_m_048_r2	270	15	4.05	7.2
cas_m_048_r3	224	15	3.36	7.2
cas_m_048_r4	318	15	4.77	7.1
cas_m_288_r1	186	14	2.604	7.1
cas_m_288_r2	90	13	1.17	7.1
cas_m_288_r3	342	15	5.13	7.4
cas_m_288_r4	132	15	1.98	7
inf_a_000_r1	1228	14	17.192	7.2
inf_a_000_r2	116	9	1.044	7
inf_a_000_r3	820	11	9.02	6.8
inf_a_000_r4	134	12	1.608	7.1
inf_a_012_r1	345	16	5.52	7.7
inf_a_012_r2	234	14	3.276	7.2
inf_a_012_r3	78	14	1.092	7.2
inf_a_012_r4	138	17	2.346	7.2
inf_a_024_r1	189	13	2.457	7.2
inf_a_024_r2	112	14	1.568	7.2
inf_a_024_r3	114	13	1.482	7.2
inf_a_024_r4	162	14	2.268	7.2
inf_a_036_r1	246	13	3.198	7.2
inf_a_036_r2	112	13	1.456	7.4
inf_a_036_r3	172	12	2.064	7

inf_a_036_r4	279	14	3.906	7.1
inf_a_048_r1	208	12	2.496	7.1
inf_a_048_r2	140	9	1.26	7
inf_a_048_r3	110	15	1.65	7
inf_a_048_r4	198	16	3.168	7.3
inf_a_288_r1	145	15	2.175	7.4
inf_a_288_r2	82	10	0.82	7.2
inf_a_288_r3	40	15	0.6	7.7
inf_a_288_r4	61	15	0.915	7.3
inf_b_000_r1	119	12	1.428	8
inf_b_000_r2	216	13	2.808	6.8
inf_b_000_r3	237	13	3.081	7.3
inf_b_000_r4	343	14	4.802	7.5
inf_b_012_r1	213	15	3.195	7.5
inf_b_012_r2	318	13	4.134	7.1
inf_b_012_r3	213	18	3.834	7.8
inf_b_012_r4	213	16	3.408	7.2
inf_b_024_r1	273	15	4.095	7.3
inf_b_024_r2	88	14	1.232	7.4
inf_b_024_r3	318	15	4.77	7.3
inf_b_024_r4	96	15	1.44	6.2
inf_b_036_r1	174	13	2.262	7.7
inf_b_036_r2	142	9	1.278	7.3
inf_b_036_r3	101	12	1.212	6.8
inf_b_036_r4	130	19	2.47	6.9
inf_b_048_r1	146	16	2.336	7.1
inf_b_048_r2	144	14	2.016	7
inf_b_048_r3	60	14	0.84	7.2
inf_b_048_r4	282	16	4.512	6.9
inf_b_288_r1	195	15	2.925	7.5
inf_b_288_r2	61	14	0.854	6.7
inf_b_288_r3	92	13	1.196	7.3
inf_b_288_r4	95	14	1.33	6.8
inf_m_000_r1	779	15	11.685	7
inf_m_000_r2	297	17	5.049	6.7
inf_m_000_r3	120	13	1.56	7.5
inf_m_000_r4	276	12	3.312	7.4
inf_m_012_r1	336	13	4.368	7.7
inf_m_012_r2	399	14	5.586	7
inf_m_012_r3	363	10	3.63	6.9
inf_m_012_r4	222	14	3.108	7.4
inf_m_024_r1	384	13	4.992	7.2
inf_m_024_r2	91	12	1.092	6.8
inf_m_024_r3	158	14	2.212	7.2
inf_m_024_r4	147	15	2.205	7.5
inf_m_036_r1	390	11	4.29	7

inf_m_036_r2	164	14	2.296	7.2
inf_m_036_r3	128	15	1.92	7.3
inf_m_036_r4	224	13	2.912	7
inf_m_048_r1	146	13	1.898	6.7
inf_m_048_r2	246	14	3.444	6.7
inf_m_048_r3	94	15	1.41	7
inf_m_048_r4	85	14	1.19	6.8
inf_m_288_r1	147	16	2.352	7.8
inf_m_288_r2	86	11	0.946	7.1
inf_m_288_r3	95	14	1.33	7.4
inf_m_288_r4	166	14	2.324	7.2

*Abbreviations: cas: Cassia, inf: Infinity, a: OP18(9), b: 44.07, m: mock, r: biological replicate. E.g.: cas_m_048_r1: Cassia, Mock treatment, 48 hpi replicate 1

Table S3.3: Quality control of mapped reads of each sample after Illumina sequencing by Novogene.

Sample name*	Raw reads	Clean reads	Error rate(%)	Q20(%)	Q30(%)	GC content(%)
caA00r1	43946047	43176355	0.03	97.82	93.51	58.94
caA00r2	41660653	41097365	0.03	97.46	92.72	58.9
caA00r3	42957312	42126284	0.03	97.15	92.01	58.1
caA00r4	41359900	40504545	0.03	97.15	92	58.49
caA12r1	40835029	39592225	0.03	97.52	92.82	56.92
caA12r2	43127089	41936142	0.03	97.4	92.57	57.44
caA12r3	42171814	40701673	0.03	97.23	92.35	56.07
caA12r4	42456128	41715614	0.03	97.21	92.29	56.2
caA24r1	61283632	60135360	0.03	97.37	92.53	58.29
caA24r2	43305903	42738304	0.03	97.38	92.54	57.63
caA24r3	47738004	47106193	0.03	97.48	92.96	57.44
caA24r4	59571471	59077278	0.03	97.23	92.27	57.55
caA288r1	45392212	44976332	0.03	97.12	92.01	56.05
caA288r2	42550630	40796907	0.02	98.46	95.47	56.94
caA288r3	54600504	53755213	0.03	97.11	91.99	56.48
caA288r4	55320410	54571462	0.03	97.27	92.32	56.75
caA36r1	48512174	47522736	0.03	97.29	92.4	55.83
caA36r2	44849903	44338606	0.03	97.13	92.03	55.62
caA36r3	44808744	44327530	0.03	97.25	92.32	56.35
caA36r4	45695903	45286337	0.03	96.98	91.7	55.65
caA48r1	40808169	40353020	0.03	97.33	92.48	56.96
caA48r2	49233197	48364684	0.03	97.53	93.13	57.6
caA48r3	41495771	41014941	0.03	97.22	92.24	57.16
caA48r4	46723191	46196362	0.03	97.19	92.09	57.31

caB00r1	43158005	42237117	0.03	97.36	92.51	58.09
caB00r2	43768060	42822946	0.03	97.37	92.51	58.68
caB00r3	42300425	41553495	0.03	97.69	93.25	58.08
caB00r4	42648817	41848872	0.03	97.45	92.7	58.59
caB12r1	43576110	42041528	0.03	97.42	92.62	57.21
caB12r2	45310420	43117939	0.03	97.44	92.69	56.88
caB12r3	40301685	39121317	0.02	97.98	94.34	57.05
caB12r4	43292368	41385041	0.02	98.04	94.41	56.38
caB24r1	54102615	52888733	0.03	97.33	92.48	57.75
caB24r2	53188036	52466389	0.03	97.44	92.89	57.33
caB24r3	42972432	41708185	0.02	98.01	94.31	58.17
caB24r4	45429123	43813859	0.02	98.12	94.72	56.92
caB288r1	42145826	40439119	0.02	98.59	95.8	56.01
caB288r2	45309857	44041839	0.03	97.24	92.39	56.53
caB288r3	41742221	41283987	0.03	97.08	91.92	56.59
caB288r4	43644433	43030933	0.03	97.15	92.06	56.39
caB36r1	40620938	40078044	0.03	97.26	92.32	55.5
caB36r2	47630994	47079287	0.03	97.15	92.11	54.93
caB36r3	46415420	45911929	0.03	97.16	92.12	56.33
caB36r4	42178782	40272837	0.02	97.96	94.27	54.96
caB48r1	47054859	45527958	0.02	98.12	94.7	57.14
caB48r2	42193241	41732577	0.03	97.12	91.97	57.11
caB48r3	40233416	38668364	0.03	96.98	91.73	57.15
caB48r4	44768897	42601956	0.02	98.1	94.69	57.17
caM00r1	43674913	43060408	0.03	97.81	93.45	59.48
caM00r2	40379187	39334739	0.03	97.4	92.5	58.71
caM00r3	42797635	41928296	0.03	97.47	92.72	58.82
caM00r4	44149441	43407571	0.03	97.36	92.5	58.47
caM12r1	42326592	41651797	0.03	97.57	92.84	57.45
caM12r2	43379050	42446643	0.03	97.41	92.63	56.5
caM12r3	42949739	41672627	0.03	97.43	92.67	57.34
caM12r4	51479413	50321990	0.03	97.22	92.27	57.38
caM24r1	44663968	44061005	0.03	97.57	93.09	57.99
caM24r2	43695867	42701092	0.03	97.4	92.65	58.01
caM24r3	44420970	43876468	0.03	97.26	92.34	57.64
caM24r4	42429064	42018278	0.03	97.24	92.3	57.89
caM288r1	51628308	51103413	0.03	97.05	91.85	55.66
caM288r2	45940842	45552384	0.03	97.25	92.34	56.76
caM288r3	50479380	49722270	0.03	97.22	92.22	56.65
caM288r4	43937626	43351782	0.03	97.12	92.01	57.18
caM36r1	42600783	42134566	0.03	97.42	92.72	55.64
caM36r2	45975166	45377073	0.03	97.4	92.7	55.51
caM36r3	56557150	55837695	0.03	96.9	91.58	56.54
caM36r4	43675462	43223007	0.03	97.03	91.82	55.84
caM48r1	45026685	44680359	0.03	97.26	92.4	57.98
caM48r2	41159738	40482202	0.03	97.3	92.45	57.72

caM48r3	47391638	46852930	0.03	97.2	92.18	57.59
caM48r4	44570460	44133723	0.03	97.43	92.71	57.86
ifA00r1	44315526	43577522	0.03	97.69	93.2	59.24
ifA00r2	43938812	43143855	0.03	97.64	93.04	58.56
ifA00r3	44718346	43656909	0.03	97.34	92.46	58.57
ifA00r4	43138634	42221085	0.03	97.51	92.77	59.13
ifA12r1	44139585	42868057	0.03	97.33	92.43	57.36
ifA12r2	45917903	44937978	0.03	97.23	92.33	56.12
ifA12r3	40168198	38984932	0.03	97.09	92	57.35
ifA12r4	45528833	44365963	0.03	97.23	92.23	56.68
ifA24r1	43428249	42351453	0.03	97.32	92.53	58.38
ifA24r2	45987550	45496610	0.03	97.23	92.29	57.41
ifA24r3	50643492	50148751	0.03	97.51	93.02	56.99
ifA24r4	45369046	44974857	0.03	97.13	92.12	58.15
ifA288r1	43396590	42665198	0.03	97.11	91.96	55.75
ifA288r2	43767058	43420737	0.03	97.22	92.21	57.39
ifA288r3	40186808	38707964	0.02	98.49	95.62	57.44
ifA288r4	48324920	47794800	0.03	97.33	92.39	57.31
ifA36r1	45543250	45127763	0.03	97.16	92.09	55.83
ifA36r2	44465586	43757809	0.03	97.11	92.05	55.34
ifA36r3	43617990	42947118	0.03	97.16	92.09	56.34
ifA36r4	51596469	51128667	0.03	97.18	92.14	56.71
ifA48r1	53237153	52780821	0.03	96.94	91.66	57.48
ifA48r2	47384341	47003972	0.03	97.14	92.08	57.88
ifA48r3	44814135	44342529	0.03	97.19	92.14	56.99
ifA48r4	40452328	40035805	0.03	97.12	91.97	57.44
ifB00r1	40044071	39313756	0.03	97.5	92.75	59.27
ifB00r2	43459450	42825500	0.03	97.33	92.41	58.11
ifB00r3	39902526	39266616	0.03	97.33	92.42	58.11
ifB00r4	45342608	44489386	0.03	97.49	92.7	59.13
ifB12r1	44374047	43418468	0.03	97.43	92.64	57.24
ifB12r2	42937334	41471697	0.03	97.31	92.34	56.68
ifB12r3	45926223	44490649	0.02	98.14	94.76	56.99
ifB12r4	44202145	43156251	0.03	97.13	91.99	56.37
ifB24r1	48195813	47579946	0.03	97.22	92.26	57.58
ifB24r2	40152226	39663256	0.03	97.18	92.15	57.28
ifB24r3	42311326	40873062	0.02	98.1	94.59	57.98
ifB24r4	42563410	40562073	0.02	98.08	94.58	57.73
ifB288r1	53475626	52943494	0.02	98.16	94.95	56.24
ifB288r2	44731904	44161892	0.03	97.49	92.78	56.84
ifB288r3	45549578	44980930	0.03	97.23	92.22	57.18
ifB288r4	41228828	40646593	0.03	97.25	92.28	57.53
ifB36r1	42411565	41715246	0.03	97.27	92.3	56.68
ifB36r2	47185072	46501955	0.03	97.2	92.21	55.3
ifB36r3	41042194	40429412	0.03	97.25	92.29	56.36
ifB36r4	41944692	39783928	0.02	98.1	94.63	55.22

ifB48r1	46652536	45710620	0.03	97.31	92.41	57.55
ifB48r2	44948474	44188418	0.03	97.34	92.54	57.42
ifB48r3	44813732	44391903	0.03	97.21	92.18	57.08
ifB48r4	42613943	40394005	0.02	98.02	94.38	57.19
ifM00r1	40609635	39972605	0.03	97.72	93.24	58.74
ifM00r2	60445830	59048613	0.03	97.24	92.26	58.74
ifM00r3	43227442	42322208	0.03	97.34	92.44	58.27
ifM00r4	49355254	48502481	0.03	97.39	92.54	58.33
ifM12r1	42775911	41887045	0.03	97.37	92.51	57.21
ifM12r2	42953815	41673347	0.03	97.32	92.46	56.97
ifM12r3	42046852	40841727	0.03	97.44	92.71	57.32
ifM12r4	44067716	43295134	0.03	97.38	92.63	57.54
ifM24r1	48486711	47067455	0.03	97.47	92.86	58.28
ifM24r2	44764448	43907060	0.03	97.36	92.64	58.2
ifM24r3	43887585	43474571	0.03	97.16	92.13	57.04
ifM24r4	43106179	42408724	0.03	97.28	92.34	57.51
ifM288r1	40924338	40472264	0.03	97.07	91.84	55.41
ifM288r2	47663767	47209518	0.03	97.06	91.87	56.39
ifM288r3	40302266	39469681	0.03	97.24	92.23	56.48
ifM288r4	41596107	41069842	0.03	97.03	91.81	56.39
ifM36r1	41052584	40536667	0.03	97.28	92.39	56.07
ifM36r2	46491318	45834117	0.03	97.3	92.5	55.78
ifM36r3	51820109	51186318	0.03	97.5	93.04	56.83
ifM36r4	43335517	42874977	0.03	97.34	92.53	55.91
ifM48r1	44232219	43935414	0.03	96.96	91.67	57.95
ifM48r2	43196107	42080278	0.03	97.21	92.28	57.66
ifM48r3	40772562	40229898	0.03	97.27	92.32	57.08
ifM48r4	44332063	43859815	0.03	97.11	91.98	57.68

*Abbreviations: ca: Cassia, if: Infinity, A: OP18(9), B: 44.07, M: mock, r: biological replicate. E.g.: ifA36r1 = Infinity, Treatment A (OP18(9)), 36 hpi, replicate 1