The University Of Sheffield.

The role of basal resistance in *Arabidopsis thaliana* against the parasitic weed *Striga gesnerioides*.

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'but those who hope in the Lord will renew their strength. They will soar on wings like eagles; they will run and not grow weary, they will walk and not be faint.' **Isaiah 40:31**

This thesis is dedicated to my parents and brother, David, without who constant love, encouragement and support I would never have reached this point. I only wish my dear mum could have lived to have seen this work finally submitted.

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Declaration:

No portion of the work referred to in this thesis has been submitted in support of another application for another degree of qualification at this or any other university of institute of learning.

Abstract:

This PhD project investigated the role of basal resistance in limiting infection by the parasitic weed *Striga gesnerioides*, in the model host *Arabidopsis thaliana*. A range of *Arabidopsis* knock-out mutants affected in different defence components were screened for altered resistance to *S. gesnerioides*. The results indicated that the hormone salicylic acid (SA) and the transcription factors WRKY70 and ORA59 may protect against initial infection, whilst camalexin may inhibit later stages of parasite development. Meanwhile, the hormones jasmonic acid and ethylene appeared to promote susceptibility. Gene expression analysis during a time-course of infection revealed strong induction of SA-associated genes (e.g. *PR5, PR2*), besides those associated with reactive oxygen species (ROS) detoxification (e.g. *GST1, PRX33*). Overall, the induced gene signature showed similarities with that induced by biotrophic microbial pathogens. Excessive ROS may be a susceptibility factor, since ROS-deficient RbohD and *RbohD/RbohF* mutants showed increased resistance: furthermore, 3,3'-Diaminobenzidine (DAB) staining revealed strong ROS activity at the host-parasite interface, apparently originating from the parasite. The gene *ERF4*, which can be alternatively expressed as a transcriptional repressor or activator appears particularly relevant since the *erf4-1* mutant showed significantly greater susceptibility to *S. gesnerioides*. Phenotypic analysis and gene expression of an independent *erf4-2* mutant and *ERF4-Activator* over expression line indicated that undisrupted *ERF4-Repressor* function is crucial for host basal resistance. Gene expression analysis suggested that altered ROS homeostasis and/or a reduced SA-associated response may be the basis of the increased susceptibility of *erf4-1* to *S. gesnerioides*. Despite a strong local defence response in the roots, *S. gesnerioides* did not induce a systemic immune response such that above-ground resistance to two foliar pathogens (the biotroph *Hyaloperonospora arabidopsidis* and the necrotroph *Plectosphaerella cucumerina*) was affected, suggesting that the parasite may suppress host immune system using effector molecules.

Summary:

Striga gesnerioides, a hemi-parasitic plant that parasitizes many dicotyledenous species, is a severe constraint on cowpea production in Sub-Saharan Africa. Little is understood about how *S. gesnerioides* overcomes the host immune system and what defence pathways contribute to host basal resistance, ultimately determining the severity of infection on susceptible cultivars. This thesis sought to investigate this by systematically applying knowledge from other plant-root pathogen interactions, using *Arabidopsis thaliana* as a model host for *S. gesnerioides*. An initial phenotypic screen was made of *Arabidopsis* mutants impaired in defence hormone signalling, the generation of reactive oxygen species (ROS) and the production of defensive secondary metabolites. This was followed by targeted analysis of defence gene expression during a time-course of infection in wild-type Col-0 plants. The results indicated that salicylic acid (SA) contributes to basal resistance, since the SA biosynthesis mutant *sid2-1* showed greater early-stage susceptibility and infected Col-0 roots showed strong upregulation of SA-inducible defence marker genes (e.g. *PR5*, *PR2*). A signature similarity search on GENEVESTIGATOR indicated that the overall gene expression response was

similar to that induced by biotrophic pathogens, which are suppressed by SA-regulated defences. Conversely, certain mutants relating to jasmonic acid (*aos1, opr3-1*) and ethylene (*etr1-1, ein3-1*) appeared more resistant to *S. gesnerioides*, whilst there was no effect for the ABA hyper-sensitive mutant *abi1-2*. Camalexin was implicated as a defence compound at late stages of parasite development, since the proportion of haustoria with developing shoots was markedly greater on camalexin-deficient *cyp79B2/B3* mutants. This was corroborated by the finding that the camalexin-biosynthesis gene PAD3 was strongly induced during late stages of infection. Overall, wound-response genes regulated by JA and JA/ethylene genes related to necrotrophic defence showed poor upregulation in response to *S. gesnerioides*. WRKY70 may be important transcription factor in this interaction, since the corresponding mutant showed increased early-stage susceptibility; in addition, *WRKY70* was induced significantly during infection.

Furthermore, ROS homeostasis appears to play a critical regulatory role in basal resistance, since the *RbohD* and *RbohD/RbohF* mutants (impaired in ROS generation) showed increased resistance at both early and late stages of infection. In addition, 3,3'-Diaminobenzidine (DAB) staining revealed strong ROS activity at the host-parasite interface and genes relating to ROS detoxification (*GST1, PRX33, PRX53*) were significantly induced during infection of Col-0 plants. The importance of ROS was further supported by the finding that the *erf4-1* mutant, which is affected in ROS homeostasis, showed markedly increased early-stage susceptibility. ERF4 can be transcribed as either an activator or repressor, and the activator variant has been shown to suppress ROS-dependent defence signalling. Indeed, over-expression of the ERF4-Activator variant induced susceptibility in a wild-type line, which is consistent with the hypothesis that ROS contributes to basal resistance to *S. gesnerioides*. Surprisingly, however, the independent *erf4-2* mutant was not affected in basal resistance to *S. gesnerioides*, despite the fact that both *erf4-1* and *erf4-2* mutants lacked the ERF4-Activator variant. Expression analysis of *ERF4* splice variants revealed that the *erf4-1* mutant, unlike the *erf4-2* mutant, retained some expression of the *ERF4-Repressor* variant and expressed wild-type levels of the *ERF4-Intron Retention* variant. Together, these results indicate that the elevated ERF4-Activator : ERF4-Repressor ratio in the *p35S:ERF4-Activator* line and the elevated ERF4-Intron Retention:ERF4-Repressor ratio in the *erf4-1* mutant both cause enhanced susceptibility to *S. gesnerioides* by interfering with the resistance-inducing activity of the ERF4-Repressor variant.

Finally, investigations were also made into whether *S. gesnerioides* induces systemic effects on host resistance that impact above-ground defences against foliar pathogens. Pre-infection with *S. gesnerioides* did not induce a systemic response against the foliar pathogens *Hyaloperonospora arabidopsidis* (a biotroph) or *Plectosphaerella cucumerina* (a necrotroph). In conclusion, the work presented in this thesis advances our understanding of the plant basal resistance response against the parasitic weed *S. gesnerioides*.

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CHAPTER ONE

An introduction to plant defence and root parasitic weeds.

ABSTRACT

Parasitic plants of the genus *Striga* are a severe constraint on food security particularly in Sub-Saharan Africa, where they infect most staple crops. This work focuses on *Striga gesnerioides*, an obligate parasite of cowpea. The lifecycle of *Striga* has evolved in a highly sophisticated manner alongside the host, making it a difficult problem to control and eradicate. Resistant phenotypes against *S. gesnerioides* are known, however these tend to be race-specific, monogenic traits that the parasite can rapidly evolve to overcome. Consequently, there is much interest in the genetic basis of basal resistance in susceptible interactions. Evidence from other host-root parasite interactions suggests that this shows overlap with basal resistance mechanisms against microbial plant pathogens, such as bacteria and fungi. Basal resistance is based on a multitude of different signalling and defence mechanisms, including defence hormones (e.g. salicylic acid, jasmonic acid), formation of structural barriers and production of toxic secondary metabolites (such as phytoalexins). Comparative analysis of basal resistance mechanisms against other root pathogens, including parasitic nematodes and *Fusarium oxysporum*, can provide promising avenues to investigate regarding the genetic basis of basal resistance against *S. gesnerioides*.

1.1 An introduction to parasitic plants

Securing a sustainable and nutritious diet for all remains one of the most pressing challenges of modern times. World hunger is rising, particularly in countries affected by climate change and increasingly extreme weather patterns. One of the most food deprived areas is Sub-Saharan Africa, where it is estimated that 23.2% of the population are undernourished (Food and Agricultural Organisation of the United Nations 2018). Farmers in this region, most of which operate at a small-scale subsistence level, face a challenging combination of pressures including drought, soil erosion, insect pests, plant diseases and poor access to markets. For many, however, the greatest constraint on crop production is actually caused by other plants (Scholes and Press 2008). These are not straightforward weeds, competing mainly for space and light; these plants are parasites that attach to and live off unwilling victims. The most notorious parasitic weeds are those of the *Striga* genus, whose sophisticated lifecycle makes them both highly effective in exploiting host plants and such a difficult problem to eradicate.

Parasitism is a highly effective life strategy, as demonstrated by its presence across all kingdoms of life and its having independently evolved at least 12 times within angiosperms (Westwood et al. 2010). Approximately 1% of angiosperms are parasitic, encompassing 3-4,000 species across 16 families (Westwood et al. 2010). These species represent a tremendous diversity of forms and sizes (including trees, shrubs and vines), whose native habitats encompass almost the entire globe, from tropical forests to the Svalbard Archipelago (Musselman and Press 1995). What they share in common is a complex, specialised absorptive organ, called a haustorium (from the Latin *haustor*, 'water-drawer'), which functionally connects the parasite to the host's vascular system (Kuijt 1969).

Parasitic plants can be broadly classified into two groups depending on where they attach to their host. Stem parasites such as the mistletoes and Dodder (*Cuscuta* and *Cassytha*) infect their host in above ground regions, while root parasites, for instance broomrape (*Orobanche*), attack below ground and infect their host via the roots (Musselman and Press 1995). Another important distinction is the degree to which the parasite depends on the host. Facultative parasites such as *Triphysaria* and *Rhinanthus* are capable of completing their life cycle independently but will exploit a host if the opportunity arises. Obligate parasites, on the other hand, depend entirely on a host plant and must attach immediately after germination in order to survive (Estabrook and Yoder 1998, Kuijt 1969). In addition, parasitic plants differ in the nutrients they aquire from the host although all functionally connect to the host's vascular system. Hemi-parasites retain photosynthetic capacity and predominantly extract water via connections with the host's xylem vessels. Holo-parasites, on the other hand, lack photosynthetic capacity and thus make connections to both xylem and phloem vessels to extract water and carbon sugars (Musselman and Press 1995). These categories are not clear-cut however; *Cuscuta reflexa* for

instance, is considered as an intermediate state between holo-and hemi-parasites since it retains low levels of chlorophyll and rubisco that appear to recycle carbon by-products from the host (Hibberd et al. 1998). In addition, hemi-parasitic root parasites could potentially be considered holo-parasites until their shoots emerge above ground. Parasitic plants also vary in their host ranges; certain *Cuscuta* and *Castellija* can infect hundreds of different hosts across many families whereas others are restricted to just one or a few host species (Musselman and Press 1995). The mistletoe species *Viscum capitellatum* for instance, is an obligate parasite primarily on the mistletoe *Dendrophthoe falcata*, a curious case of epi-parasitism (Calvin and Wilson 2009).

Whilst most parasitic plants are harmless botanical curiosities, those that infect economically important crops can become serious pests. Three obligate hemi-parasites of the *Striga* genus are the most notorious parasitic weeds, between them affecting nearly all staple crops. *Striga hermonthica* and *Striga asiatica* infect cereals (for instance rice, maize and sorghum) whilst *Striga gesnerioides* is a parasite of legumes. Infected plants typically show symptoms of chlorosis and wilting, similar to that induced by drought and vascular disease (Nweze 2015) (Figure 1.1). In addition, *Striga* exerts a marked stunting effect on the host, giving it the common name 'Witchweed' (Figure 1.1). The difference in host height cannot be accounted for by the biomass of the attached parasites (Gurney, Press and Scholes 1999), suggesting *Striga* disrupts endogenous hormone signalling pathways that normally promote growth.

Striga species are estimated to affect over 40% of the cereal producing regions of sub-Saharan Africa, affecting the lives of over 100 million people (Scholes and Press 2008). Average yield losses range from 30-90% (Ejeta , Ejeta 2007) and are worth approximately $10 billion each year (Westwood et al. 2010). This problem continues to escalate, aided by increased monocropping, reduced soil fertility and general ignorance and lack of resources regarding control methods (Oswald 2005).Whilst the scale of damage wrought by all three *Striga* spp. is large, this project focuses on *Striga gesnerioides*, a major scourge of cowpea, an economically valuable legume crop.

1.2 Cowpea: a vital resource for food security in Sub-Saharan Africa

Cowpea, *Vigna unguiculata*, a relatively inexpensive source of high-quality protein, is the most economically important legume indigenous to Africa (Langyintuo et al. 2003). Of the 3.7 million tonnes produced globally each year, 65% originates from Sub-Saharan Africa where cowpea is a major cash crop for many subsistence farmers (Langyintuo et al. 2003). Cowpea is a preferred crop in these regions as its deep taproots confer high drought tolerance, allowing it to produce a harvest with only 300 mm of annual rainfall (Carlos Gómez 2004). For the 200 million Africans that consume cowpea, it is a vital source of protein within diets otherwise based on cereals and tubers (Carlos Gómez 2004).

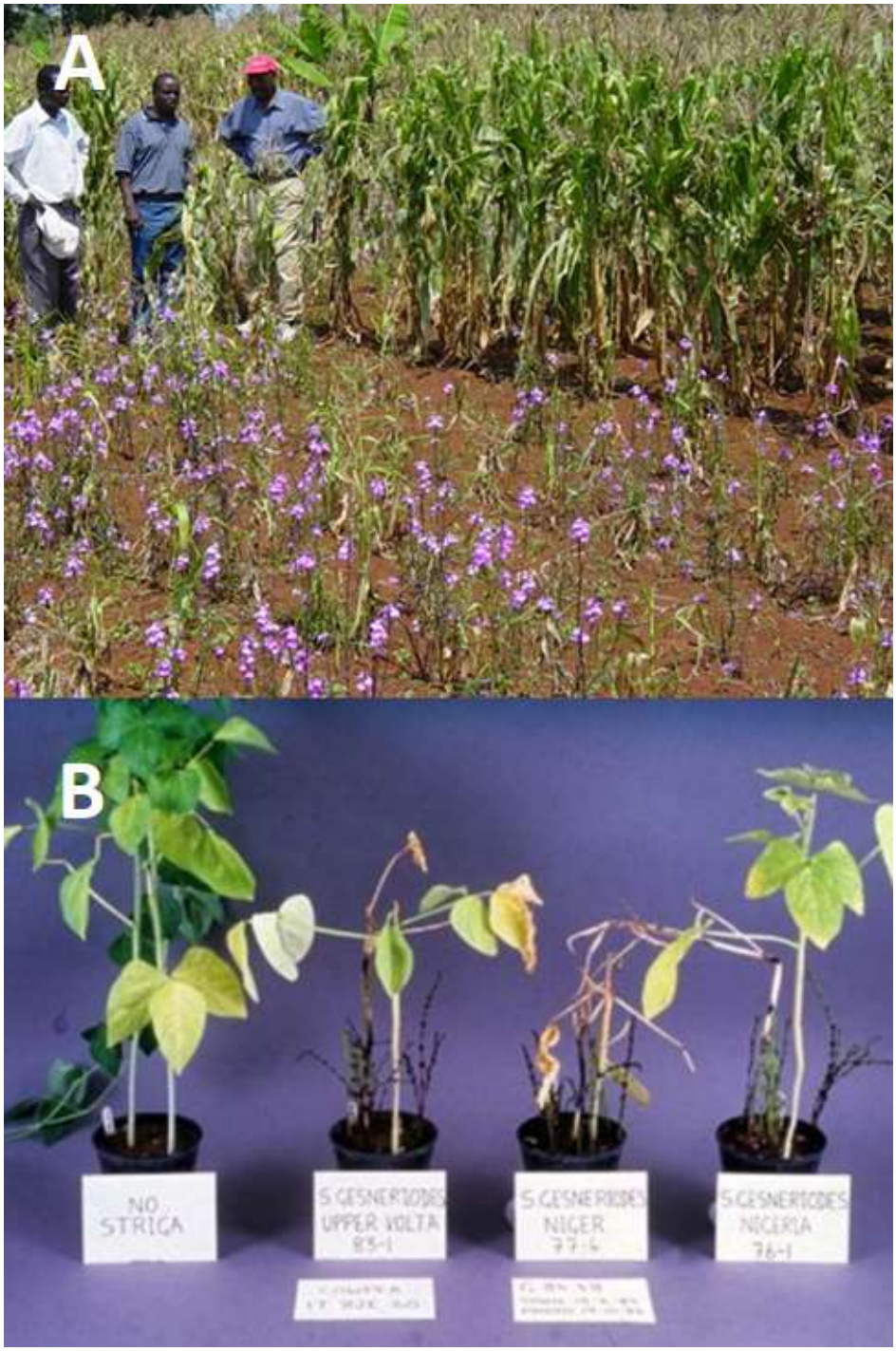


Figure 1.1: The effects of *Striga* root parasitic weeds on susceptible crop hosts. A: Maize crop infested with *Striga hermonthica*. Note the difference in height compared with the non-infected plants in the background. Image provided with kind permission by Joel Ransom, North Dakota State University, 2018. **B:** Cowpea infected with *Striga gesnerioides*. From left to right, cowpea plants have been infected as follows: No Striga; infected with *S. gesnerioides* from Upper Volta; infected with *S. gesnerioides* from Niger; infected from *S. gesnerioides* from Nigeria. Note the signs of chlorosis and wilting on the *S. gesnerioides* -infected cowpea plants. Image from the International Institute of Tropical Agriculture, IITA, 2010.

As a legume, cowpea also improves soil quality through nitrogen fixation, and is frequently used in crop rotation schemes with cereals (Tarawali et al. 2002). The leaves and stalks are an important source of fodder for ruminant livestock, particularly during the dry season. It has been estimated that a single hectare of cowpea can bring a farmer an additional 50 kg of meat each year through better-fed livestock, besides 300 kg more cereal grain through improved soil fertility (Tarawali et al. 2002). Cowpea's resilience and flexibility make it a valuable resource in achieving sustainable agricultural production in the context of a changing climate (Gómez 2004).

Cowpea producers face many challenges, including cowpea weevil, aphids, fungal pathogens, bacterial blight and viruses (Gómez 2004). One of the most devastating pests, however, is *Striga gesnerioides*, which is estimated to cause $200 million worth damage to cowpea yields across West and Central Africa each year (Singh 2002). Average annual yield losses are estimated to be 30%-40% (Gómez 2004, Omoigui et al. 2017) however this can be much more severe: in certain dry regions of Nigeria, for instance, *S. gesnerioides* infestations have been reported which reduced potential cowpea yields from 2-3 tonnes per hectare to just 0.37 tonnes (IITA 2008). As described below, the difficulty in eradicating this parasite can be attributed to various features of the *Striga* lifecycle.

1.3 Lifecycle of *Striga* parasites

As an obligate parasite, the lifecycle of *Striga* spp. has evolved to become intimately intertwined with that of its host. Parasite seeds remain dormant in the soil until stimulated to germinate by compounds in the root exudates of potential hosts (known as strigolactones) (Figure 1.2 A) (Ueno et al. 2011). As *Striga* seed have minimal reserves, this strategy ensures that germination only occurs when a potential host is in close proximity. Prior to germination, an initial "pre-conditioning phase" in a warm, moist environment is required for parasite seeds to become responsive (Rich and Ejeta 2007). Once germinated, the seed produces a finger-like projection called a radicle, which grows in a directional manner towards the host root. On contacting the host root, the radicle ceases to grow further and instead produces sticky hemicellulose hairs to aid attachment (Figure 1.2 B) (Hood et al. 1998, Reiss and Bailey 1998, Dörr 1997). Following this, chemicals from the host (termed Haustorial Initiation Factors, HIFs) trigger the development of the mature haustorium which functionally connects the host and parasite. HIFs identified from root extracts include 2,6-dimethoxy-1,4-benzoquinone (DMBQ), derived from syringic acid, a breakdown product of lignin (Yoshida et al. 2016). Subsequently identified HIFs include p-coumaric acid and vanillic acid, also components of lignin. Emerging *Striga* radicles are a potent source of hydrogen peroxide (H_2O_2), thus leading to a model whereby parasite-encoded peroxidases and H_2O_2 released by the radicle convert host lignin molecules into HIFs (Figure 1.2 A) (Kim et al. 1998, Keyes et al. 2007). This is supported by evidence that haustorial formation of germinating *S. hermonthica* seedlings can be reduced by applying reactive oxygen species (ROS) inhibitors (Wada,

Cui and Yoshida 2019) or through manipulating the lignin composition of the host (Cui et al. 2018). A wedge of intrusive cells then penetrates the cortex and endodermis (Figure 1.2 B), using a combination of mechanical pressure and enzymatic digestion of host cell walls (Hood et al. 1998, Reiss and Bailey 1998, Dörr 1997). On reaching the vascular stele, intrusive cells develop openings called oscula and penetrate the pits of xylem vessels. These and adjacent haustorial cells lose their protoplast and intervening cell walls to form continuous water-conducting elements (Figure 1.2 B) (Dörr 1997). *Striga* spp. are not known to make connections with host phloem vessels (Dörr 1997); as hemi-parasites, they retain photosynthetic capacity and appear to rely on their host mainly for water and nutrients. Following vascular penetration, the haustorium differentiates into three distinct tissues; the endophyte (the part of the parasite located within host tissues), the vascular core and the hyaline body (Hood et al. 1998, Reiss and Bailey 1998, Dörr 1997). Nutrient transfer to the parasite is promoted by high parasite transpiration rates and the production of osmoticants which maintain a favourable water potential gradient (Joel, Gressel and Musselman 2013b, Ehleringer and Marshall 1995). Accumulated starch fuels the growth of a distinct swelling, 3-4 cm in diameter, from which the apical meristem develops (Joel, Gressel and Musselman 2013a).

Several features of the *Striga* lifecycle make it particularly difficult to control. Each flowering stalk is capable of producing up to 100,000 dust-like seed which are readily dispersed by the wind (Scholes and Press 2008). Furthermore, these seed can remain dormant for at least 20 years, making it difficult to eradicate the parasite once it becomes established on land (Scholes and Press 2008). As initial germination and attachment take place underground, farmers are typically unaware that their crop is infected until the flowering shoots emerge. The subsistence farmers who are worst affected by the parasite have very limited control strategies (Oswald 2005). The most common include hand-weeding the flowering stems, crop-rotation, intercropping with non-host species and improving soil fertility through organic means (since *Striga* spp. are more prevalent in less fertile soils). More sophisticated chemical treatments, such as nitrogen fertilisers, seed-dressing herbicide resistant crops (Kanampiu et al. 2002) and artificial stimulants that induce 'suicidal germination' (Abayo et al. 1998) are prohibitively expensive for most. It is widely believed that the most effective long-term control strategy is the development of improved crop varieties showing strong resistance (Scholes and Press 2008).

1.4 Resistant responses against *Striga* parasites

Host resistance can act at various points of the parasite lifecycle, and incorporate both chemical and physical defences. Pre-attachment resistance is associated with low release of the strigolactones that stimulate parasite germination. This has been bred for in *Striga*-resistant sorghum (Haussmann et al. 2000) and assessed in carotenoid-biosynthesis impaired maize mutants (Matusova et al. 2005).

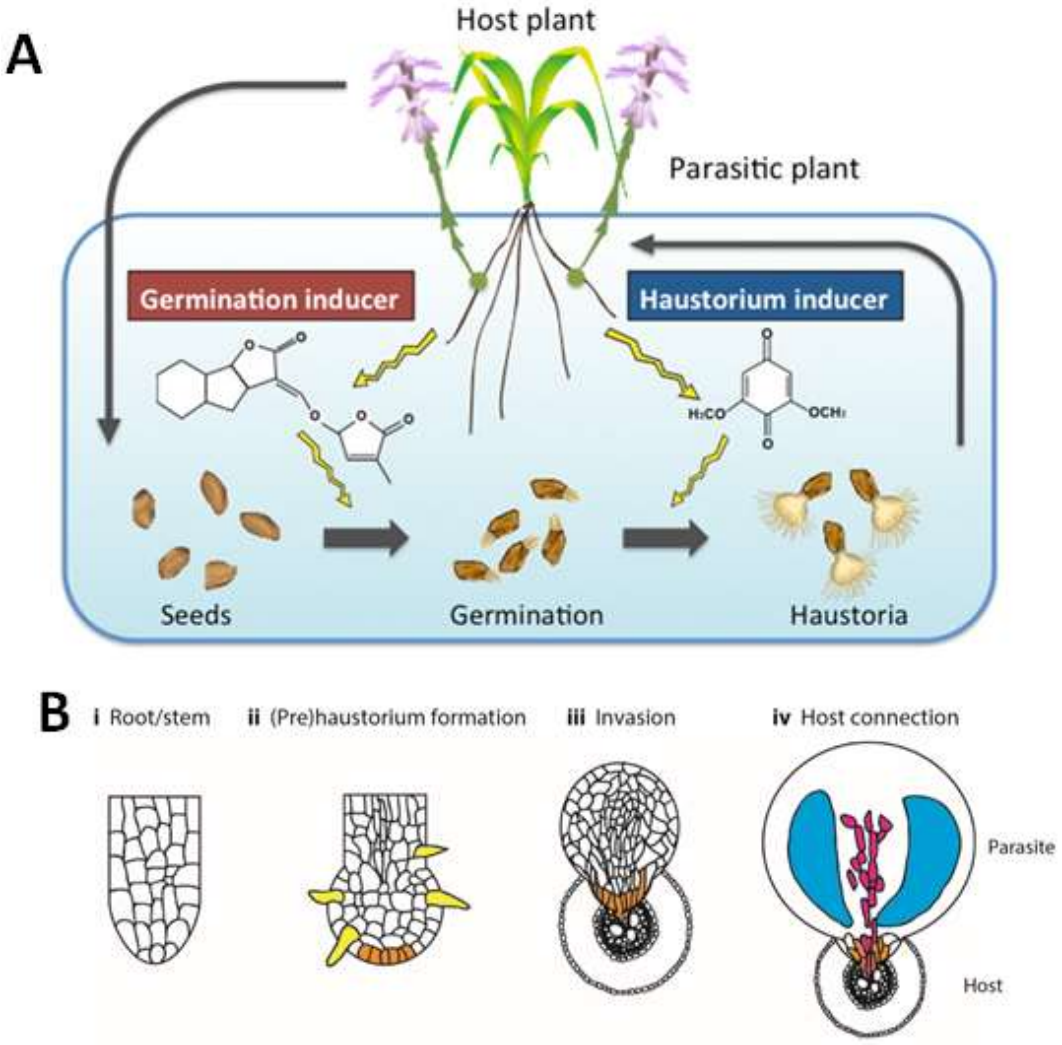


Figure 1.2: Life cycle of obligate parasite *Striga spp*. A. Chemical stimulation of parasite seed. *Striga* seed remain dormant in the soil and require a warm, moist conditioning phase to become responsive to chemical cues. Compounds known as strigolactones present in the root exudates of suitable hosts act as gemination inducers on conditioned *Striga* seed. The germinated seed produces a radicle that grows directionally to the host root. H_2O_2 produced by the radicle tip is a substrate for host peroxidase enzymes, causing them to liberate cell wall polyphenols. These diffuse towards the parasite seed where they induce a developmental transition that forms a haustorium: an absorptive organ that functionally connects host and parasite. Image provided with kind permission by Professor Satoko Yoshida. **B.** Process of host root penetration. **i)** Germinated parasite seed produce a radicle that grows directionally towards the host root. **ii)** Haustorial Initiation Factors (HIFs) from the host root promote the development of the absorptive haustorium and sticky hemicellulose hairs to aid attachment. **iii)** The haustorium penetrates the host root cortex as a wedge of intrusive cells, using a combination of mechanical pressure and enzymatic digestion of cell walls. **iv)** The haustorium penetrates the host vascular system and differentiates to form continuous xylem-xylem connections. Image from (Yoshida et al. 2016).

Resistance can also act immediately after the parasite attaches to the root in the form of a rapid hypersensitive response (HR), similar to those deployed against biotrophic pathogens. This is seen in cowpea cultivars resistant to *S. gesnerioides* (Lane et al. 1993a) (Figure 1.3 A-B) and the sorghum cultivars Framida and Dobbs against *S. hermonthica* (Mohamed et al. 2003), where rapid, localised cell death at the attachment site prevents further entry of the parasite, causing it to die. A similar response occurs in the non-host interaction between marigold (*Tagetes erecta*) and *S. asiatica*. This is associated with rapid necrosis of root cortical cells flanking the site of attempted penetration, and cell wall thickening in adjacent cells (Gowda, Riopel and Timko 1999).

In some cases, the parasite penetrates the root but fails to make connections with the host vascular system. This occurs in Nipponbare rice where *S. hermonthica* is unable to breach the endodermis, causing it to encircle the stele or pass through the cortex on the other side (Gurney et al. 2006). Similar post-attachment resistance is seen in sorghum cultivar N-13, where both *S. asiatica* and *S. hermonthica* fail to penetrate the endodermis: this phenotype is associated with lignification and thickening of endodermal and pericycle cells (Haussmann et al. 2004, Maiti et al. 1984).

Even when the parasite establishes vascular continuity, host resistance can inhibit further development. This occurs during attempted infection of *S. hermonthica* on the wild relative of maize *Tripsacum dactyloides*: here the parasite forms initial connections with the xylem but fails to differentiate further, indicating that the host either produces a compound that actively inhibits further development or lacks key differentiation factors (Gurney et al. 2003). In the sorghum cultivar SRN-4841, tylose-like occlusions are induced in the cavities of xylem vessels breached by *S. asiatica* (Maiti et al. 1984). Late-stage resistance is also known in cowpea. In cowpea cultivar B301, *S. gesnerioides* that overcome the host HR and successfully penetrate the vascular system are unable to develop beyond 1-2 mm in size and do not produce a flowering shoot (Lane et al. 1993a).

The molecular basis of post-attachment resistance responses against *Striga* remains unclear and is likely to vary between host-parasite interactions. Comparative analysis with post-attachment resistance to *Orobanche* (broomrape) holo-parasites has proposed that these mechanisms may include the accumulation of toxic phytoalexins and phenolics (Lozano-Baena et al. 2007, Serghini et al. 2001), occlusion of vessel elements (Labrousse et al. 2001, Echevarría-Zomeño et al. 2006), protein cross-linking of host cell walls (Perez-de-Luque et al. 2006, Echevarría-Zomeño et al. 2006) and lignification of the host endodermis and pericycle (Pérez-De-Luque et al. 2005, Pérez-de-Luque et al. 2007).

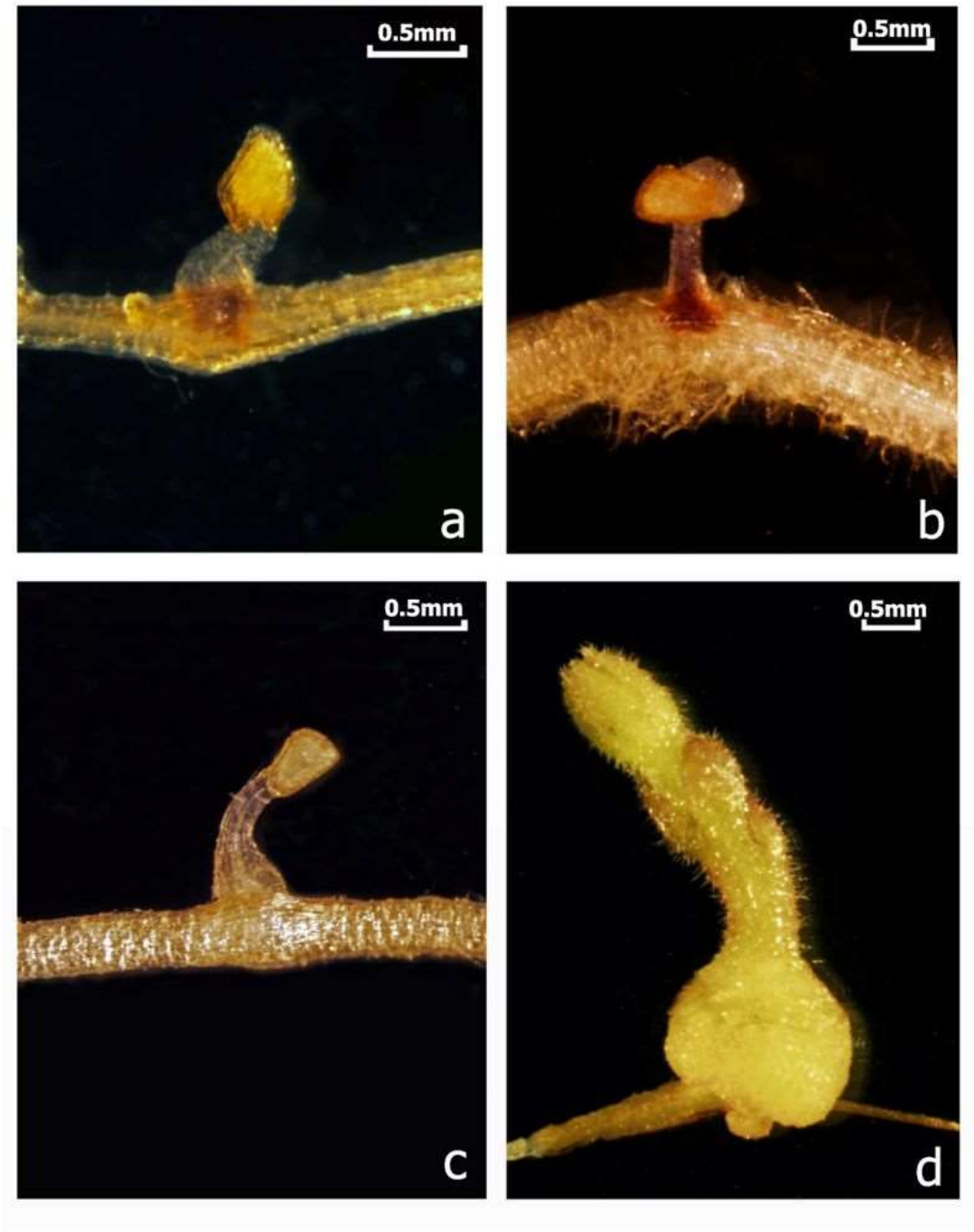


Figure 1.3: The resistant (A-B) and susceptible (C-D) response of cowpea cultivar B301 to different races of *Striga gesnerioides*. **A:** Root infected with incompatible *S. gesnerioides* race SG3 at 6 dpi. The host has already begun to mount a hypersensitive response against the parasite, with necrosis visible at the host-parasite interface. **B:** Root infected with SG3 at 13 dpi. Programmed cell death at the host-parasite interface has prevented further invasion causing the parasite to die. **C.** Root infected with the hyper-virulent *S. gesnerioides* race SG4z at 6 dpi. No hypersensitive response is evident in the host tissue and the parasite has begun to penetrate the root. **D:** Root infected with SG4z at 13 dpi. The parasite has progressed to form a large haustorium with a developing shoot. Image from (Huang et al. 2012).

1.5 Race-specific resistance to *Striga gesnerioides*

More so than *S. hermonthica* and *S. asiatica*, *S. gesnerioides* can be subdivided into distinct races, such that cowpea cultivars may be resistant to *S. gesnerioides* originating from one region, but susceptible to those from other areas. Cowpea cultivar 58-57, for instance, is resistant to *S. gesnerioides* from Burkina Faso but susceptible to strains from Mali (Lane et al. 1993b). Analysis of differential host responses and genome profiling with molecular markers has identified at least seven distinct strains of the parasite (Botanga and Timko 2006). Complete resistance is not known, yet cowpea cultivar B301 shows resistance to 6 of the 7 known races of *S. gesnerioides* and has been a major focus of improved cowpea breeding programmes (Li, Lis and Timko 2009). Nevertheless, this phenotype, and indeed most sources of *S. gesnerioides* resistance, is conferred by a single dominant gene (Touré et al. 1997, Li et al. 2009) making it relatively easy for the parasite to evolve to overcome resistance. Indeed, when originally identified B301 was thought to show complete resistance across all known *S. gesnerioides* strains (Singh and Emechebe 1990). In the early 1990s however, a new hypervirulent race (designated SG4z) was discovered to which B301 is susceptible (Figure 1.3 C-D)(Lane et al. 1993b). Consequently, there is much focus on using marker-assisted selection to screen for cultivars with multiple resistance genes and thus more durable resistance (Omoigui et al. 2017).

1.6 Introducing the concept of basal resistance

Even in susceptible interactions, levels of *S. gesnerioides* infestation vary markedly between cowpea cultivars, including trials that control for environmental factors and the race of parasite (Omoigui et al. 2017). Indeed, most plant-pathogen interactions show gradients of susceptibility (Niks and Marcel 2009). This is attributed to variations in the host's genome translating into different levels of basal resistance. According to Dangl and Jones, basal resistance can be defined as defences that restrict pathogen spread after successful infection and the onset of disease symptoms; these can be overlaid by race-specific responses (i.e. 'gene-for-gene' resistance, reviewed below) (Dangl and Jones 2001). A human analogy could be the winter flu virus: most people are susceptible to this but symptoms will vary from mild to severe among those affected. The genetic elements that underlie basal resistance in any plant-pathogen interaction can be inferred from genetic mutants which show altered levels of susceptibility compared with their wild-type counterpart. As reviewed below, plant defence is a complex system that operates at multiple levels, hence these genetic elements could act through various mechanisms including synthesising defence-related hormones, transcribing defence gene regulators and reinforcing structural barriers.

1.7 A basic overview of plant defence

Literally rooted in position, plants cannot physically flee the wide variety of fungal, bacterial and insect predators that daily assault them. Consequently, they have developed a sophisticated array of defence mechanisms frequently tailored to the specific attack they face. Producing defensive compounds is metabolically costly, hence these defences tend to be induced in response to an attack rather than constitutively expressed (van Hulten et al. 2006). The pathogen-recognition systems that trigger these defences have naturally become targets for suppression by the pathogens themselves. This has locked plants and their predators into an evolutionary 'arms race' with each evolving higher levels of defence and virulence respectively.

The most basic level of plant immunity is Pattern-Triggered Immunity (PTI) where invading organisms are recognised by conserved microbe- or pattern-associated molecular patterns (MAMPs and PAMPs) that distinguish between self and non-self. These include bacterial cell-surface molecules such as flagellin, besides chitin, a dominant component of fungal cell walls (Chisholm et al. 2006, Boller and Felix 2009) (Figure 1.4). These trigger pattern-recognition receptors (PRRs) to activate innate defences (Macho and Zipfel 2014), which can include an oxidative burst, strengthening cell walls through callose and lignin deposition, inducing lytic enzymes (e.g. chitinases) and producing antimicrobial compounds such as phytoalexins and defensins (Nürnberger et al. 2004). These responses can also be precipitated by degraded 'self-molecules' produced during the invasion process, known as damage-associated molecular patterns (DAMPs) (Boller and Felix 2009).

Many pathogens secrete effector molecules that target and suppress PTI responses, causing effector-triggered susceptibility (ETS) in the host (Figure 1.4). In turn, host plants have developed a second tier of plant immunity (effector-triggered immunity, ETI) which recognises the secreted effector molecules themselves (Figure 1.4) (Jones and Dangl 2006a, Chisholm et al. 2006). ETI is activated by immune receptors known as resistance (R) proteins, the majority of which are nucleotide-binding domain and leucine-rich repeat (NB-LRR)-type proteins. Upon recognising a pathogen effector molecule, these undergo a conformational change that induces defence signalling (Caplan, Padmanabhan and Dinesh-Kumar 2008). Given that the resistant response is based on recognition of individual effector molecules by specific plant receptors, coded by *Avr* and *R* genes respectively, it is also known as 'gene-for-gene' resistance (Glazebrook 2005). The monogenic nature of *S. gesnerioides* resistance and the parasite's apparent race structure indicate that, similar to other plant pathogens, *S. gesnerioides* may use effector molecules to suppress the host's immune system. Furthermore, in cowpea cultivar B301, a resistance gene against *S. gesnerioides* race SG3 (*RSG3-301*) was identified which encoded a NB-LRR protein (Li and Timko 2009). Virus induced silencing of this gene rendered cowpea B301 newly

susceptible to *S. gesnerioides* SG3, demonstrating that this 'Zig-Zag' model of plant immunity may operate between parasitic plants and their hosts (Figure 1.4).

In general, PTI responses are triggered non-specifically and are effective against a broad spectrum of pathogens, whilst ETI evolves to counter specific pathogen races adapted to the host species. ETI responses are considered more effective as they typically involve a rapid hypersensitive response and localised cell death (Jones and Dangl 2006a). In addition, a defence signal may be transmitted throughout the host, increasing pathogen resistance in distal areas (systemic acquired resistance, SAR) (Fu and Dong 2013, Li et al. 2016, Jones and Dangl 2006b). Once a host acquires ETI, natural selection then acts on the pathogens to either lose the responsible effectors, or to evolve additional effectors that can inhibit ETI (Jones and Dangl 2006a).

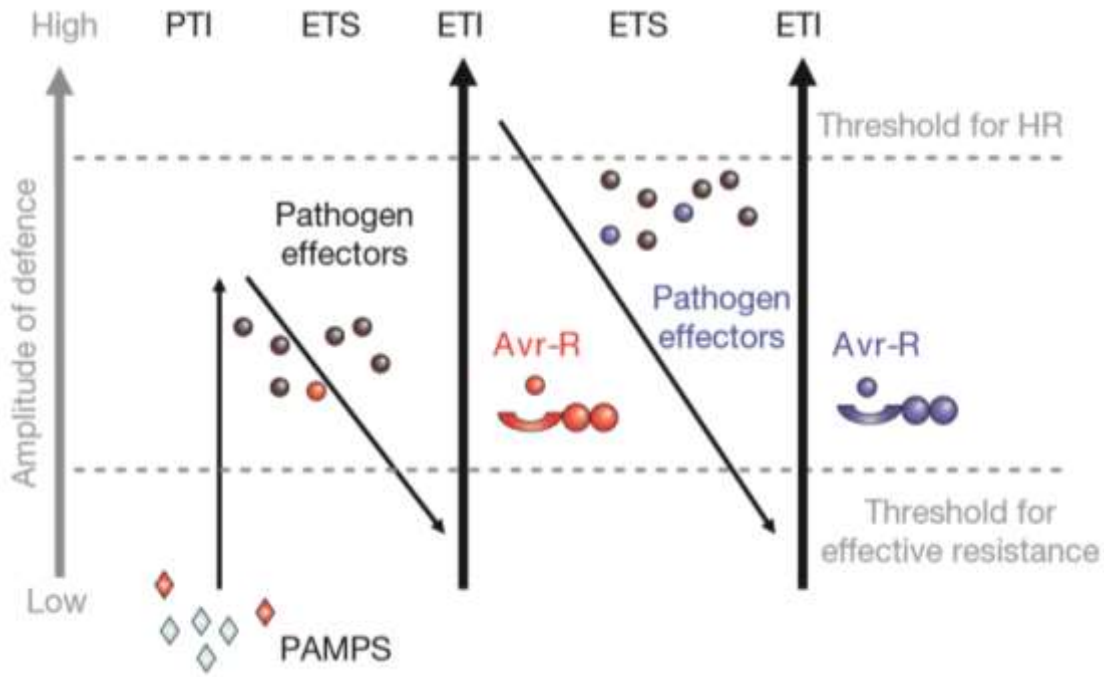


Figure 1.4: The 'Zig-Zag' model of plant immunity. The most basic level of plant immunity is Pattern-Triggered Immunity (PTI) where conserved pattern-associated molecular patterns (PAMPS) distinguish invading organisms as 'non-self', activating host defences. Virulent pathogens secrete effector molecules (coded by *Avr* virulence genes) that suppress PTI, inducing Effector-Triggered Susceptibility (ETS) in the host. In turn, plants can evolve Resistance (*R*) genes, coding receptors that recognise effector molecules and induce Effector-Triggered Immunity (ETI), also known as gene-for-gene resistance. Natural selection then favours pathogens that either lose the effector molecules that trigger host ETI, or that gain additional suppressive effectors. Image from (Jones and Dangl 2006a).

Specificity of plant defence responses is achieved in part through the coordination of multiple defence-related plant hormones, mainly salicylic acid (SA), jasmonic acid (JA) and ethylene. Extensive study on plant-pathogen interactions has established that these are differentially associated with defences against specific classes of pathogen. SA, for instance, generally induces defences effective against biotrophic pathogens, which feed off living host tissues (Glazebrook 2005). These include hypersensitive responses, pathogenesis-related (PR) proteins (Durner, Shah and Klessig 1997) and activation of SAR (Durrant and Dong 2004, Gaffney et al. 1993). The JA-signalling pathway on the other hand, is associated with defences effective against wounding/mechanical damage and insect herbivory (Reymond et al. 2000); these include proteinase inhibitors (Farmer and Ryan 1992) and the release of volatiles to attract natural predators of insect herbivores (Krumm, Bandemer and Boland 1995). Ethylene acts in concert with JA to activate downstream defences against necrotrophic pathogens (Glazebrook 2005) which can include antimicrobial thionins (Ellis and Turner 2001) and induction of the plant defensin gene *PDF1.2* (Penninckx et al. 1998). Evidence that this delineation between SA and JA exists comes from *Arabidopsis thaliana* mutants impaired in the biosynthesis or transmission of one of these defence hormones. The JA-insensitive *coronatine-insensitive 1 (coi1)* mutant, for instance, is more susceptible to the necrotrophic fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* but not to the biotroph *Peronospora parasitica*. Conversely, the opposite is the case for *NahG* and *nonexpressor of pathogenesis-related gene 1* (*npr1)* mutants impaired in SA accumulation and signalling, respectively (Thomma et al. 1998).

There is much experimental evidence which suggests these pathways are mutually antagonistic (reviewed in Pieterse et al. 2012, Li et al. 2019). Transgenic *NahG Arabidopsis* plants, for instance, fail to accumulate SA during pathogen attacks and show higher levels of JA and hyper-induction of JA-responsive genes (Spoel et al. 2003). JA-signalling mutants on the other hand, such as *coi1*, *suppressor of SA insensitivity 2 (ssi2)* and *mitogen-activated protein kinase 4 (mpk4)* show enhanced activity of SA-regulated defences (Kloek et al. 2001, Kachroo et al. 2001, Petersen et al. 2000). The mode of suppression is still not clearly understood, but transcription factors such as WRKY70 and ORA59 are thought to act as key 'nodes' within the hormone signalling network, capable of transmitting positive and negative regulation (Li, Brader and Palva 2004, Van der Does et al. 2013).

It has been supposed that this mutual antagonism is an 'adaptive tailoring' mechanism which shuts down inappropriate signalling pathways during pathogen attacks, ensuring that only effective defence responses are induced (Thaler, Humphrey and Whiteman 2012). In recent years, however, it has become apparent that this view is overly-simplistic since there is evidence which suggests that in some cases SA and JA can mutually reinforce one another (Reymond and Farmer 1998, Schweizer, Buchala and Métraux 1997, Schenk et al. 2000). This is particularly the case with pathogens whose lifestyles straddle conventional categories, such as the hemi-biotroph *Magnaporthe oryzae,* where both SA and

JA have been shown to play a role in host defence (De Vleesschauwer et al. 2016). These interactions appear to be highly concentration dependent: low concentrations of SA and JA, for instance, can synergistically induce *PR1* and *PDF1.2* (SA- and JA-reporter genes, respectively) yet higher concentrations of SA and JA antagonise expression (Mur et al. 2006). It is also apparent that cross-talk between SA and JA/ethylene can be modified by other hormones such as auxin, gibberellic acid, cytokinin and abscisic acid (ABA), as reviewed in (Robert-Seilaniantz, Grant and Jones 2011, Pieterse et al. 2012). This has given rise to the 'Tuneable Defence Model' (Figure 1.5) where the relative combination of defence hormones determines the transcriptional output of associated genes. Such a strategy would greatly expand the ability to tailor specific defence responses from only a limited suite of initial hormones (Reymond and Farmer 1998, Pieterse et al. 2012). Nevertheless, this delicate web of hormone inter-communication presents many opportunities for exploitation by pathogens, for instance through targeting critical integrating nodes (Sarris et al. 2015) or through producing compounds that mimic hormone activity (Robert-Seilaniantz et al. 2011).

Clearly plant defence is a complex, sophisticated network that will not conform to simplistic models. Much work remains to be done in order to understand how subtle variations in the relative balance of different hormones, besides the influence of temporal dynamics, is ultimately achieved to select the correct host defence response.

1.8 Basal resistance against root pathogens: parasitic nematodes and *Fusarium oxysporum*

Given the conserved nature of plant defence hormones across species, discoveries from model plant-pathogen interactions relating to host basal resistance will likely apply to commercial crops. Indeed, the novel aspect of this project is to systematically investigate the genetic components of basal resistance against *S. gesnerioides* using the model host *Arabidopsis*. The almost inexhaustive availability of *Arabidopsis* genetic mutants meant it was beyond the scope of this PhD project to test every gene associated with basal resistance against pathogens. Therefore, a review was made of the current understanding of basal resistance against root pathogens that exhibit a similar infection strategy to *Striga*. These include plant-parasitic nematodes belonging to the root-knot nematode (RKN) genus *Meloidogyne* and the two cyst nematode genera *Heterodera* and *Globodera* (Niebel, Gheysen and Van Montagu 1994), besides the ascomycete fungus *Fusarium oxysporum. Arabidopsis* is readily infected by these pathogens (Czymmek et al. 2007, Hamamouch et al. 2011) and has been used as a tool in countless studies investigating the molecular basis of host basal resistance.

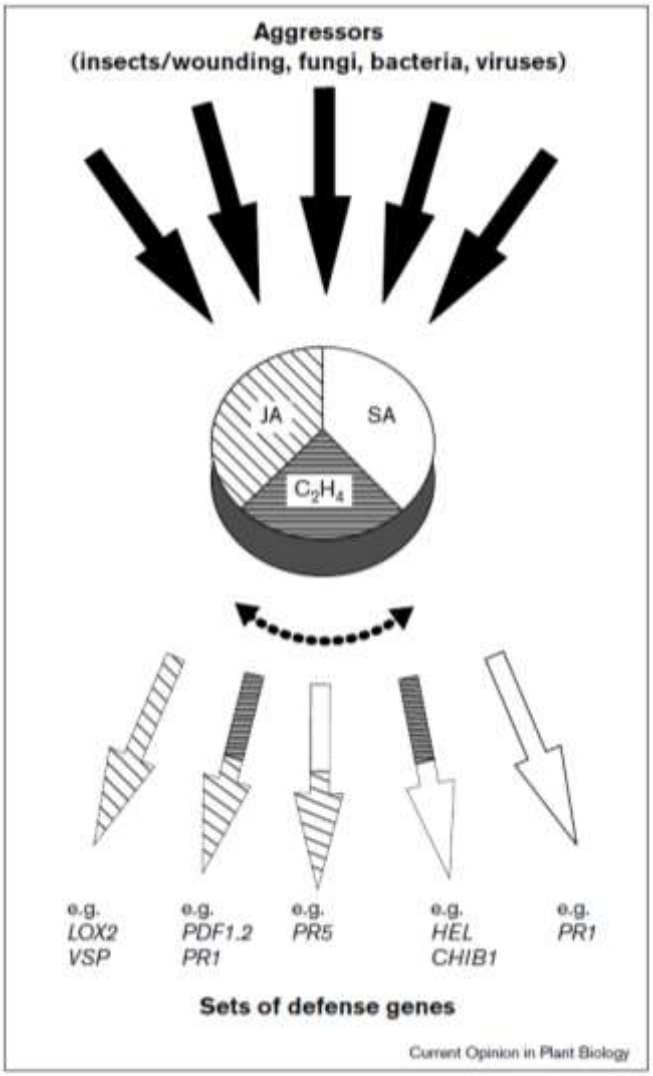


Figure 1.5: Tuneable defence model for defence gene regulation by the signalling molecules jasmonic acid (JA, hatched lines), salicylic acid (SA, white) and ethylene (C_2H_4, grey).
To activate the optimum defence response against an invading attacker, plants can fine-tune defence gene induction through using either a single hormone signal (single-pattern arrows) or a combination (multi-pattern arrow). This mechanism enables a wider range of specific downstream defences to be selected from a limited number of initial starting hormones. Nevertheless, this system could be disrupted by pathogens that destroy, produce or mimic these hormone signals. Image from (Reymond and Farmer 1998).

Parasitic nematodes are highly destructive agricultural pests that infect a wide range of crops, causing global agricultural losses of $157 billion each year (Abad et al. 2008). The larvae penetrate the host root and migrate intracellularly to reach the vascular cylinder, where they induce host cells to re-differentiate into multinucleated feeding cells from which they obtain nutrients (Niebel et al. 1994). They can maintain a biotrophic interaction with the host for up to several weeks (Jaouannet et al. 2013).

Fusarium oxysporum is a hemibiotrophic pathogen that causes wilt disease on various plant species including *Arabidopsis*. During initial infection, when *F. oxysporum* acts as a biotroph, hyphae enter the roots of the host plant and migrate towards the central vasculature (Czymmek et al. 2007, Michielse and Rep 2009). In response to fungal elicitors (such as the fungal cell wall component chitin), the host mounts a basal, PTI-driven response, which includes callose deposition, hydrogen peroxide production and an oxidative burst (Berrocal-Lobo and Molina 2008). However virulent *F. oxysporum* strains can suppress these through secreting effectors such as the SECRETED IN XYLEM (SIX) proteins (Thatcher et al. 2012). This allows the pathogen to migrate through the root cortex intercellularly, until it reaches

the vascular bundle where it migrates upwards. As fungal mycelia and host-defence products accumulate in the xylem vessels, these become blocked and cause wilting symptoms (Michielse and Rep 2009, Czymmek et al. 2007, Thatcher et al. 2012). Later, the pathogen switches to a necrotrophic state, leading to symptoms that include lesions and death of leaf tissue (Lyons et al. 2015).

Both parasitic nematodes and *F. oxysporum* show striking similarities with *Striga* spp; namely entry via the root, migration through the cortex and eventual penetration of the host vascular system. It is therefore likely that similar signalling pathways and defence compounds play a role in basal resistance against these pathogens.

1.8.1 The role of defence hormones in basal resistance against parasitic nematodes

Since parasitic nematodes are biotrophic pathogens, it is not surprising that numerous studies have associated salicylic acid (SA) with host resistance. *Arabidopsis* mutants with defective SA accumulation (including *sid2-1, pad4*-1 and *NahG* transgenics) showed greater susceptibility to the sugar beet cyst nematode *Heterodera schachti* (Wubben, Jin and Baum 2008). Furthermore, both RKN and beet cyst nematodes induced SA-associated defence genes such as *PR1* in host roots in studies on *Arabidopsis* and tomato (Martínez-Medina et al. 2016, Hamamouch et al. 2011); this appears to be relevant in host resistance since over-expression of *PR1* reduced susceptibility to both RKN and beet cyst nematodes (Hamamouch et al. 2011). In an additional study using a range of SA-affected *Arabidopsis* mutants, basal resistance against cyst nematodes correlated with the amount of *PR1* expression in the root (Wubben et al. 2008). The endophytic fungus *Trichoderma harzianum* induced protection against RKNs in host tomato plants and this was associated with more rapid induction of SA-defence genes during nematode invasion (Martínez-Medina et al. 2016). Similarly, the protective effect of *Glomus mosseae* on tomato was also associated with upregulation of chorismate synthase, which catalyses the production of SA precursors (Vos et al. 2013). There is also evidence that virulent nematode species may target host SA-regulated defences (Jaouannet et al. 2013, Wang et al. 2018). Transgenic expression of the nematode virulence effector Mi-CRT in *Arabidopsis*, for instance, suppressed the normal induction of SA-associated defence genes during RKN infection, rendering the host plants considerably more susceptible (Jaouannet et al. 2013).

The contribution of SA towards host basal resistance may be mediated via NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1), a transcription factor essential for a subset of SA-associated responses including SAR (Cao et al. 1994). *npr1 Arabidopsis* mutants were more susceptible to infestations with sugar beet cyst nematodes, whilst mutations in *SUPPRESSOR OF NPR1-1, INDUCIBLE1* (*SNI1*), a negative regulator of the SA pathway downstream of NPR1, significantly enhanced resistance (Wubben et al. 2008). In rice, transcriptomic studies have found that cultivars

resistant to RKN showed greater upregulation of SA-biosynthesis and NPR1-responsive genes (Kumari et al. 2016). Furthermore, constitutive expression of *NPR1* from *Arabidopsis* in cotton plants increased host resistance to reniform nematodes besides various fungal pathogens (Parkhi et al. 2010). Interestingly, these transgenic plants did not show increased basal activity of defence-associated genes (including *PR1*): instead these were induced more quickly upon nematode invasion, suggesting that *NPR1* over-expression primes host root defences, rather than increases basal activity (Parkhi et al. 2010).

On the other hand, there is also evidence that defence against nematodes can be activated independently of SA and/or NPR1. For instance*, PR2* and *PR5* were induced in response to *H. schachti* in both wild-type and *sid2 Arabidopsis,* suggesting that this occurs independently of SA (Wubben et al. 2008). In addition, transgenic *NahG* tomato plants, which cannot accumulate SA, did not show altered susceptibility to RKNs (Bhattarai et al. 2008).

Jasmonic acid (JA) and ethylene have also been implicated in resistance against parasitic nematodes, however these results are often contradictory. In tomato plants, mutations in *jasmonate insensitive 1 (jai1)* (equivalent to *coi1* in *Arabidopsis* where JA perception is impaired but not JA biosynthesis) increased resistance to RKNs, with the JA biosynthesis *deficient in jasmonate1 (def1)* mutant showed no effect on resistance (Bhattarai et al. 2008). In contrast, a separate study found the JA-deficient *suppressor of prosystemin-mediated responses2 (spr2)* tomato mutant to be more susceptible to RKN, whilst a transgenic line with elevated JA levels, *35S:PS,* was more resistant (Fan et al. 2015), suggesting a protective effect for JA. This seems to be the case for rice, where the JA biosynthesis mutant *hebiba* was significantly more susceptible to RKN; moreover, this was reversed if MeJA is applied (Nahar et al. 2011). Applications of MeJA were also reported to protect oats against cyst nematodes (Soriano et al. 2004) and tomatoes and rice against RKN (Fujimoto et al. 2011, Bali et al. 2018, Nahar et al. 2012). In the latter case, protection increased steadily with MeJA concentration, up to 5 mM (Fujimoto et al. 2011). Nevertheless, *lipoxygenase4 (lox4) Arabidopsis* mutants showed significantly greater susceptibility to RKN and cyst nematodes; this phenotype was associated with induction of the JA-biosynthesis genes allene oxide synthase (AOS) and allene oxide cyclase (AOC2), causing significantly greater endogenous JA levels (Ozalvo et al. 2014).

These conflicting results may be due to the ability of JA to induce both defences against mechanical damage/insect herbivores and necrotrophic pathogens. In the presence of ethylene, necrotrophic-associated defences are selected over wound-response genes (Lorenzo and Solano 2005). In *Arabidopsis*, the ethylene insensitive mutants *etr1, ein2* and *ein3* showed greater resistance to *H. schachti*, whereas mutants that overproduce ethylene (*eto1-1, eto2* and *eto3*) were hyper-susceptible (Wubben et al. 2001). Furthermore, basal resistance of wild-type Col-0 plants was compromised by

applications of the ethylene precursor ACC, but enhanced by the ethylene inhibitor 2-Aminoethoxyvinylglycine (AVG) (Wubben et al. 2001). This could suggest that tipping the balance in favour of the wound-response pathway by disabling ethylene signalling increases basal resistance against parasitic nematodes. In support of this, cytokinin-hypersensitive type-A *arr Arabidopsis* mutants showed significantly increased resistance against *H. schachtii* and this was associated with downregulation of *ETHYLENE RESPONSE FACTOR 1* and *2 (ERF1* and *ERF2)*. These transcription factors positively regulate necrotrophic-defence genes while inhibiting the wound-response pathway (Shanks et al. 2015). These mutants also showed strong upregulation of *NON-RACE DISEASE RESISTANCE 1* (*NDR1*), and *ENHANCED DISEASE SUSEPTIBILITY 1) (EDS1)*: both of these are positive regulators of SA-associated defences, whilst *EDS1* additionally acts to suppress JA/ethylene signalling (Shanks et al. 2015). Nevertheless, external applications of ethylene on rice reduced infestations of the RKN *Hirschmanniella oryzae*, although this was found to be independent of the JA-biosynthesis pathway (Nahar et al. 2012).

Potentially, SA and JA are both important in defence against nematodes but act at different stages of the pathogen lifecycle, as proposed by Martínez-Medina et al. (2016). In their studies, inoculation with the fungus *T. harzianum* induced resistance against RKN penetration and infection in tomato. This effect was lost in SA-deficient *NahG* tomato mutants, but intact and even stronger in the JA-deficient *def1* mutant. However, *T. harzanium*-mediated protection acting at a later stage of infection (root galling) was still fully expressed in the *NahG* plants but blocked in *def1.* The authors propose that *T. harzanium* protection is plastic and switches from priming SA defences during initial infection to optimising the JA defence pathway at the sedentary feeding stage of RKN infection (Martínez-Medina et al. 2016). Furthermore, a transcriptomic study identified significant upregulation of JA biosynthesis during the early stages of *H. schachtii* infection in *Arabidopsis* but not for SA-associated genes (Kammerhofer et al. 2015). At later stages of infection, JA biosynthesis and activity became downregulated, however the authors suggest that later downregulation of JA-associated signalling is due to active suppression by the parasite, rather than host activity. Downregulation of JA-biosynthesis genes also occurs during late stages of infection in the compatible soybean-cyst nematode interaction (Ithal et al. 2007). Intriguingly, the RKN effector Mi-CRT repressed the induction of JA/ethylene associated defence genes in *Arabidopsis*, such as *FRK1, WRKY33, PDF1.2*, besides SA-associated genes (Jaouannet et al. 2013). In tomato plants infected with the RKN *Meloidogyne incognita,* JA-responsive genes were downregulated however this was reversed with pre-inoculation with *T. harzianum* (Martínez-Medina et al. 2016). Curiously, watermelon plants exposed to red light showed significantly increased resistance to RKN, and this was associated with increased endogenous levels of JA and SA in the root (Yang et al. 2018), indicating that these hormones can cooperate synergistically in basal resistance.

1.8.2 The role of defence hormones in basal resistance against *Fusarium oxysporum*

Given that *F. oxysporum* acts initially as a biotroph, host defences coordinated by SA may be important in limiting the early phases of infection. Indeed, in *Arabidopsis* exogenous applications of SA to the leaves reduced disease symptoms caused by *F. oxysporum* (Edgar et al. 2006) and mutants with disrupted SA signal transduction pathways (*npr1-1*) or SA accumulation (*NahG*, *sid2* and *eds5-1*) showed increased susceptibility (Berrocal-Lobo and Molina 2004). In tomato, transgenic over-expression of *NPR1* from *Arabidopsis* significantly increased resistance to *F. oxysporum* and this was also associated with upregulation of SA-associated *PR* genes (Lin et al. 2004). Potentially, SA may work synergistically with JA in promoting basal resistance against *F. oxysporum*. *Arabidopsis erf4* mutants showed increased resistance to *F. oxysporum* and this was associated with enhanced basal expression of both SA-responsive defence genes (*PR1*, *BGL2* and *PR5*) and JA-regulated genes (*PDF1.2* and *PAD3*) (Edgar et al. 2006). Over-expression of *ERF2*, a positive regulator of JA-defence genes, also increased resistance to *F. oxysporum* (McGrath et al. 2005), whilst *jasmonate resistant1* (*jar1-1)* mutants, which have disrupted JA signalling, showed increased susceptibility (Berrocal-Lobo and Molina 2004).

Whilst these results suggest a protective effect of JA against *F. oxysporum*, this is complicated by the *coi1 Arabidopsis* mutant which is impaired in JA perception, yet demonstrated remarkably high resistance against *F. oxysporum* (Thatcher, Manners and Kazan 2009). Nevertheless, this striking phenotype only appeared effective against *F. oxysporum* strains which produce JA-leucine and JA-isoleucine conjugates in their culture filtrates in quantities sufficient to activate JA-inducible genes in the host (Cole et al. 2014). This enhanced resistance was maintained in the *coi1 NahG* double mutant, making it unlikely that the phenotype is caused by reduced suppression of the SA pathway by JA (Thatcher et al. 2009). It has been proposed that low levels of JA can have a protective effect against *F. oxysporum*, yet virulent strains hijack JA signalling downstream of COI1 through actively secreting JA-conjugates (Cole et al. 2014). This would be similar to the bacterial foliar pathogen *Pseudomonas syringae*, which secretes the JA-mimic coronatine: consequently, the *coi1* mutant was also more resistant to *P. syringae* (Kloek et al. 2001). Such a model for *F. oxysporum* is supported by the *Arabidopsis* constitutive *JASMONATE-ZIM-DOMAIN PROTEIN 7 (JAZ7)* expression mutant: this causes hyper-activation of the JA pathway and also showed increased susceptibility to *F. oxysporum* (Thatcher et al. 2016). If only a low level of JA is required for optimal defence, this could explain why *Arabidopsis* JA-biosynthesis mutants (including *fad3-2*, *fad7-1 fad8, opr3*, *aos*) do not show altered susceptibility (Thatcher et al. 2009). Furthermore, pre-treating tomato seeds with 0.1 mM MeJA enhanced seedling resistance to *F. oxysporum*, but treatment with 1 mM MeJA increased susceptibility (Król et al. 2015).

Similar to parasitic nematodes, activating the correct defence pathway downstream of JA appears to have a profound effect on basal resistance against *F. oxysporum*. In this case, however, JA/ethylene-regulated defences associated with necrotrophic pathogens seem to be more effective than wound-response genes. In *Arabidopsis*, over-expression of *ERF1*, a positive regulator of necrotrophic defences induced synergistically by JA and ethylene (Dombrecht et al. 2007, Lorenzo et al. 2003), enhanced resistance to *F. oxysporum* (Berrocal-Lobo and Molina 2004), possibly due to constitutive expression of antifungal compounds. More recently, *ERF2* was identified as another positive regulator of necrotrophic defences; over-expression of this increased expression of JA/ethylene-induced genes such as *PDF1.2* and *BASIC CHITINASE (B-CHI)* and enhanced resistance to *F. oxysporum* (McGrath et al. 2005). Conversely, *erf14 Arabidopsis* mutants were more susceptible to *F. oxysporum* and showed reduced gene-induction in response to ethylene, including *ERF1* and *ERF2* (Oñate-Sánchez et al. 2007). Similarly, the *ethylene insensitive* mutant *ein2-5* also showed increased susceptibility to *F. oxysporum* (Berrocal-Lobo and Molina 2004). Furthermore, cotton plants infected by *F. oxysporum* showed induction of ethylene-synthesis and signalling genes, and increased endogenous levels of the hormone (Dowd, Wilson and McFadden 2004). These results suggest that ethylene and JA act synergistically to promote resistance, although since ethylene levels vary across species, this may not hold true for all plants. Indeed, ethylene-insensitive *Never ripe* tomatoes showed higher survival when infected with *F. oxysporum* (Lund, Stall and Klee 1998).

Potentially, in the interaction between *F. oxysporum* and *Arabidopsis*, SA functions primarily through restricting JA signalling, rather than direct gene induction. This could explain why certain mutations in upstream modulators of pathogen-induced SA-production, such as *eds1-1* and *pad4-1*, were not majorly affected in basal resistance (Berrocal-Lobo and Molina 2004). Furthermore, the enhanced susceptibility of the SA-induction mutant *sid2* depended on functional COI1 (Cole et al. 2014), indicating that this phenotype arises through mis-regulation of the JA pathway. Clearly, basal resistance against this pathogen is determined by a fine balance in hormone signalling, maintained by complex cross-regulation.

1.8.3 The role of reactive oxygen species (ROS) against root pathogens

As reviewed by (Torres 2010), in both susceptible and resistant plant-pathogen interactions, one of the most rapid host responses is a transient oxidative burst, caused by the generation of reactive oxygen species (ROS) such as superoxide anion (O_2–) hydrogen peroxide (H_2O_2), and reactive nitrogen species such as nitric oxide (NO). In resistant hosts, a second oxidative burst is generated a few hours later and is sustained. The ROS produced in this second phase have multiple roles. Besides having direct anti-microbial activity, ROS can trigger localised cell death (as part of the hypersensitive response), promote cell wall cross-linking/lignification and activate gene transcription. ROS have also been reported to

modulate the activity of defence-related hormones including SA, ethylene and ABA (Li et al. 2019). ROS can activate SA biosynthesis for instance (Leon, Lawton and Raskin 1995), yet antagonistic interactions between ROS and SA have also been reported (Xu and Brosché 2014). In addition, H_2O_2 is reported to downregulate the expression of ethylene receptor genes (Jakubowicz et al. 2010).

Localised production of ROS is facilitated through the *RBOH (RESPIRATORY BURST OXIDASE HOMOLOG)* gene family (reviewed by (Torres and Dangl 2005)), which has ten members in *Arabidopsis* (*AtRBOHA-J*). Mutations in individual *RBOH* genes differentially affect basal resistance to *F. oxysporum*; *RbohD* and *RbohF* showed significantly increased and decreased resistance respectively in *Arabidopsis* (Zhu et al. 2013). This likely reflects the distinct functions of individual *RBOH* genes that are beginning to be deciphered: *AtRBOHD*, for instance, is thought to produce most ROS in response to pathogens (Morales et al. 2016, Perchepied et al. 2010, Fagard et al. 2007, Torres, Dangl and Jones 2002) whilst *AtROHF* appears to have a more important role in regulating the hypersensitive response and restricting cell death (Torres et al. 2002). RBOHD and RBOHF may also show temporal differences in ROS generation: RBOHD, for instance, is known to be directly activated by BOTRYTRIS INDUCED KINASE 1 (BIK1), part of the pattern recognition receptor (PRR) complex, allowing it rapidly generate apoplastic ROS during pathogen attacks (Wan et al. 2019). ROS can also be generated by class III peroxidases (Mathé et al. 2010). In tomato, the *F. oxysporum* resistant GS-12 cultivar showed strong induction of peroxidase activity on infection that was not seen in a susceptible counterpart (Ghazy and Mohamed 2007). In addition, the *Arabidopsis pub22/pub23/pub24* U-box type E3 ubiquitin ligase triple mutant which showed enhanced ROS production in response to pathogen attack was significantly more resistant to *F. oxysporum* (Chen et al. 2014). Further evidence that host-generated ROS protects against this pathogen comes from the observation that the virulence effector *Avr2* from *F. oxysporum f. sp. lycopersici* inhibited flg22-induced ROS production when expressed transgenically in tomato and *Arabidopsis* (Di et al. 2017).

There is also evidence that ROS production promotes resistance against nematodes. The *Arabidopsis RbohD RbohF* double mutant, for instance, showed significantly higher susceptibility to RKN (Teixeira, Wei and Kaloshian 2016). The protective effect of red light on watermelon plants against RKN was also associated with greater induction of H_2O_2 in both roots and leaves. Furthermore, this was coupled with enhanced redox homeostasis capacity through increased levels of key antioxidant enzymes (e.g. catalase, superoxide dismutase) and total reduced glutathione (GSH) (Yang et al. 2018). Furthermore, both RKN and cyst nematodes express antioxidant enzymes including ascorbate peroxidase, catalase and peroxiredoxins, presumably to protect against host-generated ROS (Molinari and Miacola 1997, Dubreuil et al. 2011). When these were targeted by RNAi in *M. incognita*, this resulted in 60% less gall formation on tomato plants (Dubreuil et al. 2011).

1.8.4 Physical obstruction: the role of lignin and callose in combatting root pathogens

Besides being directly toxic and coordinating hypersensitive responses, ROS can also be used to reinforce cell walls, particularly through the oxidation of lignin monomers (Passardi, Penel and Dunand 2004). Lignin is a waterproof aromatic polymer deposited mainly in cell walls to provide rigidity (Vanholme et al. 2010). In combination with callose, a β-1,3-glucan polymer, it can form cell wall appositions (papillae) between the cell wall and plasma membrane at sites of attempted pathogen penetration (Underwood 2012). There is evidence that physical reinforcement through lignin and callose deposition is a strategy used by plants to restrict invading parasitic nematodes. A transcriptomic study on rice found greater induction of callose synthase and lignin biosynthesis genes in a cultivar resistant to RKN compared to a susceptible cultivar (Kumari et al. 2016). In the compatible soybean-cyst nematode interaction, infected roots showed upregulation of genes in the phenylpropanoid pathway, suggesting that cell wall fortification and lignin deposition is a host defence response (Ithal et al. 2007, Alkharouf et al. 2006). The amino acid β-amino-butyric acid (BABA) increased resistance of rice plants to subsequent infection by RKN, with the host plants showing upregulation of lignin and callose biosynthesis and deposition around nematode feeding sites. Furthermore, BABA-induced resistance was compromised by suppression of PHENYL AMMONIA LYASE (PAL), which synthesises polyphenols and ultimately lignin. Curiously, BABA-induced resistance was maintained in mutants with impaired JA, SA and ethylene biosynthesis (Ji et al. 2015). The virulence effector Mi-CRT from *M. incognita* appears to target callose-associated defences, as expression of this in *Arabidopsis* inhibited callose deposition in response to pathogenic triggers (Jaouannet et al. 2013). Similarly, over-expression of the effector molecule 30C02 from soybean cyst nematode *H. glycines* induced susceptibility in *Arabidopsis*: this molecule appears to physically interact with a β-1,3-endoglucanase. It has been proposed that this limits callose formation or the production of signal molecules that may induce defence responses (Hamamouch et al. 2012).

There is some evidence that lignin deposition also forms part of host basal resistance against *F. oxysporum*. Treatment of tomato plants with *F. oxysporum* mycelium extract significantly increased lignin deposition, preceded by induction of lignin-synthesis enzymes (Mandal and Mitra 2007). In addition, a banana cultivar resistant to panama disease caused by *F. oxysporum f. sp. cubense* demonstrated stronger lignin deposition in response to fungal elicitor (De Ascensao and Dubery 2000). Callose, on the other hand, may be a susceptibility factor since in a susceptible interaction between chickpea and *F. oxysporum f.sp. ciceris*, callose degradation products accumulated in and blocked xylem vessels; this was not observed in a resistant cultivar (Gupta et al. 2013). Nevertheless, the virulence effector *Avr2* from *F. oxysporum f. sp. lycopersici* inhibited flg22-induced callose deposition when expressed transgenically in tomato and *Arabidopsis* (Di et al. 2017), suggesting that callose deposition was actively targeted by the pathogen.

1.8.5 Chemical warfare: phytoalexins and glucosinolates

Phytoalexins are low molecular weight antimicrobial compounds produced in plants specifically during pathogen infection and include terpenoids, phenolics, fatty acid derivatives and polyacetylenes (Hammerschmidt 1999). In *Arabidopsis*, the phytoalexin camalexin is an integral component of PTI responses against various foliar fungal and bacterial pathogens, and there is evidence that this extends to defence against nematodes. RKN infection triggers camalexin biosynthesis genes, such as the cytochrome P450 *CYP71A12* (Teixeira et al. 2016) and the camalexin-deficient *phytoalexin deficient 3 (pad3)* mutant was significantly more susceptible to both RKN and cyst nematodes (Teixeira et al. 2016, Ali et al. 2014). Overexpressing positive regulators of camalexin biosynthesis, including *WRKY33* in *Arabidopsis* (Ali et al. 2014) and *AtPAD4* in soybean enhanced resistance to both classes of parasitic nematodes (Youssef et al. 2013). Another phytoalexin identified in banana, anigorofone, showed high anti-nematodicial activity (Hölscher et al. 2014). On the other hand, camalexin does not seem important for basal resistance against *F. oxysporum* as *Arabidopsis pad3* mutants did not show altered susceptibility (Kidd et al. 2011). Nevertheless, other phytoalexins active against *F. oxysporum* have been isolated from cotton (Zhang et al. 1993), carnation (Curir, Dolci and Galeotti 2005) and chickpea (Stevenson, Turner and Haware 1997).

Glucosinolates are a large class of sulphur and nitrogen containing secondary metabolites predominantly found in the *Brassicaceae* family. They have been demonstrated to act as chemical defences against both herbivores and foliar microbial pathogens (Buxdorf et al. 2013), and evidence also suggests they protect against root pathogens. In *Arabidopsis*, RKN induced transcription factor *MYB51*, which regulates glucosinolate biosynthesis, and the *myb34 myb51* double mutant (completely impaired in glucosinolate production) was significantly more susceptible (Teixeira et al. 2016). Furthermore, the *pgip1 Arabidopsis* mutant, which showed increased susceptibility to the cyst nematode *H. schachtii,* demonstrated impaired induction of indole-3-glucosinolate biosynthesis during infection (Shah et al. 2017). The *cyp79B1/B2* double mutant, which has a strong defect in the production of both indole-glucosinolates and camalexin, also showed increased susceptibility to *H. schachtii* (Shah et al. 2017). Cytochrome P450 genes are strongly induced in *Arabidopsis* by *F. oxysporum*, suggesting that indole glucosinolates may have a role in basal host defence (Zhu et al. 2013). Furthermore, *F. oxysporum* grew more aggressively and causeed more disease symptoms on *Arabidopsis gsm1* mutants, which lack many of the aliphatic glucosinolates (Tierens et al. 2001).

1.8.6 Hydrolytic enzymes: chitinases and glucanases

Hydrolytic enzymes such as chitinases and glucanases, have strong defensive action by hydrolysing pathogen cell walls, particularly those of bacterial and fungal pathogens (Spoel and Dong 2012). The

tomato cultivar GS-12 was highly resistant to *F. oxysporum* and showed significantly greater induction of β-1,3-glucanase when challenged with the pathogen (Ghazy and Mohamed 2007). Simultaneous over-expression of a tobacco chitinase and β-1,3-glucanase gene increased resistance to *F. oxysporum* in tomato; curiously, expression of the individual genes had no impact on host basal resistance (Jongedijk et al. 1995). Meanwhile, transgenic expression of *NPR1* from *Arabidopsis* in cotton enhanced resistance to both *F. oxysporum* and reniform nematodes: this may be due, in part, to more rapid induction of glucanase and chitinase enzymes upon pathogen invasion (Parkhi et al. 2010).

1.8.7 Basal resistance mechanisms against *Striga gesnerioides* and other root parasitic weeds

The evidence from nematodes and *F. oxysporum* suggests that hormone signalling pathways, physical barriers and the production of repellent chemicals all have a role in plant defences against invading root pathogens. A fundamental difference with parasitic weeds however is that, being plants, they may lack the 'non-self' molecules that typically trigger these pathways. Nevertheless, there is some limited evidence that similar mechanisms underpin host basal resistance against parasitic weeds. For *Striga gesnerioides*, this has been investigated by microarray analysis of resistant and compatible interactions using resistant cowpea cultivar B301 (Huang et al. 2012). This cultivar is broadly resistant to many species of *S. gesnerioides* (including SG3, used in this study), against which it mounts a hypersensitive response, however the hyper-virulent strain SG4z overcomes this and successfully attaches to the host vasculature (Figure 1.3). Attempted parasitism by strain SG3 prompted marked upregulation in genes relating to programmed cell death, cell wall biogenesis, oxidative stress responses and biotic stress-associated signalling pathways. In contrast, the virulent strain SG4z caused wide gene-downregulation in the host, including those relating to cell wall biogenesis; phenylpropanoid and lignin biosynthesis; the SA and JA signal transduction pathways and the plant growth regulators auxin and gibberellin (Huang et al. 2012).

1.8.8 Hormone defences and basal resistance against parasitic weeds

As the complete cowpea genome was unavailable at the time of the study described above (Huang et al. 2012), there was limited scope to investigate specific hormone signalling components. However, in an earlier study this group demonstrated that resistant responses of cowpea against *S. gesnerioides* are associated with stronger upregulation of the SA-responsive *PR-5* transcript, compared with susceptible interactions (Li et al. 2009). On the other hand, *COI1* showed high upregulation in non-host and susceptible interactions, but not in resistant hosts. The authors suggest that *S. gesnerioides* may use effector molecules to actively induce *COI1*, thus suppressing defences coordinated by SA (particularly the hypersensitive response, a hallmark of effective resistance). This bears much similarity

to the infection strategy used by *F. oxysporum* (Cole et al. 2014, Thatcher et al. 2009) and fits the gene-for-gene resistance model that the race structure of this parasite implies.

There are many parallels between these results and compatible/resistant interactions for the closely related *S. hermonthica* and its monocot hosts. Similar to the susceptible interaction between cowpea B301 and SG4z, infection of susceptible rice IAC1 with *S. hermonthica* caused widespread gene downregulation; particularly, in this case, for genes relating to auxin and gibberellic acid signalling, and cell division (Swarbrick et al. 2008). Highly susceptible sorghum cultivars showed induction of JA-responsive genes but not SA-related defences in response to *S. hermonthica* whereas moderately resistant cultivars showed upregulation of both pathways. Pre-treatment of both cultivars with SA reduced *S. hermonthica* infection, demonstrating that suppression of SA-related responses is relevant in basal resistance (Hiraoka and Sugimoto 2008). These results provide further evidence that virulent *Striga* parasites actively suppress host defences, particularly those relating to SA and the hypersensitive response. In contrast, the resistant rice cultivar Nipponbare demonstrated a rapid hypersensitive response against *S. hermonthica* and showed large-scale gene upregulation of genes including hypersensitive response protein homologs, PR proteins and enzymes in phenylpropanoid metabolism (Swarbrick et al. 2008). In addition, various *WRKY* transcription factors showed marked upregulation, several of which have previously been linked to pathogen-defence and SA signalling (Swarbrick et al. 2008).

Studies on other host-parasitic plant systems also indicate that susceptible hosts show a lack of SA–associated activity relative to JA/ethylene signalling. This includes the interactions between *Orobanche ramosa* and *Arabidopsis*, (Dos Santos et al. 2003) and tomato with *Phelipanche ramosa* (Torres-Vera et al. 2016). Furthermore, the SA-analogue benzothiadiazole (BTH) had a protective effect against *O. cumana* on sunflower (Sauerborn et al. 2002), *O.ramosa* on hemp & tobacco (Gonsior et al. 2004) and *O. crenata* on pea (*Pisum sativum*) (Pérez-de-Luque, Jorrín and Rubiales 2004) and faba bean (Sillero et al. 2012). In addition, Kusumoto et al. found that treating the roots of red clover (*Trifolium pratense*) with SA or BTH dramatically reduced the frequency of *O. minor* attachment (Kusumoto et al. 2007).

Nevertheless, it is not clear in these interactions whether SA-signalling primarily functions by directly activating defence genes, or by restricting JA-signalling, as it has been proposed for *F. oxysporum*. One study in particular indicates that downstream responses against parasitic weeds are highly sensitive to the overall balance of hormone activity (Mutuku et al. 2015). Their results demonstrated that the rice mutant *hebiba*, which lacks the JA biosynthesis gene *ALLENE OXIDE CYCLASE (AOC),* was more susceptible to *S. hermonthica:* moreover, this was reversed with external applications of Me-JA (Mutuku et al. 2015). Furthermore, greater resistance was also seen in rice plants with the *NahG* transgene, which cannot accumulate endogenous SA. Nevertheless, the functional analogue BTH,

which activates defences downstream of SA accumulation (Lawton et al. 1996), reduced susceptibility to *S. hermonthica* for both *hebiba* and the susceptible cultivar Koshihikari. In certain plant-pathogen interactions (e.g. *P. syringae pv. Phaseolicola* and *Arabidopsis* (Van Wees and Glazebrook 2003)) the phenotype of *NahG* plants has been attributed to the accumulation of catechol, rather than the lack of SA. This does not appear be the case in this study however, as treating wild-type rice cultivars with catechol did not affect susceptibility to *S. hermonthica*. To explain these results, the authors propose a model whereby the effects of SA and JA in this interaction are mediated by WRKY45, since *wrky45* knock-down plants showed considerably greater susceptibility to *S. hermonthica*. BTH is known to act on WRKY45 (Shimono et al. 2007), which positively regulates JA biosynthesis, with *wrky45* plants showing significantly reduced JA content (Mutuku et al. 2015). According to this model, greater activity of downstream JA responses enhances resistance to *S. hermonthica.* This can be achieved through removing SA-mediated pathways that suppress JA-signalling (as in *NahG* transgenics) or through external applications of BTH (which induces JA-biosynthesis via WRKY45) or Me-JA. Nevertheless, it should be borne in mind that *hebiba* is a large genomic deletion, affecting at least 27 genes (Riemann et al. 2003); hence these effects may be caused by alternative mechanisms. Also, rice is known to have higher endogenous levels of SA than other crops and *Arabidopsis* (Raksin et al. 1990), hence the effect of inhibiting SA accumulation (e.g. via *NahG*) may not be applicable to other species. Despite this, it appears likely that WRKY transcription factors may have important roles in modulating hormone cross talk during infection by parasitic plants, as appears to be the case for other pathogens (as reviewed by (Pandey and Somssich 2009)). Curiously, *WRKY45* was one of the *WRKY* transcription factor genes upregulated in Nipponbare rice by *S. hermonthica*, in comparison with a susceptible cultivar (Swarbrick et al. 2008). Furthermore, AtWRKY70, which is thought to regulate crosstalk between the SA and JA pathways (Li et al. 2004), has also been implicated in the nonhost interaction between *Arabidopsis* and pre-germinated *S. hermonthica* seeds (Vasey 2005). Given the large size (at least 74 in *Arabidopsis*) and multiple redundancy of the WRKY transcription factor family (Rushton et al. 2010), it remains an ongoing challenge to characterise the effect of individual components on the plant defence-signalling network.

Host defence signalling during infection with *Striga* may also be affected by ABA. Although typically associated with abiotic stresses, ABA is becoming increasingly appreciated as a driver and modulator of plant defences (Asselbergh, De Vleesschauwer and Höfte 2008, Cao, Yoshioka and Desveaux 2011, Ton, Flors and Mauch-Mani 2009). In particular, ABA appears to suppress SA accumulation, ROS generation, the production of antimicrobial compounds and JA/ethylene signalling; conversely, ABA is a positive regulator of JA biosynthesis and callose deposition. Foliage-derived ABA also inhibits shoot growth and promotes higher root : shoot ratios during water stress (Saab et al. 1990, Sharp et al. 1994, McAdam, Brodribb and Ross 2016), hence it has been proposed that the stunting effect of *Striga* may

be mediated in part through this hormone (Taylor, Martin and Seel 1996). In support of this, one study found that ABA concentrations in the leaves and xylem of sorghum plants increased by 57% and 108% respectively when the host was infected with *S. hermonthica* (Frost et al. 1997). Furthermore, one study found ABA concentrations an order of magnitude greater in *S. hermonthica* shoot tissue compared to the host maize plant (Taylor et al. 1996). A microarray analysis of the susceptible rice-*S. hermonthica* interaction revealed the upregulation of an ABA response element binding factor by over 5 fold (Swarbrick et al. 2008). Nevertheless, there is no overwhelming evidence so far that ABA transport occurs between the parasite and the host and it has been suggested that ABA signalling is a consequence of mechanical damage caused by the parasite invading the root (Swarbrick et al. 2008). Alternatively, ABA may be induced as a drought-response due to the parasite withdrawing water from the host (Taylor and Seel 1998); this would explain why the stomata of host plants close soon after *S. hermonthica* attaches (Frost et al. 1997). In any case, increased ABA signalling would likely prioritise abiotic defence responses over biotic ones (Asselbergh et al. 2008), potentially compromising host basal resistance against invading *Striga* parasites.

Auxin and cytokinin have also been implicated in plant parasitism, particularly in haustorium formation. Cytokinin transfer has been demonstrated from the hemi-parasitic root parasite *Phtheirospermum japonicum* and appears necessary for hypertrophy of host roots surrounding the infection site (Spallek et al. 2017). This was also found to depend on the host cytokinin-signalling genes *AHK3,4*, but not the cytokinin-biosynthesis genes *IPT1,3,5,7*. *ahk3,4* mutants were resistant to *P. japonicum*-induced hypertrophy and grew larger than wild-type counterparts, suggesting that parasite-derived cytokinin has a relevant role in infection. Auxin also appears to have a role in *P. japonicum*, since this accumulates at the haustorium apex apparently through the induction of the auxin-biosynthesis gene *YUC3*, expressed specifically during infection (Ishida et al. 2016). Silencing of *YUC3* reduced haustorial formation, whilst transgenic expression at ectopic sites caused haustorial-like structures to develop. It has been proposed that auxin may promote the dedifferentiation and differentiation of cells required to form haustorial hairs at the infection site.

1.8.9 Reactive oxygen species and physical barriers in host defence against parasitic weeds

As described previously, cowpea cultivars resistant to *S. gesnerioides* typically show a rapid hypersensitive response against the parasite where programmed cell death at the host-parasite interface prevents further invasion (Figure 1.3) (Huang et al. 2012). This is likely to be driven by local ROS production. In support of this, resistant cowpea cultivar B301 showed induction of cytochrome P450 enzymes as part of its defence response. These enzymes catalyse various detoxification pathways and may act to minimise tissue damage from ROS production (Huang et al. 2012). On the other hand, ROS may also act as substrates for peroxidase enzymes to promote lignin biosynthesis and the

reinforcement of cell wall barriers; indeed cowpea B301 also showed greater expression of cell wall biosynthesis genes compared with a susceptible cultivar (Huang et al. 2012). In this study, the resistant cultivar also showed increased upregulation of hydrolytic enzymes, such as narbonin, that may limit parasite growth by altering cell wall extensibility (Huang et al. 2012).

An oxidative burst and/or the generation of physical barriers is also associated with host resistance in other host-parasite interactions. The resistant sunflower cultivar LR1 showed upregulation of genes related to ROS detoxification (e.g. methionine synthase, glutathione S-transferase, a quinone oxidoreductase) and increased callose deposition at the host-parasite interface during attempted penetration by *O. cumana* (Letousey et al. 2007). Callose deposition has also been observed during early-stage resistance against *O. crenata* in faba bean: these authors suggest that, besides physical reinforcement, callose may provide a source of β-glucans as elicitors for defence responses (Pérez-de-Luque et al. 2007). Lignin deposition has also been implicated in resistance against *O. crenata* on legumes (Pérez-De-Luque et al. 2005), and *O. aegyptiaca* on vetch (Goldwasser et al. 1999). Nevertheless, despite the inability of *S. hermonthica* to penetrate the vascular system of Nipponbare rice, this cultivar did not show upregulation of NADPH oxidases and peroxidases, suggesting that physical barriers are formed independently of ROS (Swarbrick et al. 2008).

Vessel occlusion has also been observed as a defence against root parasites. During attempted parasitism by *O. crenata* on incompatible legumes, the host appeared to respond to a parasite secretion by blocking xylem vessels with a carbohydrate-based substance (Pérez-De-Luque et al. 2005). Besides barring access to the nutrient stream, this may have prevented the parasite obtaining host-derived growth hormones essential for haustorial development (Joel et al. 2013a).

1.8.10 Chemical resistance against parasitic weeds.

Although there is currently limited evidence that toxic phytoalexins have a role in defence responses against *S. gesnerioides,* resistant Nipponbare rice showed upregulation of genes related to the production of defensive secondary metabolites, including chalcone synthase and PAL, in response to *S. hermonthica* (Swarbrick et al. 2008). In sunflower, greater accumulation and excretion of 7-hydroxylated simple coumarins was associated with stronger basal resistance against *O. cernua* (Serghini et al. 2001). A vetch cultivar resistant to *O. aegyptiaca* showed a four- and eight- fold increase in free and bound phenolics respectively, which may form the basis of a chemical response against the parasite (Goldwasser et al. 1999). Similarly, phenolic compounds accumulated in parasite tissues and contacting host cells in the incompatible interaction between *O. cumana* and sunflower cultivar HE-39999 (Echevarría-Zomeño et al. 2006), besides that of *O. crenata* and *Medicago truncatula* (Lozano-Baena et al. 2007), yet this was absent in comparative susceptible interactions.

1.8.11 Hydrolytic enzymes in basal defence against *Striga* spp.

Compared to defence against *F. oxysporum* and parasitic nematodes, there is little evidence so far that hydrolytic enzymes are a strong component of basal resistance against root parasitic weeds. Perhaps this is not surprising, given that have the same cell wall composition as their hosts. Nevertheless, *S. hermonthica* induced genes encoding endochitinases (*PR-3*) and glucanases (*PR-2*) in resistant Nipponbare rice, but not in a susceptible cultivar (Swarbrick et al. 2008). It may be, however, that induction of these genes is an unavoidable consequence of hormone activity activating other, more relevant, responses.

1.9 PhD project aims and objectives

In order to breed durable resistance against *Striga gesnerioides* into crops, thorough understanding is needed of how different elements of plant defence – including hormone signalling, structural barriers and chemical defences – can be coordinated to increase host resistance. This thesis applies existing knowledge of host immune responses against other plant pathogens to systematically test whether a range of specific basal resistance defence components also have a relevant role against *S. gesnerioides*.

The overall aims of this project are:

1. To test a range of *Arabidopsis* mutants impaired in different components of plant defence for altered basal resistance against *S. gesnerioides* (**Chapter 3**).
2. To profile changes in host gene expression throughout the course of infection by *S. gesnerioide*s (**Chapter 4**).
3. To study the mechanisms by which the *ERF4* gene controls both resistance and susceptibility responses to *S. gesnerioides* (**Chapter 5**).
4. To investigate whether infection by *S. gesnerioides* induces systemic changes in host defence, such that basal resistance to foliar pathogens is affected (**Chapter 6**).

Detailed aims and context are provided in the introduction to each chapter.

Full materials and methods are described in **Chapter 2** whilst **Chapter 7** reviews and discusses the results of the preceding chapters in the context of current understanding of plant immunity.

CHAPTER TWO

Materials and Methods.

2.1 Introduction

The key advantage in using *Arabidopsis thaliana* as a model host for *Striga gesnerioides* is the ability to rigorously investigate the genetic basis of basal resistance. This is due to the wide availability of knock-out mutant lines for almost every known gene; transgenic over-expresser and reporter lines for genes of interest and publicly accessible databases for designing gene-expression assays. Furthermore, it allows ready comparison with the extensive body of published work investigating basal resistance of *Arabidopsis* to other root pathogens. This chapter describes the optimisation of a rhizotron system for growing *Arabidopsis* and infecting with *S. gesnerioides*, including quantifying the severity of infection. In addition, full methods are presented for the experimental work detailed in the following chapters including gene expression using reporter constructs; 3,3'-Diaminobenzidine (DAB) staining for reactive oxygen species; real-time quantitative polymerase chain reaction (qPCR) to assess gene expression and infection assays with foliar pathogens to investigate systemic effects of *S. gesnerioides* on host basal resistance.

2.2 Sterilisation, conditioning and germination of *S. gesnerioides* seed

A protocol for growing *Arabidopsis* in rhizotrons and infecting the roots with *Striga hermonthica* was adapted (Vasey 2005). *S. gesnerioides* is endemic to sub-Saharan Africa and requires a warm, moist conditioning phase to break dormancy and make the seed responsive to germination stimulants (Scholes and Press 2008). Once germinated, the seeds produce a radicle which grows directionally towards the host root, where they attach. For optimal infection, the radicle should only just protrude from the seed (Figure 2.1): if it must grow longer than this to reach the host root, the likelihood of successful penetration decreases. It was therefore important to determine the laboratory conditions that would replicate an optimum conditioning and post-germination phase, including duration of conditioning, temperature and type of germination stimulant.

2.2.1 Selection of *S. gesnerioides* ecotype

A preliminary investigation was carried out to identify a suitable *S. gesnerioides* ecotype to use for this project. Three ecotypes, 'Tobacco Ecotype B', collected from tobacco in Zimbabawe, ecotype '87/12' collected from cowpea in Nigeria, and ecotype 'IITA' (also collected from cowpea in Nigeria) were tested for their ability to germinate in the presence of the synthetic strigolactone GR24, which acts as an artificial germination stimulant. Two batches of *S. gesnerioides* seeds of Tobacco ecotype B, (labelled Tobacco B1 and Tobacco B2) collected from tobacco at different times, were tested. For each batch of seed, 4 samples of 20 mg seed were prepared. The seeds were sterilised by soaking in 10 % sodium hypochlorite for 7 min, then washing repeatedly with distilled water. They were then placed in Petri dishes containing 9 cm diameter discs of moistened glass-fibre filter paper (Whatman GF/A,

Whatman International, Maidstone, UK). The Petri dishes were sealed with parafilm, wrapped in foil and kept in darkness in a 25°C incubator. After 12 days, 3 ml of GR24 were applied to each plate: for each ecotype, 2 plates were treated with 0.1 ppm GR24 and 2 with 1 ppm GR24. These concentrations were selected on the basis that *S. hermonthica* is known to respond to 0.1 ppm GR24, but *S. gesnerioides* is considered less sensitive. Germination was assessed 40 h later by scoring four aliquots of 100 seeds for each plate, using a compound microscope (Prior, Model Z6T222): these data were used to calculate the average % germination (Figure 2.2).

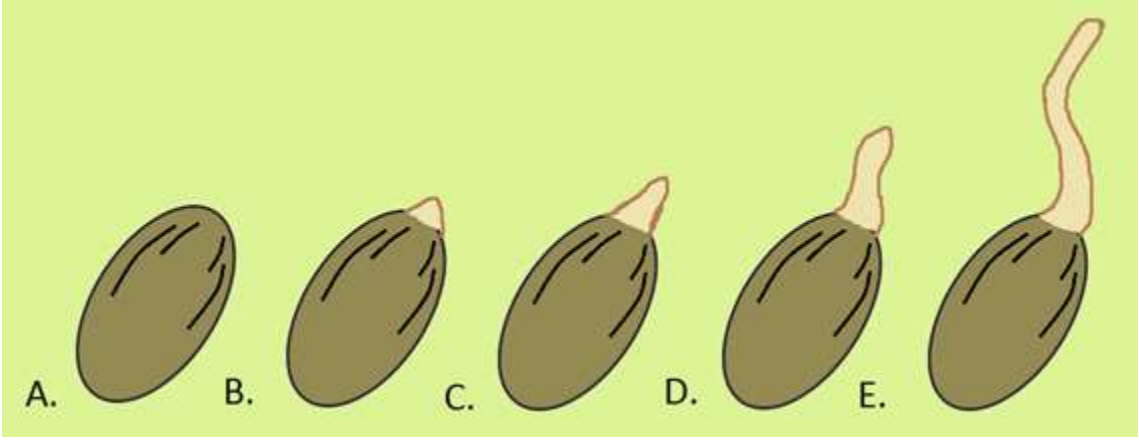


Figure 2.1: Diagrammatic representation of *Striga gesnerioides* germination. *S. gesnerioides* seed are approximately 0.2- 0.5 mm in length. A. Dormant, non-germinated seed. B. Radicle just emerged, too short for optimum infection. C. Optimum radicle length for infection. D. Radicle length beyond the optimum for infection. E. Radicle too long for optimum infection.

Seeds from ecotypes 87/12 and IITA did not germinate and were thus unsuitable for this project. For Tobacco B1 and B2, % germination was higher when 1 ppm GR24 was used, compared to 0.1 ppm GR24. A two-way ANOVA test showed that both the sample seed type and the GR24 concentration significantly affected germination (ANOVA significance < 0.0001) and that there was a significant interaction between these (ANOVA significance < 0.0001). When 1 ppm GR24 was used, % germination was considerably higher in Tobacco B1 (Mean = 68.5) than B2 (Mean = 32.5): an independent samples t-test showed that this difference was significant ($p < 0.0001$). The difference in germination between 0.1 and 1 ppm GR24 was significantly different for both Tobacco B1 ($p < 0.0001$) and Tobacco B2 ($p = 0.014$). These results confirm that viable *S. gesnerioides* seed are extremely sensitive to concentration differences in a germination stimulant, and that the Tobacco B1 ecotype was the most suitable to use for infection assays. GR24 (1 ppm) was the suitable for germination of *S. gesneri*oides seeds.

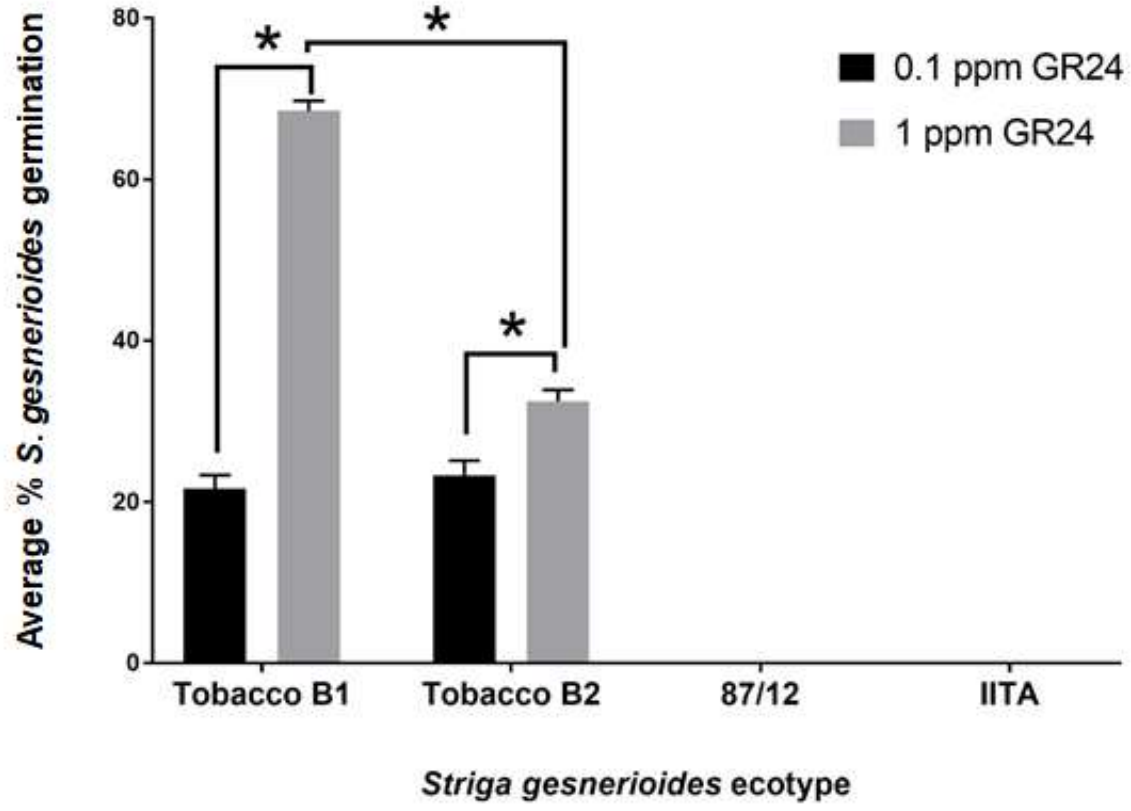


Figure 2.2: Percentage germination for three different *Striga gesnerioides* ecotypes (Tobacco B1, Tobacco B2, 87/12 and IITA), in response to 0.1 and 1 ppm GR24. Error bars: mean +/- standard error. * denotes a significant difference according to an independent samples t-test ($p<0.05$). Preconditioned parasite seeds were treated with the germination stimulant GR24 at either 0.1 or 1.0 ppm. Germination was assessed 40 h later by scoring four aliquots of 100 seed for each plate, from which an average % was calculated. Parasite seeds with an emerging radicle were counted as having germinated.

2.2.2 Optimisation of conditioning of *S. gesnerioides* seeds and incubation of seeds with GR24

A time-course was conducted to find the optimum temperature at which to condition the seeds and the incubation time in the artificial germination stimulant GR24, to allow optimal infection of the host roots. Conditioning was performed at both 25 and 30°C, to investigate if a higher temperature increased % germination. Aliquots of 10 mg of Tobacco B1 seed were sterilised and prepared onto 36 Petri dishes as described above: half of these were kept at 25°C and the rest at 30°C for 12 days. Three ml of 1 ppm GR24 were applied to each plate and germination quantified 41, 45, 48, 49, 52 and 56 h later. At each time point, 3 replicate Petri dishes were taken from each incubator. For each Petri dish, germination was assessed by scoring the number of germinated seeds out of 100: this was repeated 3 times to calculate an average % germination. Each time point/ temperature combination therefore had 3 biological replicates (3 Petri dish samples), with 3 pseudo replicate counts from each: these

were used to calculate an average % germination. A short description of the length of the radicle was also made for each sample.

The temperature of the conditioning phase affected how quickly *S. gesnerioides* seeds responded to GR24 (Figure 2.3). For all time points/temperatures tested, parasite seed germination was at least 40% or above. Repeated measures ANOVA demonstrated that % germination did not alter significantly between 40-56 h following GR24 application when the seed were incubated at 25°C (ANOVA significance = 0.314), but that it did when a 30°C incubation temperature was used (ANOVA significance < 0.0001, F = 72.696). Between 40-48 h, there was no discernible difference in the average % germination between the two temperature regimes (Figure 2.3). Beyond 48 h however, the % germination of seeds incubated at 25°C remained approximately the same, but began to increase for the seeds incubated at 30°C. At 50 h and beyond, % germination was significantly higher at 30°C than 25°C (for 50, 52 and 56 h, $p < 0.0001$). Furthermore, % germination appeared to be still increasing for seed incubated at 30°C. Nevertheless, as described previously, the optimum point to use *S. gesnerioides* seed for infection is when the radicle has just emerged from the seed coat (Figure 2.1C). At and beyond 48 h, most of the parasite seed incubated at either 25 and 30 °C had passed beyond the optimum stage for infection because the radicles had become longer than approximately 0.1mm (Figure 2.1,D-E). At all time points tested, a greater proportion of the germinated seed were at the optimum length when preconditioned at 25°C compared with 30 °C. In conclusion, these results indicate that 44 h post-germination was the maximum time point at which *S. gesnerioides* seed should be used for infection, and that 25°C was a suitable temperature for conditioning.

2.2.3 Selecting a germination stimulant for *S. gesnerioides*

GR24 was originally developed as an artificial germination stimulant for *S. hermonthica* (Johnson, Rosebery and Parker 1976). Thus, alternatives were tested for their efficacy on *S. gesnerioides* seed germination. Cowpea is a natural host for *S. gesnerioides*, and chemicals in the root exudates stimulate the parasite to germinate (Müller, Hauck and Schildknecht 1992). In addition, Gibberellic Acid (GA) has anecdotally been reported to increase levels of *S. gesnerioides* germination. Therefore, both cowpea exudates and GR24 with GA were tested to see if they were more successful than GR24 alone in germinating conditioned *S. gesnerioides* seed. Cowpea seeds were germinated on M3 compost, then transplanted into a hydroponic system, with the roots immersed in 40% Long Ashton nutrient solution containing 1 mol m^{-3} ammonium nitrate (Hewitt 1966). Twenty-four plants were kept in a 5 L container and samples of the solution collected every 2-3 days from when the plants were approximately three weeks old, and frozen until used (Figure 2.4).

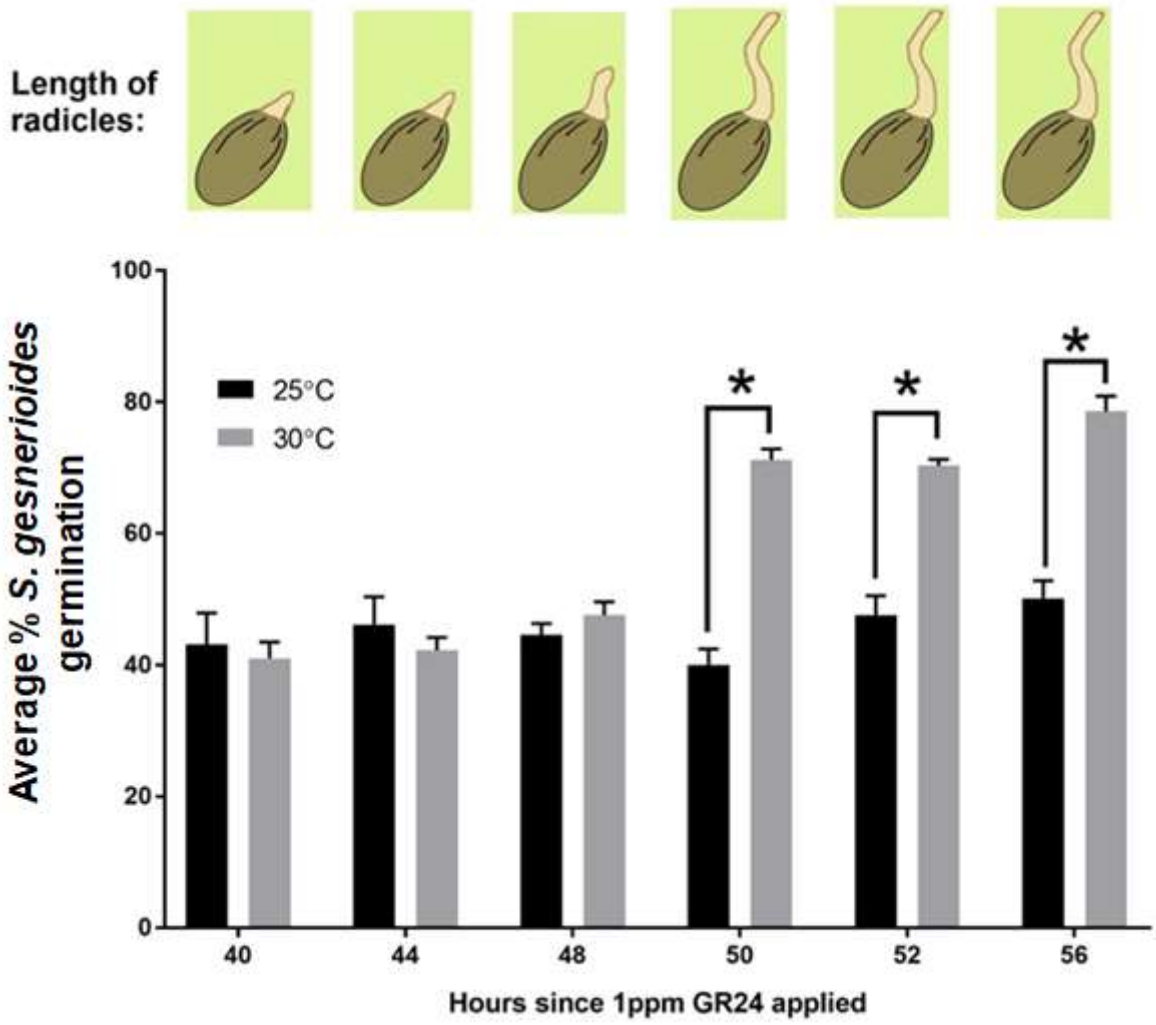


Figure 2.3: The change in responsiveness to 1ppm GR24 germination stimulant in *Striga gesnerioides* over time, when incubated at either 25°C or 30°C. Error bars: mean +/- standard error. * denotes a significant difference ($p<0.001$) in % germination scores between the 25 and 30°C assays, according to an independent samples t-test. Top panel illustrates the length of the emerging radicles from the germinated parasite seed.

Figure 2.4. Hydroponic system for collecting root exudates from cowpea. Twenty four plants were grown in a container filled with 40% Long Ashton nutrient solution, replenished as needed.

A solution was prepared containing 100ppm GR24 and 1 mM GA. *S. gesnerioides* seed were sterilised with 10% bleach, sealed in moist Petri dishes and conditioned at 25°C for 12 days as described in Section 2.2.2. Three replicate Petri dishes were set up for each germination stimulant. Three ml of each germination stimulant was added to each of the replicate plates. The plates were resealed with parafilm and returned to the incubator. Percentage germination was quantified after 40 and 44 h (Figure 2.5).

Cowpea exudates gave significantly higher % germination than either GR24 alone or GR24 in combination with Gibberellic Acid (GA) (Figure 2.5). At 40 h post-germination, the average % germination was significantly higher for samples treated with cowpea exudates compared with GR24 (Mean values: GR24 = 15.92%, cowpea exudates = 38.33%, $p < 0.0001$). At 44 h post-germination, this difference was even more pronounced (Mean values: GR24 =38.00%, cowpea exudates = 66.0%, $p < 0.0001$). At both +40 and +44 h, the addition of 1 mM GA did not significantly increase % germination compared to GR24 alone. As the highest germination rate was seen on samples treated with cowpea exudates at 44 h post-germination, with most of the parasite radicles at the optimum length for infection, these conditions were selected for use in all future infection assays.

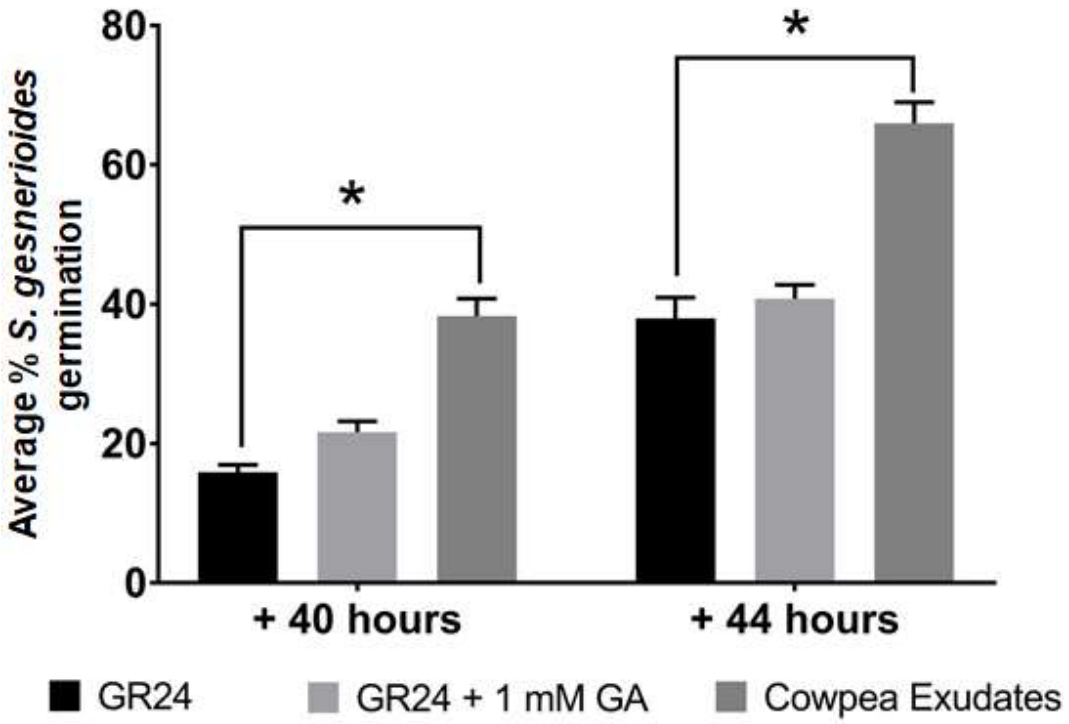


Figure 2.5. Percentage germination of conditioned *S. gesnerioides* seed in response to different germination stimulants at 40 h and 44 h after application. GR24 is a synthetic germination stimulant, developed for the root parasite *Striga hermonthica.* GA = Gibberellic Acid. Data are the mean +/- standard error. * denotes a significant difference ($p<0.05$) in % germination scores, according to an independent samples t-test.

2.3 Optimising a rhizotron system for growth and infection of *Arabidopsis thaliana* roots

2.3.1 Selecting the rhizotron packing media

A series of experiments were performed to determine the optimum growth conditions for the parasite and host, including a packing medium for the rhizotrons that would allow adequate drainage. Rhizotrons were constructed from 12 cm square Petri dishes. A hole was cut through the lid and base at the top (to accommodate the seedling) and the bottom (to allow drainage). Two media were tested: the base of the rhizotrons was either packed with rockwool only or with vermiculite in the centre with a strip of rockwool at the top and bottom (Figure 2.6A). For half of the rhizotrons in each group, a layer of GF/A filter paper was placed on top to see if this would promote more even uptake of moisture by the roots. All rhizotrons then had a layer of 35 µm mesh placed on top to prevent the *Arabidopsis* roots from growing into the packing medium. This made four experimental groups (Figure 2.6A) with 10 replicate rhizotrons in each group. *Arabidopsis* Col-0 seeds were sterilised in 10% bleach for 10 min, washed several times with distilled water then individually placed onto 9 cm diameter round Petri dishes filled with sterile Murashige and Skoog (MS) agar, using a 200 µl pipette with sterile tips (Figure

2.6B). The Petri dishes were sealed with micropore tape, wrapped in foil and cold stratified for 3 nights at 4°C. They were then moved into a Conviron Growth Cabinet with a 9-h photoperiod (photon flux density at plant height approximately 120 µmol m^{-2} s^{-1}) and temperature regime of 26/24˚C day/night and 60% humidity. The Petri dishes were placed vertically to encourage downward root growth across the surface of the agar. The seedlings were transplanted into rhizotrons when they were 17 days old and the main root was approximately 5 cm long, by carefully removing them with tweezers and placing them on top of the layer of mesh. The lid was then placed over this and sealed with waterproof tape. The rhizotrons were wrapped in foil to prevent light exposure and placed upright in the growth cabinet (Figure 2.7A). The rhizotrons were watered every 4-5 days from below with 40% Long Ashton Nutrient Solution (Hewitt 1966).

The *Arabidopsis* plants were infected when they were 43 days old. *S. gesnerioides* seeds were sterilised as described in 2.2.1 and preconditioned at 25 °C for 12 days (2.2.2). Forty hours prior to infection, 3 ml of cowpea root exudates was added to each Petri dish of parasite seeds. The Petri dishes were then returned to the 25°C incubator. To infect the *Arabidopsis* hosts, the germinated parasite seeds were washed into a beaker using distilled water. The seeds were allowed to settle and the water carefully removed. An aliquot of clean water was then added. This ensured that the germination stimulant was removed prior to infection of the roots. *Striga* seeds were suspended in distilled water and placed onto the root systems of the *Arabidopsis* with a paintbrush (~6 mg of seed for each root system) (Figure 2.7B). Following this, the rhizotrons were re-sealed and wrapped in foil and returned to the growth cabinet. The level of parasite infestation was quantified 3 weeks later by scanning the root systems using a Canon 9000F Mark II scanner at a resolution of 2400 dpi. The number of haustoria on each root system was quantified from the images using the CellCounter function on ImageJ software. A parasite haustorium was defined as an attachment to the host root that had begun to swell (Figure 2.8).

The results demonstrated that rock wool with a layer of mesh alone was unsuitable as none of the seedlings survived (Table 2.1). In the rhizotrons containing vermiculite with a layer of mesh only, 3 of the 10 seedlings survived, however only one of these was successfully infected by the parasite. Adding a layer of filter paper improved seedling survival for both rock wool and vermiculite-filled rhizotrons (Table 2.1). Although the survival rate was similar, the average number of *S. gesnerioides* haustoria on the *Arabidopsis* hosts was much greater for rhizotrons prepared with vermiculite, filter paper and mesh, rather than rockwool, filter paper and mesh (Table 2.1), although this was not statistically significant according to an independent samples t-test. As the rhizotrons prepared using vermiculite with a layer of filter paper and mesh gave both the highest *Arabidopsis* survival and the ighest level of *S. gesnerioides* infection, this packing system was used in all subsequent assays.

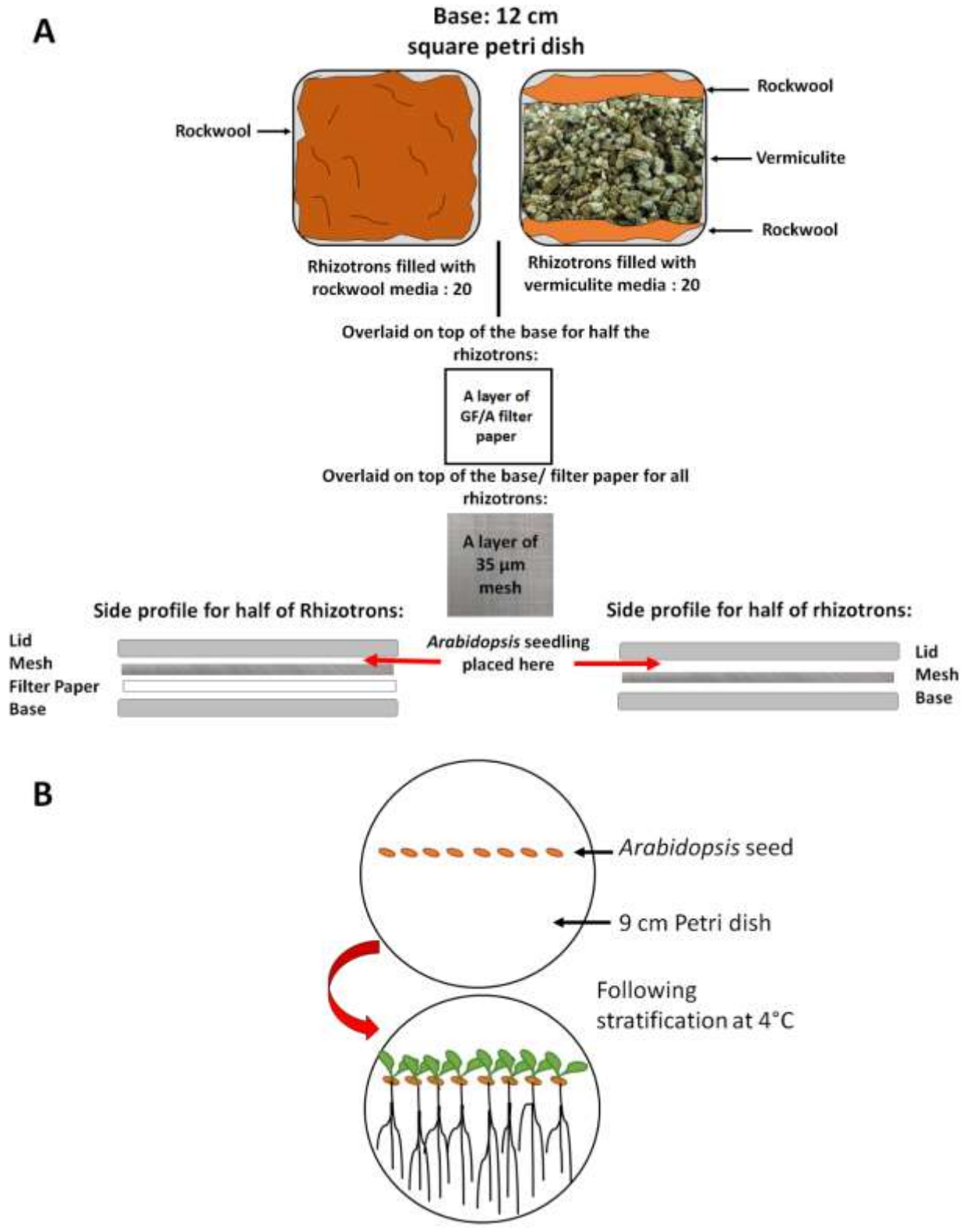


Figure 2.6: Optimisation of the *Arabidopsis* rhizotron system. **A:** 40 rhizotrons (12cm square Petri dishes) were prepared: half of these were filled with rockwool only, the remainder with vermiculite media in the centre with a strip of rockwool at the top and bottom. For half of the rhizotrons in each group, a layer of GF/A filter paper was added over the base layer. All rhizotrons had a sheet of 35 µm mesh as the top layer, overlaying either the base or the filter paper. The Petri dish lid was then sealed on top**. B:** *In vitro* germination of *Arabidopsis* seed in 9 cm diameter round Petri dishes filled with sterile Murashige and Skoog (MS) agar.

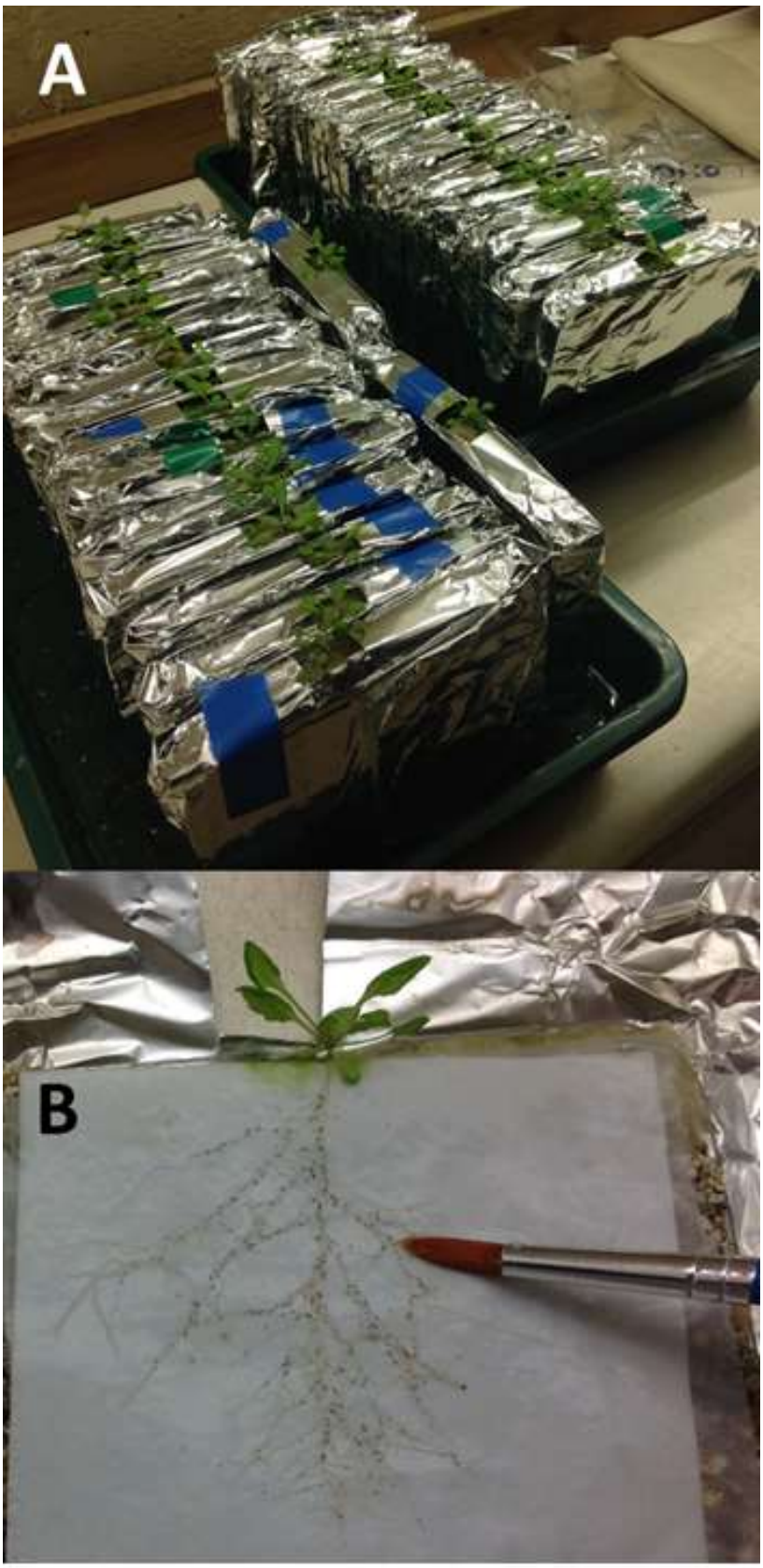


Figure 2.7: Rhizotron system for growing *Arabidopsis thaliana* and infecting the roots with germinated seeds of the parasite *Striga gesnerioides*. **A**. Rhizotrons were wrapped in foil to prevent light exposure around the roots and stood upright in a Conviron Growth Cabinet. **B**. To infect the host with *Striga gesnerioides*, the lid of the rhizotron was carefully removed and the germinated parasite seed applied directly onto the root using a paintbrush.

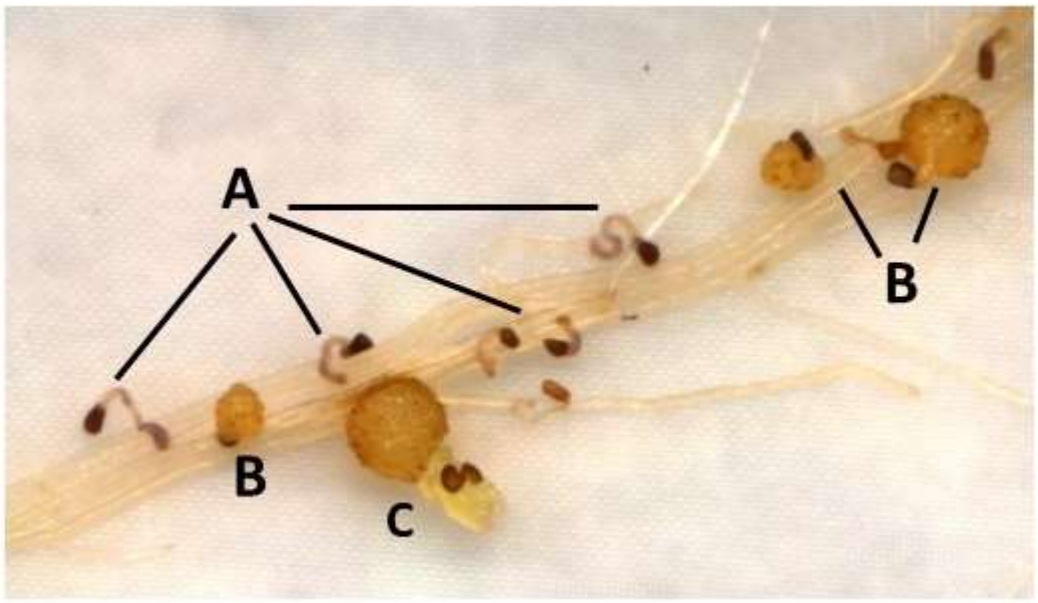


Figure 2.8: Examples of germinated *S. gesnerioides* seeds applied to an *Arabidopsis* host root system. **A.** *S. gesnerioides* seed that had failed to penetrate the host root and infect successfully. The germinated seed had produced a radicle which was in contact with the host root but a haustorium did not develop. These seeds were scored as unsuccessful infections. **B.** *S. gesnerioides* seeds that had successfully attached, penetrated the host root and developed a haustorium, visible as a round swelling on the host root. These were scored as successful infections. **C.** A more advanced-stage *S. gesnerioides* haustorium that had begun to develop a shoot.

Table 2.1: Survival rate and levels of infection by *Striga gesnerioides* for *Arabidopsis* seedlings grown in rhizotron systems with different packing materials

	Percentage *Arabidopsis* survival	**Average S. *gesnerioides* haustoria per host**	**SEM S. *gesnerioides* haustoria per host**
Rock wool and mesh	0	/	/
Rock wool + filter paper + mesh	50	4.6	2.293
Vermiculite + mesh	30	1.33	1.333
Vermiculite + filter paper + mesh	**60**	**20.67**	**10.588**

2.3.2 Determining the optimum host development stage for infection with *S. gesnerioides*

An experiment was conducted to determine when the host *Arabidopsis* roots were at the optimum length to infect with *S. gesnerioides*. Nucleic acid yields are generally higher for younger tissue, hence for gene expression assays it was important to find the earliest time point possible at which the host *Arabidopsis* plants could be infected. Five Col-0 *Arabidopsis* plants were grown in rhizotrons and photographed every two days (between 16-30 days old) to record the growth of the root system. As it was difficult to distinguish the white roots against the white filter paper, these were converted into diagrammatic sketches (Figure 2.9).

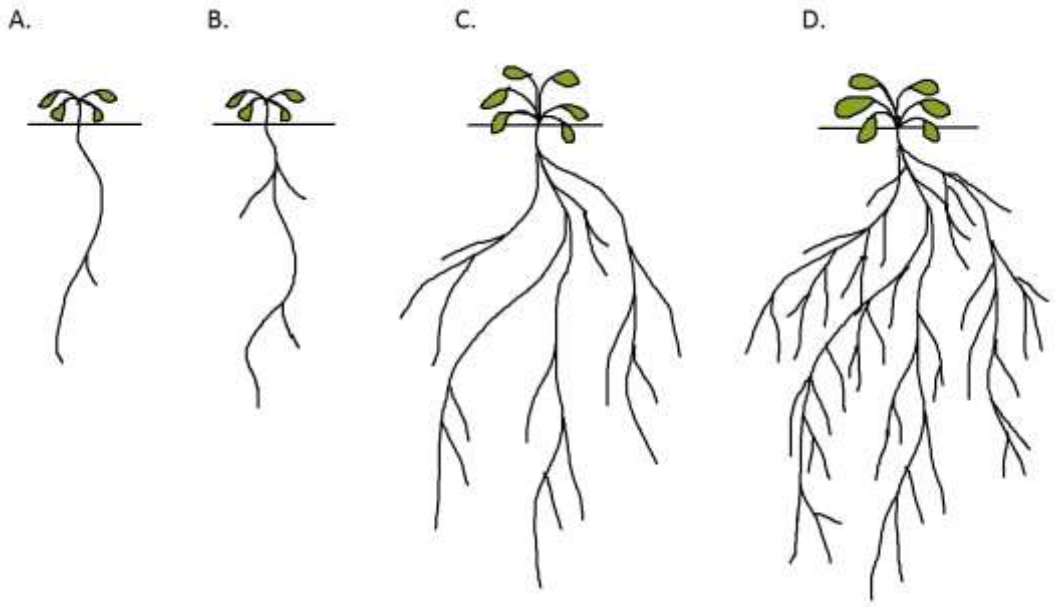


Figure 2.9: Diagram to show the development of *Arabidopsis* roots in rhizotrons. Days were numbered from the point at which the cold stratified seeds were sown onto agar to germinate. The seedlings were transplanted into rhizotrons when they were 14 days old. A. Root system at 18 days old. B. Root system at 20 days old. C. Root system at 27 days old. D. Root system at 30 days old.

Based on these results, an assay was then performed to determine the earliest time point at which infection gave reproducible results. Twenty-four rhizotrons were prepared. Ten *Arabidopsis* plants were infected with 6 mg of germinated *S. gesnerioides* seeds when they were 27 days old, and 14 were infected when they were 37 days old. Conditions for preconditioning and germinating the parasite seed followed those described above. The number of haustoria was compared between seedlings infected at 27 or 37 days old (Figure 2.10). There was no significant difference in the average number of haustoria on the roots of *Arabidopsis* infected at 27 or 37 days ($p > 0.05$). These results confirmed that infection with *S. gesnerioides* could be carried out when the *Arabidopsis* hosts were 27 days old without affecting the success of the parasite.

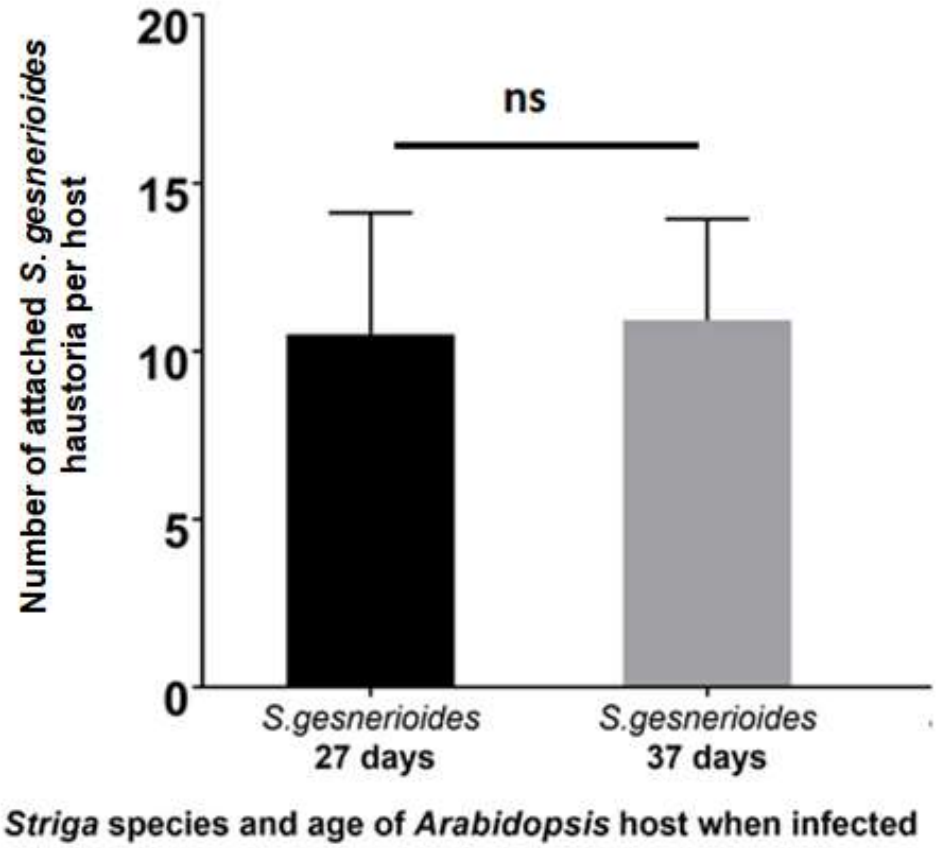


Figure 2.10: Average numbers of attached haustoria of *Striga gesnerioides* on *Arabidopsis* hosts infected at either 27 or 37 days old. Data show mean +/- standard error. ns indicates no significant difference ($p<0.05$) in the average number of attached parasite haustoria per host, according to an independent samples t-test.

2.4 Quantifying resistance / susceptibility of *Arabidopsis* plants against *S. gesnerioides*

Chapter 3 describes the results of a phenotypic screen of a range of *Arabidopsis* mutants for altered basal resistance against *S. gesnerioides*. Thus, an accurate measure to quantify the amount of infection and development of haustoria on the *Arabidopsis* root systems was required. *S. gesnerioides* and *Arabidopsis* seed were sterilised with 10% bleach as described in 2.2.1 and 2.3.1. *Arabidopsis* seed were cold stratified at 4°C for 3 nights, and the *S. gesnerioides* seed were preconditioned at 25 °C for 12 days. *Arabidopsis* seedlings were transplanted when they were 14 days old into rhizotrons packed with vermiculite medium (with a strip of rockwool at the top and bottom), with an overlying layer of GF/A filter paper and 35 µm mesh. Plants were grown in Conviron cabinets under a 9-h photoperiod cycle (photon flux density approximately 120 µmol m^{-2} s^{-1} at plant height) and temperature regime of 26/24˚C day/night and 60% humidity. Infection with *S. gesnerioides* took place when the seedlings

were 27 days old: cowpea exudates were applied to the *S. gesnerioides* seed 44 hours prior to infection. To quantify the level of infection on each host, the rhizotrons were scanned 3 weeks after infection using a Canon 9000F Mark II scanner at resolution 2400 dpi. The images were then scored using the CellCounter function on ImageJ software. The number of unsuccessful and successful attachments was counted and expressed as the % of successful attachments out of all the parasite seed applied, as an indicator of early-stage host resistance (henceforth termed as the '% infection rate'). This normalised for differences in root length (especially between Col-0 and the mutant line) and for slight differences in the amount of parasite seeds applied. Any attached parasite seed that had formed a visible haustorium was counted as a successful infection (Figure 2.8).

To quantify host resistance acting after parasite attachment (late-stage resistance), the size of each haustorium was scored. This was done by overlaying black circles on top of the haustoria in ImageJ then subtracting the image background using the default thresholding option. The size was calculated using the Analyse Particles function and this figure converted into cm^2. In addition, the number of haustoria showing a developing shoot was also scored as this is an indicator of more advanced parasite development (Figure 2.8): this number was expressed as a proportion of the total number of attached haustoria.

2.5 Microscopic analysis of *Arabidopsis* roots infected with *S. gesnerioides*

A microscopic analysis was performed to identify the extent of parasite penetration into the *Arabidopsis* roots at specific times after infection, in order to perform an analysis of changes in gene expression at key stages of the parasite lifecycle (Chapter 4, Section 4.4.1). Twenty rhizotrons containing *Arabidopsis* (Col-0) plants were prepared and infected with *S. gesnerioides* as described in Section 2.4. Small root sections with attached parasite haustoria were harvested from three host plants at 7, 10, 12, 14, 16, 18 and 20 days post-infection. Sections were first placed in Carnoy's fixative (100% EtOH: acetic acid in a 4:1 ratio) overnight. The following day, samples were washed twice in 100% ethanol for 30 min each time. The samples were then returned to 100% ethanol. Samples were embedded in Technovit 7100 solution following the manufacturer's instructions. For pre-infiltration, the Samples were incubated in Technovit 1: EtOH in a 1:1 ratio for at least 2 h, before being transferred to 100% Technovit 1 for at least 15 min. The samples were then placed into the lids of 1.5 ml Eppendorf tubes in a solution of Technovit 1 and Hardener 2. As this solution set, the roots sections were manipulated so that they stood up longitudinally to allow a series of sections to be taken. At least a week was allowed for the sections to harden before these were mounted onto Histoblocs (Manufacturer). Sections, 8-12 µm thick, were cut using a Leica RM 2145 microtome and dried onto glass slides. Sections were stained with dilute toluidine blue (which stains acidic tissue components) by laying the slides in shallow trays containing the dye for 2 min and then repeatedly washing with

distilled water. Slides were air-dried overnight then examined using an upright Olympus Epifluorescence Microscope Model BX51 (Olympus Optical.Co.UK, London). Images were captured using an Olympus high resolution digital camera DP71 (Olympus Optical.Co.UK, London).

Sections of infected roots were also taken and viewed under UV light to visualise phenolic compounds. Long sections of infected root were cut with a scalpel and placed in 15 ml falcon tubes containing chloral hydrate ($C_2H_3Cl_3O_2$). This clearing solution increases the transparency of plant tissues without disrupting cellular contents. A stock solution was prepared using 100 g chloral hydrate and 30 ml water with 5 ml glycerol to prevent crystallisation. Root samples were left in the clearing solution for a week at room temperature then mounted on glass slides and viewed under UV light using the Olympus Epifluorescence Microscope Model BX51 (Olympus Optical.Co.UK, London). Fluorescence was generated by a LED-excitation, provided by a CoolLED pE-2 excitation system (CoolLED Ltd.Andover UK, www.coolled.com). UV light causes phenolic compounds to fluoresce (Bennett et al. 1996, McLusky et al. 1999); this includes lignin-containing elements such as cell walls and xylem. Consequently, UV light allows host-parasite vascular connections to be seen.

2.6 β-glucuronidase (GUS) assays for reporter gene expression

Chapter 4 details how transgenic *Arabidopsis* GUS reporter lines were used to investigate whether parasitism by *S. gesnerioides* activates jasmonic acid (JA) or salicylic acid (SA)-associated signalling in *Arabidopsis* roots. GUS staining solution containing X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) was prepared in advance and stored at -20°C. Table 2.2 lists the quantities of reagents used. For all experiments, *Arabidopsis* plants were grown in rhizotrons and infected with *S. gesnerioides* according to the optimised protocol described in Section 2.4. At the point of harvest, the central part of the root systems was excised with a scalpel and placed in 15 ml falcon tubes containing GUS-staining solution. The tubes were vacuum infiltrated (lids removed) for approximately 2 min, repeated 6 times in total. These were then incubated at 37°C overnight and transferred into 70% ethanol at room temperature the following day, then into fresh 70% ethanol for storage the day after.

Table 2.2: Reaction mix used for GUS-reporter assays for *Arabidopsis* infected with *S. gesnerioides*

	For 20 ml of reaction mix:	**For 50 ml of reaction mix:**
20 mg/ml X-GlcA (dissolved in DMSO)	0.55 ml	1.375 ml
0.5 M NaH_2PO_4 / Na_2HPO_4	4.00 ml	10.00 ml
100 mM $K_4Fe(CN)_6$	0.20 ml	0.50 ml
100 mM $K_3Fe(CN)_6$	0.20 ml	0.50 ml
Triton X-100 10% v/v	0.20 ml	0.50 ml
H_2O (distilled)	14.85 ml	37.125 ml
Total:	20 ml	50 ml

~ 8 ml of reaction mix was aliquoted into 15 ml falcon tubes (each containing one root sample).

2.7. 3,3'-Diaminobenzidine (DAB) staining of infected roots

Chapter 4 describes how 3,3'-Diaminobenzidine (DAB) staining was used to investigate ROS activity in the host and parasite during the initial stages of infection. DAB $(H_2N)_2C_6H_3$ C_6H_3 $(NH_2)_2$ is an organic compound derived from benzene that can be used to detect intercellular ROS (Thordal-Christensen et al. 1997). In its unoxidized state, the compound is soluble and is readily taken up by plant leaf and root tissue. At sites of peroxidase activity, where H_2O_2 is generated, the compound polymerises to form an insoluble, dark-brown substrate.

Arabidopsis plants were prepared in rhizotrons and infected with *S. gesnerioides* following the optimised protocol described in Section 2.4. Root systems were harvested at 7, 10, 12, 15, 18 and 21 days post-infection. A 1mg/ml DAB solution was prepared the day before each harvest by dissolving 0.05 g of 3,3'-Diaminobenzidine in 50 ml of distilled water. Hydrochloric acid was added to adjust the pH to 3.8, then the solution mixed for several hours with a magnetic stirrer to dissolve the DAB. After this, the solution was stored at 4°C. On the day of use, the pH of the DAB solution was checked and adjusted to 5.5 using hydrochloric acid or sodium hydroxide if necessary. *Arabidopsis* roots are light sensitive and known to produce ROS when exposed to light (Yokawa et al. 2011), hence harvesting and staining of root samples took place in darkness. For each time point, three *S. gesnerioides*-infected *Arabidopsis* roots were harvested, and also the root systems from two control, non-infected *Arabidopsis* plants. The central portions of the host root systems were excised and placed in a shallow tray containing DAB solution. In addition, root systems harvested from two different *S. gesnerioides*-infected *Arabidopsis* plants were placed in a beaker containing distilled water, to act as a control. Both DAB and water treated samples were vacuum infiltrated for 20 min in a chamber covered in foil to prevent light exposure. The samples were incubated for a further four hours then washed in 50:50 ethanol:distilled water. Following this, samples were stored in fresh 50:50 ethanol:distilled water and later placed on glass slides to view under brightfield illumination using an upright Olympus Epifluorescence Microscope Model BX51.

2.8 Quantitative PCR (qPCR) for measuring gene expression

Chapters 4, 5 and 6 present the results of quantitative PCR (qPCR) analysis of defence gene expression changes in *Arabidopsis* leaf and root tissue during infection by *S. gesnerioides*. A key advantage of qPCR over reporter-staining techniques is that it gives quantitative fold-changes in gene expression that are normalised against both a control treatment (which represents baseline gene expression) and the expression of a reference gene (to correct for variations in nucleic acid concentration between samples). The starting point is RNA extraction, to distinguish actively transcribed genes from all genetic material. RNA is then reverse-transcribed into double-stranded complementary DNA (cDNA), which is

amplified according to the conventional PCR cycles of denaturation, annealing and elongation. In qPCR, the amplification reaction includes a fluorescent dye which binds to double (but not single) stranded molecules. During amplification, the dye is incorporated into newly synthesised double-stranded molecules, producing a fluorescent signal. The signal strength is converted into a quantitative value by measuring the number of cycles required for the fluorescence to reach a pre-defined threshold (termed as the 'take-off' value; Ct value). The higher the amount of starting cDNA, the more rapidly fluorescence is generated, resulting in a lower take-off value. These values can be subject to absolute and/or relative quantification. In absolute quantification, the take-off value is compared with a standard curve for solutions of known RNA concentrations (Livak and Schmittgen 2001). Relative quantification, meanwhile, determines fold gene expression differences between a control, reference group and the condition under test (Livak and Schmittgen 2001) (in this case, *Arabidopsis* plants infected with *S. gesnerioides* vs non-infected *Arabidopsis*).

2.8.1 RNA extraction

RNA extraction was performed according to the QIAGEN RNA Extraction Plant-Mini Kit protocol. Samples were lysed by grinding with autoclaved pre-frozen pestles, whilst in liquid nitrogen. To the ground material, 600 µl of highly lysing guanidine-thiocyanate containing buffer RLT (with added 2-mercaptoethanol) was added. This simultaneously disrupts cell membranes and inactivates any RNase enzymes to keep the RNA intact (QIAGEN June 2012). The samples were ground further using a QIAGEN TissueLyser with two grinding balls (2mm diameter) in each tube, shaken at 25 Hz for 1 min, repeated three times. The samples were vortexed to mix then homogenised by centrifugation at 20,000 g in a QIAshredder spin column: this removes insoluble material and shears high-molecular weight cellular compounds into a homogenous lysate (QIAGEN June 2012). To this lysate, 0.5 volume of ethanol was added and mixed by pipetting; the solution was then centrifuged in a RNeasy Mini Spin column. Ethanol promotes the selective binding of RNA to the RNeasy membrane in the RNeasy Mini Spin column. The captured RNA was then washed of contaminants using the RW1 and RPE buffers supplied in the kit. RNA was then eluted in 30 µl nuclease-free water. RNA concentration was tested by applying 1.5 µl of the eluted solution to a ThermoScientific NanoDrop 8000 Spectrophotometer (220 – 360 nm wavelength) pre-blanked with nuclease-free water which measures RNA quantity on the basis of UV absorbance. The concentration was measured twice for each sample and the average calculated. A280/A260 ratios were checked to ensure they were approximately 2.0, as this indicates an acceptably pure sample (Scientific 2010, ThermoFisherScientific 2010). These results were verified by running 3 µl of sample on a 1.5% agarose gel, made visible with 1:5 DNA Loading Buffer. Relative differences in concentration were visible as different brightness in the bands.

2.8.2 cDNA synthesis

The ThermoScientific Maxima First Strand cDNA Synthesis Kit with dsDNase (Catalogue Number K1671) was used to reverse transcribe RNA into complementary DNA (cDNA). To ensure the cDNA products would have equal concentrations, the amount of starting RNA solution (in µl) required for a yield of 500 ng was calculated using the average NanoDrop result. The difference was made up to an 8 µl volume using nuclease-free water. The samples were then treated with a double stranded DNAse enzyme (dsDNase) to remove any genomic DNA contaminants that remained after RNA extraction. To each sample,1 µl of dsDNase and 1 µl of 10x dsDNase buffer were added. All samples were then incubated at 37°C for 10 min in a Techne Prime Thermocycler. In order to check the efficiency of the dsDNase treatment step, a PCR reaction was performed on an aliquot of the DNase-treated RNA samples to check for any gDNA contamination. A genomic DNA sample was used as a control. Following successful removal of gDNA, samples were reverse transcribed. The following reagents were then added to each sample: 4 µl 5x Reaction Mix; 2 µl Maxima Enzyme Mix; 4 µl nuclease-free water. Samples were spun down and incubated as follows: 10 min at 25°C; 30 min at 50°C; 5 min at 85°C. cDNA was either used for qPCR analysis immediately, or frozen at -20 ^{o}C.

2.8.3 Primer design for target genes

The genes selected for qPCR were selected on the basis of the results from Chapter 3 or established association with defence hormone activity and/or defence against root pathogens. Where primers were not already available from laboratory supplies, these were designed using Primer3 (http://primer3.ut.ee/) or QuantPrime (http://quantprime.mpimp-golm.mpg.de/) or they were based on sequences provided from published qPCR studies, (Table 2.4). Complete coding sequences (CDS) for each target gene were obtained from TAIR (https://www.arabidopsis.org/) to act as the template for the design process. Key criteria were: primer length of around 20 base pairs; a GC content of 50-60%; an annealing temperature of 55-65 °C and a product size between 150-200 base pairs. Where possible, primers were designed to span the junctions between two exons, so that any DNA contaminants would not be amplified. Primers were also checked to ensure there was low potential for primer dimers or hairpin products forming. Sequences were BLASTed against the *Arabidopsis* genome on TAIR 10 to check that there was no complementarity with unrelated genes. For most genes, at least two different primer pairs were ordered in case one failed. Primers were ordered from Invitrogen as dry pellets. These were reconstituted in nuclease-free water to a stock concentration of 100 µM and stored at -20°C degrees. Primer sequences used for qPCR are shown in Table 2.3.

Primers were tested on gDNA and cDNA samples from control *Arabidopsis* plants to check that they amplified effectively. These were also tested to make sure that they were specific for *Arabidopsis*, and

not *Striga gesnerioides*. To do this, shoot material was harvested from *S. gesnerioides* plants growing on tobacco (*Nicotiana tabacum* L. cv. Samsun) (Figure 2.11). The tobacco seed was a gift from Professor Marc Knight, Durham University, UK. RNA from *Arabidopsis* and *S. gesnerioides* was extracted using the QIAGEN kit method as outlined above; this was then used to produce cDNA as before. Standard PCR conditions were used (according to QIAGEN Taq PCR Master Mix Kit, Catalogue Number 201443). For each primer pair the following mix was used: 25µl Taq PCR Master Mix; 5µl of forward/reverse primer mix at 2 µM; 18 µl nuclease-free water and 2 µl of template cDNA. The Thermocycler programme was set as follows: initial denaturation: 3 min at 94 °C; 35 cycles of 0.5 min at 94°C, 0.5 min at the average annealing temperature, 1 min at 72°C; final extension of 10 min at 72°C. Due to the large number of primers to test, multiple PCR runs were required. For each run, the optimum annealing temperature was calculated for each run using the Thermofisher Tm calculator (www.thermofisher.com/uk/en/home.html).

Many of the gene primers were unsuitable as they either failed to amplify a product on control *Arabidopsis* cDNA samples (Figure 2.12A); showed secondary product formation (Figure 2.12B) or amplified a product on cDNA samples from *Striga gesnerioides* (Figure 2.12A). *LOX2* was removed from the analysis as the primers failed to amplify a product (Figure 2.12A). *PGIP1* was also removed as the primers were not specific to *Arabidopsis* as they also amplified a product on cDNA from *S. gesnerioides* (Figure 2.12A).

Figure 2.11: *Striga gesnerioides* growing on tobacco (*Nicotiana tabacum* L. cv. Samsun). Non-flowering shoot material was harvested from the parasite to test qPCR primers for specificity to *Arabidopsis*.

Table 2.3: List of final primer sequences used for qPCR analysis

Gene name	Locus	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Source
Salicylic Acid (SA) -associated genes					
PR1 (PATHOGENESIS RELATED PROTEIN 1)	At2g14610.1	CCTGGGGTAGCGGTGACTTGTC	CGTTCACATAATTCCCACGAGG	222	(Choi et al. 2012)
PR2 (PATHOGENESIS RELATED PROTEIN 2)/ β-1,3-GLUCANASE	At3g57260.1	AGCTTCCTTCTTCAACCACACAGC	TGGCAAGGTATCGCCTAGCATC	70	QuantPrime
PR5 (PATHOGENESIS RELATED PROTEIN 5)	At1g75040	CCGGAGGATCGGGAGATTG	CTCCACGGCAGCAATATTG	153	(Liu et al. 2013)
PAD4 (ARABIDOPSIS PHYTOALEXIN DEFICIENT 4)	At3g52430.1	GTTGGATGAGGCGAGAAAAG	TCGCATAACTCTCGAATGGA	86	(Li, Zhong and Palva 2017)
Jasmonic acid (JA) -associated defence genes					
VSP2 (VEGETATIVE STORAGE PROTEIN 2)	At5g24770.2	GGACTTGCCCTAAAGAACGACACC	GTCGGTCTTCTCTGTTCCGTATCC	114	QuantPrime, David Pardo.
PAD3 (PHYTOALEXIN DEFICIENT 3)	At3g26830.1	TTCCTCTGTTTCCTCGTCCT	ATGATGGGAAGCTTCTTTGG-3	104	(Edgar et al. 2006)
Jasmonic-acid / ethylene coregulated					
THI2.1 (THIONIN2.1)	At1g72260	CCAATGAGCACTGCAAGTTAGGG	CACTTGCATCAGAGTTCTGGAGAG	78	Quantprime
PDF1.2 (PLANT DEFENSIN 1.2)	At5g44420	CTTGTTCTCTTTGCTGCTTTCGAC	TTGGCTCCTTCAAGGTTAATGCAC	140	QuantPrime, David Pardo.
PR4/ HEL (PATHOGENESIS RELATED PROTEIN 4: HEVEIN-LIKE)	At3g04720.1	GCGGCAAGTGTTTAAGGGTGAAG	TCCAAATCCAAGCCTCCGTTGC	94	QuantPrime, David Pardo.
B-CHI (BASIC CHITINASE)	At3g12500	ACTACAGGTGGATGGGCTACAG	TCCTCTTCCGTAGTAGCGTTTGC	144	Quantprime
Signalling nodes and defence-regulatory transcription factors					
ERF1 (ETHYLENE RESPONSE FACTOR 1)	At3g23240.1	AGTCGACAGCGAGTTCGGTTAC	AGCTAGGGTTTCGTCCGTACAC	78	QuantPrime, David Pardo.

ERF2 (ETHYLENE RESPONSE FACTOR 2)	At5g47220.1	ACACGTCATCATCGGACTTGAGC	TCGCCGTAAAGTTCTCAGTTGGC	78	QuantPrime, David Pardo.
ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59)	At1g06160.1	ATCAGGCGGCTTTCGCTTTG	CTTCCGGAGAGATTCTTCAACGAC	80	QuantPrime, David Pardo.
WRKY70 (ARABIDOPSIS THALIANA WRKY DNA-BINDING PROTEIN 70)	At3g56400.1	TGAGCTCGAACCCAAGATGTTCAG	TGCTCTTGGGAGTTTCTGCGTTG	80	QuantPrime, David Pardo.
ERF4 (ETHLYENE RESPONSE FACTOR 4)	At3G15210	GCGGCTCGTGTTATCAGATC	CTAAACGCCGATGTCACAGG	109	Primer3
ERF4-Repressor isoform	At3G15210	TTGCCTCCTCCATCGGAACAGG	CAAAAAGAAGAAGAAACGCATGCGC	80	(Lyons et al. 2013)
ERF4-Activator isoform	At3G15210	GGCTTGTGGTGCCCAAAGCG	TCACACCCTCTTATACGTCGTCGT	Unknown	(Lyons et al. 2013)
AtMYC2/JIN1 (JASMONATE INSENSITIVE 1)	At1g32640.1	AACCACGTCGAAGCAGAGAGAC	TTGGTACAACCGCTCGTAACGC	76	QuantPrime, David Pardo.
Reactive oxygen species (ROS)-associated					
GST1 (GLUTATHIONE S-TRANSFERASE 1)	At1g02930.1	CCTTCTCTCAACTGGCAAGG	TCCCAAACAAGCTTTGAACC	96	(Li et al. 2017)
PRX33 (PEROXIDASE 33)	At3g49110.1	TAACGCAAATCTTCCAGCTCCA	GGTCAGGTAATCCAGTGTTGC	182	(Li et al. 2017)
PRX53 (PEROXIDASE 53)	At5g06720.1	TACAAACGATCTGGTAGCCTTATCTGGT	GTCCCGCTGAAGTTAAATAGTCTGTTGTTG	93	(Jin 2010)
Inhibitors of cell wall degrading enzymes					
PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1)	At5g06860.1	GTCATTTGGGTCGTTTCCAG	ATCGAAGCATCACCTTGGAG	144	(Li et al. 2017)

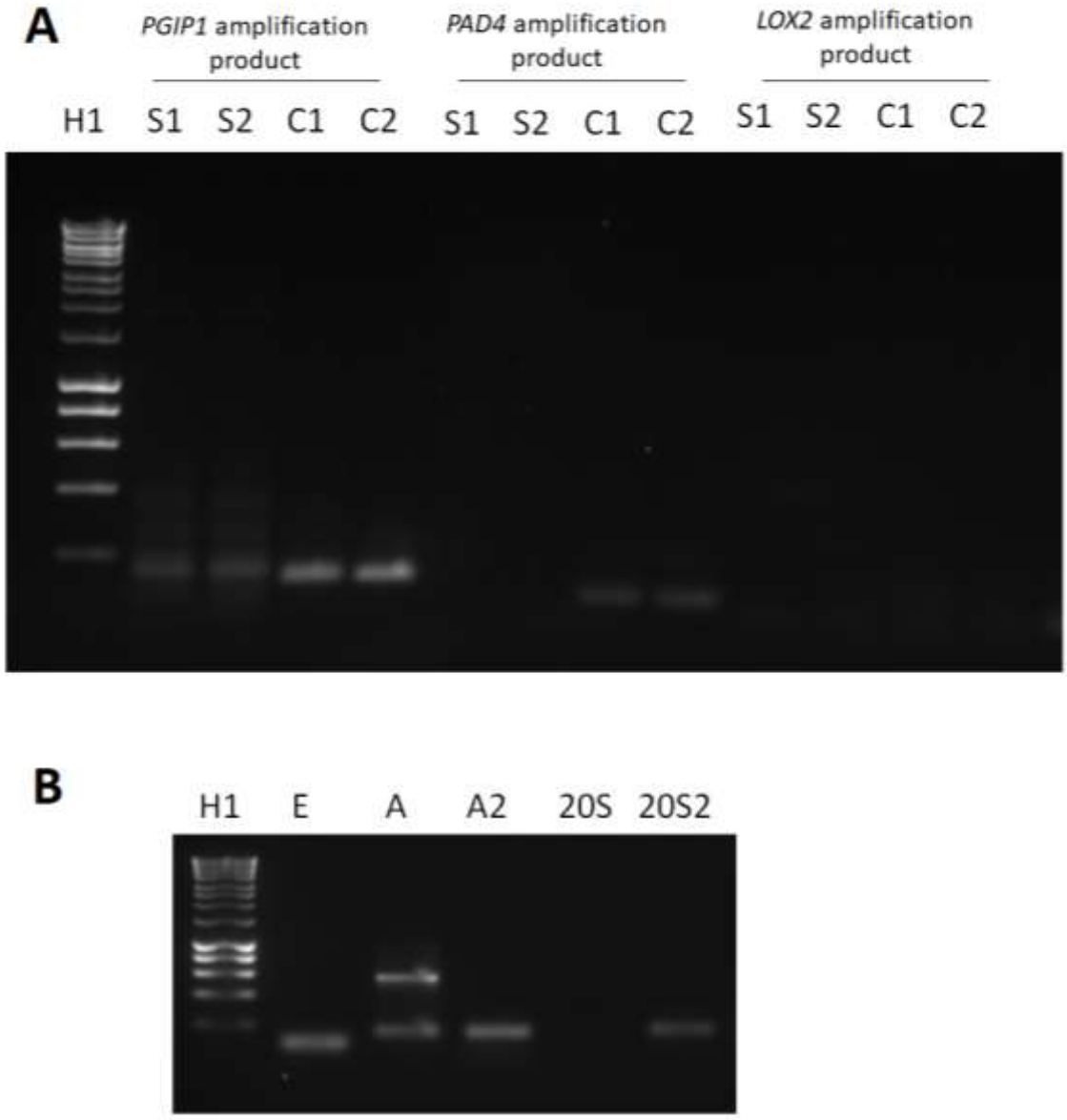


Figure 2.12: Examples of primers showing poor amplification or non-selective amplification. **A.** Amplification products of primers for the *Arabidopsis* genes *PGIP1*, *PAD4* and *LOX2* on *Striga gesnerioides* (S1, S2) and *Arabidopsis* (C1, C2) cDNA. A clear product for *PGIP1* is seen on both *S. gesnerioides* samples, indicating that the primer pair is not suitable for host gene expression analysis. The primers for *LOX2* failed to amplify on either *Arabidopsis* or *S. gesnerioides,* making them also unsuitable. **B.** Amplification products for five primer pairs on *Arabidopsis* cDNA. Primer pairs are as follows: E = ERF4, A = β-Actin, A2 = β-Actin 2 (second primer pair), 20S = 20S Proteasome, 20S2 = 20S Proteasome (second primer pair). The first primer pair for β-Actin is unsuitable because it produces a secondary product. The first primer pair for 20S Proteasome is unsuitable because it failed to amplify. H1 = Hyperladder I.

2.8.4 Selecting reference ('housekeeping') genes for standardisation

If the total cDNA concentration between samples is different, this can affect the calculation of relative expression for the genes of interest. For this reason, it is necessary to include reference genes (also known as 'housekeeping genes') against which the take-off values for the target genes can be standardised. These reference genes should be consistently expressed, with very little variation over time and under different conditions. For this assay in particular, they should remain stable throughout the time-course of infection and not differ significantly between control and infected samples. Ideally, at least two different reference genes should be used as studies have indicated that using only a single reference gene can still result in quantification errors of up to 6 fold (Vandesompele et al. 2002). Primers for a number of different control genes were tested (Table 2.4), based on those described in previous studies using *Arabidopsis*. Many of these proved unsuitable for reasons that included poor amplification on *Arabidopsis* root cDNA or secondary product formation (Table 2.4). The most promising, *β-ACTIN* (second primer pair), *GADPH* and *20S PROTEASOME* (second primer pair) were investigated further using qPCR on 18 cDNA samples: 9 each from control and infected *Arabidopsis* roots. The samples were further divided equally between the three time points at which the material was harvested: 7, 10 and 12 dpi. *β-ACTIN* was expressed too robustly across all samples to allow accurate quantification and was thus not selected. Both *GADPH* and *20S PROTEASOME* showed consistent expression between control/non-infected conditions across all time points. Additionally, their Ct values contrasted with each other: this range of difference increased the accuracy of normalising the Ct values of target genes. Consequently, *GADPH* and *20S PROTEASOME* were chosen as the two reference genes to use in the main assay.

2.8.5 Diluting cDNA samples for optimal amplification

Because qPCR is such a sensitive method, high concentrations of cDNA can produce background signal and/or primer-dimer products, making it necessary to dilute the starting sample. Furthermore, diluting the cDNA samples allows considerably more genes to be tested than would be allowed by the initial 20 µl product. To determine the optimal cDNA dilution for this study, qPCR was performed on a range of cDNA dilutions, using primers for the reference gene *20S proteasome beta subunit PBG1* (*20S*). The dilution ratios tested were 1:2, 1:4, 1:8, and 1:16 for cDNA : nuclease-free water. For each dilution, four control and four *S. gesnerioides*-infected *Arabidopsis* root tissue samples were included, with two technical replicates for each sample. Based on these results, the dilution factor 1:16 was chosen as this produced the most consistent results, with the highest amplification efficiency.

Table 2.4: Candidate reference genes for qPCR analysis

Gene	Locus	Reason selected	Function	Primer pairs (5'-3')	Product size (bp)	Source	Reason rejected:
SMALL NUCLEAR RIBONUCLEOPROTEIN (SNP)	**At3g07590**	Shows minimal variation in control and *Striga hermonthica*-infected *Arabidopsis* root tissue (Vasey 2005)	RNA Processing	**Primer pair 1:** **FW**: GCTGAAGAATGGGACTGTGG **RV:** GTCTCGAGGTTCAAGCTATCAG	180	Primer3	Amplified multiple loci on *Arabidopsis* root cDNA samples.
				Primer pair 2: **FW:** AGACAGTGAAGATGAGCCTGA **RV:** ACAGCTTTCCCAGCAACAG	172		Poor amplification on *Arabidopsis* root cDNA samples.
20S PROTEASOME BETA SUBUNIT PBG1 (20S)	**At1g56450**	Shows minimal variation in control and *Striga hermonthica*-infected *Arabidopsis* root tissue (Vasey 2005)	Protein catabolism	**Primer pair 1:** **FW:** GGAACACTCTCGTCCTTGG **RV:** AAACTCAGGTCTGCATGCC	160	Primer3	Poor amplification on *Arabidopsis* root cDNA samples.
				Primer pair 2: **FW:**TGAGCTCACCCTGAATGATAAC **RV:**TTGACACCATTCCAAGGTAAC	176		Selected for reference gene.
B-ACTIN	**At3g18780**	Traditional *Arabidopsis* reference gene (Huggett et al. 2005)	Cytoskeletal component	**Primer pair 1:** **FW:** GCTGGATTCTGGTGATGGTG **RV:** AATTTCCCGCTCTGCTGTTG	169	Primer3	Amplified multiple loci on *Arabidopsis* root cDNA samples.
				Primer pair 2: F**W:** GCACCCTGTTCTTCTTACCG **RV:** AGAATCCAGCACAATACCGG	166		Expression too robust
TAP42-INTERACTING PROTEIN OF 41 KDA (TIP41-LIKE)	**At4g34270**	'Superior' *Arabidopsis* reference gene (Czechowski et al. 2005)	Signalling component: Target-of-Rapamycin (TOR) pathway	**FW:** GTGAAAACTGTTGGAGAGAAGCAA **RV:** TCAACTGGATACCCTTTCGCA	61	(Czechowski et al. 2005)	Primers showed poor amplification on *Arabidopsis* root cDNA
PROTEIN PHOSPHATASE 2A (PP2A) 65 KD REGULATORY SUBUNIT	**At1g13320**	'Superior' *Arabidopsis* reference gene (Czechowski et al. 2005)	Ser/Thr protein phosphatase	**FW:** TAACGTGGCCAAAATGATGC **RV:** GTTCTCCACAACCGCTTGGT	61	(Czechowski et al. 2005)	Amplified product on *S. gesnerioides* cDNA
UBIQUITIN-CONJUGATING ENZYME (UBC)	**At5g25760**	Traditional *Arabidopsis* reference gene (Czechowski et al. 2005).	Catalyses ubiquitin transfer	**FW:** CTGCGACTCAG^GGAATCTTCTAA **RV:** TTGTGCCATTGAATTGAACCC	61	(Czechowski et al. 2005)	Primers showed poor amplification on *Arabidopsis* root cDNA
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GADPH)	**At1g13440**	Traditional *Arabidopsis* reference gene (Huggett et al. 2005)	Cellular metabolism/ glycolysis	**FW:** TTGGTGACAACAGGTCAAGCA **RV:** AAACTTGTCGCTCAATGCAATC	62	(Czechowski et al. 2005)	Selected as reference gene

2.8.6 Cycle programme for qPCR

For each run, the instrument used was a Corbett Rotor-Gene 6000 model, fitted with a 72-well rotor for 0.1 ml tubes (QIAGEN). This spins the samples in a centrifugal rotor illuminated from below by a light emitting diode which excites the fluorescent dye in the reaction solution. The emitted fluorescence passes through an emission filter on the other side of the chamber and is quantified by a photo multiplier. The fluorescence signal is used to calculate the Ct value for each sample: the cycle at which the rate of fluorescence increase reaches 20% of the maximum. Sample tubes were prepared in a 72-well metal plate, cooled on ice, using 2-20 µl and 20-200 µl electronic pipettes. The reaction mixture in each well comprised of 4 µl cDNA template, 1 µl forward/reverse primer mix at 2.5 µM concentration and 5 µl SYBR®Green 1. Within each of the three time points, each target gene was tested on at least three control and three infected root samples, as well as a no-template control. For each gene tested, two technical replicates were included for every biological sample, to calculate an average Ct value. The Rotor-Gene program for all assays was set as follows: 95°C for 10 min; 40 cycles of 95°C for 15 sec, 60°C for 15 sec, 72°C for 15 seconds, acquiring to SYBR Green fluorescence signal at the end of each 72°C cycle. Each run finished with a melt analysis starting at 60°C and raising by 1°C degree to 93°C to produce a melting curve describing the dissociation kinetics for each sample. This is an effective way to check for primer-dimer formation, since there is a sudden decrease in fluorescence at the melting point when the double-stranded cDNA molecules dissociate. For each sample, the amplification efficiency was calculated, with values of at least 1.7 being acceptable.

2.9 Genotyping *erf4-1* and *erf4-2*

Chapter 5 explores the molecular basis of the increased susceptibility of the *erf4-1 Arabidopsis* mutant (SALK_073394C) to *S. gesnerioides.* This involved comparison with *erf4-2* (SALK_200761C), which has more severely reduced *ERF4* expression than *erf4-1* (see Chapter 5, Section 5.4.5-5.4.6). Both mutant lines were genotyped using PCR primers for the wild-type sequence flanking the approximate site of the T-DNA insertion (Left and Right primer). In the presence of an intervening T-DNA insertion, no product can be amplified as the primers become separated by too great a distance (Figure 2.13). The T-DNA insertion can be confirmed using a primer that recognises part of the insertion sequence (Left Border primer) with the Right primer. Primer sequences and expected product sizes were obtained from the T-DNA primer design service of the SALK Institute Genomic Analysis Laboratory (http://signal-genet.salk.edu/tdnaprimers.2.html). Since both mutants were originally produced using a pROK2 vector, the same Left Border primer was used for each. Primer sequences were as follows (5'-3'): Left Primer: TCCAATTAATTTTCTCATTGCC, Right Primer: TGAAACGACACCGTTAAAAGC, Left Border Primer: GCGTGGACCGCTTGCTGCAACT. Leaf samples (approximately 5mm diameter) were taken from 6-week-old Col-0, *erf4-1* and *erf4-2 Arabidopsis* plants and treated according to the Thermo Scientific Phire

Plant Direct PCR Kit (Catalogue number: F130WH). Samples were crushed gently with a sterile pipette tip and left overnight in 20 µl dilution buffer. The following day, the samples were spun down and PCR was performed according to the kit protocol. The following reaction mix was used for each sample: 10 µl of 2 x Phire Plant PCR Buffer, 0.4 µl Phire Hot Start II DNA Polymerase, 1 µl of each primer (10 µM concentration), 1 µl of plant tissue supernatant, 6.6 µl nuclease-free water. The PCR cycle was as follows: initial denaturation (5 mins at 98°C); 35 cycles of 5 seconds at 98°C, 5 seconds at 66°C, 22 seconds at 72°C; final extension (1 min at 72°C). 10 µl of product was mixed with 3 µl of 1:5 DNA loading dye and run on a 1.5% Agarose gel, alongside 3 µl of the product size reference Hyperladder I.

The combination of the Left and Right genotyping primers only amplified a product on the wild-type (Col-0) samples, demonstrating the presence of an intervening T-DNA insertion in *erf4-1* and *erf4-2* (Figure 2.14A). The wild-type product matched the expected size of 1182 base pairs. In contrast, the Left Border and Right primer amplified a product on both *erf4-1* and *erf4-2* (Figure 2.14B). Crucially, the amplified product was larger on *erf4-2*, which is to be expected as the T-DNA insertion is further upstream from the Right primer recognition sequence. No product was formed on Col-0 with the Left Border and Right primer, confirming that this line had no T-DNA insertions of the pROK2 vector in the *ERF4* gene (Figure 2.14B).

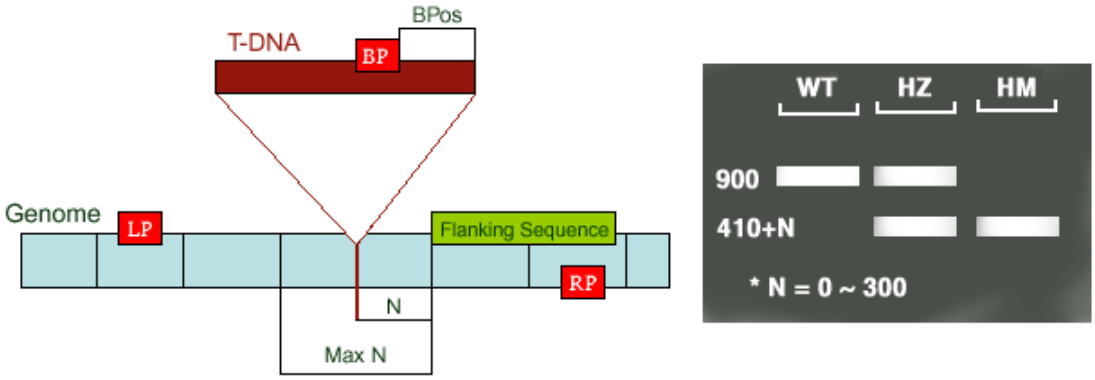


Figure 2.13: Genotyping T-DNA insertion mutants using alternative primer combinations. The Left primer (LP) and Right Primer (RP) are designed to flank the site of the T-DNA insertion, and can thus amplify a product on the wild-type gene sequence. Where a T-DNA insertion is present, however, the Left and Right primer become separated by too great a distance to amplify a product. The Left Border primer (BP) recognises a sequence within the T-DNA insertion and, in combination with the Right primer, produces a product that is typically smaller than the wild-type Left and Right primer product. N: difference between the T-DNA insertion site position and flanking sequence, typically 0-300 bases. Image modified from the T-DNA primer design service of the SALK Institute Genomic Analysis Laboratory (http://signal-genet.salk.edu/tdnaprimers.2.html). WT = wild-type; HZ= heterozygous for T-DNA insertion, HM= homozygous for T-DNA insertion.

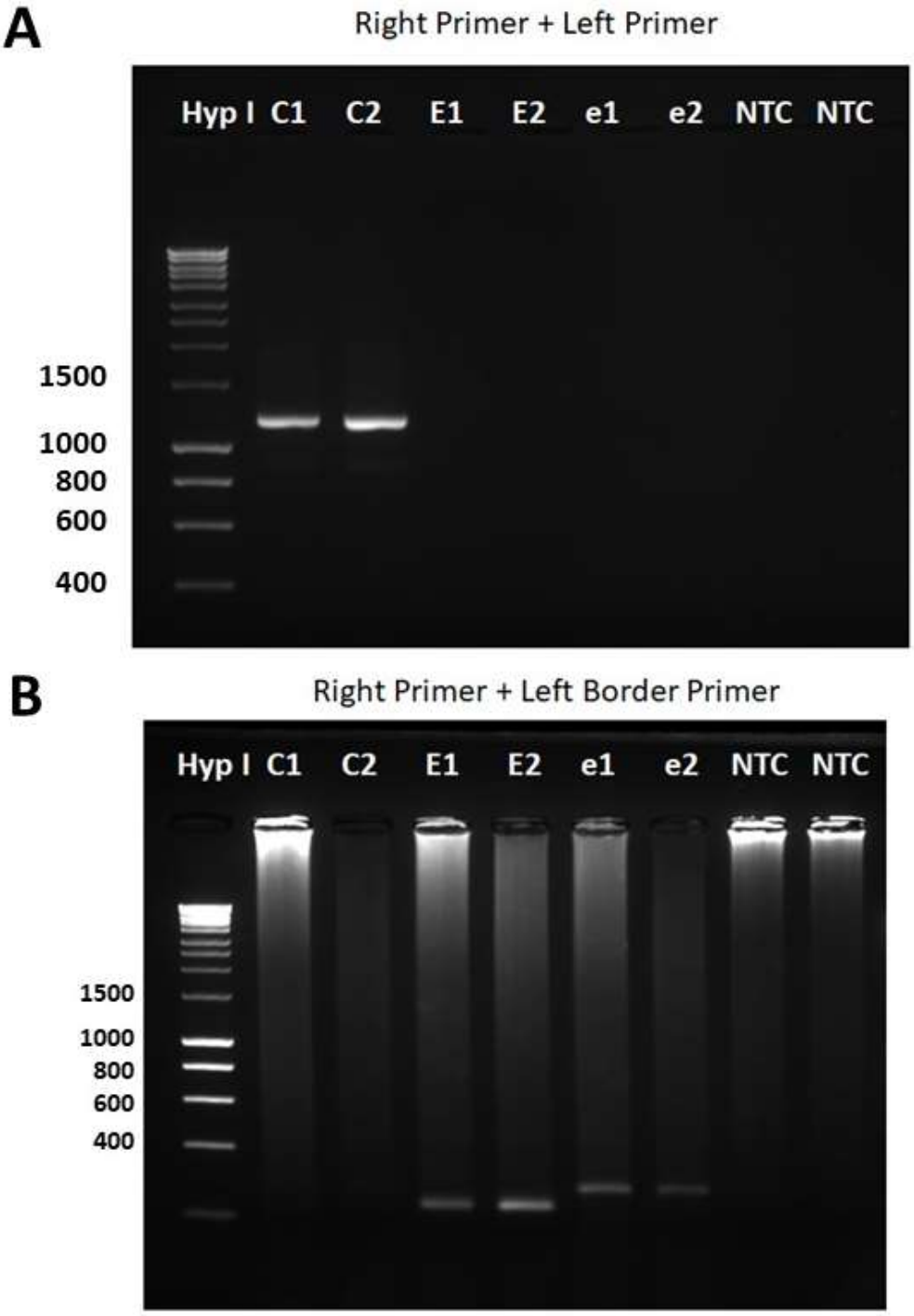


Figure 2.14: Confirmation of T-DNA insertions in *Arabidopsis* mutants *erf4-1* (SALK_073394C) and *erf4-2* (SALK_200761C). PCR was performed on leaf samples using either primers that flank the T-DNA insertion site (Left and Right primer, A) or that would recognise a site within the T-DNA insertion (Left Border and Right primer, B). The Left and Right primer can only amplify a product in the absence of an intervening T-DNA insertion. In contrast, the Left Border Primer will only amplify a product where the T-DNA insertion occurs in close proximity to the Right primer recognition sequence. Hyp I: Hyperladder I size reference (in base pairs). C: Col-0 samples 1 and 2: E: *erf4-1*, samples 1 and 2. e: *erf4-2*, samples 1 and 2, NTC: No template control. 10 µl of product was mixed with 3 µl of 1:5 DNA loading dye.

2.10 Preparation of *Hyaloperonospora arabidopsidis* spores and infection of *Arabidopsis* leaves

Chapter 6 contains the results of two experiments to investigate whether below-ground infection with *S. gesnerioides* affects above-ground resistance of *Arabidopsis* to the foliar biotrophic pathogen *Hyaloperonospora arabidopsidis*. In both experiments, *H. arabidopsidis* spores (strain WACO9) were collected from Col::*NahG* transgenic *Arabidopsis* hosts (at least 2 weeks old) grown at high density on 80:20 M3 peat: sand, under sealed propagator lids to maintain high humidity. This line is deficient in SA accumulation (Delaney et al. 1994), causing basal resistance to *H. arabidopsidis* to be impaired and allowing the pathogen to reach a high density. Spores were transplanted between hosts every 4-6 days to maintain pathogen vitality. *H. arabidopsidis* was moved to a new Col::*NahG* host, 5 (experiment 1) and 6 (experiment 2) days before infecting the experimental Col-0 *Arabidopsis* hosts, to ensure that the pathogen would be sporulating when transferred for the final time.

Spore inoculum was prepared by removing infected leaves with tweezers and washing these in distilled water in a falcon tube. The liquid was then strained through miracloth and the concentration of spores assessed using a Neubauer Chamber Cell Counting Grid under a light microscope. The solution was diluted with distilled water to a concentration of 5×10^4 spores per ml/ 50 spores per µl. This solution was then evenly sprayed onto the Col-0 hosts using a 50 ml spray bottle. In both experiments, this took place when the Col-0 *Arabidopsis* hosts were 38 days old. Hosts with and without *S. gesnerioides* were randomised between the trays. All plants were covered with propagator lids and placed in a dedicated Conviron growth chamber.

Five days after infection, eight leaves were removed from each plant (including both small and large leaves) and placed in 15 ml falcon tubes. These were stained with lactophenol-trypan blue solution: lactophenol destains leaves whilst trypan blue stains dead cells, the vasculature and fungal hyphae. Leaf tissue was infiltrated by placing the sample tubes in a boiling water bath for 2 x 1 min intervals, until the chlorophyll had been completely removed. Trypan blue was removed after 3.5 h and replaced with chloral hydrate to further destain the leaves. The following day the samples were placed in fresh chloral hydrate for long-term storage.

2.11 Preparation of *Plectosphaerella cucumerina* and infection of *Arabidopsis* leaves

Chapter 6 contains the results of three experiments to investigate whether below-ground infection with *S. gesnerioides* affects above-ground resistance of *Arabidopsis* to the foliar necrotrophic pathogen *Plectosphaerella cucumerina*. The inoculum was prepared from *P. cucumerina* (originally isolated from naturally infected *Arabidopsis* accession Landsberg erecta), grown on 19.5 g l−1 potato dextrose agar at room temperature for 2 weeks. Five ml of distilled water was spread across the plate and collected with a pipette. This was strained through miracloth and analysed using a Neubauer Chamber Cell Counting Grid under a light microscope to determine the spore concentration. The solution was diluted with deionised water to a spore concentration of 5×10^6 per ml. To each *Arabidopsis* plant, 5 µl droplets were applied to 5 fully expanded leaves using a multipipette. The plants were sealed with parafilm under propagator lids to ensure 100% relative humidity for optimal pathogen growth.

CHAPTER THREE

Quantification of basal resistance against *Striga gesnerioides* in *Arabidopsis* mutants compromised in various defence pathways.

ABSTRACT

A range of *Arabidopsis* mutants were tested for altered basal resistance against the root parasitic plant *Striga gesnerioides*. These included mutants affected in the biosynthesis and signalling of major defence-associated hormones (salicylic acid SA, jasmonic acid JA, ethylene and abscisic acid ABA); key defence signalling transcription factors (e.g. ORA59, WRKY70); the generation of reactive oxygen species (ROS) and the production of defensive secondary metabolites. In these assays, early-stage resistance/susceptibility was measured by the percentage of parasite seeds applied to the roots that resulted in successful attachments, whilst late-stage resistance/susceptibility was quantified by the haustorium size and the proportion of attached haustoria that had progressed to shoot development. The results indicated that SA may have a protective effect during the early stages of infection, as indicated by the *sid2-1* mutant. The effect of JA was ambiguous, since contrasting phenotypes were seen between biosynthesis and signalling mutants, indicating a potential role of the intermediary signalling compound OPDA. The phenotype of *etr1-1* and *ein3-1* suggested that ethylene signalling promotes infection at both early and late stages. The proportion of parasites that had transitioned to shoot development was significantly greater for the *abi1-2* mutant, which has enhanced ABA sensitivity. Mutants of WRY70, which positively regulates downstream SA-signalling but negatively regulates SA accumulation, showed significantly decreased early-stage resistance, but were not affected in late-stage resistance. *ora59* and *erf4-1* also showed significantly decreased early-stage resistance, despite these transcription factors acting antagonistically on downstream JA/ethylene defences. The *RbohD/RbohF* double mutant showed significantly greater early-stage resistance and a higher proportion of parasites that had transitioned to shoot development, indicating that ROS are a susceptibility factor. *pmr4-1* showed significantly increased late-stage resistance: it is not clear whether this was due to increased SA or loss of callose biosynthesis in this line. In contrast, *cyp79B2/B3*, deficient in camalexin and indole-3-glucosinolates, showed a significantly greater proportion of haustoria with a developing shoot.

3.1 Introduction

A key advantage to using *Arabidopsis* as a model host is the ready availability of loss-of-function and over-expressor lines for virtually every identified gene, so that individual genetic components in host basal resistance can be investigated. This chapter presents the results of a series of experiments investigating whether specific mutations in a range of defence pathways significantly affect basal resistance against *Striga gesnerioides*. Both early-stage resistance (that prevents the parasite from forming a visible attachment organ, or haustorium) and late-stage resistance (that restricts further haustorium development and the transition to producing a shoot) were investigated. Table 3.1 details the full list of the mutant lines selected for analysis.

3.1.1 Mutants affected in the salicylic acid (SA) pathway

As outlined in Chapter 1, Section 1.8.8, various interactions between parasitic plants and their hosts have indicated a protective effect of SA. This may be due to toxic effects of the hormone itself or defences induced by downstream signalling. To investigate this, *Arabidopsis* mutants impaired in both SA biosynthesis and signalling were selected.

The SA-biosynthesis mutant chosen for this study was *sid2-1*. *SALICYLIC ACID INDUCITON DEFICIENT 2 (SID2)* encodes an isochorismate synthase that produces SA from chorismate specifically during pathogen challenge (Wildermuth et al. 2001). This biosynthetic pathway is parallel to the production of SA from phenylalanine, hence basal SA levels are unchanged in *sid2-1* mutants (Wildermuth et al. 2001). *sid2-1* is compromised in basal resistance against numerous biotrophic pathogens, including *P. syringae* and the oomycete *Hyaloperonospora parasitica* (Nawrath and Métraux 1999). Conversely, defences against necrotrophic pathogens such as *Botrytis cinerea* (Ferrari et al. 2003a) and *Alternaria brassicicola* (van Wees et al. 2003) are generally unaffected, which fits general understanding that SA-mediated defences are not effective against this class of pathogen (Reymond and Farmer 1998).

NONEXPRESSOR OF PR GENES1 (NPR1), an ankyrin-repeat containing protein (Cao et al. 1997), is a key defence signalling node that both regulates a subset of SA-induced genes and has a role in mediating JA/SA cross talk. *npr1 Arabidopsis,* allelic to *nim1* (*no immunity 1*) and *sai1* (*SA insensitivity 1*)), does not demonstrate SAR or express *PR* genes in response to pathogen challenge or chemical inducers, but accumulates wild-type levels of SA (Cao et al. 1994, Glazebrook, Rogers and Ausubel 1996, Delaney, Friedrich and Ryals 1995, Shah, Tsui and Klessig 1997). This allows the contribution of SA-signalling in basal resistance to be dissected from SA accumulation, hence *npr1-1* was also selected for this study. NPR1 has distinct functions based on its location in either the cytoplasm or nucleus. Under non-induced conditions, NPR1 forms a large oligomer in the cytosol, held together with disulphide bonds, that is too large to enter the nucleus (Mou, Fan and Dong 2003). Cytoplasmic location of NPR1 seems

essential for mediating SA-driven antagonism of JA-gene induction and herbivore defence (Spoel et al. 2003, Yuan et al. 2007). SA accumulation, for instance during pathogen challenge, induces a rapid oxidative burst followed by a reducing phase: this reduces the intermolecular disulphide bonds of the NPR1 oligomer, releasing the monomer form (Mou et al. 2003). NPR1 monomers move into the nucleus to activate *PR* genes (Kinkema, Fan and Dong 2000), apparently via TGA transcription factors (Zhang et al. 2003b). Microarray analysis has shown that *npr1-1 Arabidopsis* have differential expression of both SA-associated genes and those that require JA and ethylene (Glazebrook et al. 2003), indicating a role in direct regulation and hormonal cross talk. Not all SA-associated responses are mediated by NPR1 however: accumulation of the antimicrobial compound camalexin, for instance, requires SA but is independent of NPR1 (Thomma et al. 1999).

A subset of SA-mediated defences is facilitated by *ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)* and *PHYTOALEXIN DEFICIENT 4 (PAD4*). These encode lipase-like proteins (Falk et al. 1999, Jirage et al. 1999) but there is currently no evidence that lipid hydrolysis is essential for either EDS1 or PAD4 function (Wiermer, Feys and Parker 2005). EDS1 and PAD4 form a complex *in vivo,* which induces SA accumulation and downstream responses that promote basal resistance responses against biotrophic pathogens (Rietz et al. 2011, Zhou et al. 1998, Feys et al. 2001). Unlike NPR1, EDS1 and PAD4 are both important in stimulating camalexin biosynthesis against certain pathogens (Mert-Türk et al. 2003). Furthermore, SA induces EDS1 and PAD4 expression (Jirage et al. 1999, Falk et al. 1999), suggesting a positive feedback loop mechanism. Most known functions of PAD4 rely on EDS1, although EDS1-independent functions have recently been discovered, including a phloem-based defence response against green peach aphids (Pegadaraju et al. 2007).

EDS1 can also function independently of PAD4 in transmitting effector-triggered immunity (ETI) responses downstream of TIR NBS/LRR receptors (R genes) including rapid hypersensitive response (HR) and local cell death (Rietz et al. 2011, Parker et al. 1996, Aarts et al. 1998). EDS1 can also form an independent complex with SAG101, however this appears to be less important than the EDS1/PAD4 complex for immune function (Rietz et al. 2011). It has been proposed that EDS1 independently transmits rapid ETI responses, then forms a complex with PAD4 to 'reinforce' the defence signal through SA accumulation and transcriptional amplification of target genes (Rietz et al. 2011).

Not all basal- and ETI-related functions of EDS1/PAD4 appear to depend on SA however since blocking SA accumulation only partially affected the phenotype of *snc1*, which shows constitutive R gene-mediated resistance dependent on EDS1 and PAD4 (Zhang et al. 2003a) and gene targets of EDS1/PAD4 have been identified that are expressed independently of ICS1-generated SA (Bartsch et al. 2006).

In a role separate from the oxidative burst that occurs during HR and ETI, the EDS1/PAD4 complex can amplify reactive oxygen species (ROS) signals to promote cell death, for instance surrounding infection sites and during high-light stress (Rustérucci et al. 2001, Mateo et al. 2004). It has thus been proposed that, besides transducing SA-signals, EDS1/PAD4 can integrate stimuli from a diverse range of environmental stresses. EDS1/PAD4 may also be implicated in antagonistic cross-talk between JA and SA since the *eds1* mutant (and to a lesser extent, *pad4*) suppressed the phenotype of *mpk4*, which shows constitutive expression of SA-associated defences, but impaired induction of JA and ethylene-associated genes (Brodersen et al. 2006). This indicates that EDS1/PAD4 function downstream of and antagonistically to MPK4 by activating SA defences and repressing JA/ethylene defence. Given the different functions ascribed to the EDS1/PAD4 complex compared with the individual proteins, the following three mutants were included to test for altered basal resistance against *S. gesnerioides*: *eds1-5, pad4-1* and *eds1-5/pad4-1.*

3.1.2 Mutants affected in the Jasmonic Acid (JA) pathway

To investigate whether endogenous JA contributes to basal resistance against *S. gesnerioides*, the JA biosynthesis mutants *aos1* (*allene oxide synthase*) and *opr3-1 (oxophytodienoate-reductase 3)* were selected. Jasmonates are derived from linolenic acid, through oxidative cyclation involving numerous enzyme-mediated steps (Wasternack and Song 2016). ALLENE OXIDE SYNTHASE, located in the chloroplast, acts early in the JA biosynthesis pathway, converting fatty acid hydroperoxides into unstable allene epoxides which form cyclopentenone acids via allene oxide cyclase or spontaneously (Park et al. 2002, Laudert et al. 1996). OPR3 (OXOPHYTODIENOATE-REDUCTASE 3), located in the peroxisome, acts later on the substrate 12-oxo-phytodienoic acid (OPDA) (Stintzi 2000). Between these steps, OPDA is transported from the chloroplast to the peroxisome. Although a precursor for JA, OPDA has been demonstrated to have independent signalling activity (as reviewed in (Dave and Graham 2012)). Microarray analysis has identified 157 genes induced by OPDA but not by methyl-jasmonate (MeJA) and independently of COI1, a downstream regulator of JA signalling. Almost half of these genes were also induced by wounding (Taki et al. 2005). To distinguish between effects mediated solely by JA and those caused by OPDA, both *aos1* and *opr3-1* were selected for the mutant assay.

jar1-1 (*jasmonate resistant 1*) (Staswick, Yuen and Lehman 1998) was selected as a JA-signalling mutant for this assay. *JAR1* encodes a JA-amino-synthetase and catalyses the conjugation of JA with isoleucine to form the bioactive compound jasmonoyl-L-isoleucine (JA-Ile) (Staswick and Tiryaki 2004, Staswick, Tiryaki and Rowe 2002). *jar1-1* mutants show insensitivity to JA and MeJA (Staswick et al. 1998), increased susceptibility to the opportunistic oomycete pathogen *Pythium irregulare* (Staswick et al. 1998)and blocked induced systemic resistance (Pieterse et al. 1998). Unlike *coi1, jar1-1* mutants do

not show increased basal resistance against the biotroph *P. syringae* (Kloek et al. 2001), suggesting that it does not have a role in JA-mediated antagonism of SA-associated defences.

3.1.3 Mutants affected in ethylene and abscisic acid (ABA)

As reviewed in Chapter 1, Section 1.7, the outcome of JA signalling on host defence is affected by the presence of the hormone ethylene. In *Arabidopsis* five ethylene receptors (ETR1, ETR2, EIN4, ERS1, and ERS2) on the endoplasmic reticulum actively repress ethylene responses in the hormone's absence. Mutations in the hydrophobic region of these receptors confer dominant ethylene insensitivity (Hua et al. 1998, Sakai et al. 1998, Rodrıguez et al. 1999), as is the case with *etr1-1* (*ethylene response 1*) (Bleecker 1999). Normally, when ethylene interacts with these receptors, their repressive activity is lifted and the signal is relayed to the nuclear-localised transcription factor EIN3 (ETHYLENE INSENSITIVE 3). This induces *ERF1* (*ETHYLENE RESPONSE FACTOR 1*), which in turn activates genes containing GCC-box promoter elements including *CHIB* and *PDF1.2* (Chao et al. 1997, Solano et al. 1998). *ein3-1* mutants accordingly show impaired induction of genes normally responsive to ethylene and pathogens (Solano et al. 1998). Recently, EIN3 was found to act as an integration point for JA and ethylene signalling, since it can be bound and repressed by JAZ repressor proteins (Zhu et al. 2011b). For this mutant assay, *etr1-1* and *ein3-1* were chosen to investigate the contribution of ethylene to basal resistance against *S. gesnerioides.*

Besides ethylene, a number of other plant hormones modulate defence responses triggered by SA and JA, including abscisic acid (ABA), normally associated with abiotic stress. In particular, ABA appears to modify JA-signalling activity to promote defences against wounding and insect herbivores over necrotrophic responses (Dinh, Baldwin and Galis 2013, Bodenhausen and Reymond 2007, Anderson et al. 2004). As such, ABA mutants show increased resistance against certain necrotrophs, such as *Fusarium oxysporum* (Anderson et al. 2004). However positive effects of ABA on necrotrophic defences are also known, suggesting the role of ABA in plant defence is specific for each interaction. Treating *Arabidopsis* with ABA, for instance, induced callose accumulation and resistance to the fungal necrotrophic pathogens *A. brassicicola* and *Plectosphaerella cucumerina* (Ton and Mauch-Mani 2004). Meanwhile *aba1-5*, impaired in ABA biosynthesis, showed defective callose deposition in response to *P. cucumerina* (Ton and Mauch-Mani 2004). Bi-directional antagonism has also been reported between ABA and SA, such that ABA mutants can show increased resistance against biotrophic pathogens (reviewed by (Cao et al. 2011)).

ABA may have particular relevance against *Striga*, given the water stress this parasite imposes on its hosts and evidence of increased ABA concentrations in infected hosts (Frost et al. 1997). Both an ABA biosynthesis and signalling mutant were selected for this study since certain downstream signalling

pathways do not appear relevant for all the effects of ABA on disease resistance. The ABA-deficient mutants *aba2-1* and *aba1-1*, for instance, are more resistant to *Pseudomonas syringae pv. tomato* DC3000 *(Pst* DC3000*), H. parasitica* and *F. oxysporum*, while ABA-insensitive *abi1-1* and *abi1-2* do not differ from the wild-type (Mohr and Cahill 2003, Anderson et al. 2004). This suggests that the effects of ABA in this interaction are mediated by directly interfering with other biotic defence signalling pathways (Mohr and Cahill 2003). Furthermore, the tomato *sitiens* mutant, which has defective ABA biosynthesis, shows increased resistance to *B. cinerea* apparently due to greater ROS accumulation during tissue penetration (Audenaert, De Meyer and Höfte 2002). It has been proposed that the accumulation of ABA precursors in the xanthophyll cycle decreases the level of the antioxidant ascorbate, allowing augmented ROS generation (Ton et al. 2009). The *aba1-5 (ABA deficient 1-5)* biosynthesis mutant (Koornneef et al. 1982) was chosen for this assay. ABA is derived from carotenoids (Wasilewska et al. 2008), and *aba1* mutants are affected in the enzyme zeaxanthin epoxidase which converts zeaxanthin into violaxanthin (Audran et al. 2001). *abi1-2 (ABA insensitive 1-2)* was chosen to investigate the influence of ABA signalling. *ABI1*, and also *ABI2*, encodes a protein phosphatase that negatively regulates ABA signalling (Gosti et al. 1999). *abi1-2* contains a loss-of-function T-DNA insertion which confers ABA hypersensitivity (Saez et al. 2006).

3.1.4 Transcription factors involved in defence-hormone crosstalk

WRKY70:

There is much evidence for cross-regulation between different plant defence hormones, mediated in part by a diverse array of transcription factors and integrating nodes. Effective resistance against root parasitic weeds may depend on the relative balance of these hormones, particularly JA and SA (reviewed in Chapter 1, Section 1.8.8). Increasingly, WRKY transcription factors are becoming recognised as key mediators of this cross-regulation. One of the most well studied is WRKY70, induced by biotrophic pathogens and SA downstream of NPR1 (Wang, Amornsiripanitch and Dong 2006). There was particular interest in testing *wrky70* in this assay since *WRKY70* was identified in a screen of upregulated genes during the non-host response of *Arabidopsis* to *Striga hermonthica* (Vasey 2005). WRKY70 is a positive regulator of biotrophic defences since *WRKY70* over-expression increases resistance to biotrophic pathogens such as *Pst* DC300 (Li et al. 2017) and *Golovinomyces cichoracearum* (Li et al. 2006). In particular, WRKY70 appears to be a limiting factor for *PR* gene (e.g. *PR2, PR5*) induction by SA (Li et al. 2004). However WRKY70 additionally functions as a negative regulator of SA accumulation, through suppressing *ICS1*: as such *wkry70* shows increased SA accumulation both under basal conditions and in response biotrophic pathogens (Wang et al. 2006) and increased resistance to *P. syringae pv. maculicola* (Zhou et al. 2018). Nevertheless, *wrky70* mutants are more susceptible to

G. cichoracearum (Li et al. 2006) and oomycete *H. parasitica* (Knoth et al. 2007), indicating that in these interactions, raised SA levels do not compensate for the role of WRKY70 in signal transduction.

In addition, WRKY70 may facilitate SA-mediated antagonism of JA signalling since it has been shown to suppress basal and induced expression of JA-regulated genes including *PDF1.2* and *VSP2*, in a manner partly dependent on NPR1 (Li et al. 2004, Li et al. 2006). Furthermore, *WRKY70* over-expressors show increased susceptibility to the necrotroph *A. brassicicola*, with the opposite trend seen in *wrky70* knock out mutants (Li et al. 2006). WRKY70 has also been reported to inhibit biosynthesis of camalexin and indole glucosinolates (Li et al. 2006). WRKY70 is also a positive regulator of ROS accumulation and cell death, with *WRKY70* over-expressors being more susceptible to the necrotrophs *B. cinerea* and *A. brassicicola* as a result of increased cell death (Li et al. 2017, Li et al. 2006). It was intended to test this in *35S:WRKY70* in parallel with *wrky70*; unfortunately, only *wrky70* was available.

AP2/ERF-domain proteins:

Besides WRKY transcription factors, another important gene family which regulates plant defence pathways is the AP2/ERF-domain proteins, also known as Ethylene Response Factors (ERFs) (Figure 3.1). These can act as transcriptional repressors or activators, specifically recognising the sequence AGCCGCC (GCC box), present in the promoters of many defence-related genes, including *PDF1.2* (Fujimoto et al. 2000). Certain ERFs distinguish between the different defence responses downstream of JA, and integrate the influence of ethylene on this pathway. One of these pathways is induced synergistically by MeJA and ethylene and is associated with defence against necrotrophic pathogens (Broekgaarden et al. 2015). Two of the most well-studied positive regulators of necrotrophic defence are ERF1 (ETHYLENE RESPONSE FACTOR 1) and ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59), although others, such as ERF5 and ERF6, are known (Moffat et al. 2012, Broekgaarden et al. 2015). *ORA59* and *ERF1*, both induced synergistically by MeJA and ethylene, bind independently to the *PDF1*.2 promoter and have an additive effect on expression (Zarei et al. 2011). Furthermore, RNAi silencing of *ORA59* has shown that this is crucial for *PDF1.2* induction (Pré et al. 2008). Over-expression of *ERF1* or *ORA59* increases resistance to necrotrophic pathogens, while *ORA59* RNAi-silenced plants are more susceptible (Berrocal-Lobo, Molina and Solano 2002, Pré et al. 2008). These transcription factors are also an entry point for inhibition of necrotrophic defences by SA since presence of the GCC box alone is sufficient for SA-mediated suppression (Van der Does et al. 2013). In addition, SA inhibits ORA59 accumulation, both through transcriptional repression and protein degradation (Van der Does et al. 2013, Zander, Thurow and Gatz 2014).

AP2/ERF transcription factors can also act as repressors. ERF4 (ETHYLENE RESPONSE FACTOR 4), for instance, counteracts ERF1 and ORA59 by negatively regulating necrotrophic defences in favour of wound-associated genes downstream of JA (Figure 3.1). *ERF4* is induced by ethylene, JA, ABA, wounding and *F. oxysporum* but not SA (Delessert et al. 2004, Yang et al. 2005, McGrath et al. 2005). Over-expression of *ERF4* in *Arabidopsis* impairs induction of JA/ethylene co-regulated genes such as *PDF1.2, HEL* and *B-CHI*, although basal levels are not affected (McGrath et al. 2005, Pré 2006). JA induction of the wound-associated genes *VSP1* and *CYP79B2*, on the other hand, is enhanced in these plants (Pré 2006). *erf4 Arabidopsis* show greater basal *PDF1.2* expression and increased resistance to *F. oxysporum,* whilst over-expressor lines show increased susceptibility to this necrotroph (McGrath et al. 2005). ERF4 is hypothesised to suppress a negative regulator of the JA-induced wounding/insect herbivory signalling pathway; this regulator may be ERF1 since *ERF1* over-expression inhibits *VSP2* induction in response to JA (Lorenzo et al. 2004).

MYC-like transcription factors:

Aside from necrotrophic defences, the other main branch downstream of JA signalling promotes resistance against wounding and insect herbivores and is synergistically induced by ABA (Bodenhausen and Reymond 2007, Dinh et al. 2013). This is also known as the MYC-branch since it is positively regulated by MYC-like transcription factors. These recognise the G-box motif (CACGTG) which is present in the promoters of wound-response genes such as *VSP2* and *LOX* (Lorenzo et al. 2004, Dombrecht et al. 2007, Toledo-Ortiz, Huq and Quail 2003). One of the best studied of these is the basic helix-loop-helix transcription factor MYC2 (allelic to JASMONATE INSENSITIVE 1, JIN1), recognised as a 'master regulator' of downstream JA defences (Kazan and Manners 2013) (Figure 3.1). MYC2 appears to regulate a similar spectrum of genes as ERF4, however this acts as a transcriptional activator since *MYC2*-overespression is sufficient to activate *VSP2* in the absence of JA (Lorenzo et al. 2004), whilst over-expression of *ERF4* does not (Pré 2006). The presence of conjugated JA-Ile liberates MYC2 from a repressor complex with JAZ proteins, allowing it to induce a large number of early-response JA genes, including other transcription factors (Woldemariam, Baldwin and Galis 2011). Comparative microarray analysis has demonstrated that, besides positively regulating insect/wound defences, MYC2 promotes oxidative stress tolerance and flavonoid metabolism, whilst suppressing pathogen defence and secondary metabolism (including camalexin and indole glucosinolates) (Dombrecht et al. 2007).

jin1/myc2 mutants are more resistant to *F. oxysporum* (Anderson et al. 2004), further illustrating the apparent antagonism between the two downstream JA-pathways. MYC2 suppresses the activity of EIN3, both through physical interaction that inhibits EIN3 DNA-binding and induction of the F-box gene *EIN3 BINDING F-BOX PROTEIN 1* which targets EIN3 for degradation (Zhang et al. 2014, Song et al. 2014). Conversely, EIN3 interacts with and represses MYC2 activity (Song et al. 2014).

The suppressive effect of SA on JA signalling may also act via MYC2 (Zander et al. 2010), which in turn suppresses SA-associated defences (Laurie-Berry et al. 2006, Nickstadt et al. 2004). This has made MYC2 a target of biotrophic pathogens such as *P. syringae* which secrete the JA-mimic coronatine to activate MYC2 and suppress host defence (Zheng et al. 2012). Repression appears to act via induction of suppressive NAC (NAC: petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2) domain transcription factors that inhibit expression of SA-biosynthesis genes (e.g. *ICS1/SID2*) (Zheng et al. 2012). Gibberellic Acid (GA) can also have synergistic or antagonistic effects on JA signalling and this appears to be mediated by MYC2. DELLA repressors (targeted to destruction by GA) can bind and repress the activity of either MYC2 (Hong et al. 2012) or the JAZ1 repressors that inhibit MYC2 in the absence of JA (Hou et al. 2010).

The NAC-domain transcription factor ARABIDOPSIS THALIANA ACTIVATION FACTOR 2 (ATAF2) appears to act similarly to MYC2 and is induced by wounding, MeJA and SA but not ABA (Delessert et al. 2004, Delessert et al. 2005) (Figure 3.1). Over-expressor and loss of function mutants indicate that ATAF2 represses both necrotroph defence genes (*PDF1.2, PR4*) and SA-associated *PR* genes (*PR1, PR2, PR5*), with ATAF2-overexpressors being more susceptible to *F. oxysporum* (Delessert et al. 2005). Recently it has been demonstrated that ATAF2 can function as a transcriptional activator, depending on the promoter sequence (Nagahage et al. 2018).

Parasitic plants do not comfortably fit within the conventional biotroph/necrotroph categories for plant pathogens, and published studies (reviewed in Chapter 1, Section 1.8.8-1.8.11) indicate that host basal resistance may incorporate elements of multiple defence pathways. As such, downstream transcription factors may have a critical role in tailoring a specific and effective response. To investigate this, loss-of-function T-DNA insertion mutants affected in ORA59, ERF4, MYC2/JIN1, and ATAF2 were selected to include in this assay (Table 3.1).

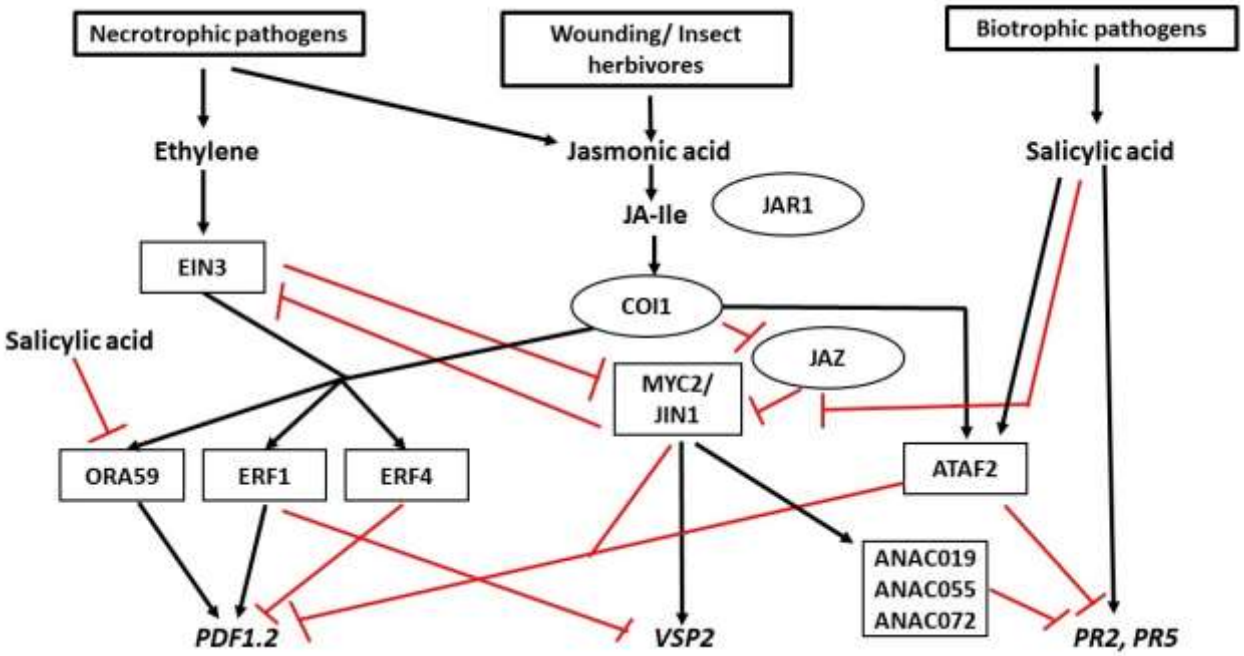


Figure 3.1: Role of transcription factors in the downstream regulation of signalling pathways induced by jasmonic acid (JA), ethylene and salicylic acid (SA). Jasmonic acid is synthesised in response to wounding/insect herbivores and converted to biologically active JA-isoleucine (JA-Ile) by JAR1. JA activates downstream transcription factors via COI1; in the case of MYC2, this is via removal of repressive JAZ proteins. Certain stresses, such as necrotrophic pathogens, simultaneously induce ethylene, which activates the transcription factor EIN3. Ethylene and JA co-induce *ORA59*, *ERF1* and *ERF4*. ORA59 and ERF1 act as transcriptional activators of necrotrophic defences (e.g. *PDF1.2*); ERF1 additionally inhibits wound-associated genes (e.g. *VSP2*). Conversely MYC2 and ERF4 promote the wound-response pathway over necrotrophic defences. MYC2 and EIN3 antagonise each other's function. MYC2 also suppresses biotrophic defences induced downstream of SA such as *PR2* and *PR5*. This is apparently mediated by NAC-domain transcription factors (e.g. ANAC019). In turn, SA suppresses MYC2 activity. SA additionally suppresses *ORA59*. The transcription factor ATAF2, induced by JA, inhibits both biotrophic and necrotrophic defences. See text for further details. Figure based on (Memelink 2009).

3.1.5 Mutants affected in reactive oxygen species (ROS) production/detoxification

Reactive oxygen species (ROS) act in concert with plant hormones to mediate defence responses, besides having direct toxic effects on invading pathogens (reviewed in Chapter 1, Section 1.8.3). ROS have a fundamental role in the HR, programmed cell death (PCD), cell wall reinforcement and gene induction (Torres 2010). Nevertheless, as reviewed in Chapter 1, Section 1.3, during infections by *Striga* spp., ROS catalyse the conversion of host-derived lignin molecules into Haustorial Initiation Factors, HIFs, thus promoting parasite development (Kim et al. 1998, Keyes et al. 2007, Wada et al. 2019).

Hence, mutations that disrupt ROS generation may have a positive or negative effect on host basal resistance. Four ROS-associated mutants were selected for the mutant assay: *prx33, RbohD, RbohF* and *RbohD/RbohF*. PEROXIDASE 33 (PRX33) is a haem-containing class III peroxidase, a multi-functional enzyme that catalyses both the reduction of H_2O_2 to water and its production through the hydroxylic cycle (Mathé et al. 2010). Peroxidases are associated with a diverse range of roles including auxin catabolism (Gazaryan et al. 1996), wound healing (Bernards et al. 1999, Allison and Schultz 2004) lignin production (Barceló and Pomar 2001) and cell wall cross linking (Passardi et al. 2004). In *Arabidopsis* , PRX33 and PRX34 appear particularly relevant in defence against pathogens, having been found to be a major source of ROS generated during pathogen invasion (Mammarella et al. 2015). Accordingly, *prx33* mutants show reduced oxidative burst and callose deposition in response to MAMPS and greater susceptibility to *Pst* DC3000 (Daudi et al. 2012).

NAPPH oxidases encoded by the *RESPIRATORY BURST OXIDASE HOMOLOG (RBOH)* gene family are another important source of host-generated ROS and generate superoxide anions by transferring electrons to molecular oxygen (reviewed by (Torres and Dangl 2005). Of the entire *RBOH* gene family, *RBOHD* has the highest basal expression (Zhu et al. 2013). Its known functions include regulating cell death (Torres, Jones and Dangl 2005), lignification (Denness et al. 2011) and relaying systemic signals during both abiotic and biotic challenges (Miller et al. 2009). A number of these roles show overlap with those of RBOHF as double *RbohD/RbohF* mutants have more pronounced phenotypes than single mutants, including stunted growth (Torres et al. 2002) and impaired ROS-dependent ABA signalling in guard cells (Kwak et al. 2003). Synergistic activity between RBOHD and RBOHF also occurs during pathogen interactions, for instance in the HR against avirulent *P. syringae* (Torres et al. 2002). and basal resistance against the necrotrophic fungus *Sclerotinia sclerotiorum* (Perchepied et al. 2010) as double mutants are more susceptible than either of the single mutants. They may also have a role in non-host resistance by recognising non-adapted pathogens: the non-virulent Pc2127 isolate of *P. cucumerina***,** normally unable to grow on wild-type *Arabidopsis*, shows over five-fold higher abundance on *RbohD/RbohF* double mutants but not on either of the single mutants (Morales et al. 2016).

Nevertheless, RBOHD and RBOHF show some discrepancies for certain pathogen interactions, particularly *F. oxysporum*. Whilst *RbohD* is significantly more susceptible*, RbohF* shows increased resistance and *RbohD/RbohF* has an intermediate phenotype similar to wild-type (Zhu et al. 2013). Although the mechanism for this is not clear, it has been proposed that ROS production following pathogen recognition is primarily mediated by RBOHD whereas RBOHF has a more prominent role in regulating the HR (Torres et al. 2002, Fagard et al. 2007, Morales et al. 2016, Perchepied et al. 2010). Against weakly virulent strains of *H. parasitica* for instance, *RbohF* shows an enhanced HR response and greater resistance than wild-type, whereas *RbohD* has reduced peroxide formation and no enhanced HR (Torres et al. 2002). Furthermore, fusions between *RBOH* promoters and the GUS

reporter gene have demonstrated that *RBOHD* and *RBOHF* show distinct spatial expression patterns (Morales et al. 2016), consistent with their apparent functional division. These discrepancies were the basis for selecting both *RbohD* and *RbohF*, besides the *RbohD/RbohF* double mutant, to test for altered basal resistance against *S. gesnerioides.*

3.1.6 Mutants affected in the production of defensive secondary metabolites: callose, camalexin and indole-glucosinolates

Defensive glucosinolates and phytoalexins have been associated with host defence against various root pathogens and may also protect against invasion by parasitic weeds (reviewed in Chapter 1, Section 1.8.5). To investigate whether this is the case for *S. gesnerioides*, the *cyp79B2/B3* double mutant was selected; this line is affected in the cytochrome P450 enzymes CYP79B2 and CYP79B3 that metabolise tryptophan to form indole-3-acetaldoxime (Mikkelsen et al. 2002). Both indole-glucosinolates and the phytoalexin camalexin are derived from this compound, hence *cyp79B2/B3* is completely devoid of both (Zhao et al. 2002, Glawischnig et al. 2004). The regulation of these genes parallels those of wound-response genes, such as *VSP2*, since they are induced by MeJA and suppressed by ethylene. Induction in response to pathogen elicitors is abolished in JA-insensitive *coi1*, but not the ethylene- and SA-insensitive mutants *ein2-1* and *NahG*, suggesting this downstream response depends largely on JA (Mikkelsen et al. 2003). Conversely, SA overproducing mutants (e.g. *cpr1* and *mpk4*) show reduced total basal glucosinolate production, suggesting that SA negatively regulates this pathway (Mikkelsen et al. 2003, Mewis et al. 2005).

As described in Chapter 1, Section 1.8.4, deposition of the β-1,3-glucan polymer callose has been implicated in defence responses against parasitic weeds, particularly *Orobanche* species (Letousey et al. 2007, Pérez-de-Luque et al. 2007). *pmr4-1* was selected to test this with *S. gesnerioides*. PMR4 (POWDERY MILDEW-RESISTANT 4 also known as GLUCAN SYNTHASE-LIKE 5) is the primary callose synthase responsible for pathogen-induced callose accumulation, with *pmr4-1* mutants being completely defective in this (Nishimura et al. 2003). Nevertheless, *pmr4-1* is more resistant to biotrophic mildew pathogens (*G. cichoracearum, Erysiphe orontii* and *H. parasitica);* this is restored in *pmr4-1*/*npr1* double mutants indicating that this resistance results from increased SA activity (Nishimura et al. 2003). It remains to be seen whether callose or the PMR4 protein itself negatively regulates SA-associated defence responses. It has been hypothesised that callose is rapidly synthesised as part of the early defence response then inhibits later responses that may cause negative effects on the host plant (Nishimura et al. 2003). Given the increased SA signalling activity in *pmr4-1,* the *pmr4-1/npr1-1* double mutant (Nishimura et al. 2003) was the preferred choice for this study, however this was unfortunately not available.

PMR4 may be regulated by ABA since this appears to be a positive regulator of callose deposition. ABA deficient and insensitive mutants (e.g. *aba1-3* and *abi1-1*), are more susceptible to the fungus *Leptosphaeria maculans*, associated with significantly decreased callose deposition (Kaliff et al. 2007). Furthermore, priming of *Arabidopsis* for increased resistance to a range of pathogens by the non-protein amino acid β-amino-butyric acid (BABA) requires a functional ABA pathway (Ton and Mauch-Mani 2004). Furthermore, *ocp3 Arabidopsis* show enhanced basal and pathogen-induced ABA accumulation, and accelerated callose deposition in response to the necrotrophs *B. cinerea* and *P. cucumerina* (García-Andrade et al. 2011). This appears to be an independent pathway to BABA-induced callose deposition, since *ocp3* plants still respond to BABA-mediated callose induction; furthermore, increased resistance in *ocp3* depends on both ABA synthesis and JA perception via COI1, although the latter is not essential for basal callose synthesis (García-Andrade et al. 2011). *pmr4-1* also has a synergistic effect on mutations in the protein kinase EDR1 (ENHANCED DISEASE REISTANCE 1), which enhances resistance to *G. cichoracearum* (Wawrzynska, Rodibaugh and Innes 2010). Unlike either of the individual mutants, *edr1/pmr4* double mutants have increased constitutive expression of SA-signalling and biosynthesis related genes, and also greater expression of ERF1 than *edr1*. This suggests that PMR4 and EDR1 have a synergistic, negative effect on both the SA and JA pathway (Wawrzynska et al. 2010).

3.2 Aims and objectives

Given that plant defence pathways are typically effective against a broad range of pathogens, the hypothesis of this chapter is that genes demonstrated to have a role in host defence for other interactions may contribute to basal resistance against the parasitic weed *Striga gesnerioides*.

Aim:

To identify genes and defence hormones that may contribute to host basal resistance against *Striga gesnerioides*.

Objectives:

1. To test a range of *Arabidopsis* mutants affected in different defence-related pathways for altered basal resistance against *S. gesnerioides*. These include mutants affected in both defence-associated hormone signalling pathways (SA, JA, ethylene and ABA), and the production of specific defence compounds (glucosinolates, callose and reactive oxygen species, ROS).

2. To distinguish between early- and late-stage resistance in these lines.

3.3 Experimental design

Table 3.1 contains the complete list of mutant lines tested for altered basal resistance against *S. gesnerioides*. In each experiment, Columbia (Col-0) *Arabidopsis* served as the wild-type control. *Arabidopsis* seedlings were grown, transplanted into rhizotrons and infected according to the protocol described in Chapter 2, Section 2.3. Col-0 and mutant *Arabidopsis* lines were grown in a Fitotron Growth Cabinet with a 9-hour photoperiod cycle, temperature regime of 26/24˚C day/night, 60% humidity and photon flux density of 120 µmol m^{-2} s^{-1} at plant height. Where there was enough seed, two screens were performed within the assay. In these cases, plants were germinated and transplanted together but infected in two batches, staggered one day apart. Plants were transplanted into rhizotrons when 14 days old, and infected with *S. gesnerioides* when 26 (batch 1) or 27 days (batch 2) old. For each experiment (and batch, where two batches of plants were screened), between 10 and 20 plants were infected for both the Col-0 wildtype and mutant line (equal numbers of each). Sterilised *S. gesnerioides* seed were conditioned for 12 (batch 1) or 13 days (batch 2) in a 25°C incubator and germinated 44 hours prior to infection by applying 3 mls of cowpea root exudates (see Chapter 2, Section 2.2).

For the following Arabidopsis mutants, only one batch was infected: *npr1-1, aos1, opr3, jar1-1, ein3-1, abi1-2, wrky70, RbohD/RbohF* and *cyp79B2/B3.*

For the following mutants, two batches were infected: *sid2-1, pad4-1, eds1-2, eds1-2/ pad4-1, etr1-1, AtAF2, jin1-7/ myc2, erf4-1, ora59, RbohD, RbohF*, *prx33* and *pmr4-1*.

Statistical analyses

To quantify early-stage host resistance, the % infection of *S. gesnerioides* seed was quantified (see Chapter 2, Section 2.4). Late-stage resistance was quantified by calculating the average haustorium size and the proportion of haustoria with a developing shoot (see Chapter 2, Section 2.4). The % infection, average haustorium size and proportion of haustoria with a developing shoot were tested for significant differences ($p < 0.05$) between Col-0 and the mutant line using Student's T-tests. For mutants where plants were screened in two batches, the two batches were assessed independently for differences between Col-0 and the mutant line. Since the plants in both batches were germinated and transplanted together (but infected one day apart), two-way ANOVA analysis was performed on the combined results to quantify the extent of batch-to-batch variation. Tests were carried out using IBM SPSS Statistics 23 software and normality checks performed for each experiment. For both the Student's T-tests and ANOVA analysis, normality checks were carried out and homogeneity of variances was verified using Levene's test for Equality of Error variances: where this was not met, significance was taken as $p < 0.01$, rather than $p < 0.05$.

3.4 Results

3.4.1 *Arabidopsis* mutants impaired in SA-associated defences: *npr1-1*, *sid2-1*, *pad4-1*, *eds1-2*, *eds1-2/pad4-1*

There was no significant difference in either early- or late-stage resistance for the *npr1-1* mutant compared to wildtype Col-0 *Arabidopsis* plants (Figures 3.2 A, C). For the SA-biosynthesis mutant *sid2-1*, differences were seen between the two batches of plants. For batch 2, the mutant showed a significantly higher % infection ($p = 0.003$) (Figure 3.2A) and haustorium size ($p = 0.003$) (Figure 3.2C) compared to wildtype Col-0 plants. In batch 1, however, neither of these parameters varied significantly compared with the Col-0 control. For both *sid2-1* batch 1 and 2, there was no significant difference in the proportion of haustoria that had transitioned to shoot development (Figure 3.2C). For the % infection, two-way ANOVA analysis for the combined data indicated a significant genotype effect ($p = 0.02$) and also an interaction between the batch and genotype ($p = 0.034$). For the average haustorium size, two-way ANOVA indicated a significant batch effect ($p = 0.006$), but no genotype effect.

For both batches of *pad4-1*, there was no significant difference in % infection compared to wildtype Col-0 (Figure 3.2A). For the haustorium size, there was a significant batch effect ($p = 0.009$) since in batch 1, *pad4-1* did not differ significantly from the control, whilst for batch 2, the haustorium size was significantly higher for *pad4-1* ($p = 0.041$) (Figure 3.2B). Two-way ANOVA analysis of the combined data indicated a significant interaction between the batch and genotype for the haustorium size ($p = 0.048$). For the proportion of haustoria with a developing shoot, neither batch of *pad4-1* differed significantly to the control (Figure 3.2C).

Table 3.1: *Arabidopsis* mutants selected to screen for altered basal resistance against *Striga gesnerioides*

Mutant	Affected in	Gene function	Background	Reference
Salicylic acid (SA) - associated mutations				
npr1-1	SA signalling	NPR1 acts as a key downstream signalling node and activates a subset of SA-dependent responses.	Col-0	(Cao et al. 1994)
sid2-1	Pathogen-induced SA biosynthesis	This has impaired SA responses due to a mutation in the gene isochorismate synthase (ICS1). This specifically inhibits the pathogen-induced accumulation of SA that occurs during a hypersensitive response, but does not overly affect basal levels of SA.	Col-0	(Wildermuth et al. 2001).
pad4-1	SA signalling	Together with EDS1, PAD4 promotes basal resistance and SA accumulation.	Col-0	(Jirage et al. 1999, Zhou et al. 1998)
eds1-2	SA signalling	EDS1 is a positive regulator of basal resistance and of effector-triggered immunity via TIR-NB-LRR (TNL) resistance proteins. Also interacts with PAD4 to induce SA accumulation and downstream defence responses.	Col-0	(Parker et al. 1996, Glazebrook et al. 1996)
eds1-2/ pad4-1			Col-0	
Jasmonic acid (JA) – associated mutations				
aos1	JA biosynthesis	Defective in the production of 12-oxo-phytodienoic acid (OPDA) which acts as an independent signalling molecule and as a precursor for JA.	Col-0	(Park et al. 2002)
opr3-1	JA biosynthesis	Defective in the production of JA from OPDA.	Col-0	(Stintzi 2000)
jar1-1	JA signalling	JAR1 biochemically modifies JA to allow interaction between JAZ7 and COI1 in the JA signalling pathway. Mutant shows blocked JA signalling.	Col-0	(Staswick et al. 2002, Staswick, Su and Howell 1992)
Ethylene and abscisic acid (ABA) – associated mutations				
etr1-1	Ethylene signalling	Ethylene-insensitive mutation: ETR1 is an ethylene receptor located on the endoplasmic reticulum.	Col-0	(Bleecker et al. 1988)
ein3-1	Ethylene signalling	Nuclear-located transcription factor required for ethylene signalling.	Col-0	(Chao et al. 1997)

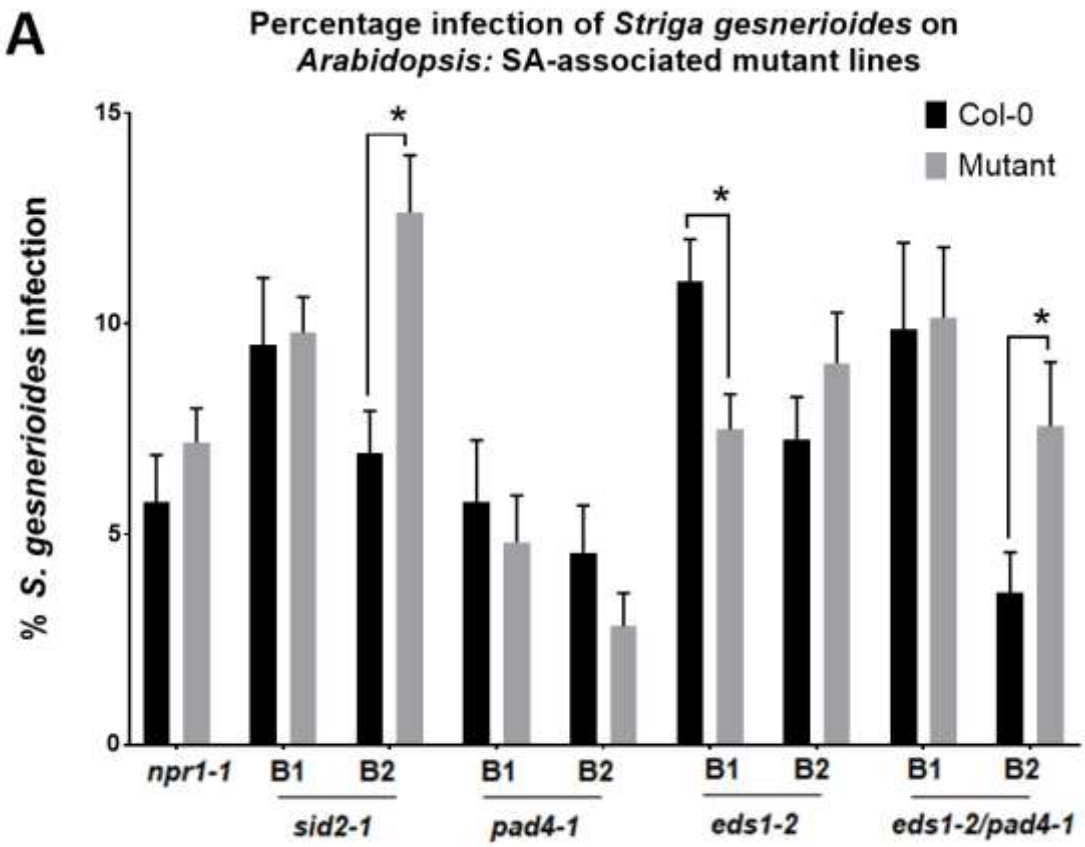
aba1-5	ABA biosynthesis	Catalyses the first step in the biosynthesis of ABA.	Col-0	(Koornneef et al. 1982)
abi1-2	ABA perception	*ABI1* functions as a negative regulator of ABA signalling. *abi1* mutants show enhanced ABA sensitivity.	Col-0	(Saez et al. 2006)
Mutants affected in defence-associated transcription factors				
AtAF2	Downstream JA signalling	ATAF2 acts as a negative regulator: mutant shows upregulation of genes co-regulated by JA/ethylene, e.g. *PDF1.2*	Col-0	(Delessert et al. 2005)
jin1-7/ myc2	Downstream JA signalling	MYC2 acts as a positive regulator of the wound response and a negative regulator of genes co-regulated by JA/ethylene, e.g. *PDF1.2.*	Col-0	(Lorenzo et al. 2004, Abe et al. 2003)
erf4-1	Downstream JA signalling	ERF4 is a transcriptional repressor of JA-Ethylene regulated genes, including *PDF1.2.*	Col-0	(McGrath et al. 2005)
wrky70	SA / JA signalling	Transcription factor that facilitates SA-signalling and SA-mediated antagonism of JA-signalling. Negatively regulates SA accumulation.	Col-0	(Li et al. 2006)
ora59	JA/ Ethylene signalling	ORA59 is a member of the ERF/AP2 transcription factor family. Integrates JA and ethylene signalling cascades to positively regulate genes including *PDF1.2* and *CHI-B.*	Col-0	(Zander et al. 2010, Zander et al. 2014)
Mutations associated with the production of defensive compounds: reactive oxygen species (ROS), glucosinolates and callose				
RbohD	ROS generation	These function as NADPH oxidases that catalyse the generation of ROS, including in response to pathogens.	Col-0	(Torres et al. 2002)
RbohF	ROS generation		Col-0	
RbohD/RbohF	ROS generation		Col-0	
prx33	ROS generation	PRX33 is a class III peroxidase, expressed in roots. Located in the cell wall; appears to generate apoplastic H202 during pathogen attack.	Col-0	(Bindschedler et al. 2006)
pmr4-1	Callose synthesis and SA signalling	PMR4 is a stress-induced callose synthase, however *pmr4* mutants show greater resistance to biotrophic pathogens such as powdery mildew as a result of enhanced basal SA activity.	Col-0	(Vogel and Somerville 2000)
cyp79B2/B3	Glucosinolate and camalexin production	These catalyse the conversion of tryptophan into indole-3-acetaldoxime, a precursor for both indole glucosinolates and camalexin.	Col-0	(Zhao et al. 2002)

Different results were seen between the two batches performed for *eds1-2*. For batch 1, the % infection was significantly lower for *eds1-2* compared with the control (p =0.012), however there was no significant difference for batch 2 (Figure 3.2A). Two-way ANOVA analysis indicated no significant genotype or batch effect, but a potential interaction between the two (p = 0.011). The results for the average haustorium size were similar to those of *pad4-1*: *eds1-2,* batch 1 did not differ significantly from the control, whilst for batch 2, the haustorium size was significantly higher on the mutant line (p = 0.005) (Figure 3.2B). For this parameter, the ANOVA results indicated a significant genotype effect (p = 0.001) and batch effect (p < 0.0001), besides an interaction between the two (p = 0.024). The proportion of haustoria with a shoot was moderately higher in batch 1 (p = 0.055) but significantly higher in batch 2 (p=0.036) (Figure 3.2C).

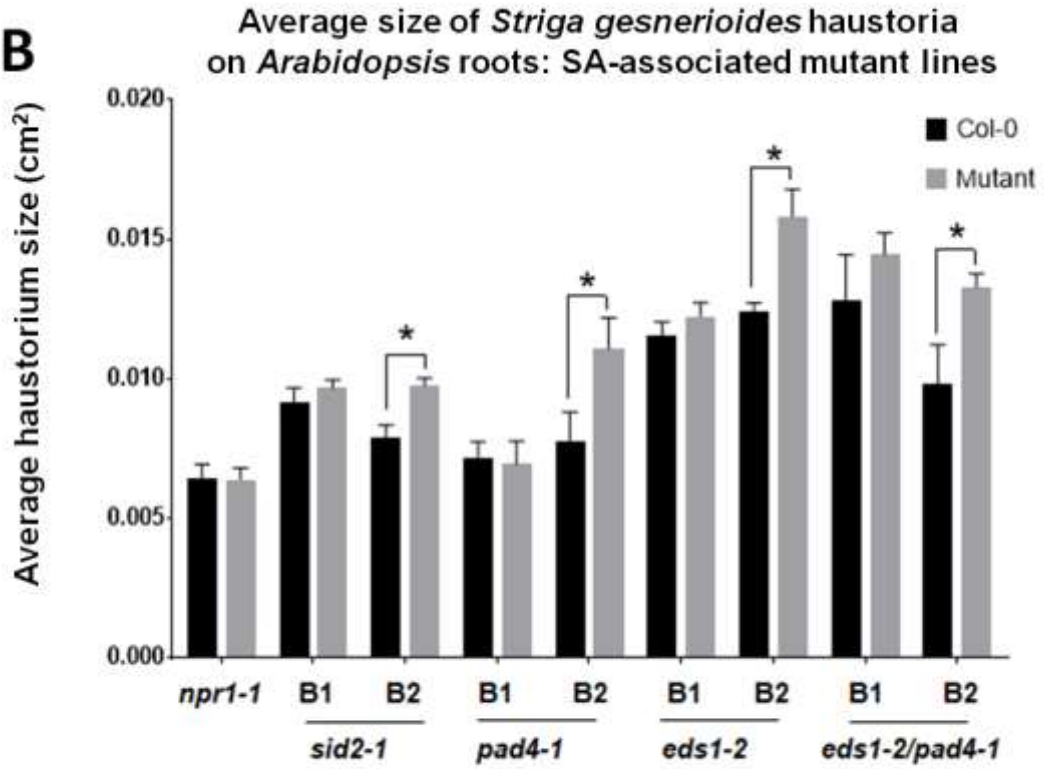
Differences were also seen between the two batches of the double mutant *eds1-2*/ *pad4-1*. The % infection was significantly higher on the mutant for batch 2 (p = 0.035), however there was no significant difference for batch 1 (Figure 3.2A). ANOVA analysis indicated a significant batch effect (p = 0.007) but not a significant genotype effect or interaction between the two. Similarly, haustorium size was significantly higher on *eds1-2*/ *pad4-1* for batch 2 (p = 0.034), but not batch 1 (Figure 3.2B). In this case, no significant effects or interactions were indicated by the ANOVA analysis. No significant differences or effects were found between *eds1-2*/ *pad4-1* and Col-0 in the proportion of haustoria with a developing shoot (Figure 3.2C).

3.4.2 *Arabidopsis* mutants impaired in JA-associated defences: *aos1*, *jar1-1*, *opr3*

The % infection was significantly lower on the JA-biosynthesis mutant *aos1* compared with the Col-0 control (p = 0.01) (Figure 3.3A), however there was no significant difference in haustorium size (Figure 3.3B) or the proportion of haustoria with a developing shoot (Figure 3.3C). *opr3-1* which is defective in JA biosynthesis but maintains the intermediate signalling molecule OPDA, showed no significant difference compared with the control regarding either the % infection (Figure 3.3A) or the proportion of haustoria with a developing shoot (Figure 3.3C). Haustorium size, however, was significantly smaller on this mutant (p < 0.0001) (Figure 3.3B). Similarly, the JA-signalling mutant *jar1-1* showed no significant difference compared with the control regarding the % infection (Figure 3.3A) or the proportion of haustoria with a developing shoot (Figure 3.3C). In contrast to *opr3-1*, however, haustorium size was significantly higher on *jar1-1* than wildtype Col-0 (p = 0.001) (Figure 3.3B).



Genotype	0.306	0.020	0.267	0.401	0.181
Batch		0.912	0.190	0.289	0.007
Batch * Genotype		0.034	0.740	0.011	0.243



Genotype	0.988	0.162	0.088	0.001	0.107
Batch		0.006	0.009	0.000	0.244
Batch * Genotype		0.108	0.048	0.024	0.977

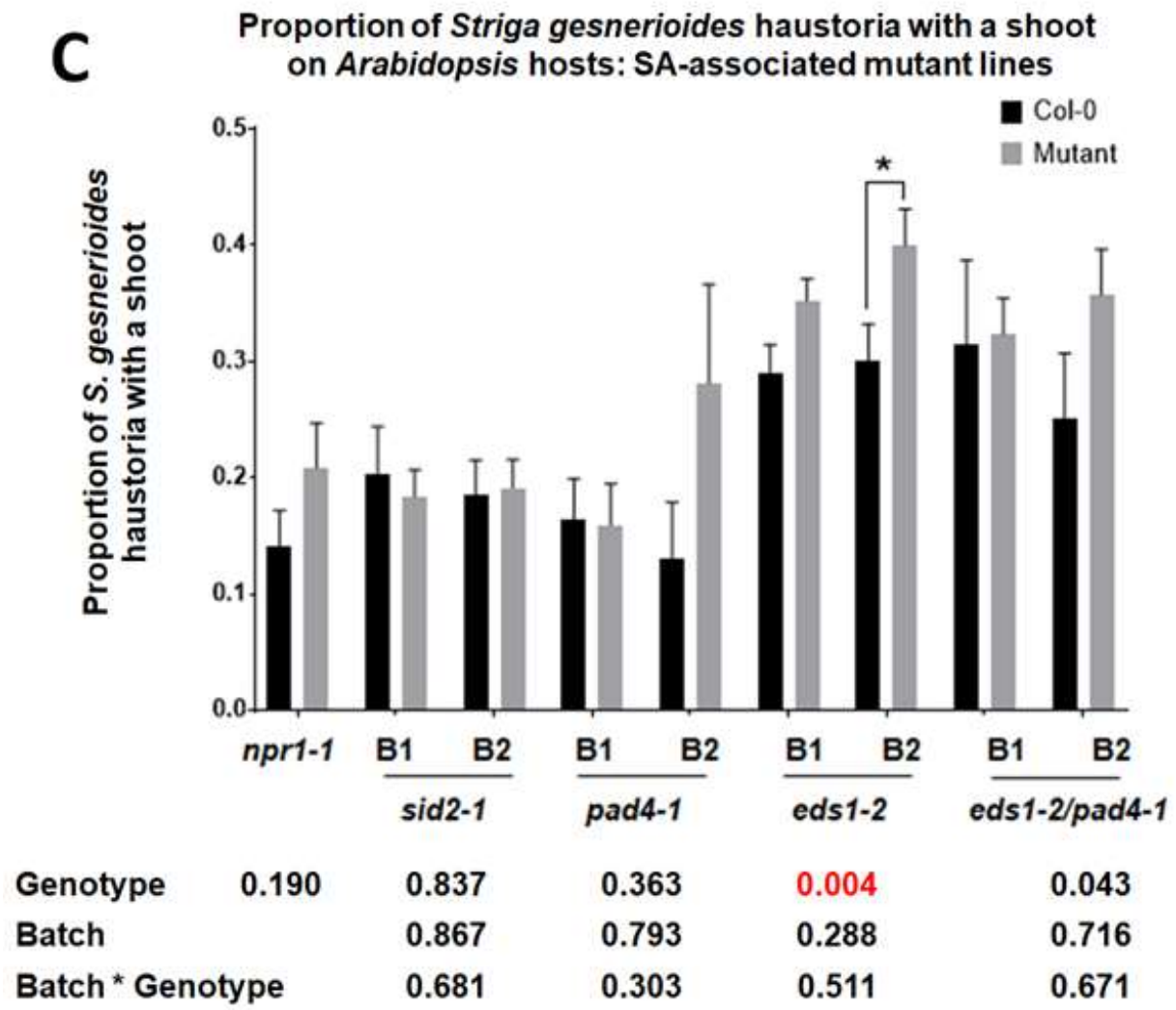
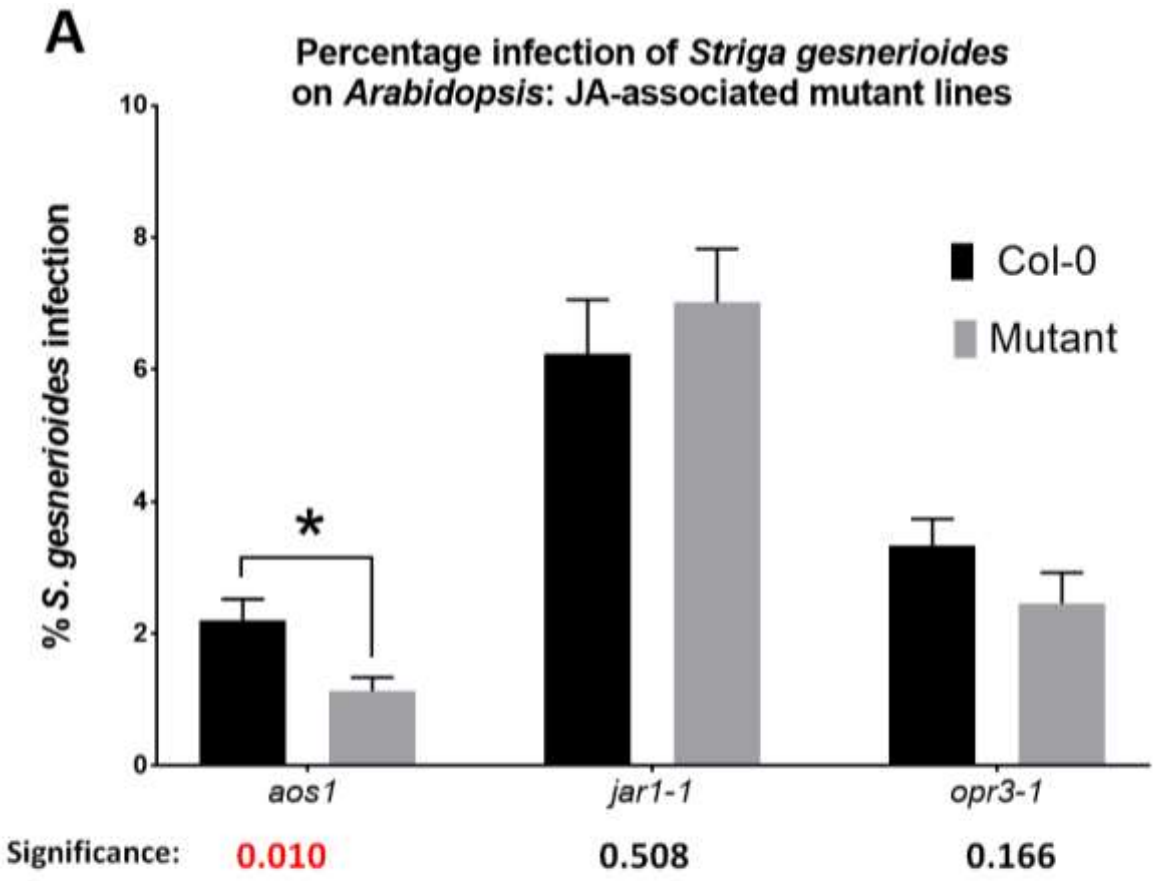
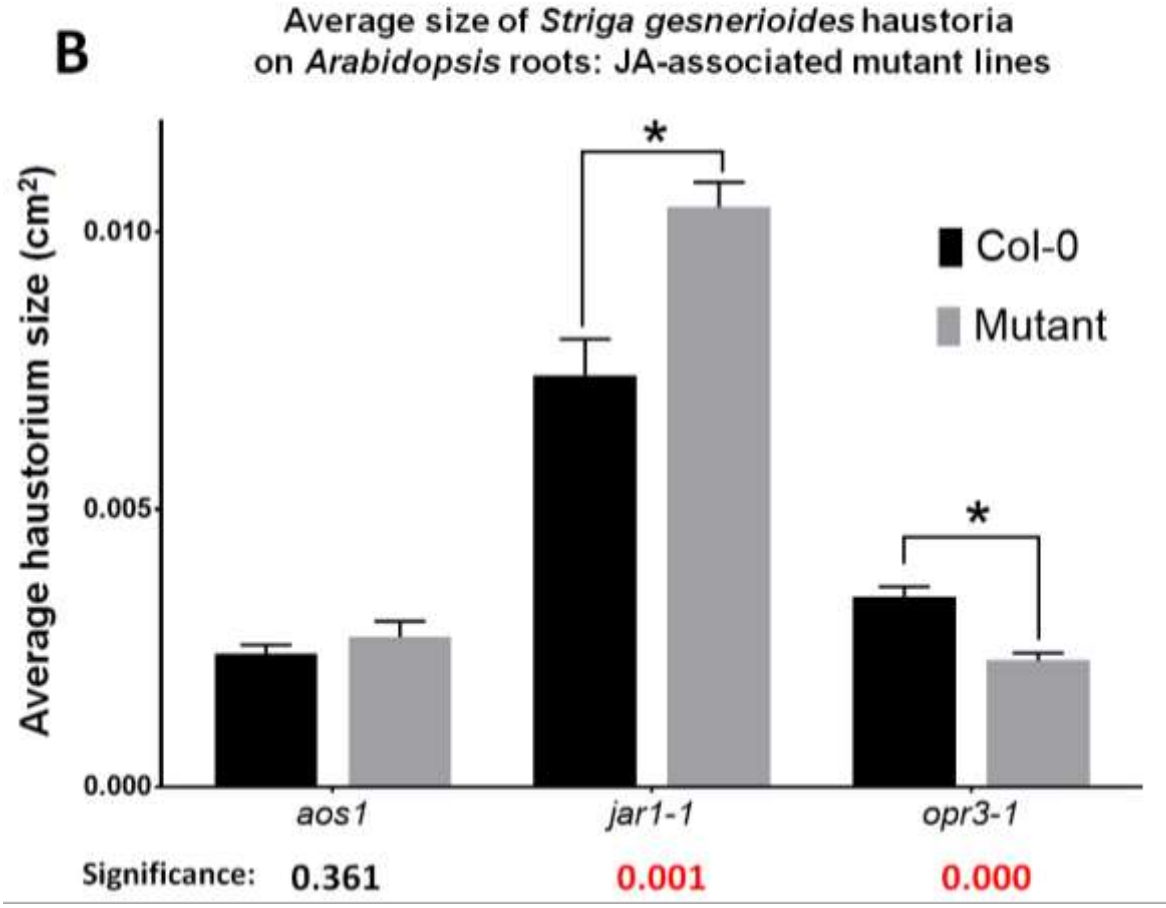


Figure 3.2: Basal resistance phenotypes of *Arabidopsis* mutants with defects in salicylic-acid (SA) biosynthesis or signalling infected with *Striga gesnerioides*, at 3 weeks post-infection compared with wild-type (Col-0). **A:** Number of attached *S. gesnerioides* haustoria per host expressed as a % of total parasite seed applied. **B:** Average size of each individual attached parasite haustorium. **C:** Proportion of attached haustoria with a developing shoot, an indicator of advanced development. Error bars show mean +/- standard error. B1, B2 = Batch 1, Batch 2 (for genotypes where two batches were screened). * denotes a significant difference ($p \leq 0.05$) between the mutant line and the Col-0 control according to Student's T-test. Figures beneath each graph describe the results of Student's T-test (where the experiment only contained one batch of plants) or two-way ANOVA for significant batch and genotype effects (where two batches of plants were screened as part of the experiment). Significant figures are indicated in red. For each batch, between 10 and 20 plants were infected for both the Col-0 wildtype and mutant line (equal numbers of each). Normality and homogeneity of variance checks were carried out in each case.


A
Percentage infection of Striga gesnerioides on Arabidopsis: JA-associated mutant lines
% S. gesnerioides infection
10
8
6
4
2
0
Col-0
Mutant
*
aos1
jar1-1
opr3-1
Significance:
0.010
0.508
0.166


B
Average size of Striga gesnerioides haustoria on Arabidopsis roots: JA-associated mutant lines
Average haustorium size (cm²)
0.010
0.005
0.000
Col-0
Mutant
*
*
aos1
jar1-1
opr3-1
Significance:
0.361
0.001
0.000

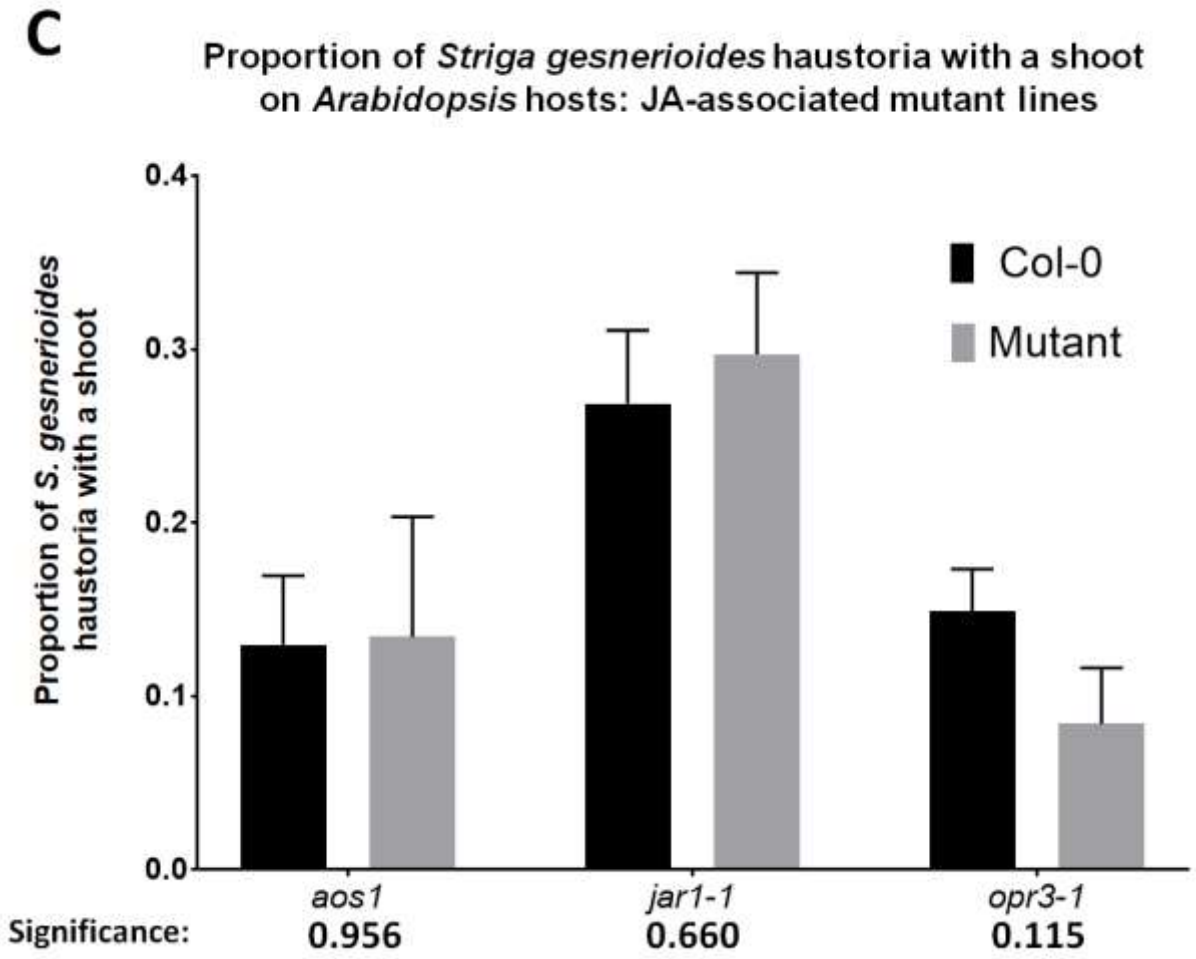


Figure 3.3: Basal resistance phenotypes of *Arabidopsis* mutants with defects in jasmonic acid (JA) biosynthesis or signalling infected with *Striga gesnerioides*, at 3 weeks post-infection compared with wild-type (Col-0). **A:** Number of attached *S. gesnerioides* haustoria per host expressed as a % of total parasite seed applied. **B:** Average size of each individual attached parasite haustorium. **C:** Proportion of attached haustoria with a developing shoot, an indicator of advanced development. Error bars show mean +/- standard error. B1, B2 = Batch 1, Batch 2 (for genotypes where two batches were tested). * denotes a significant difference ($p \leq 0.05$) between the mutant line and the Col-0 control according to Student's T-test. Figures beneath each graph describe the results of Student's T-test for differences between Col-0 and the mutant line. Significant figures are indicated in red. For each batch, between 10 and 20 plants were infected for both the Col-0 wildtype and mutant line (equal numbers of each). Normality and homogeneity of variance checks were carried out in each case.

3.4.3 *Arabidopsis* mutants affected in defence-associated transcription factors: *AtAF2*, *jin1-7*, *ora59*, *wrky70*, *erf4-1*

For the defence-associated transcription factors AtAF2, JIN1/MYC2, ORA59, WRKY70 and ERF4, two batches of plants were infected, apart from *wrky70*. No significant differences in early-stage host resistance (i.e. the % infection), were seen for either batch of *AtAF2* or *jin1-7* (Figure 3.4A). For *ora59*, the % infection was significantly higher for batch 1 (Mean values: Col-0 = 7.18%, *ora59* = 12.08%, p = 0.005), but not batch 2. The ANOVA analysis indicated a significant genotype effect (p = 0.033) and a potential interaction between batch and genotype (p = 0.033). A similar result was seen for *erf4-1,* the % infection was significantly higher only on batch 1 (p = 0.002). In this case, two-way ANOVA indicated a significant genotype effect (p =0.001) but no significant interaction between the genotype and batch. In the only batch of *wrky70*, the % infection was also significantly higher on the mutant line (p = 0.021).

The results for late-stage host resistance (as measured by haustorium size), differed compared to early-stage resistance. No significant differences were seen for *wrky70, erf4-1* or *AtAF2* compared to wildtype Col-0 (Figure 3.4B). For *jin1-7*, haustorium size was significantly higher for the mutant line in batch 2 (p= 0.016), but not batch 1. ANOVA analysis indicated a significant interaction between batch and genotype for *jin1-7* (p = 0.020), but not an overall significant genotype or batch effect. For *ora59*, there was no significant difference in haustorium size compared with the wildtype, nevertheless ANOVA analysis indicated a significant batch effect (p = 0.033). Regarding the proportion of haustoria with a developing shoot, no significant differences were seen at all for *wrky70, ora59, AtAF2, erf4-1* or *jin1-7* (Figure 3.4C).

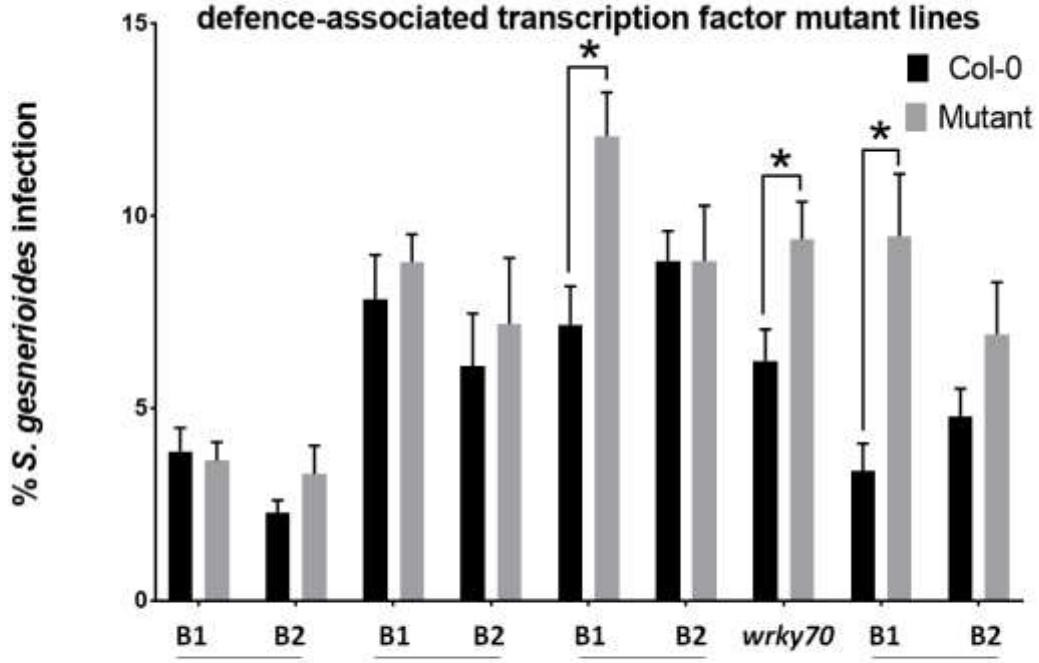
3.4.4 *Arabidopsis* mutants impaired in ethylene and abscisic acid (ABA) signalling: *etr1-1*, *ein3-1*, *abi1-2*

The results for both batches of *etr1-1* were highly similar, yet more pronounced for batch 2 than batch 1. The % infection was lower on *etr1-1* for both batches, but only significantly so for batch 2 (p = 0.047) (Figure 3.5A). ANOVA analysis indicated a significant genotype effect for this parameter (p = 0.017). Similarly, the proportion of haustoria with developing shoots was lower for *etr1-1* in both batches tested, however this was only significant in batch 2 (p = 0.045) (Figure 3.5B). Again, ANOVA analysis indicated a significant genotype effect for this parameter (p = 0.017). There was no significant difference in haustorium size, however, between *etr1-1* or the Col-0 control (Figure 3.5C).

Despite *ein3-1* also being an ethylene-signalling mutant, the results for this mutant varied somewhat with *etr1-1* in that there was no difference in the % infection between the mutant and control (Figure 3.5A). Similar to *etr1-1*, however, there was no significant difference between *ein3-1* and Col-0 in

A

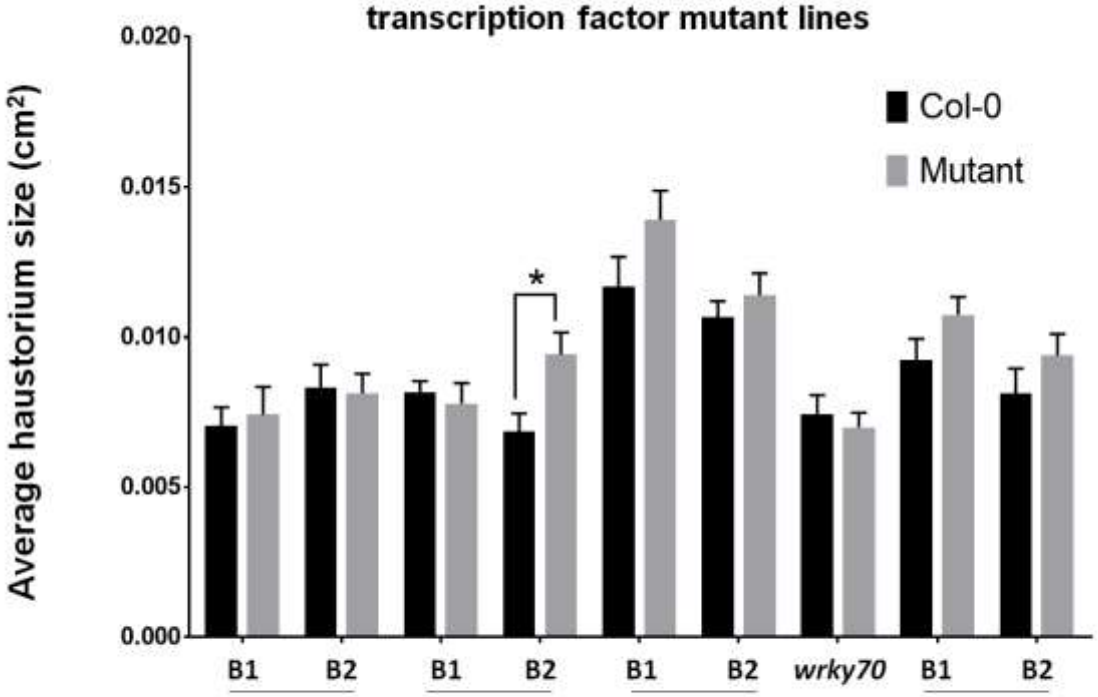
Percentage infection of *Striga gesnerioides* on *Arabidopsis*: defence-associated transcription factor mutant lines



	AtAF2	jin1-7	ora59	wrky70	erf4-1
Genotype	0.494	0.420	0.033	0.021	0.001
Batch	0.093	0.193	0.477		0.636
Batch * Genotype	0.270	0.954	0.033		0.098

B

Average size of *Striga gesnerioides* haustoria on *Arabidopsis* roots: defence-associated transcription factor mutant lines



	AtAF2	jin1-7	ora59	wrky70	erf4-1
Genotype	0.900	0.076	0.072	0.599	0.062
Batch	0.195	0.798	0.033		0.092
Batch * Genotype	0.717	0.020	0.360		0.871

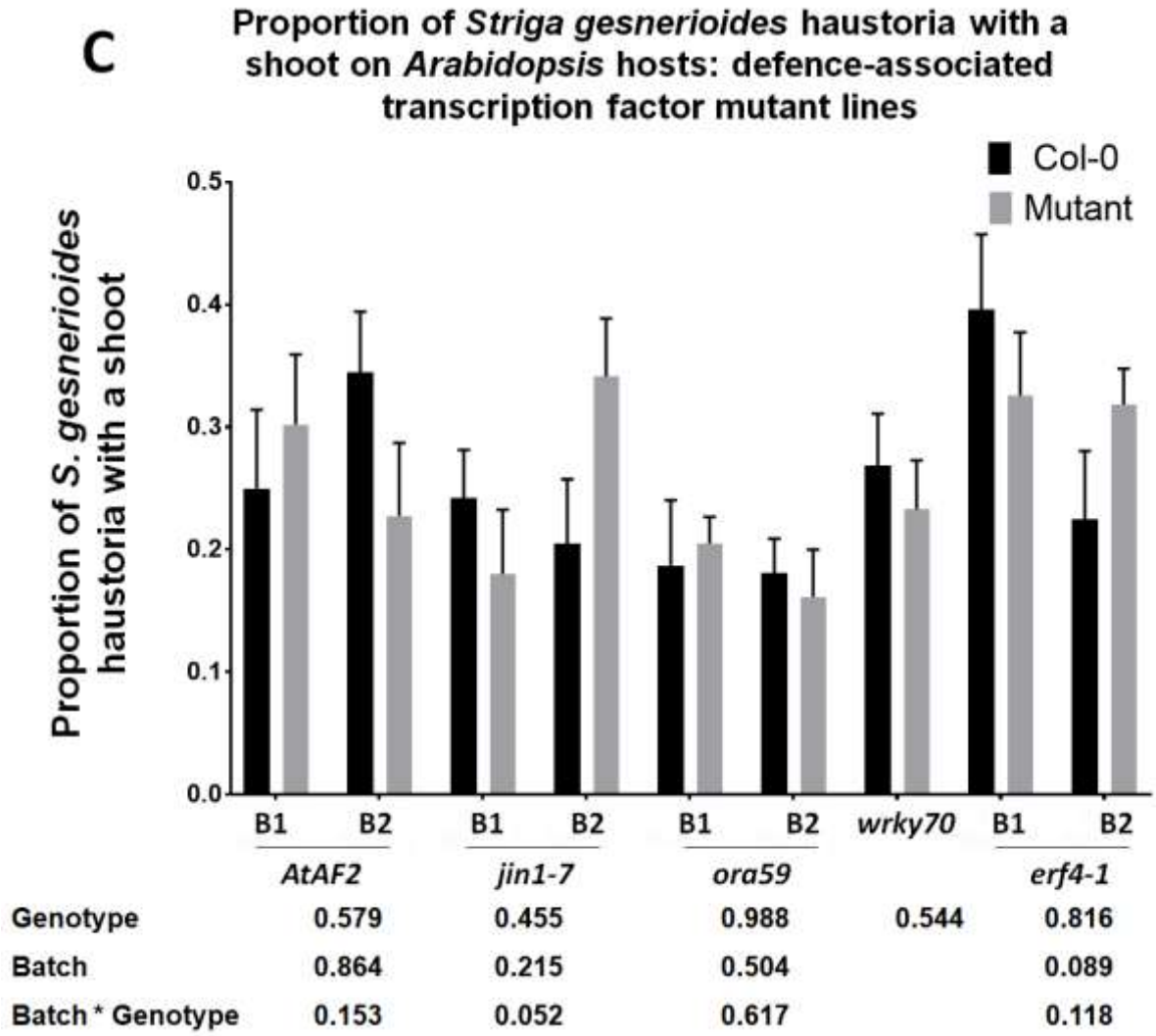


Figure 3.4: Basal resistance phenotypes of *Arabidopsis* mutants with defects in key defence-associated transcription factors infected with *Striga gesnerioides*, at 3 weeks post-infection compared with wild-type (Col-0). **A:** Number of attached *S. gesnerioides* haustoria per host expressed as a % of total parasite seed applied. **B:** Average size of each individual attached parasite haustorium. **C:** Proportion of attached haustoria with a developing shoot, an indicator of advanced development. Error bars show mean +/- standard error. B1, B2 = Batch 1, Batch 2 (for genotypes where two batches were screened). * denotes a significant difference ($p \leq 0.05$) between the mutant line and the Col-0 control according to Student's T-test. Figures beneath each graph describe the results of Student's T-test (where the experiment only contained one batch of plants) or two-way ANOVA for significant batch and genotype effects (where two batches of plants were screened as part of the experiment). Significant figures are indicated in red. For each batch, between 10 and 20 plants were infected for both the Col-0 wildtype and mutant line (equal numbers of each). Normality and homogeneity of variance checks were carried out in each case.

haustorium size (Figure 3.5B). The proportion of haustoria with a developing shoot, however, was even more reduced on *ein3-1* than *etr1-1* compared with the control (p = 0.00) (Figure 3.5C).

Similar to *ein3-1*, the ABA-hypersensitive mutant *abi1-2* showed no significant differences in the % infection or haustorium size (Figures 3.5A and B). In contrast to *etr1-1* and *ein3-1*, however, the proportion of haustoria with a developing shoot was slightly higher on *abi1-2* (p = 0.065) (Figure 3.5C).

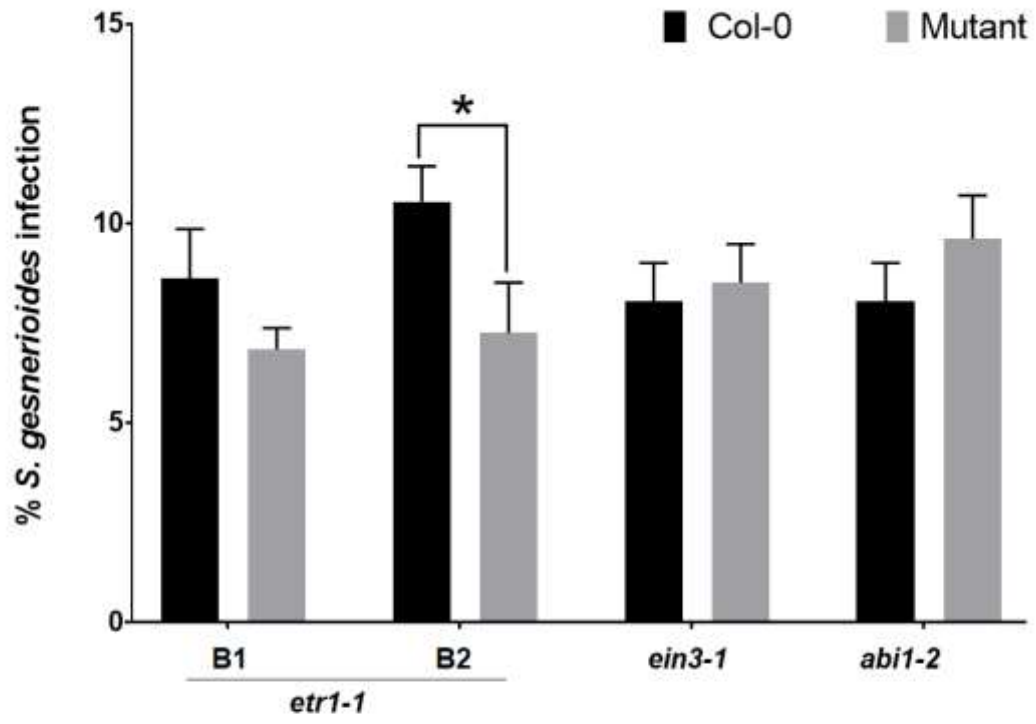
3.4.5 *Arabidopsis* mutants impaired in reactive oxygen species (ROS) generation/detoxification: *RbohD*, *RbohF*, *RbohD/RbohF*, *prx33*

For *RbohD*, different results were seen in the two batches regarding the % infection (Figure 3.6A). In batch 2 the % infection was significantly lower for *RbohD* (p = 0.013), however there was no significant difference for batch 1. Nevertheless, ANOVA analysis indicated a significant effect for genotype (p = 0.017), but not batch. In contrast to *RbohD*, there was no significant difference in the % infection for *RbohF* compared with the control. ANOVA analysis indicated a significant batch effect for *RbohF* (p = 0.006), possibly since higher levels of overall infection were seen in batch 2. *RbohD/RbohF* meanwhile, showed a stronger phenotype than either of the single mutants, with the % infection being significantly lower than the control (p = 0.012).

Regarding haustorium size, no significant difference was seen for *RbohF* or *RbohD/RbohF* (Figure 3.6B). Strikingly, haustorium size was significantly lower on *RbohD* for both batch 1 (p = 0.034) and batch 2 (p = 0.014). ANOVA analysis indicated a significant genotype effect for *RbohD* for haustorium size (p = 0.001). For the proportion of haustoria with a developing shoot, no significant differences were seen for the single *RbohD* and *RbohF* mutants. For the *RbohD/RbohF* double mutant, however, the proportion of haustoria that showed shoot development was significantly lower compared to Col-0 (p = 0.038) (Figure 3.6C).

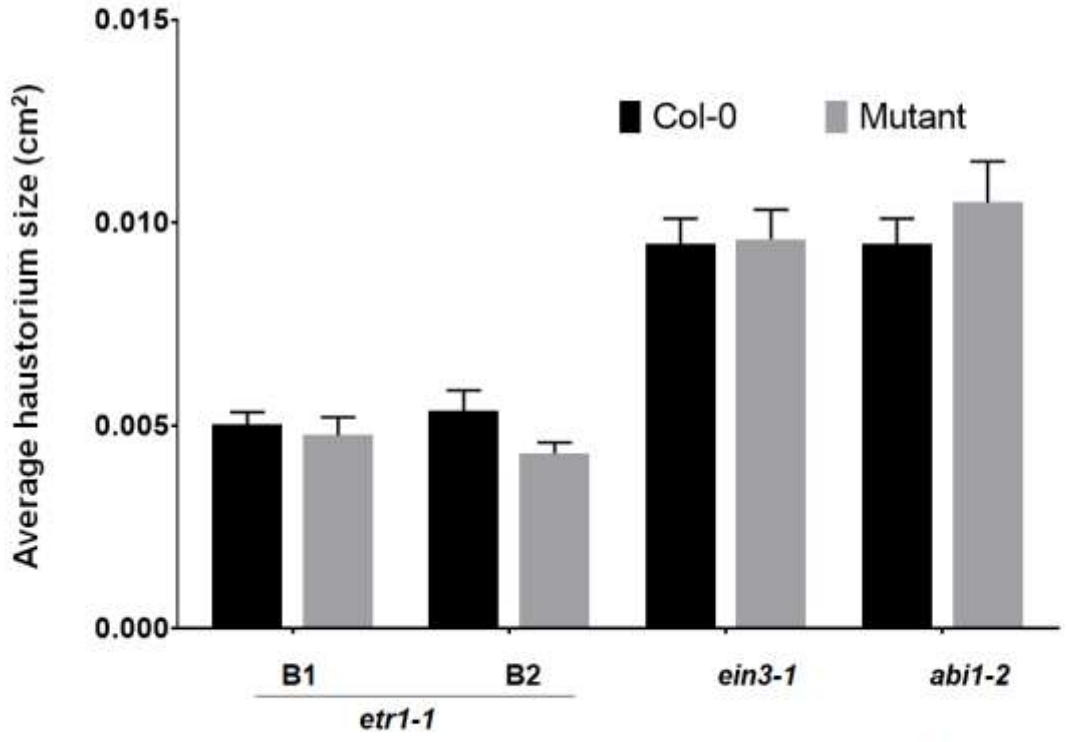
Neither batch of *prx33* showed any significant difference to the control regarding the % infection or haustorium size (Figures 3.6A and B). A significant batch effect however was observed regarding the proportion of haustoria with a developing shoot (p = 0.045) (Figure 3.6C). Specifically, the proportion of haustoria with a developing shoot was significantly higher on *prx33* compared with the wildtype for batch 1 (p = 0.024), however there was no discernible difference for batch 2.

A Percentage infection of *Striga gesnerioides* on *Arabidopsis*: ethylene- and ABA-associated mutant lines



Genotype	0.017	0.735	0.289
Batch	0.258		
Batch * Genotype	0.461		

B Average size of *Striga gesnerioides* haustoria on *Arabidopsis* roots: ethylene and ABA-associated mutant lines



Genotype	0.104	0.904	0.390
Batch	0.896		
Batch * Genotype	0.314		

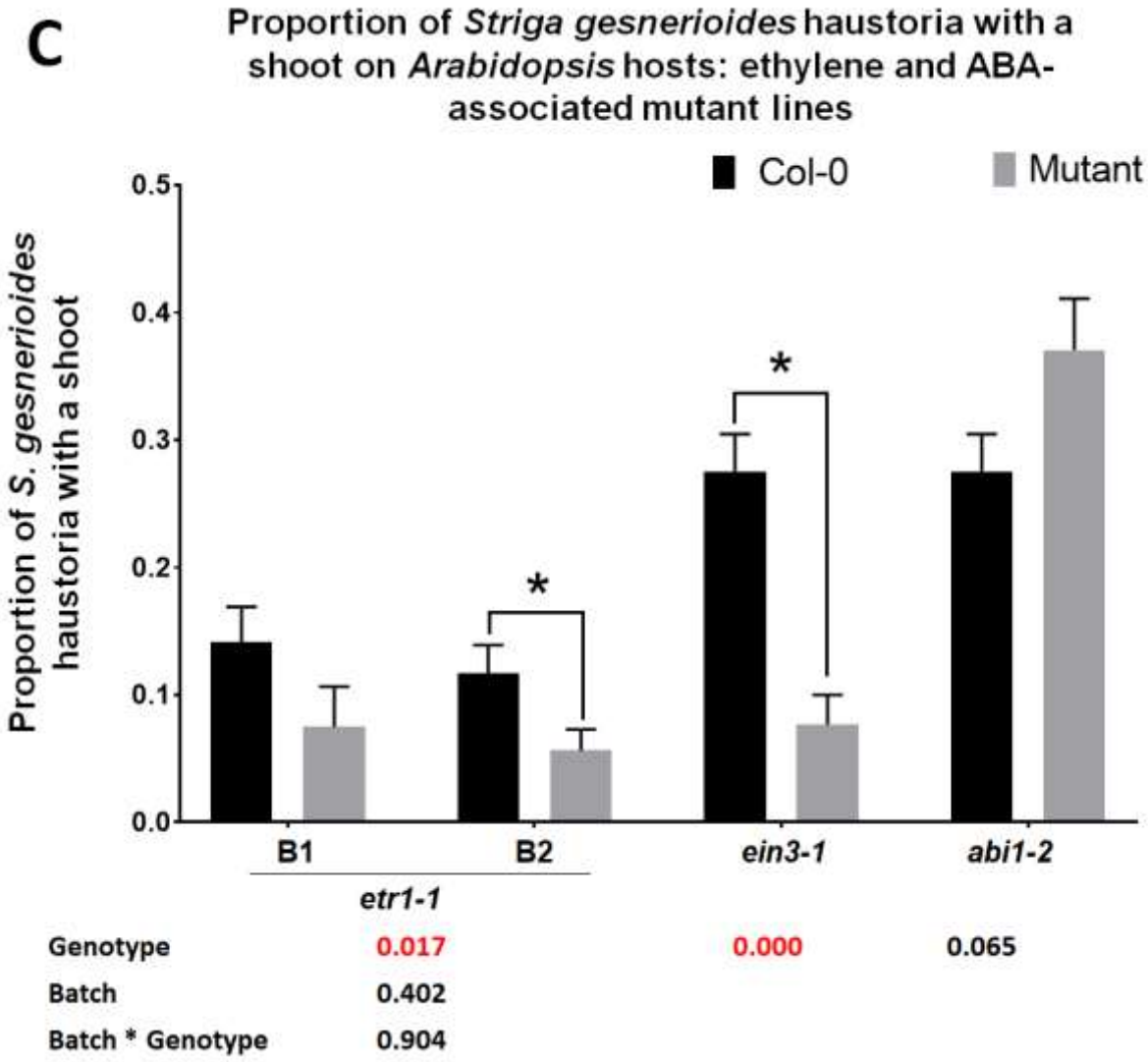
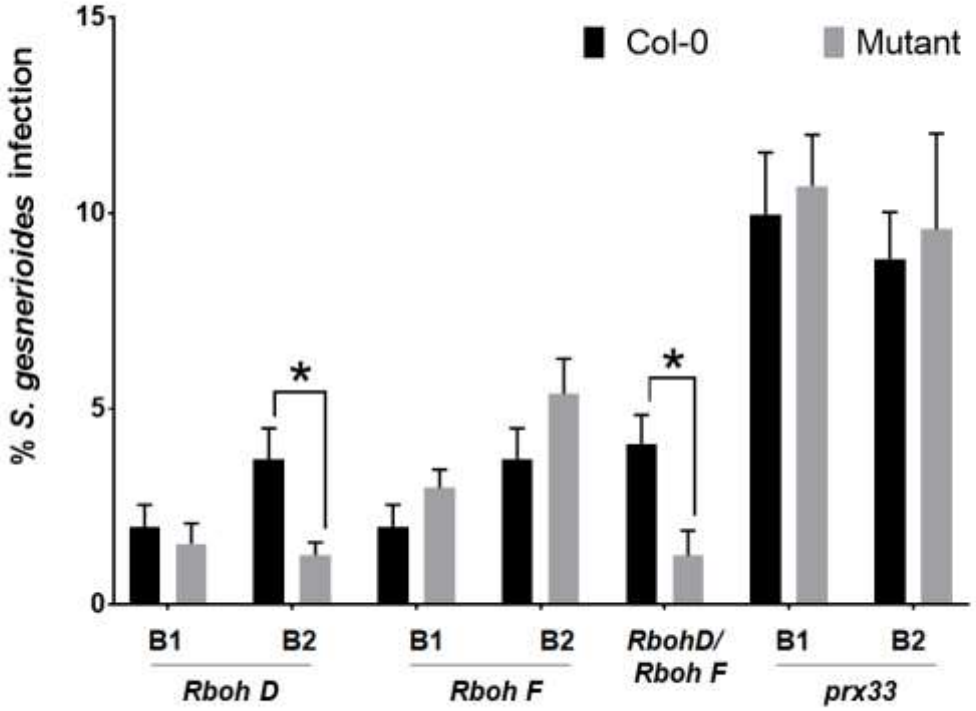


Figure 3.5: Basal resistance phenotypes of *Arabidopsis* mutants with defects in ethylene or abscisic acid (ABA) biosynthesis/signalling, infected with *Striga gesnerioides* at 3 weeks post-infection compared with wild-type (Col-0). **A:** Number of attached *S. gesnerioides* haustoria per host expressed as a % of total parasite seed applied. **B:** Average size of each individual attached parasite haustorium. **C:** Proportion of attached haustoria with a developing shoot, an indicator of advanced development. Error bars show mean +/- standard error. B1, B2 = Batch 1, Batch 2 (for genotypes where two batches were screened). * denotes a significant difference ($p \leq 0.05$) between the mutant line and the Col-0 control according to Student's T-test. Figures beneath each graph describe the results of Student's T-test (where the experiment only contained one batch of plants) or two-way ANOVA for significant batch and genotype effects (where two batches of plants were screened as part of the experiment). Significant figures are indicated in red. For each batch, between 10 and 20 plants were infected for both the Col-0 wildtype and mutant line (equal numbers of each). Normality and homogeneity of variance checks were carried out in each case.

A

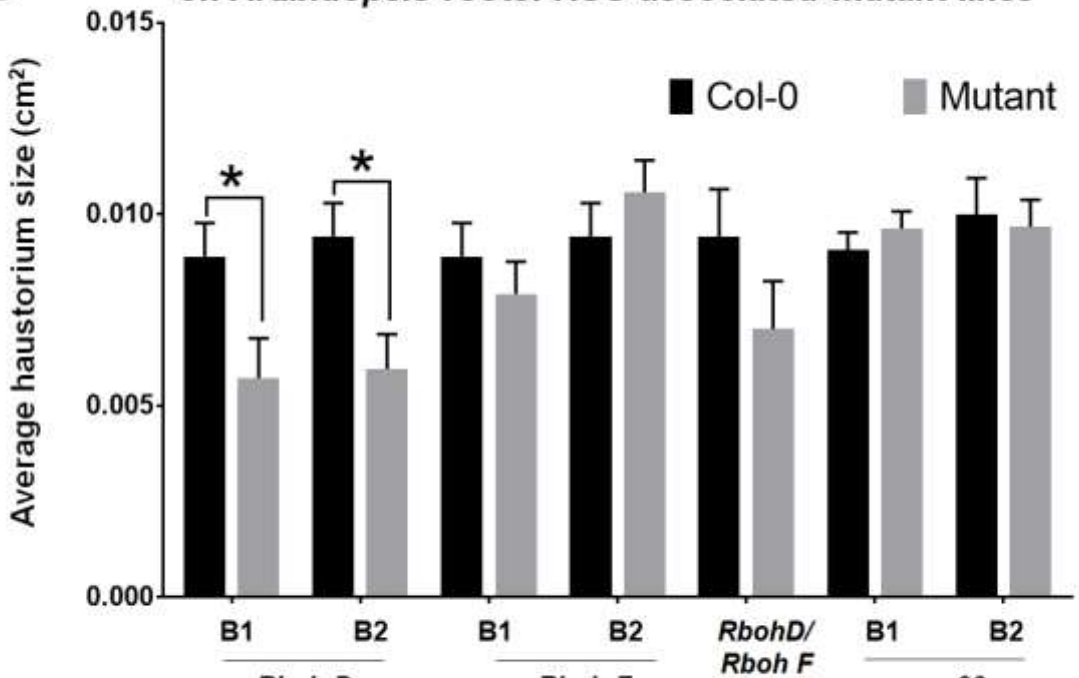
Percentage infection of *Striga gesnerioides* on *Arabidopsis*: ROS-associated mutant lines



	Rboh D	Rboh F	RbohD/ Rboh F	prx33
Genotype	0.017	0.068	0.012	0.647
Batch	0.217	0.006		0.490
Batch * Genotype	0.088	0.640		0.992

B

Average size of *Striga gesnerioides* haustoria on *Arabidopsis* roots: ROS-associated mutant lines



	Rboh D	Rboh F	RbohD/ Rboh F	prx33
Genotype	0.001	0.906	0.191	0.852
Batch	0.688	0.074		0.476
Batch * Genotype	0.886	0.222		0.523

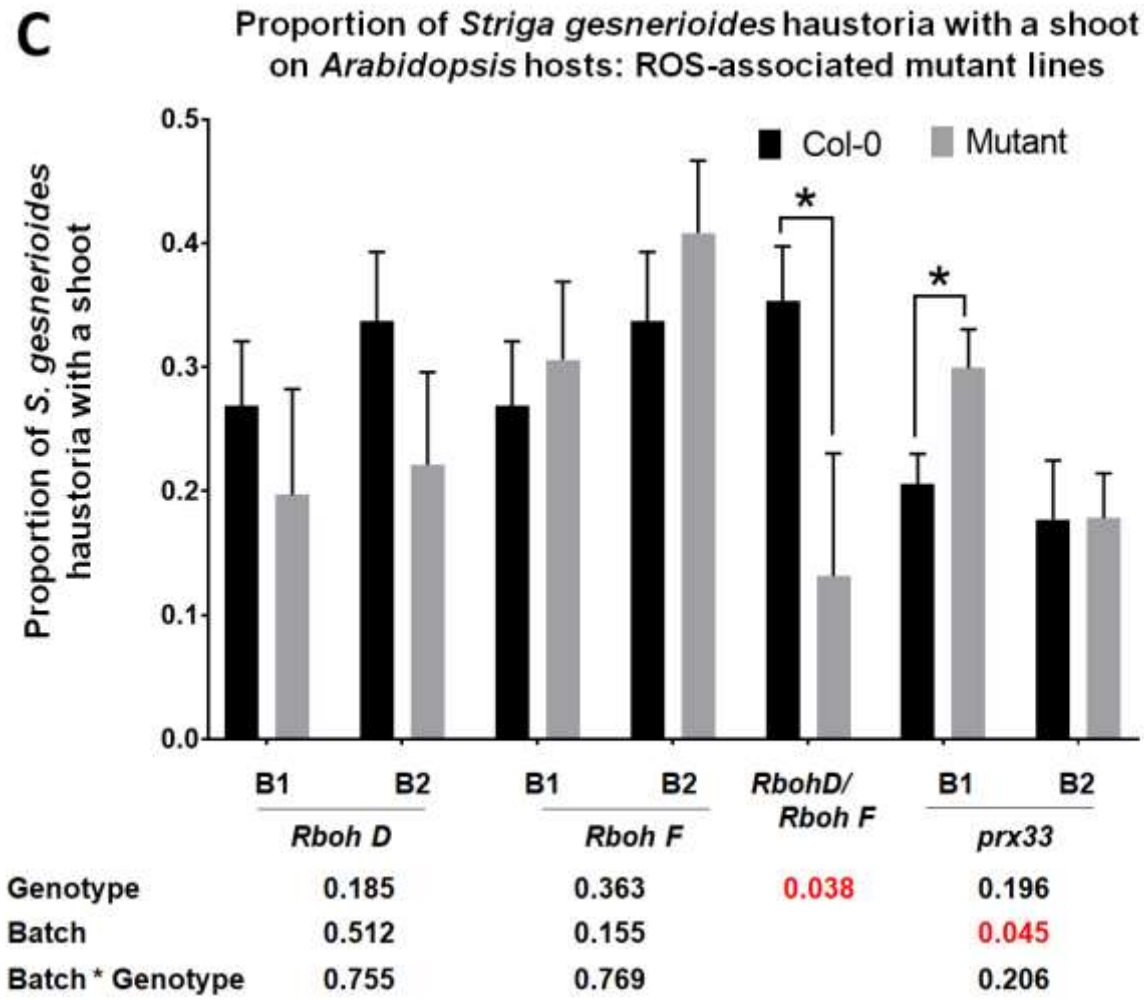


Figure 3.6: Basal resistance phenotypes of *Arabidopsis* mutants with defects in reactive oxygen species (ROS) generation/detoxification, infected with *Striga gesnerioides* at 3 weeks post-infection compared with wild-type (Col-0). **A:** Number of attached *S. gesnerioides* haustoria per host expressed as a % of total parasite seed applied. **B:** Average size of each individual attached parasite haustorium. **C:** Proportion of attached haustoria with a developing shoot, an indicator of advanced development. Error bars show mean +/- standard error. B1, B2 = Batch 1, Batch 2 (for genotypes where two batches were screened). * denotes a significant difference ($p \leq 0.05$) between the mutant line and the Col-0 control according to Student's T-test. Figures beneath each graph describe the results of Student's T-test (where the experiment only contained one batch of plants) or two-way ANOVA for significant batch and genotype effects (where two batches of plants were screened as part of the experiment). Significant figures are indicated in red. For each batch, between 10 and 20 plants were infected for both the Col-0 wildtype and mutant line (equal numbers of each). Normality and homogeneity of variance checks were carried out in each case.

3.4.6 *Arabidopsis* mutants impaired in the production of callose, camalexin and indole-glucosinolates

Neither batch of *pmr4-1* differed from the control regarding the % infection (Figure 3.7A), nevertheless, two-way ANOVA analysis indicated a significant batch effect ($p = 0.028$). Haustorium size, however, was significantly smaller on *pmr4-1* for both batch 1 ($p = 0.001$) and 2 ($p < 0.0001$) (Figure 3.7B). ANOVA analysis indicated a significant genotype effect for this parameter ($p < 0.0001$). A striking result was seen regarding the proportion of haustoria with a developing shoot for *pmr4-1* in batch 1: almost none of the attached haustoria on the mutant line showed any signs of shoot development, unlike the control ($p < 0.0001$) (Figure 3.7C). For batch 2, however, the proportion was only slightly reduced for *pmr4-1* ($p = 0.092$). Once again, two-way ANOVA indicated a significant genotype effect for the proportion of haustoria with a developing shoot ($p = 0.001$).

Similar to *pmr4-1*, *cyp79B2/B3* showed no difference in early-stage host resistance against the parasite, as indicated by the % infection (Figure 3.7A). Unlike *pmr4-1*, however, *cyp79B2/B3* also showed no difference regarding the haustorium size (Figure 3.7B). Curiously, in marked contrast to *pmr4-1*, the proportion of haustoria with a developing shoot was significantly higher on *cyp79B2/B3* ($p < 0.0001$) (Figure 3.7C).

3.5 Discussion

The hypothesis of this chapter was that genes demonstrated to have a role in defence against plant pathogens may also contribute to basal resistance against the parasitic weed *Striga gesnerioides*. In this respect, screening a range of *Arabidopsis* mutants was successful in identifying genes that significantly affected host susceptibility to this parasite. Furthermore, the assay distinguished genes that act specifically at an early- or late-stage of the host basal resistance response. This indicates that the effectiveness of certain defence-related compounds and signalling pathways varies depending on the lifecycle stage of *S. gesnerioides*.

Nevertheless, despite these results, it is clear that this phenotypic screen has limitations. The plants were maintained in a climate-controlled chamber set to the same conditions throughout, yet the overall level of infection varied markedly on the wild-type Col-0 control between individual experiments. This is not likely to be caused by the parasite seed gradually losing viability over time, since the level of virulence did not decrease steadily over the course of experiments. Furthermore, a significant batch effect was observed for many of the mutant lines, where the results differed between batch 1 and batch 2 (e.g. *eds1-2, ora59*). The only difference between batch 1 and 2 was that the host *Arabidopsis* were infected one day apart, and the parasite seeds for batch 2 were preconditioned for an additional day: all other conditions were equal. However, the batch effect was not consistent: for

some mutants, batch 2 was more resistant, and in other cases the reverse was true. This indicates that additional factors may have been influencing the results. Since both batches tended to be watered at the same time, there may have been slight differences in the host hydration status on the day of infection; it is known that *Striga* spp. perform best on drier soils (Julie Scholes, personal communication). Other possible factors include temporary disruptions to the Conviron growth cabinet conditions, including losses of humidity, temperature control and power, which occurred on an infrequent basis over the course of these studies. Greater population sizes of infected plants could mitigate for these effects; however, the final numbers are restricted by the narrow time-window for optimum infection.

3.5.1 Salicylic acid may protect against *S. gesnerioides* under certain conditions

Overall, the SA-biosynthesis mutant *sid2-1* showed significantly reduced early-stage resistance compared to wildtype Col-0 (as measured by the % infection) (Figure 3.2A). This suggests that SA may contribute to basal resistance against *S. gesnerioides* during the early stages of infection. This supports the evidence reviewed previously (Chapter 1, Section 1.8.8) that resistant responses against *S. gesnerioides* and *S. hermonthica* are associated with greater SA-signalling activity, and that pre-treatment with SA or SA-analogues can protect hosts against both *Orobanche* and *Striga* parasites. Nevertheless, because there was no significant overall genotype effect for late-stage host resistance, SA may not restrict the parasite's development once it has connected to the host vasculature. Since the interaction between *Arabidopsis* and *S. gesnerioides* is a largely compatible interaction, further increases in host susceptibility are fairly subtle phenotypes. As such, it may be more informative to test a mutant with enhanced SA-activity such as *cpr1 (constitutive expression of PR proteins)* or *mpk4 (map kinase 4)* (Bowling et al. 1994, Petersen et al. 2000). Both these mutants show increased SA accumulation, constitutive *PR* gene expression and greater resistance to virulent *Pseudomonas* spp; *mpk4* is also insensitive to JA signalling.

Despite the result with *sid2-1*, the *npr1-1* mutation did not significantly affect either early- or late-stage basal resistance. This indicates that any protective effects of SA may be mediated through an NPR1-independent pathway. Indeed, while NPR1 appears critical for *PR1* gene expression, SAR and basal resistance against virulent biotrophs (Glazebrook et al. 1996, Delaney et al. 1995, Cao et al. 1994) it is known that certain cases of *R*-gene mediated resistance are affected by SA-dependent, but NPR1-independent pathways (Rairdan and Delaney 2002). These results are paralleled by the interaction between *Arabidopsis* and the wilt pathogen *F. oxysporum f. conglutinans*. SA-deficient *Arabidopsis* mutants (e.g. *sid2*, *eds5*) show increased susceptibility to the pathogen, but not mutants affected in *NPR1* (Diener and Ausubel 2005).

EDS1 and PAD4 are important orchestrators of downstream SA-dependent responses in R-gene mediated and basal resistance (Feys et al. 2001, Falk et al. 1999). In these experiments, the results for *eds1-4*, *pad4-1* and *eds1-4/pad4-1* are not straightforward to interpret, particularly when significant batch effects occurred. For *eds1*-2, for instance, batch 1 showed significantly greater early-stage resistance in the mutant line, whereas in batch 2, the mutant had significantly reduced late-stage resistance (Figures 3.2 A, B, C). Furthermore, for *pad4-1, eds1-2* and *eds1-2/pad4*-1, in batch 2 the haustorium size was significantly greater for the mutant line compared with the wildtype, but not for batch 1 (Figure 3.2B). Using two-way ANOVA to account for batch effects indicated that the only significant genotype result regarding late-stage resistance was reduced late-stage resistance in *eds1-2*, as measured by both the haustorium size and the proportion of haustoria with a developing shoot (Figure 3.2 B and C). However, there was no overall significant difference in either early- or late-stage resistance for the double mutant *eds1-2/pad4-1*. Given the intrinsic difficulty in performing these infection assays, batch effects may be attributed to differences in the progression of parasite infection, caused by variations in the time taken to infect all *Arabidopsis* plants.

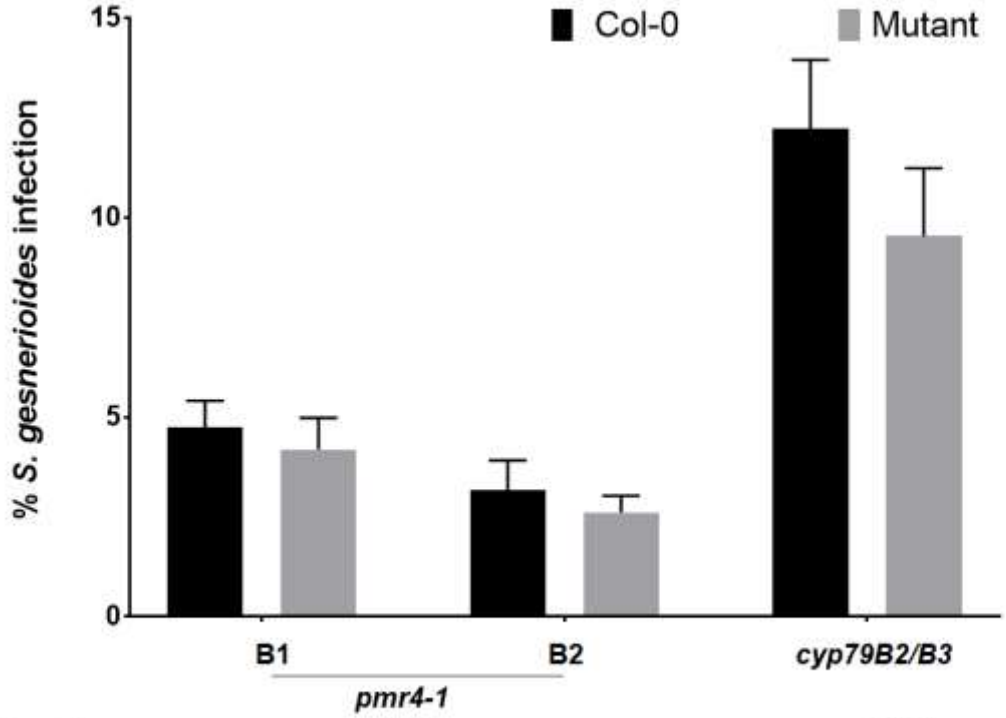
The different results for *eds1-2* and *eds1-2/pad4-1* could potentially be explained by a model whereby EDS1 has multiple functions, a subset of which depend on PAD4. Indeed, this is supported by current evidence. EDS1 acts independently in *Arabidopsis* to trigger rapid HR and local cell death against avirulent *Hyaloperonospora arabidopsidis* (Rietz et al. 2011). However EDS1 must form a complex with PAD4 to promote basal resistance against virulent *H. arabidopsidis* and *P. syringae*, which is characterised by SA-accumulation and induction of downstream responses that restrict pathogen growth (Rietz et al. 2011). Potentially, the early-acting PAD4-independent function of EDS1 causes an oxidative burst that favours *S. gesnerioides* infection. Indeed, the increased early-stage susceptibility of the *RbohD* and *RbohD/RbohF* mutants (Figure 3.6) implies that host ROS generation promotes parasite entry. A later-acting role of EDS1, executed in combination with PAD4, may trigger long-term defences effective against further development of the parasite. This may explain why *eds1-2* showed significantly reduced late-stage resistance but there was no overall effect on early-stage resistance. If EDS1/PAD4 function together to promote late-stage resistance however, one might expect a stronger phenotype for *pad4-1* which, unlike *eds1-2*, was not significantly affected in late-stage resistance. Possibly, related proteins function redundantly with PAD4 in partnering with EDS1. A candidate for this is Senescence Associated Gene 101 (SAG101); this forms a complex with EDS1 separately to PAD4, the role of which appears to be to reinforce responses induced by EDS1/PAD4 (Rietz et al. 2011), although independent functions for SAG101 in R-gene mediated resistance have been reported (Zhu et al. 2011a). It is intriguing that late-stage resistance was significantly reduced for *eds1-2,* but not the double mutant *eds1-2/pad4-1*. It may be that, in the absence of EDS1, PAD4 antagonises SAG101 activity through physical interaction. When both EDS1 and PAD4 are removed, SAG101 may then be

able to partially compensate for the loss of EDS1 function. Indeed, an EDS1-PAD4-SAG101 ternary complex has been reported (Zhu et al. 2011a) indicating a potential interaction between PAD4 and SAG101. Nevertheless, signalling activity is thought to be mediated exclusively by EDS1-PAD4 and EDS1-SAG101 heterodimers (Wagner et al. 2013). Recently, a function of PAD4 has been identified that requires neither EDS1 or SAG101, in regulating a phloem-based defence mechanism against feeding green peach aphids, apparently limiting sap uptake (Pegadaraju et al. 2007). The authors propose that, since PAD4 transduces ROS signals to promote cell death in response to pathogens and photo-oxidative stress, PAD4 may increase ROS in sieve-tube elements to promote protein coagulation that blocks the phloem vessels. Although *S. gesnerioides* is thought to only connect to the xylem and not the phloem (Dörr 1997), potentially this vascular-mediated defence mechanism may contribute towards some of the batch effect seen with *pad4-1* (Figure 3.2B).

The significant batch effects observed across this group of mutants may also result from subtle differences in environmental conditions modifying EDS1 activity. EDS1 is known to be sensitive to ROS levels and to promote SA-accumulation in response to singlet oxygen (Ochsenbein et al. 2006). As described in Chapter 1, *Striga* radicles are potent sources of H_2O_2 (Keyes et al. 2007, Kim et al. 1998, Wada et al. 2019); under conditions where parasite-generated ROS reach a certain threshold, EDS1 may be induced to promote SA accumulation which inhibits parasite entry. In genotypes where this is prevented (e.g. *sid2-1*), the mutant would be significantly more susceptible than the wild-type control. If the environmental conditions are such that *Striga*-generated ROS do not accumulate sufficiently around the root to activate EDS1 and SA-accumulation, there may be no noticeable difference between control and mutant lines. This could explain why for *eds1-2/pad4-1* batch 2, the significant difference between the control and mutant for both the % infection and haustorium size appears to be caused by increased resistance in the Col-0 (compared to Col-0 in batch 1), rather than increased susceptibility in *eds1-2/pad4-1*. Potentially in batch 2, but not batch 1, parasite ROS generation was higher, so that SA-accumulation via EDS1/PAD4 was induced, causing a protective effect in the wild-type. This could not occur in *eds1-2*, hence the virulence level on the mutant between batch 1 and 2 was similar. This does not explain, however, why in batch 1, *eds1-2* showed a significantly lower % infection than the control.

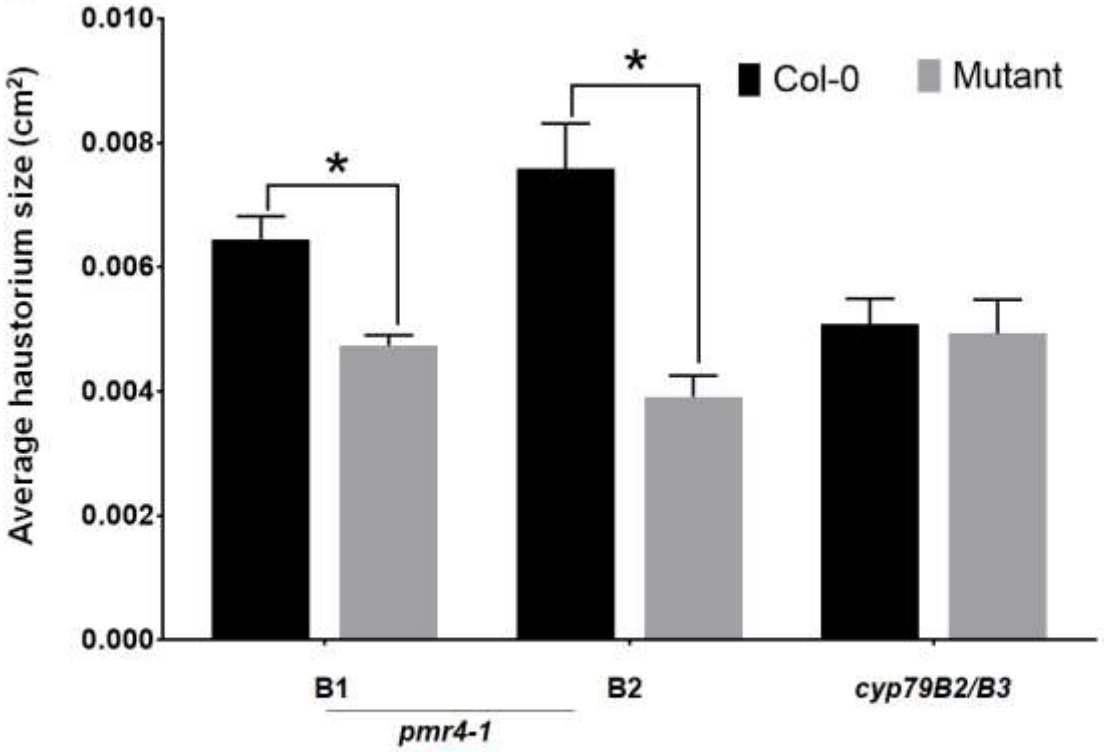
Interestingly, in a gene expression study on cowpea infected with different strains of *S. gesnerioides*, *EDS1* was found to be highly induced during non-host interactions, but not during a race-specific resistance response characterised by a rapid hypersensitive response. This indicates that certain resistant mechanisms effective against *S. gesnerioides* can act independently of *EDS1* (Li et al. 2009).

A Percentage infection of *Striga gesnerioides* on *Arabidopsis*: mutants impaired in defensive metabolites



Genotype	0.425	0.284
Batch	0.028	
Batch * Genotype	0.990	

B Average size of *Striga gesnerioides* haustoria on *Arabidopsis* roots: mutants impaired in defensive metabolites



Genotype	0.000	0.829
Batch	0.729	
Batch * Genotype	0.041	

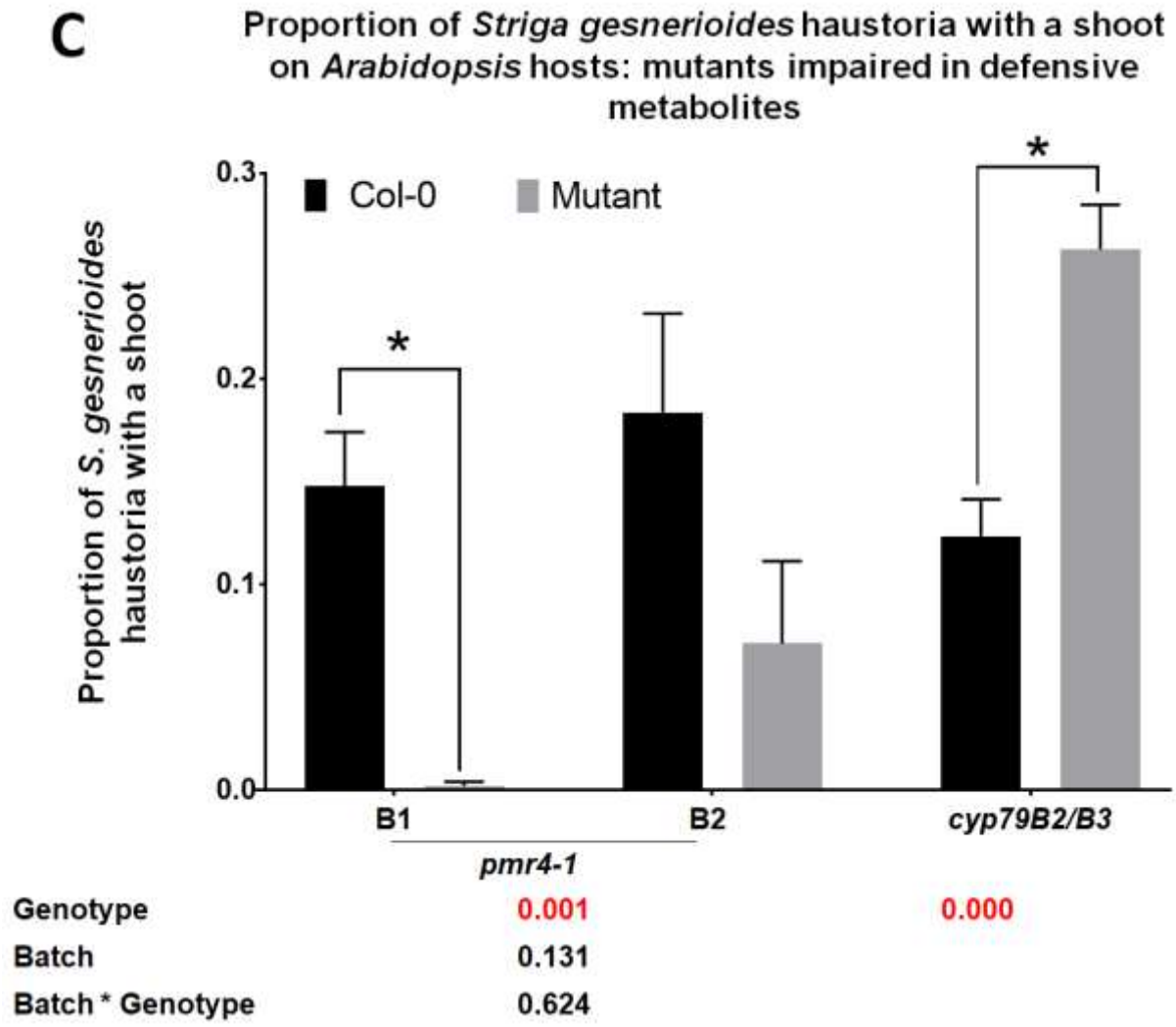


Figure 3.7: Basal resistance phenotypes of *Arabidopsis* mutants with defects in the production of callose (*pmr4-1*) or indole glucosinolates/camalexin (*cyp79B2/B3*), infected with *Striga gesnerioides* at 3 weeks post-infection compared with wild-type (Col-0). **A:** Number of attached *S. gesnerioides* haustoria per host expressed as a % of total parasite seed applied. **B:** Average size of each individual attached parasite haustorium. **C:** Proportion of attached haustoria with a developing shoot, an indicator of advanced development. Error bars show mean +/- standard error. B1, B2 = Batch 1, Batch 2 (for genotypes where two batches were screened). * denotes a significant difference ($p \leq 0.05$) between the mutant line and the Col-0 control according to Student's T-test. Figures beneath each graph describe the results of Student's T-test (where the experiment only contained one batch of plants) or two-way ANOVA for significant batch and genotype effects (where two batches of plants were screened as part of the experiment). Significant figures are indicated in red. For each batch, between 10 and 20 plants were infected for both the Col-0 wildtype and mutant line (equal numbers of each). Normality and homogeneity of variance checks were carried out in each case.

3.5.2 Reactive oxygen species (ROS) may favour *S. gesnerioides* invasion

Overall, both *RbohD* and *RbohD/RbohF* showed significantly greater early-stage resistance to *S. gesnerioides*, although there was no difference for *RbohF* (Figure 3.6A). Haustorium size meanwhile was significantly lower in *RbohD* and slightly reduced in *RbohD/RbohF* but not affected in *RbohF* (Figure 3.6B). The proportion of haustoria with a developing shoot was significantly reduced in *RbohD/RbohF*, but not affected in either *RbohD* or *RbohF* (Figure 3.6C). These results indicate that *RbohD* has a prominent role in basal resistance against *S. gesnerioides*, but not so much *RbohF*, and that ROS generated by this NADPH oxidase promotes both initial attachment and later development of the parasite. This is supported by current understanding that RBOHD and RBOHF have distinct functions, with RBOHD thought to be the main source of ROS generated upon pathogen entry (Torres et al. 2002, Fagard et al. 2007, Morales et al. 2016, Perchepied et al. 2010). RBOHF, meanwhile, is believed to function mainly in regulating and restricting subsequent HR that leads to cell death (Proels et al. 2010, Torres et al. 2002). As described above (Section 3.1.5), RBOHD and RBOHF do seem to act synergistically in basal resistance against certain pathogens (Perchepied et al. 2010, Morales et al. 2016, Torres et al. 2002), which may explain the more pronounced phenotype of *RbohD/RbohF* (compared with *RbohD*) regarding the % infection and the proportion of haustoria with a developing shoot. Interestingly, RBOHD is positively regulated by the Ca2+-dependent protein kinase CPK5, which is itself stimulated by H_2O_2 treatment (Dubiella et al. 2013). Potentially, H_2O_2 generated by *S. gesnerioides* radicles induces further ROS production from RBOHD in a positive amplification loop that overwhelms host defences.

Nevertheless, no significant genotype effect was observed for *prx33* regarding any of the parameters tested, although in batch 2 the proportion of haustoria with a shoot was significantly greater than the control line (Figure 3.6C). Given the results with the *Rboh* mutants, it is perhaps surprising that *prx33* did not show enhanced resistance, particularly as host cell wall peroxidases are thought to act on H_2O_2 released by *Striga* radicles, liberating polyphenols that induce the transition to haustorium development (xenognosins or Haustorial Initiation Factors, HIFs)(Keyes et al. 2007). It should be borne in mind, however, that unlike RBOH NADPH oxidases, peroxidases act in both detoxification and ROS generation (Mathé et al. 2010). The lack of detoxifying activity in *prx33* could negate any positive effect from reduced production of xenognosins or ROS. In any case, the functions of PRX33 in pathogen defence overlap with those of PRX34 and potentially with other peroxidases (Daudi et al. 2012). Indeed, a gene expression analysis of the non-compatible interaction between *S. hermonthica* and *Arabidopsis* found *PRX34* to be induced over 4-fold in the host root during the early stages on infection (Vasey 2005), suggesting that PRX34 may have a greater role in this interaction. ROS generation and pathogen resistance are more severely impaired in *Arabidopsis* where both PRX33 and PRX34 are disabled, for instance through transgenic expression of an antisense heterologous peroxidase gene

from French Bean (asFBP1.1) (Daudi et al. 2012). In future experiments, it could be informative to test the *prx33/prx34* double mutant, or experimentally manipulate the level of ROS through the addition of catalase or the NADPH oxidase inhibitor diphenylene iodonium (DPI), for instance.

3.5.3 Jasmonic acid may promote host susceptibility *to S. gesnerioides*

In contrast to *sid2-1*, *aos1* had a significantly reduced % infection (Figure 3.3A), indicating that JA biosynthesis hinders early-stage resistance to *S. gesnerioides*. This fits the understanding that JA and SA are mutually antagonistic, yet is in marked contrast to the interaction between *hebiba* rice and *S. hermonthica*, where deletion of the JA-biosynthesis gene *ALLENE OXIDE CYCLASE* increased host susceptibility (Mutuku et al. 2015). Nevertheless, the % infection of *opr3-1* roots was not significantly affected; this mutation disrupts JA production from 12-oxo-phytodienoic acid (OPDA). Certain studies indicate that *opr3-1* is only a partial mutation since it accumulates a reduced level of JA in response to *B. cinerea* (Chehab et al. 2011) and shows resistance to insect herbivores and *A. brassicicola* (Stintzi et al. 2001). This could explain why the phenotype of *opr3-1* is not as severe as *aos1*. Enhanced early-stage resistance was not seen in the JA-signalling mutant *jar1-1*, which cannot produce conjugated JA-Ile. This suggests that the effect of JA on early-stage resistance is not mediated by genes downstream of the JAZ7/COI1 complex. Potentially, the effect of JA is mediated via different conjugated forms, possibly through interaction with key transcription factor nodes. Alternatively, the increased resistance of *aos1* may primarily be caused by the absence of OPDA, rather than JA. Indeed, OPDA has signalling activity independent of JA, since OPDA-treatment of *opr3-1* induces many of the known COI1-regulated wound-response genes, in addition to a suite of genes not thought to be regulated by JA, MeJA and COI1; curiously, half of these genes are activated in *Arabidopsis* during wounding (Taki et al. 2005, Stintzi et al. 2001). Interestingly, the results of this present study mirror those of an investigation into the interaction between *Arabidopsis* and the RKN *Meloidogyne hapla* (Gleason et al. 2016). Whilst *delayed dehiscence2* (*dde2-2*) mutants (which have defective AOS function) showed increased susceptibility to *M. hapla*, *opr3* plants had wild-type levels of resistance, causing the authors to conclude that OPDA perception is required to maintain basal resistance. Accordingly, knock out mutants in the OPDA receptor peptidyl-prolyl cis-trans isomerase 3 (CYP20-3) showed increased susceptibility. Testing the phenotype of *cyp30-2 Arabidopsis* mutants when infected with *S. gesnerioides* could help to shed light on the exact distinction between the roles of OPDA and JA in this interaction.

The JA-insensitive mutant *jar1-1*, however, did show a phenotype regarding the haustorium size, as this was significantly larger compared with the control (Figure 3.3B). Whilst this indicates that signalling via JAZ7/COI1 promotes late-stage resistance to *S. gesnerioides*, this is countered by haustorium size being significantly lower for *opr3-1*. Potentially, where JA cannot be conjugated to JA-Ile, alternative

forms accumulate that disrupt other defence pathways, causing the increased late-stage susceptibility in *jar1-1*. In support of this, one study found that although levels of JA-Ile are reduced by 12-fold in *jar1-1* roots, total JA concentrations are only slightly reduced, indicating the presence of other JA-conjugated forms (Suza and Staswick 2008). Since haustorium size was not affected on *aos1,* this argues against OPDA having an effect independent of JA that promotes late-stage susceptibility (causing the lower haustorium size for *opr3-1*). Alternatively, if *opr3-1* is indeed a partial mutation, the residual level of JA may have a synergistic effect with SA for late-stage resistance, rather than the antagonistic effect seen with higher concentrations, as indicated by certain studies (Mur et al. 2006). This is supported by the observation that the SA-analogue BTH partly rescued the increased susceptibility of the rice *hebiba* mutant (Mutuku et al. 2015). Indeed, the authors of this study concluded that correct activation of downstream JA-responses is critical for effective host resistance, and this is achieved through careful balance of SA/JA levels (see Chapter 1, Section 1.8.8). Given that rice has higher endogenous levels of SA than *Arabidopsis* (Raksin et al. 1990), this may explain the different results seen when JA-biosynthesis enzymes are mutated: JA levels may need to be reduced in *Arabidopsis* for the optimum defence response, but increased in rice. Curiously, neither *opr3-1, jar1-1* or *aos1* showed a significant difference regarding the number of haustoria with a developing shoot (Figure 3.3C), indicating that this developmental transition is not influenced by JA-mediated defences in the host.

Ultimately, the relative contributions of JA biosynthesis, JA-Ile signalling via COI1 and OPDA signalling to host basal resistance could be further dissected by assessing the phenotype of the *coi1* mutant. Most *coi1* plants are male sterile and thus challenging to propagate (Feys et al. 1994), hence why *coi1* was not included in this study. The *coi1-16* mutant line, however, is conditionally fertile and produces viable pollen at 16°C but not 22°C (Ellis and Turner 2002): this could be an option for future assays. In addition, a more strongly affected allele of *opr3* could also be investigated, such as *opr3-2* which has an early stop codon in the cDNA sequence (Qu et al. 2010).

3.5.4 Ethylene and ABA may promote *S. gesnerioides* attachment and development

The ethylene-insensitive mutant *etr1-1* showed significantly greater early-stage resistance to *S. gesnerioides*, compared with wildtype Col-0. Since *aos1* also had a lower % infection, this could indicate that pathways co-regulated by JA/ethylene promote susceptibility to *S. gesnerioides* during attachment. The *ein3-1* mutant, however, did not show a significant phenotype yet the regulatory effects of ethylene on JA signalling are generally considered downstream of EIN3-1 (Figure 3.1). Furthermore, *jar1-1* was also not affected in early-stage resistance, yet induction of ethylene/JA co-regulated pathways is thought to be via COI1 (Figure 3.1). Interestingly, a study reported similar results for *Arabidopsis* infected with the cyst nematode *Heterodera schachtii*, where *etr1-1* showed

significantly increased resistance, whereas no difference was seen for *ein3-1* or *ctr1-1*, which functions upstream of EIN3 (Piya, Binder and Hewezi 2019). Using a series of double and triple mutants, the authors developed a model whereby ETR1 can function in a non-canonical pathway independent of CTR1 and EIN3, in which ETR1 appears to be the sole receptor. In this pathway, ETR1 kinase activity on cytokinin signalling components activates transcriptional programs that favour nematode infection. Indeed, cytokinin is transiently induced in *H. schachtii* at infection sites and *Arabidopsis* cytokinin signalling mutants (e.g. type-B ARR mutants and the *ahp1/2/3* triple mutant) show reduced susceptibility to nematode infection (Siddique et al. 2015, Shanks et al. 2015). It has thus been proposed that cytokinin has a critical role in inhibiting host defences and establishing sink activity during the formation of nematode feeding sites.

There is also evidence that cytokinin is important for parasitic plants to establish the haustorium. In the interaction between the hemi-parasite *Phtheirospermum japonicum* and *Arabidopsis*, transfer of cytokinin has been demonstrated from the parasite to the host once vascular connectivity is established (Spallek et al. 2017). This appears to induce hypertrophy in the host root around the infection site; a process dependent on the cytokinin signalling genes *AHK3* and *AHK4*. Similarly, exogenous cytokinin promotes aggressiveness and haustorial development of *Phelipanche ramosa* on *Brassica napus*, whilst treatment with the cytokinin receptor inhibitor PI-55 inhibits this (Goyet et al. 2017). Exogenous cytokinin and cytokinin-like compounds have also been shown to induce haustoria formation in *S. asiatica* and *S. hermonthica* (Keyes et al. 2000, Babiker, Parker and Suttle 1992), although one study concluded that there was no effect for *S. gesnerioides* (Babiker et al. 1992). Collectively, these results indicate that the significantly greater early- and late-stage resistance of the *etr1-1* mutant may be due to disruption of the non-canonical signalling pathways that involves cytokinin. This could be explored further using cytokinin-signalling mutants such as *ahk3* and *ahk4*.

Although an overall genotype effect was not observed for *etr1-1* for haustorium size, the proportion of haustoria with a developing shoot was significantly reduced. This was also the case for *ein3-1*, indicating that ethylene signalling through the canonical-EIN3 dependent pathway promotes the transition to shoot development in *S. gesnerioides*. The fact that neither of the JA-associated mutants tested had a significant phenotype in this parameter indicates that this effect results from a function of ethylene that is independent of JA. Indeed, ethylene is known to suppress the flowering transition and appears to do this through EIN3-dependent modulation of DELLA transcription factors, which inhibit plant growth (Achard et al. 2007).

Curiously, *abi1-2*, which shows enhanced ABA sensitivity (Saez et al. 2006), was similar to *ein3-1* in that no significant effect on the % infection or haustorium size was observed (Figures 3.4 A-B), however the proportion of haustoria with a developing shoot was slightly higher on *abi1-2* compared with the

control (Figure 3.4C). ABA is generally regarded as an inhibitor of shoot development (Davies 2013), hence this result may be due to altered source : sink relationships with the parasite, rather than direct hormonal activity. *S. hermonthica* is known to contain high concentrations of ABA in shoot tissue and to induce ABA accumulation in the host (Taylor et al. 1996), presumably as a means to increase water transfer. In the interaction between *S. gesnerioides* and *Arabidopsis* therefore, increased ABA sensitivity in the host may allow the parasite to generate a stronger sink, thus fuelling more rapid development of shoot tissues. There is evidence that ABA and JA/ethylene signalling pathways are antagonistic, with ABA supressing induction of JA/ethylene regulated defence pathways (Anderson et al. 2004, Nahar et al. 2012). In addition, *etr1-1 Arabidopsis* show reduced ABA sensitivity in the roots, as measured by root-growth inhibition (Beaudoin et al. 2000). Thus, the significantly reduced proportion of haustoria with a shoot seen on *etr1-1* and *ein3-1* may be caused in part by reduced ABA sensitivity. It would be informative to investigate this further using ABA-insensitive mutants, e.g. *abi1-1, abi2-1*.

3.5.5 *ora59* shows impaired early-stage resistance against *S. gesnerioides*

ora59 showed, overall, significantly impaired early-stage resistance (Figure 3.5A) although a strong interaction effect between batch and genotype suggests this is only present under certain conditions. Although *ORA59* is induced synergistically by ethylene and JA downstream of JAR1 and EIN3 (Zhu et al. 2011b), neither *jar1-1* or *ein3-1* showed a significantly different % infection. It may be that in these mutants, the remaining functional signalling pathway is able to stimulate *ORA59* to an adequate level of expression. This result suggests that necrotrophic-associated defence genes downstream of ORA59, such as *PDF1.2* (Pré et al. 2008), may have a protective role against *S. gesnerioides* attachment. However, this would counter the result with *aos1* and *etr1-1*, which both showed a significantly reduced % infection. At present, it is not clear whether ORA59 has any functions outside of this pathway, although it has been proposed to act downstream of ethylene to make the JA pathway insensitive to future suppression by SA (Leon-Reyes et al. 2010a). The result with *sid2-1*, however, suggests that SA protects against *S. gesnerioides* attachment so it seems unlikely that the *ora59* phenotype is caused by de-repression of SA signalling. This could be investigated further using an over-expressor line of either *ORA59* (He et al. 2017) or *ERF1* (Berrocal-Lobo et al. 2002), which is also a positive regulator of necrotrophic defences.

3.5.6 The MYC-branch of JA signalling may not be important in basal resistance against *S. gesnerioides*

MYC2/JIN1 and ATAF2 overlap somewhat in that both appear to suppress defences downstream of SA, besides necrotrophic-associated genes co-induced by JA/ethylene (Figure 3.1). Neither *AtAF2* or *jin1-7* showed any significant overall effects on basal resistance to *S. gesnerioides*, relative to the control. The lack of a result with *AtAF2* may reflect redundancy with other transcription factors, for instance the various NAC transcription factors that are induced by MYC2 to suppress SA signalling (Figure 3.1). This is supported by previous studies which found *AtAF2* knock-out mutants to show very few gene expression differences compared with wild-type Col-0 and no significant difference in resistance against *F. oxysporum* (Delessert et al. 2005). Potentially, a role for *AtAF2* in basal resistance against *S. gesnerioides* would be revealed by the *35S:AtAF2* over-expression mutant since this shows pronounced suppression of various pathogenesis-related genes (including *PR1*, *PR2*, *PR4*, *PR5*, *PDF1.1* and *PDF1.2*) and significantly greater susceptibility to *F. oxysporum* (Delessert et al. 2005).

In contrast to *AtAF2*, *jin1/myc2* knock-out mutants have significant gene expression differences (particularly upregulation of necrotrophic genes, e.g. *PDF1.2, B-CHI* and *HEL/PR4*), besides increased resistance to *F. oxysporum* (Anderson et al. 2004, Lorenzo et al. 2004). Furthermore, MYC2 is a positive regulator of wound-response genes such as *VSP2* (Lorenzo et al. 2004) that could be expected to be induced as *S. gesnerioides* penetrates the root cortex. Possibly, *S. gesnerioides* is able to suppress the host wound response, or this may be induced via other transcription factors. Alternatively, as discussed above, wound-response genes may be induced via OPDA. Induction of wound-response genes during infection by *S. gesnerioides* is investigated in Chapter 4.

3.5.7 *erf4-1* mutants are significantly more susceptible to *S. gesnerioides*

One of the most striking results was seen with *erf4-1*, since this showed a significantly increased % infection, but was not affected in late-stage resistance. ERF4 functions as a negative regulator of necrotroph-associated defence genes downstream of JA/ethylene, with *erf4* reported to show 30-fold higher basal *PDF1.2* expression and increased resistance to *F. oxysporum* (McGrath et al. 2005). The result with *erf4-1* therefore suggests that enhanced expression of JA/ethylene co-regulated genes promotes initial attachment and infection by *S. gesnerioides*, however this contradicts the result seen with *ora59*, suggesting that the basis for the *erf4-1* phenotype is caused by another function. ERF4 is regarded as a transcriptional repressor, due to the presence of an EAR (Ethylene-responsive element binding factor-associated Amphiphilic Repression) domain (Nakano et al. 2006). It was recently discovered, however, that under certain conditions alternative polyadenylation of the *ERF4* gene transcript generates a novel variant that lacks the EAR domain and acts as a transcriptional activator

(Lyons et al. 2013). Over-expression of *ERF4-Activator* suppresses flg22-triggered ROS burst and enhances basal expression of *PDF1.2.* Conversely, over-expression *ERF4-Repressor* augments flg22-triggered ROS burst and reduces basal *PDF1.2* expression (Lyons et al. 2013). The location of the T-DNA insertion of the mutant line used in this study (SALK_073394C) is such that only *ERF4-Activator* may be affected. In this case, the mutant line may still express *ERF4-Repressor* but be unable to induce *ERF4-Activator*. Therefore, the increased susceptibility of *ora59* and *erf4-1* in these assays may be linked by a reduced ability to induce *PDF1.2* and similarly regulated genes. This scenario is investigated further in Chapter 5, where the relative contributions of *ERF4-Activator* and *ERF4-Repressor* to this phenotype are investigated further.

3.5.8 WRKY70 may be an important component of early host resistance

It is curious that the % infection was significantly increased on *wrky70* (Figure 3.5A) but not *npr1-1*, since WRKY70 is a direct target of NPR1 (Wang et al. 2006) although studies indicate that *WRKY70* function is not completely abolished in *npr1* mutants (Ülker, Mukhtar and Somssich 2007). It has been proposed that WRKY70 induction by SA during pathogen challenge follows two distinct phases; an early response which is independent of NPR1 and a later amplification loop involving both SA and NPR1 (Li et al. 2004). The various functions attributed to WRKY70 make it challenging to decipher the basis of this phenotype. The fact that *ora59* also showed, overall, an increased % infection indicates that de-repression of necrotrophic associated defences is not the basis of impaired early-stage resistance in *wrky70*. As *wrky70* showed no significant effect on late-stage resistance, this could indicate that initial penetration by *S. gesnerioides* is favoured by altered basal conditions in this mutant, rather than WRKY70 signalling activity. *wrky70* mutants have been demonstrated to show increased basal expression of a broad range of defence-associated genes, both those regulated by SA (*PR1, PR2, PR5, PAD4*) and JA/ethylene-associated genes (*PDF1.2, COR1*) besides *PR3, GST1* and *PAD3* (Li et al. 2017, Ülker et al. 2007). For many of these genes, however, induction in response to the bacterial necrotroph *Pectobacterium carotovorum* is similar between wild-type plants and *wrky70* (Li et al. 2017). This again suggests that altered basal conditions, rather than WRKY70 signalling, may be the basis of the *wrky70* phenotype regarding basal resistance against *S. gesnerioides*.

wrky70 mutants also show increased accumulation of SA, under both basal conditions and in response to biotrophic pathogens (Wang et al. 2006). Nevertheless, the result with *sid2-1* indicates that SA has a protective effect against infection by *S. gesnerioides*. In any case, the increased SA accumulation in *wrky70* is not enough to confer greater resistance to biotrophic pathogens, including *P. syringae pv. maculicola* (Wang et al. 2006) and *Pst* DC3000 (Li et al. 2017, Li et al. 2004). Furthermore, WRKY70-mediated suppression of SA-accumulation is thought to act downstream of NPR1 (Wang et al. 2006) and *npr1*/*nim1* mutants accumulate more SA during pathogen challenge than wild-type (Delaney et al.

1995, Shah et al. 1997). Since *npr1-1* was not affected in early-stage resistance, this again suggests that altered SA-accumulation is not the sole basis for the *wrky70* phenotype.

Potentially, the result with *wrky70* is best explained using the model proposed by (Li et al. 2017), where the influence of the *wrky70* mutation depends on the prevailing level of ROS. In their study, both *wrky54/wrky70* double mutants and *WRKY70* over-expressor lines showed increased ROS accumulation, although this was much higher and more localised in the over-expressor lines. In *WRKY70* over-expressor plants, the increased ROS caused enhanced HR and cell death thus greater resistance to *Pst* DC3000. In *wrky54/wrky70* however, the more moderate level of ROS induced defence-related gene expression, antimicrobial compound synthesis and cell wall fortification that increased resistance against necrotrophic pathogens, but was not enough to promote HR and resistance to biotrophic pathogens. In this study, *wrky70* single mutants also showed greater basal ROS accumulation and expression of *GST1* (indicative of oxidative stress), although not as severely as *wrky54/ wrky70* (Li et al. 2017). Since *Striga* radicles are sources of ROS (Kim et al. 1998, Keyes et al. 2007, Wada et al. 2019), these may generate a positive feedback loop with the accumulated ROS in *wrky70* that overwhelms host defence. This could explain the increased early-stage resistance of *RbohD* and *RbohD/RbohF* as these mutations inhibit ROS production by host NADPH enzymes. In wild-type plants without the *wrky70* mutation, ROS levels may be maintained at a low enough level to activate the appropriate host defence. It could be informative to test the *wrky70/sid2-1* mutant which eliminates many of the basal effects of the *wrky70* mutation including high basal gene expression and ROS accumulation, besides the *wrky54/ wrky70* double mutant and a *WRKY70*-overexpressor line, as these show even greater ROS accumulation (Li et al. 2017).

3.5.9 Opposing effects of callose and glucosinolates/camalexin in late-stage resistance to *S. gesnerioides*

pmr4-1 showed no difference in early-stage resistance, however both haustorium size and the proportion of haustoria with a flowering shoot were significantly lower compared with the control (Figures 3.7A-C). It is difficult to assess whether this is due to the absence of callose deposition or increased SA accumulation in *pmr4-1* (Vogel and Somerville 2000, Nishimura et al. 2003). Callose deposition has been associated with post-attachment resistance against various species of parasitic plants (Letousey et al. 2007, Pérez-de-Luque et al. 2007), which would argue against the *pmr4-1* phenotype being caused by defective callose production. Nevertheless, the ABA-hypersensitive mutant *abi1-2* showed a significantly higher proportion of haustoria with a shoot and ABA has been demonstrated to positively regulate callose deposition (Ton and Mauch-Mani 2004, Ton et al. 2005), although under certain conditions it acts as a negative regulator (Luna et al. 2011). When considered with the result for *pmr4-1*, this could suggest that host-derived callose promotes shoot development

of attached *S. gesnerioides* haustoria, although the result with *abi1-2* may also be due to stronger sink activity in the parasite, as discussed above. If enhanced late-stage resistance in *pmr4-1* was due to increased SA activity however, it is curious that early-stage resistance was not also increased, given the result seen with *sid2-1*. Ultimately, the basis of the *pmr4-1* phenotype could be further assessed using the *pmr4-1/NahG* double mutant, which is unable to accumulate SA due to the presence of bacterial salicylate hydroxylase enzyme, or *pmr4-1/npr1-1* (Nishimura et al. 2003).

The *cyp79B2/B3* mutant, defective in indole glucosinolates and camalexin, was not significantly affected in the parasite % infection or haustorium size (Figures 3.7A-B), however the proportion of haustoria with a developing shoot was significantly higher (Figure 3.7C). This indicates that defensive glucosinolates and/or camalexin may delay *S. gesnerioides* shoot emergence, although since haustorium size was not affected, this argues against an overriding role in late-stage resistance. Potentially, these compounds may only be effective after the parasite has connected to the host vasculature. Glucosinolates have a biochemical structure compatible with phloem transport (Brudenell et al. 1999) and radio-labelled glucosinolates have been traced from sites of production in leaves to distant tissues via the phloem (Chen et al. 2001). Particularly high glucosinolate concentrations are found in specialised *Arabidopsis* cells located between the phloem and endodermis (Koroleva et al. 2000). If glucosinolate production is concentrated around the phloem, this could explain why the *cyp79B2/B3* mutant only differs from the Col-0 control at the very latest stage of infection measured, when the parasite is connected to the host vascular system.*cyp77B2/B3* mutants also show increased susceptibility to the fungus *Verticillium longisporum* which, like *Fusarium* spp. is a soil-borne vascular pathogen (Iven et al. 2012). Interestingly, although camalexin inhibits the growth of *V. longisporum* in vitro, *pad3*, which cannot perform the final step of camalexin biosynthesis, does not show increased susceptibility; this is also the case for *cyp81f2*, specifically inhibited in the production of indole glucosinolates. This suggests that other tryptophan-derived secondary metabolites may have antifungal activity in this interaction, or that camalexin and indole glucosinolates may have an additive effect (Iven et al. 2012). Ultimately, further genetic dissection using mutants such as *pad3* and/or *cyp81f2* is needed to decipher the basis of the *cyp79B2/B3* phenotype.

Collectively, these results indicate that multiple host defence-pathways may contribute to basal resistance against *S. gesnerioides*. The sheer complexity of host defence pathways and redundancy between regulatory nodes however, makes it difficult to determine the underlying basis for the phenotypes observed. Analysis of the genes expression changes that occur in host roots during the compatible interaction may provide greater insight. This is the focus of Chapter 4, which presents a gene-expression analysis of a time-course comparing Col-0 *Arabidopsis* roots infected with *S. gesnerioides* with uninfected controls.

CHAPTER FOUR

Analysis of gene expression changes in host *Arabidopsis* plants during infection with *Striga gesnerioides*.

ABSTRACT

To further investigate the nature of the *Arabidopsis* host response against *S. gesnerioides*, gene expression analyses were performed on infected root samples. β-glucuronidase (GUS) reporter assays proved unsuitable due to a coloured reaction product forming in *S. gesnerioides*, even though the parasite did not express the reporter transgene. 3,3'-Diaminobenzidine (DAB) staining indicated reactive oxygen species (ROS) at the host-parasite interface. Quantitative polymerase chain reaction (qPCR) was performed on root systems harvested from control and infected *Arabidopsis* at 7, 10 and 12 days post-infection, testing a range of defence-associated genes. This identified three broad clusters of genes: strongly induced by infection; slightly induced and genes showing minimal variation between control and infected samples. Genes that were strongly induced (i.e. showing more than 5-fold upregulation for at least one time point) had functions related to salicylic acid (SA) defences (*PR2, PR5*), camalexin biosynthesis (*PAD3*) and ROS detoxification (*PRX33*). *GST1, WRKY70, B-CHI, PR4, ORA59, ERF2* and *PRX53* showed some induction in response to *S. gesnerioides*, but never more than 3-fold compared with the reference control. Meanwhile *ERF4, ERF1, PAD4, PR1, PDF1.2, VSP2* and *MYC2* expression showed little variation between control and infected samples. A signature similarity search using the GENEVESTIGATOR database indicated that the response of *Arabidopsis* to infection by *S. gesnerioides* is highly similar to that induced by biotrophic pathogens and SA treatment.

4.1 Introduction

The results of Chapter 3 indicate that the plant defence hormones salicylic acid (SA), jasmonic acid (JA) and ethylene contribute to basal host resistance against the parasitic weed *Striga gesnerioides*. This suggests that genes regulated by these hormones are likely to show differential expression during infection. Furthermore, the enhanced susceptibility of the *RbohD* and *RbohD/RbohF* mutants suggests that an excess of reactive oxygen species (ROS) favours the parasite's entry. Thus, it may be expected that genes associated with ROS detoxification may also show upregulation during infection. Understanding the transcriptional differences between compatible and non-compatible interactions is of great interest to those seeking the molecular basis of resistant responses. Various *Striga*-host interactions have already been the subject of such analysis. In many cases, the results indicate that different host phenotypes are often associated with subtle quantitative and temporal variations of gene activity, rather than striking qualitative differences.

The enhanced susceptibility of the SA-deficient mutant *sid2-1* (Chapter 3, Figure 3.2) indicates that SA has a protective effect against *Striga* parasites, which has been suggested by previous studies. This includes an investigation which used suppression subtractive hybridisation (SSH) to identify 30 genes upregulated in susceptible sorghum cultivars during infection by *Striga hermonthica* (Hiraoka and Sugimoto 2008). Many of these upregulated genes differed in their response to external applications of SA between the two cultivars, showing reduced expression in the more susceptible cultivar and enhanced expression in the more resistant cultivar, suggesting that enhanced SA signalling activity could contribute to basal host resistance. The gene responses to MeJA, however, were largely similar. Nevertheless, two genes regulated by jasmonic acid (JA), *LOX* (lipoxygenase) and *FAD* (fatty acid desaturase), were induced to a much greater extent in the more susceptible cultivar, indicating that JA may promote susceptibility to *Striga*.

Following this, these authors used SSH to compare gene expression in *Lotus japonicus* infected with *Orobanche aegyptiaca* (a compatible interaction) and *S. hermonthica* (a non-compatible interaction) (Hiraoka, Ueda and Sugimoto 2008). Despite being applied to the same host (using a split root system), these parasites induced distinct gene responses. Specifically, *S. hermonthica* activated genes relating to phytoalexin biosynthesis, whilst *O. aegyptiaca* induced genes associated with JA synthesis. However, both parasites induced genes relating to detoxification of ROS and cell wall fortification.

In a study on the interaction between rice and *S. hermonthica*, whole genome microarrays were used to profile gene expression changes in a susceptible and resistant cultivar (Swarbrick et al. 2008). This analysis revealed that the response of the resistant cultivar (Nipponbare) was characterised by

upregulation of genes associated with pathogenesis-related (PR) proteins (e.g. endochitinases, glucanases and thaumatin-like proteins), WRKY transcription factors, secondary metabolism and ROS detoxification. In the susceptible cultivar, however, *S. hermonthica* caused wide-scale suppression of gene expression, especially genes associated with cell division, the synthesis of cellular compartments and growth regulator signalling (particularly gibberellins and auxins). On the other hand, genes related to amino acid metabolism, abiotic stress and nutrient transport were upregulated in the susceptible cultivar (Swarbrick et al. 2008). This indicates that, during incompatible interactions with *S. hermonthica,* a range of defences that are commonly induced by plant pathogens are activated, which may be suppressed by the parasite during the compatible (susceptible) interaction.

Distinct gene expression differences have also been found for three classes of interactions between cowpea and *S. gesnerioides* (Li et al. 2009). These included a compatible interaction; an incompatible interaction based on race-specific resistance and an incompatible interaction based on non-host resistance (using a race of *S. gesnerioides* which specifically infects the wild legume *Indigofera hirsutum*). High induction of pathogenesis-related protein *PR5* occurred during race-specific resistance, but not in the compatible or non-host interactions. *PR5* is induced by SA and is considered a maker for systemic acquired resistance (SAR) (Uknes et al. 1992). Conversely, *EDS1*, a signal transduction element of the SA pathway (Falk et al. 1999), was highly induced during the non-host interaction, slightly induced during the compatible interaction but remained unchanged during expression of race-specific resistance. Meanwhile, *COI1*, a component of the JA signalling pathway that inhibits SA signalling (Xie et al. 1998), was induced in the compatible and non-host interactions, but not during race-specific resistance (Li et al. 2009).

More recently, a comprehensive gene-expression analysis was conducted on cowpea, using a custom microarray to compare the host response during race-specific resistance to *S. gesnerioides* with the compatible interaction (Mellor 2013). During the race-specific resistance response, genes associated with wounding, oxidative stress, the hypersensitive response, abiotic and biotic stimuli, and JA and ethylene signalling pathways were upregulated. In addition, genes with functions that could prevent further parasite entry were also upregulated; for instance, genes involved in secondary cell wall strengthening (e.g. lignification) and cell death (Mellor 2013). On the other hand, infection with virulent S*. gesnerioides* caused wide-scale gene downregulation, including genes relating to SA and JA signalling, cell wall biosynthesis, and phenylpropanoid and lignin biosynthesis. Similar with compatible interactions between rice and *S. hermonthica* (Swarbrick et al. 2008), genes related to auxin and gibberellin function were downregulated in the host (Mellor 2013).

Of particular interest to this investigation is a study on the transcriptomic response of *Arabidopsis* during the non-host interaction with *S. hermonthica* (Vasey 2005). Using whole genome oligonucleotide arrays, this study detected gene upregulation related to various processes, including cell wall biosynthesis, oxidative stress and antimicrobial compounds. Interestingly, genes relating to both SA signalling (e.g. *EDS1, PR2, PR5*) and JA signalling (e.g. *PDF1.2, PAD3*) were upregulated. Besides this, the regulatory transcription factor *WRKY70* was significantly induced during infection. In the *Arabidopsis* mutant screen described in Chapter 3, the *wryk70* mutant showed significantly reduced early-stage resistance to *S. gesnerioides* (Chapter 3, Figure 3.4), providing further evidence that this gene be relevant in host basal resistance.

This chapter describes the results of two methods to assess gene expression changes in *Arabidopsis* during the compatible interaction with *S. gesnerioides*: the β-glucuronidase (GUS) reporter system and quantitative PCR (qPCR). Taking into account the results described in Chapter 3, genes relating to the SA and JA/ethylene signalling pathways, ROS detoxification and key defence-associated transcription factors were of particular interest. In addition, diaminobenzidine (DAB) staining was used to investigate ROS activity at the host-parasite interface.

Based on the results of the mutant assay in Chapter 3, the following hypotheses were made:

1. SA-associated genes may show strong upregulation during the early stages of parasite attachment, since the *sid2-1* phenotype indicates a protective effect of SA.

2. Conversely, JA genes may be repressed during parasite infection since the decreased % infection of *aos1* indicates that JA may promote *S. gesnerioides* attachment.

3. The phenotype of *RbohD* and *RbohD/RbohF* indicates that ROS promote *S. gesnerioides* infection. Therefore, ROS detoxification genes, e.g. *GST1*, may be highly elevated in the host. DAB staining may reveal ROS at the host-parasite interface.

4. *WRKY70, ORA59* and *ERF4* may show strong induction as the corresponding mutants showed significantly impaired early-stage resistance.

5. The weak phenotype of *jin1-7* and *AtAF2* suggests that *S. gesnerioides* does not induce a strong wound-response (e.g. *VSP2*).

4.2 Aim and objectives

Aim:

To confirm whether genes regulated by SA, JA and/or ethylene are induced in Col-0 *Arabidopsis* roots during infection by the parasitic weed *Striga gesnerioides*. In addition, to ascertain whether there is extensive ROS activity at the host-parasite interface, which may favour infection.

Objectives:

1. To carry out a microscopic analysis of the time-course of *S. gesnerioides* infection to determine the appropriate points to measure gene expression.

2. To assess the activity of SA and JA signalling pathways in *Arabidopsis* during infection with *S. gesnerioides* using β-glucuronidase (GUS) assays with transgenic plants expressing reporter genes.

3. To use 3,3'-Diaminobenzidine (DAB) staining to investigate ROS activity at the host/parasite interface.

4. To use quantitative PCR (qPCR) to determine quantitative changes in expression of a suite of defence related genes in *Arabidopsis* hosts at different stages of infection by *S. gesnerioides*.

5. To compare the gene expression responses of *Arabidopsis* to *S. gesnerioides* with those described for other plant pathogens.

4.3 Experimental design

4.3.1 Microscopic analysis of *Arabidopsis* roots infected with *S. gesnerioides*

A microscopic analysis was performed to identify the extent of parasite penetration into the *Arabidopsis* roots at specific times after infection, in order to perform an analysis of changes in gene expression at key stages of the parasite lifecycle. Twenty rhizotrons containing *Arabidopsis* (Col-0) plants were prepared and infected with *S. gesnerioides* as described in Chapter 2, Section 2.3. Small root sections with attached parasite haustoria were harvested from three host plants at 7, 10, 12, 14, 16, 18 and 20 days post-infection. These were prepared according to the Technovit protocol described in Chapter 2, Section 2.5. Slides were examined using an upright Olympus Epifluorescence Microscope Model BX51 (Olympus Optical, London) and images were captured using an Olympus high resolution digital camera DP71 (Olympus Optical, London).

4.3.2 β-glucuronidase (GUS) assays

To investigate whether parasitism by *S. gesnerioides* activates defence-associated signalling in *Arabidopsis* roots, an infection assay was performed using transgenic β-glucuronidase (GUS) reporter lines. GUS converts the substrate X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) into a coloured product that is proportional to the rate at which the enzyme is translated. The reporter lines used were *LOX2-GUS* and *PR1-GUS Arabidopsis*, which contain reporter genes activated in response to JA and SA, respectively. These were investigated in separate assays, but in each case *35S: GUS* plants, in which the *GUS* gene is constitutively expressed by the 35S cauliflower mosaic virus promoter, were included as a positive control. *Arabidopsis* plants and *S. gesnerioides* seed were prepared and hosts infected according to the optimised protocol described in Chapter 2, Section 2.3. See Chapter 2, Section 2.6 for the details of GUS solution preparation and the root harvesting and staining methods. Root samples were harvested and stained at three time points: 7, 14 and 21 days post-infection. At each time point, a total of 12 LOX/*PR1-GUS* plants were harvested: six infected with *S. gesnerioides* and six non-infected. As a positive control, six *35S-GUS* plants were also harvested at each time point (three infected, three non-infected).

4.3.3. 3,3'-Diaminobenzidine (DAB) staining of infected roots

DAB staining was performed on whole root systems harvested at 7, 10, 12, 15, 18 and 21 days post-infection. For each time point, three *S. gesnerioides*-infected *Arabidopsis* roots were harvested, and also the root systems from two control, non-infected *Arabidopsis* plants. Following staining and washing in 50:50 ethanol: water, samples were viewed under brightfield illumination. *Arabidopsis* plants and *S. gesnerioides* seed were prepared and hosts infected according to the optimised protocol described in Chapter 2, Section 2.3. See Chapter 2, Section 2.7 for the full method of harvesting, staining and washing samples.

4.3.4 Quantitative PCR (qPCR) of target genes

Experimental design and plant material:

Quantitative polymerase chain reaction (qPCR) quantitatively measures gene expression based on the amplification of a fluorescent product. The number of cycles needed for the fluorescence generated during amplification to reach a predetermined threshold is termed as the 'take-off' or Ct value. The higher the amount of starting cDNA, the more rapidly fluorescence is generated, resulting in a lower Ct value. Genes were selected on the basis of the results from Chapter 3 or previously reported marker activity for defence hormone activity or defence against root pathogens. Chapter 2, Section 2.8 describes the full methods for qPCR including primer design, optimisation, selection of reference genes

and programme settings. A description of each tested gene is provided in Table 4.1. Primer sequences are contained in Chapter 2, Table 2.3. *Arabidopsis* (accession Col-0) were planted, grown in rhizotrons and infected with *Striga gesnerioides,* according to the methods described in Chapter 2, Section 2.3. The only notable difference to the protocol used for the mutant phenotyping assays is that the *Arabidopsis* roots were infected at a younger age (24 days instead of 27-28 days) as optimisation assays demonstrated that this improved RNA yields. For each time point, both control (non-infected) and *S. gesnerioides* infected tissue was harvested. Root tissue was harvested at three time points: 7, 10 and 12 days post infection. Three separate batches of 75 Col-0 *Arabidopsis* plants were prepared, one for each time point. These were staggered one day apart in terms of germinating, transplanting and infecting. In each batch, 30 plants were infected with pre-germinated *S. gesnerioides*.

To achieve maximum RNA yields using the QIAGEN RNA Extraction kit, a starting sample of 100mg is optimum (QIAGEN June 2012). Therefore, three *Arabidopsis* plants were combined to form one biological replicate. For each timepoint, target gene expression was measured on three uninfected biological replicates and three infected biological replicates. Within each qPCR run, each biological replicate was represented twice (i.e. two technical replicates) and an average calculated from these. To harvest the samples, the central portion of the root was quickly cut with a disposable razor blade. For *S. gesnerioides*-infected plants, only areas of the root which had been treated with parasite seed were excised. The root systems were lifted with tweezers and washed in distilled water in a Petri dish to remove unattached parasite seeds. Non-infected roots from the control samples were washed in the same way. The harvested roots were placed in 1.5 ml RNase free Eppendorf tubes and weighed: samples greater than 1.5 mg were cut to size using a razor blade. The samples were flash frozen in liquid nitrogen before being stored at -80°C.

Statistical analysis:

qPCR data were analysed using a modified version of the 2-ΔΔCt method established by Livak and Schmittgen (Livak and Schmittgen 2001). For each time point, fold change in gene expression was calculated relative to the control samples for the first time point (7 dpi). Thus, the control samples for 7dpi provide a reference point against which both basal gene expression over time and change induced by *S. gesnerioides* can be inferred. For each biological sample, the average Ct value for the two technical replicates (for each biological replicate) was calculated and entered into a spreadsheet. If one of the technical replicates had failed to amplify, the average was not calculated and instead the remaining technical replicate value was used. The difference in Ct values between samples was first converted into a relative quantity using the exponential function with the estimated PCR efficiency reaction as the base (Hellemans et al. 2007). For each gene tested, the first control sample (7dpi) acted

as the calibrator against which differences in expression were converted to normalised values according to the following equation:

normalized relative quantity (NRQ) = efficiency of PCR ^ (Ct calibrator sample – Ct sample n)

The efficiency of the PCR was calculated by averaging the amplification efficiency of the primer pairs for the gene across all samples tested. For each biological sample, an average was calculated of the NRQ for both reference genes. This value was used to standardise the NRQ for each target gene according to the following formula:

NRQ for target gene / average NRQ from reference genes

This corrects any apparent differences in gene expression between samples caused by variation in the concentration of cDNA template. Student's T-tests were performed on these final values to identify whether genes showed statistically significant differences in expression between the control and *S. gesnerioides*-infected root samples for each time point. A two-way ANOVA was also performed on the data to assess interaction effects between treatment and time point. For results that were not normally distributed (e.g. *THI2.1*) a non-parametric Mann–Whitney U test was used instead of Student's T-test. Homogeneity of variances was verified using Levene's test for Equality of Error variances: where this was not met, significance was taken as $p<0.01$, rather than $p<0.05$. Cluster analysis (Euclidean distance with average linkage) was performed on the Log_2-transformed fold induction values for each gene using TICR Multi-experiment Viewer software, version 4.9.0. (Saeed et al. 2003).

4.4 Results

4.4.1 Microscopic analysis of *Arabidopsis* roots infected with *S. gesnerioides*

At 7 days post-infection (dpi) the invading cells of the attached *S. gesnerioides* haustorium had breached the host root cortex and begun to advance towards the central vascular system (Figures 4.1A and B). Similar to *S. gesnerioides* on cowpea, the parasite advanced as a cone-shaped wedge of cells, with little apparent damage to surrounding host cells (Reiss and Bailey 1998). At this point the host's vascular elements were intact and there was no sign of any differentiation of the parasite cells into vascular elements. This was clear under UV light (Figure 4.1C), as there were no lignin-containing elements visible between the host root and parasite haustoria. At 10 dpi, the invading parasite cells had almost reached the host vascular system and begun to differentiate to form the xylem vessels that will connect the host and the parasite. These were visible as elongated cells (Figure 4.2A and B). Under UV light, thread-like connections were seen forming between the host *Arabidopsis* root and the parasite haustorium (Figure 4.2C).

Table 4.1: Target genes chosen for analysis with qPCR

Gene name:	Locus:	Function:	References:
Salicylic Acid (SA) -associated genes			
PR1 (PATHOGENESIS RELATED PROTEIN 1)	At2g14610.1	Antimicrobial defence protein induced by SA and pathogens. A marker gene for the hypersensitive response and systemic acquired resistance (SAR).	(Uknes et al. 1992)
PR2 (PATHOGENESIS RELATED PROTEIN 2)/ β-1,3-GLUCANASE	At3g57260.1	Defence gene induced by SA and pathogens, predicted to encode a β-1,3-glucanase enzyme. Proposed functions include degrading fungal cell walls.	(Uknes et al. 1992)
PR5 (PATHOGENESIS RELATED PROTEIN 5)	At1g75040	Marker gene for the SA-induced SAR response, encoding a thaumatin-like antimicrobial protein.	(Uknes et al. 1992)
PAD4 (ARABIDOPSIS PHYTOALEXIN DEFICIENT 4)	At3g52430.1	A lipase-like gene required for SA biosynthesis and SA-dependent camalexin synthesis and *PR1* expression during infection with virulent pathogens.	(Glazebrook et al. 1996, Zhou et al. 1998)
Jasmonic Acid (JA) -associated defence genes			
VSP2 (VEGETATIVE STORAGE PROTEIN 2)	At5g24770.2	Encodes an acid phosphatase with anti-insect activity. Induced by abscisic acid, JA, salt, water deficiency and wounding; suppressed by SA. Commonly used JA response marker gene.	(Leon-Reyes et al. 2010b, Liu et al. 2005)
LOX2 (ARABIODOPSIS THALIANA LIPOXYGENASE2)	At3g45140.1	A chloroplast-targeted lipoxygenase necessary for JA accumulation upon wounding.	(Bell, Creelman and Mullet 1995)
Jasmonic Acid / ethylene co-regulated			
THI2.1 (THIONIN2.1)	At1g72260	Encodes a cysteine-rich peptide with antimicrobial properties. Expressed in response to various pathogens and induced by ethylene and JA.	(Epple, Apel and Bohlmann 1995, Loeza-Angeles et al. 2008, Ellis and Turner 2001)
PDF1.2 (PLANT DEFENSIN 1.2)	At5g44420	Encodes a plant defensin (cysteine-rich antimicrobial peptide) induced synergistically by JA and ethylene but unresponsive to SA.	(Penninckx et al. 1996, Ellis and Turner 2001)
PR4/ HEL (PATHOGENESIS RELATED PROTEIN 4: HEVEIN-LIKE)	At3g04720.1	Antimicrobial protein similar to the antifungal chitin-binding protein hevein. Induced by JA/ethylene but thought to require SA for full induction.	(Potter et al. 1993)
B-CHI (BASIC CHITINASE)	*At3g12500*	Encodes a basic chitinase that degrades the fungal cell wall component chitin. Induced by JA and ethylene.	(Samac et al. 1990, Ellis and Turner 2001)
Signalling nodes and defence regulatory transcription factors			
ERF1 (ETHYLENE RESPONSE FACTOR 1)	At3g23240.1	Transcription factor in the ethylene-signalling cascade, downstream of EIN2 and EIN3. A node of convergence for the ethylene and JA defence signalling pathways that can be activated by ethylene, JA or synergistically by both. Positively regulates ethylene-dependent defences against necrotrophic pathogens (for instance *PDF1.2*).	(Berrocal-Lobo et al. 2002, Solano et al. 1998, Lorenzo et al. 2003, Fujimoto et al. 2000)
ERF2 (ETHYLENE RESPONSE FACTOR 2)	At5g47220.1	Transcriptional activator that positively regulates JA-dependent defences, including the genes *PDF1.2* and *B-CHI*.	(McGrath et al. 2005, Fujimoto et al. 2000)

ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59)	At1g06160.1	A member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family. Integrates JA and ethylene signalling cascades to positively regulate genes including *PDF1.2* and *B-CHI.*	(Atallah 2005, Pré et al. 2008)
WRKY70 (ARABIDOPSIS THALIANA WRKY DNA-BINDING PROTEIN 70)	At3g56400.1	Member of the WRKY Transcription Factor, Group III that integrates the JA and SA signalling pathways. Expression is induced by SA and repressed by JA. Acts as an activator of SA-responsive genes and a repressor of JA-responsive genes. Also acts as an inhibitor of SA biosynthesis in an apparent negative feedback loop.	(Li et al. 2004, Wang et al. 2006)
ERF4 (ETHLYENE RESPONSE FACTOR 4)	At3G15210	A negative regulator of JA-dependent defence gene expression, particularly those associated with necrotrophic pathogens (e.g. *PDF1.2, PR1*).	(McGrath et al. 2005)
ERF4-REPRESSOR isoform	At3G15210	Normally transcribed variant of *ERF4*, containing the classic ERF-associated amphiphilic repression (EAR) motif. Acts as a transcriptional repressor that negatively regulates *PDF1.2* expression and promotes pathogen-induced reactive oxygen species (ROS) generation.	(Lyons et al. 2013)
ERF4-ACTIVATOR isoform	At3G15210	Alternatively spliced variant of *ERF4* that lacks the EAR motif. Acts as a transcriptional activator that promotes expression of *PDF1.2* and inhibits pathogen-induced ROS generation.	(Lyons et al. 2013)
AtMYC2/JIN1 (JASMONATE INSENSITIVE 1)	At1g32640.1	Encodes a MYC-related transcriptional activator with a typical basic helix-loop-helix leucine zipper motif DNA binding domain. Regulates diverse developmental and defence responses and positively regulates ABA signalling. Negatively regulates JA/ethylene-dependent defence genes that act against necrotrophs.	(Anderson et al. 2004)
Reactive oxygen species (ROS)- associated			
GST1 (GLUTATHIONE S-TRANSFERASE 1)	At1g02930.1	Detoxifying enzyme induced in response to oxidative stress, e.g. H_2O_2. Catalyses the conjugation of reduced glutathione to electrophilic substances.	(Wagner et al. 2002, Levine et al. 1994)
PRX33 (PEROXIDASE 33)	At3g49110.1	Class III peroxidase, expressed in roots. Located in the cell wall; appears to generate apoplastic H_2O_2 during pathogen attack.	(Bindschedler et al. 2006)
PRX53 (PEROXIDASE 53)	At5g06720.1	A class III peroxidase induced by wounding, JA and nematode attack. Implicated in the wound response, floral development and nematode resistance.	(Jin, Hewezi and Baum 2011)
Camalexin biosynthesis			
PAD3 (PHYTOALEXIN DEFICIENT 3)	At3g26830.1	Encodes a cytochrome P450 enzyme which catalyses the final reaction in the production of the phytoalexin camalexin.	(Zhou, Tootle and Glazebrook 1999, Schuhegger et al. 2006)
Inhibitors of cell wall degrading enzymes			
PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1)	At5g06860	A defence-associated polygalacturonase inhibitor. Induced during attack by fungal pathogens to suppress cell wall pectin degrading enzymes.	(Ferrari et al. 2003b)

By 12 dpi, *S. gesnerioides* had connected its xylem vessels with those of the host, forming a continuous connection between the host and the parasite (Figure 4.3A). At this point, the cells at the tip of the advancing front were large, with little cytoplasm, while cells that were further back were smaller with a dense cytoplasm, as reported for *S. gesnerioides* on cowpea (Reiss and Bailey 1998). In addition, the host cells at the host-parasite interface appeared compact and folded (Figure 4.3A), similar to observations on infected cowpea (Reiss and Bailey 1998). Under UV light, this connection was visible as lignin-containing elements forming between the *Arabidopsis* root and the parasite haustorium (Figure 4.3B). Having established a connection with the host vascular system, the parasite haustorium grew rapidly after this point. When viewed under UV light, the lignin-containing vessel elements connecting the host and the parasite haustorium became progressively more visible (Figure 4.4A). By 16 dpi, the parasite had completely disrupted the host vascular system and showed highly differentiated vessel elements (Figure 4.4B). In summary, these results demonstrate that by 12 dpi, without an effective host defence response, an invading *S. gesnerioides* parasite gains access to the vascular system of *Arabidopsis*.

4.4.2 GUS-reporter assays for SA and JA signalling

PR1-GUS and *LOX2-GUS* transgenic *Arabidopsis* plants were used to investigate whether *S. gesnerioides* activates salicylic acid (SA) or jasmonic acid (JA) signalling respectively. Neither reporter showed any activity at any of the time points tested (7, 14 and 21 dpi) (Figures 4.5 and 4.6). In every experiment the positive control *35S-GUS* plants reacted strongly to the GUS stain, confirming that there was no defect in the prepared GUS solution or incubation stage. It was apparent however, that for samples harvested at 14 and 21 dpi, most of the attached *S. gesnerioides* haustoria had a purple colouration, particularly in the emerging shoots (Figures 4.5 and 4.6, arrows). This was especially noticeable on the *35S-GUS* lines. This suggested that the parasite may express enzymes that can act upon the X-Gluc substrate. To investigate this, root samples from infected Col-0 plants (containing no GUS transgene at all) were harvested and stained following the same procedure as before, except the X-Gluc substrate was not included in the mixture. On removing the samples from the 37 °C incubator, strong purple deposits could be seen in the solution (Figure 4.7A). Before the roots were harvested, the parasite haustoria had appeared healthy (Figure 4.7B), but were now a dark colour, similar to the results seen when the X-Gluc substrate was included (Figure 4.7C). A further assay confirmed that overnight incubation in 0.5 mM $K_4Fe(CN)_6$ / $K_3Fe(CN)_6$ solution or with heat treatment was sufficient to induce the colour change in the parasite haustoria (Figure 4.8). Considering that the $K_4Fe(CN)_6$ / $K_3Fe(CN)_6$ solution acts as an oxidation catalyst, the colouration response of the haustoria suggests an altered oxidative environment compared to the other tissues. This suggests that *S. gesnerioides* has oxidative activity and is consistent with previous studies that have shown *Striga* radicles to be sources of H_2O_2 (Keyes et al. 2007, Wada et al. 2019).

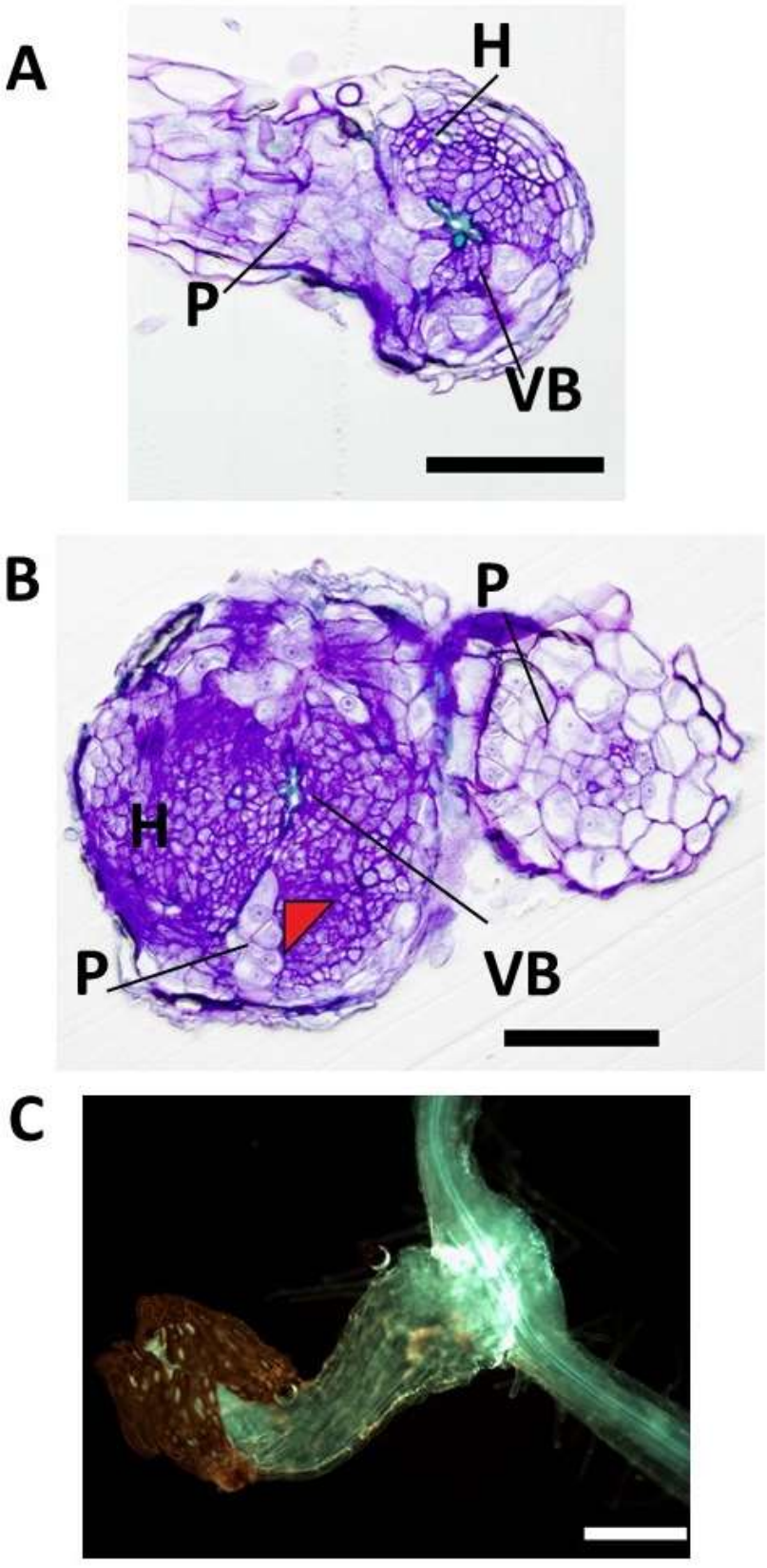


Figure 4.1: Sections of *Arabidopsis* root infected with *Striga gesnerioides*, 7 days post-infection. **A and B:** sections prepared in Technovit, stained with toluidine blue. H = Host *Arabidopsis* tissue, P = parasite tissue, VB = host vascular bundle. Scale bar: 100 µm. Intrusive parasite cells (red arrows) can clearly be seen advancing towards the host vascular bundle. **C:** Root section treated with the clearing agent chloral hydrate, viewed under UV light which causes phenolic containing compounds, including lignin, to fluoresce. Scale bar: 200 µm.

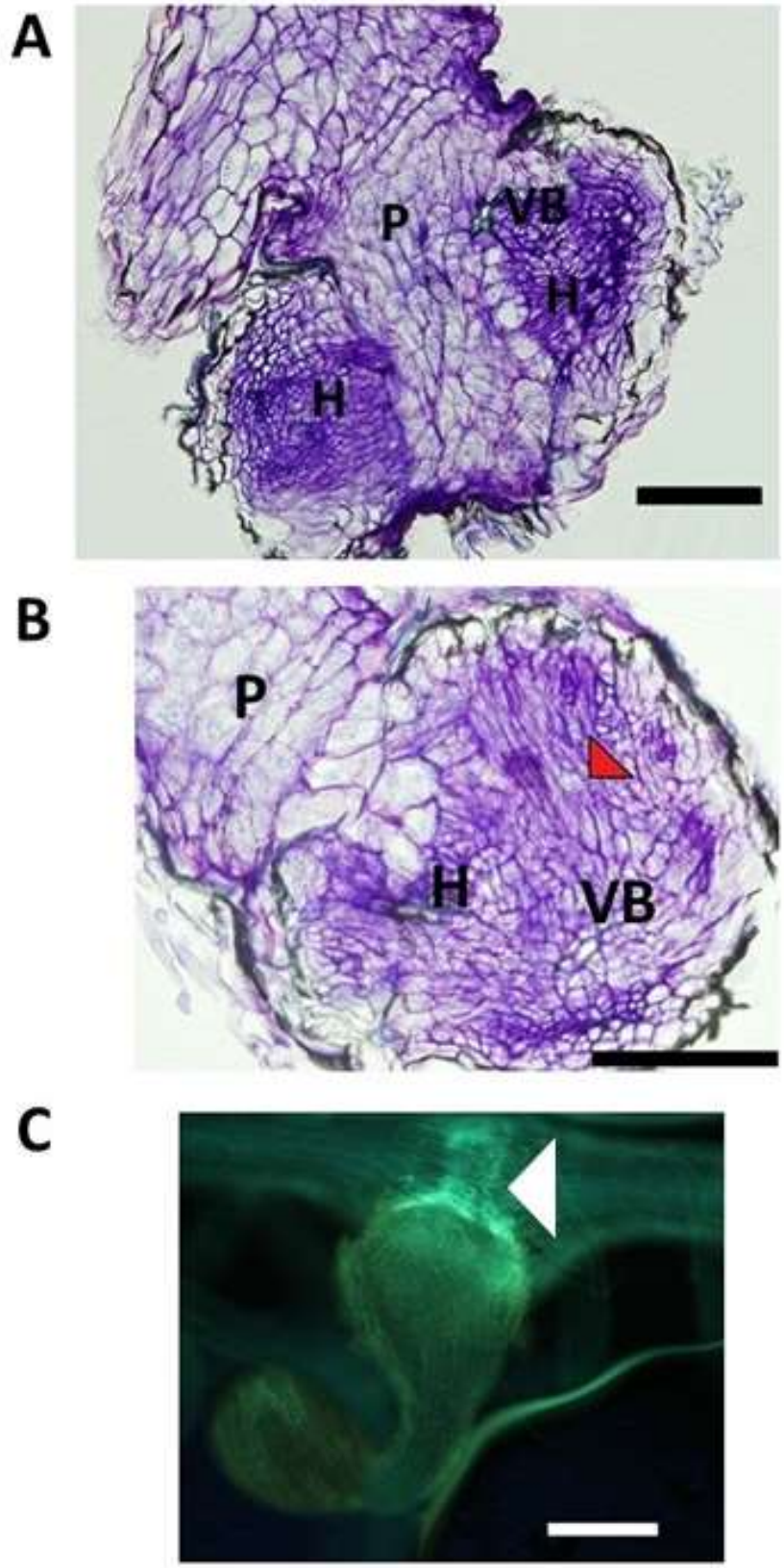


Figure 4.2: Sections of *Arabidopsis* root infected with *Striga gesnerioides*, 10 days post-infection. **A and B:** sections prepared in Technovit, stained with toluidine blue. H = Host *Arabidopsis* tissue, P = parasite tissue, VB = host vascular bundle. Scale bar: 100 µm. At this point, parasite cells begin to differentiate into xylem vessels to connect with the host vascular system (red arrows). **C:** Root section treated with the clearing agent chloral hydrate, viewed under UV light, showing developing vascular connections between the host and parasite (white arrow). Scale bar: 200 µm.

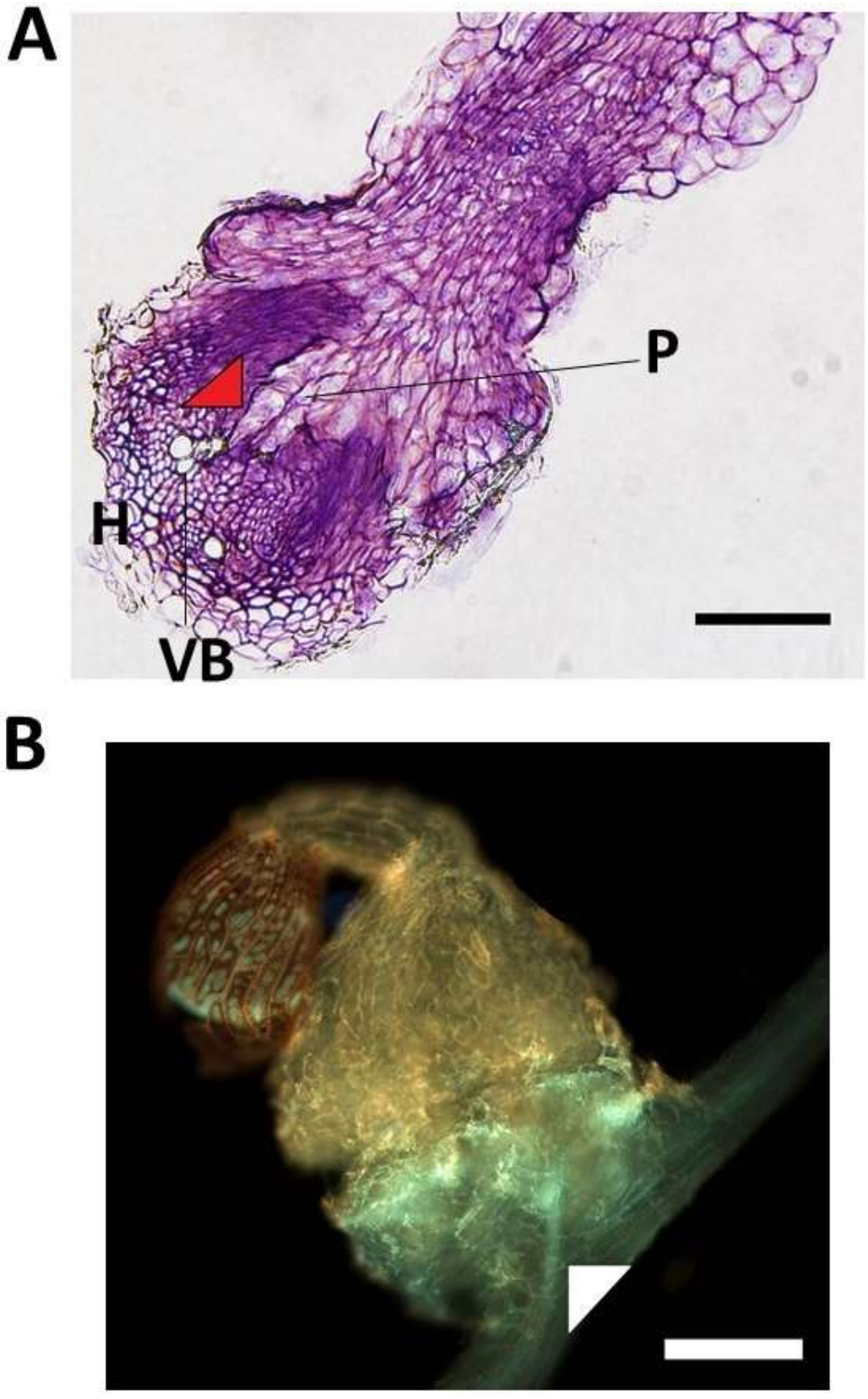


Figure 4.3: Sections of *Arabidopsis* root infected with *Striga gesnerioides*, 12 days post-infection. **A**: Infected root section prepared in Technovit, stained with toluidine blue. H = Host *Arabidopsis* tissue, P = parasite tissue, VB = host vascular bundle. Scale bar: 100 µm. By this point, the invading parasite has established a continuous connection with the host vascular system (red arrow). **B**: Root section treated with the clearing agent chloral hydrate, viewed under UV light. Lignin-containing elements (fluorescent under UV light) can be seen between the host and parasite haustorium (white arrow) Scale bar: 200 µm.

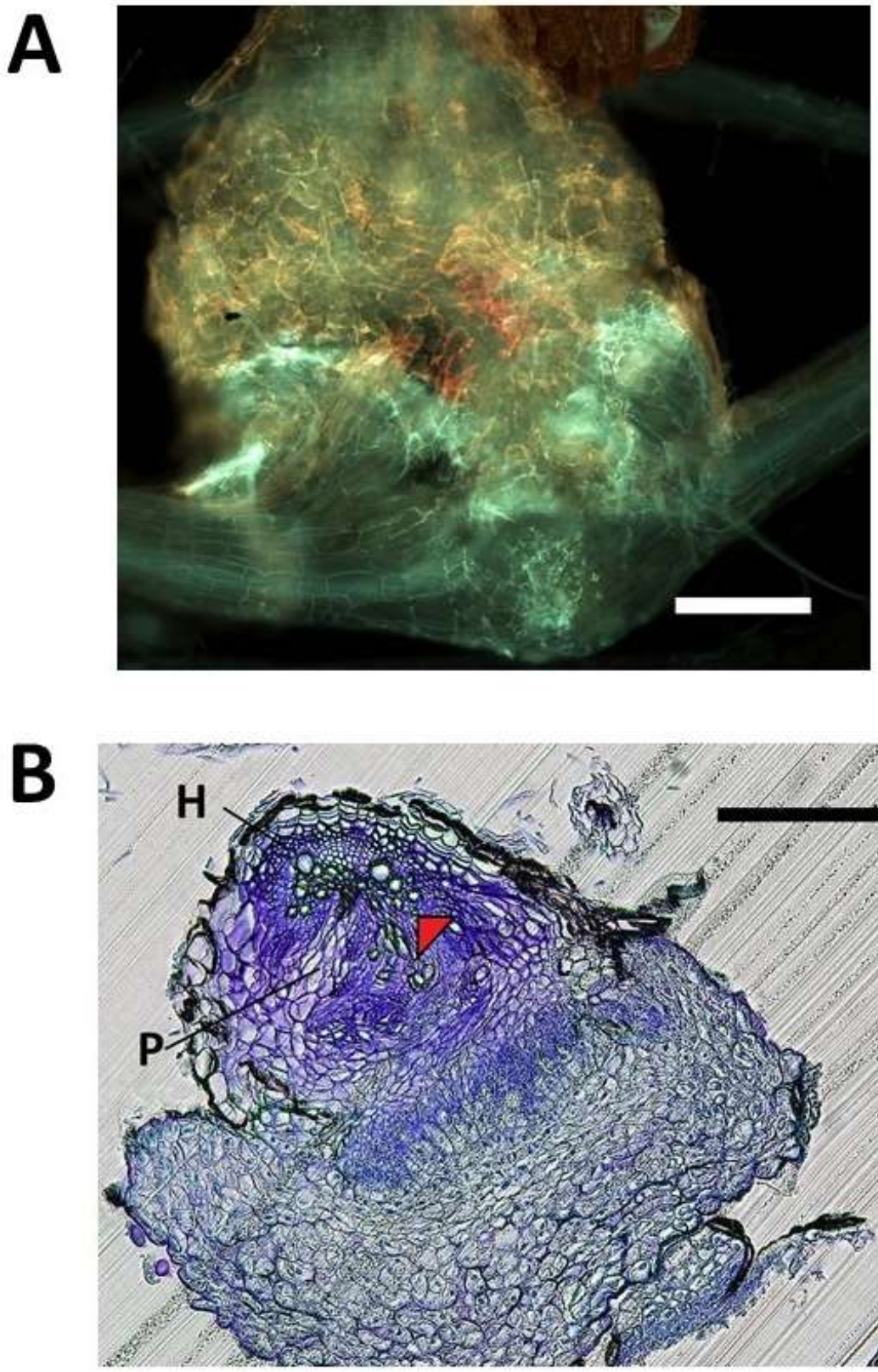


Figure 4.4: Sections of *Arabidopsis* root infected with *Striga gesnerioides*, 14 (**A**) and 16 (**B**) days post-infection. **A:** Root section 14 days post-infection treated with the clearing agent chloral hydrate, viewed under UV light. Lignin-containing elements (fluorescent under UV light) can clearly be seen connecting the parasite haustorium and the host root. Scale bar: 200 µm. **B:** Infected root section prepared in Technovit, stained with toluidine blue, 16 days post-infection. H = Host *Arabidopsis* tissue, P = parasite tissue, VB = host vascular bundle. Scale bar: 200 µm. The parasite has completely disrupted the host vascular system and has differentiated to form new xylem elements (red arrow).

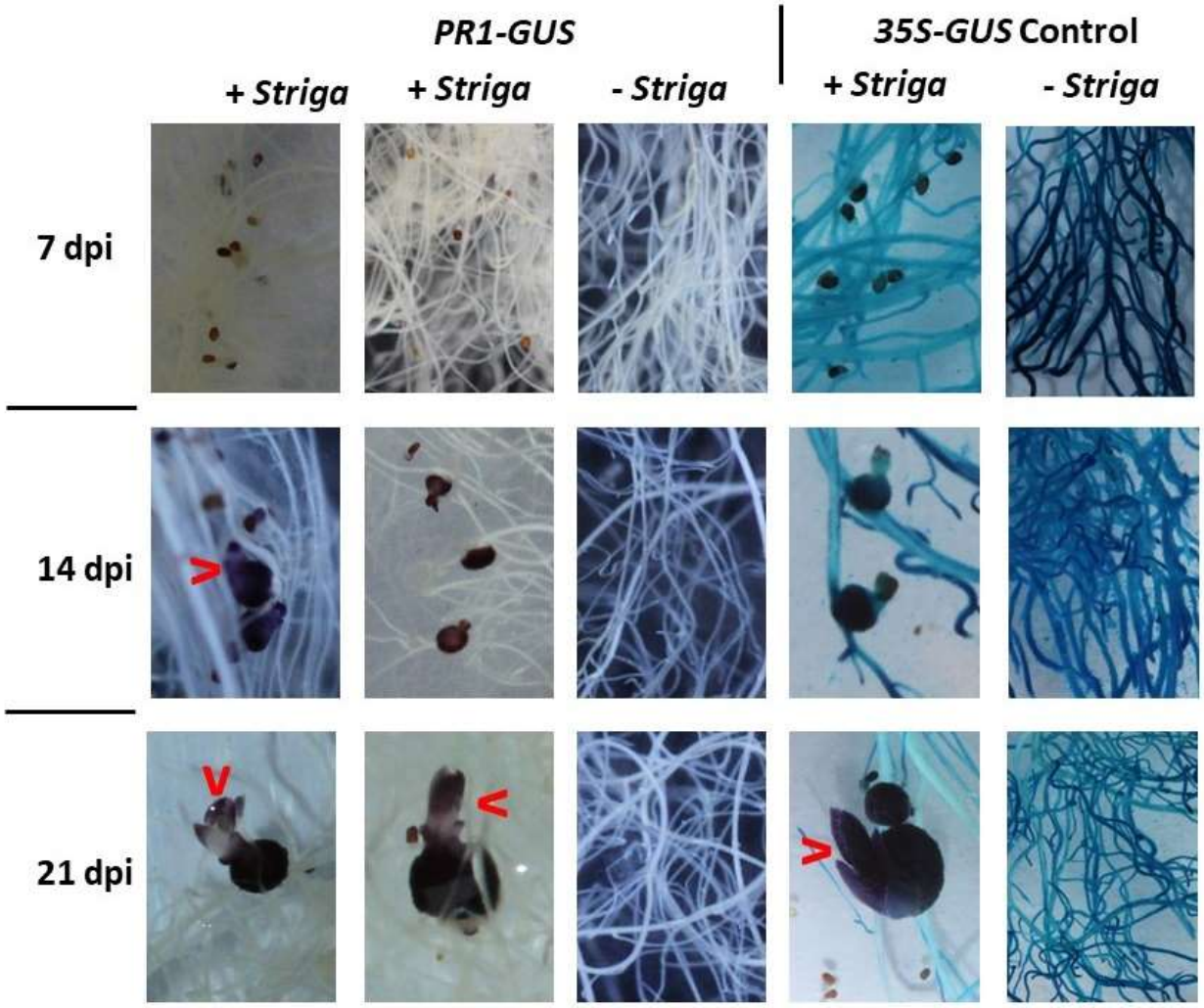


Figure 4.5: Activity of β-glucuronidase (GUS) reporter constructs in transgenic *Arabidopsis* roots, infected with *Striga gesnerioides. PR1-GUS* is a proxy for salicylic acid signalling, whilst the *35S-GUS* construct is a constitutive, positive control. Samples were treated with X-Gluc staining solution and incubated overnight at 37°C. Positive activity is visible as a blue precipitate. Red arrows indicate parasite haustoria showing atypical purple colouration.

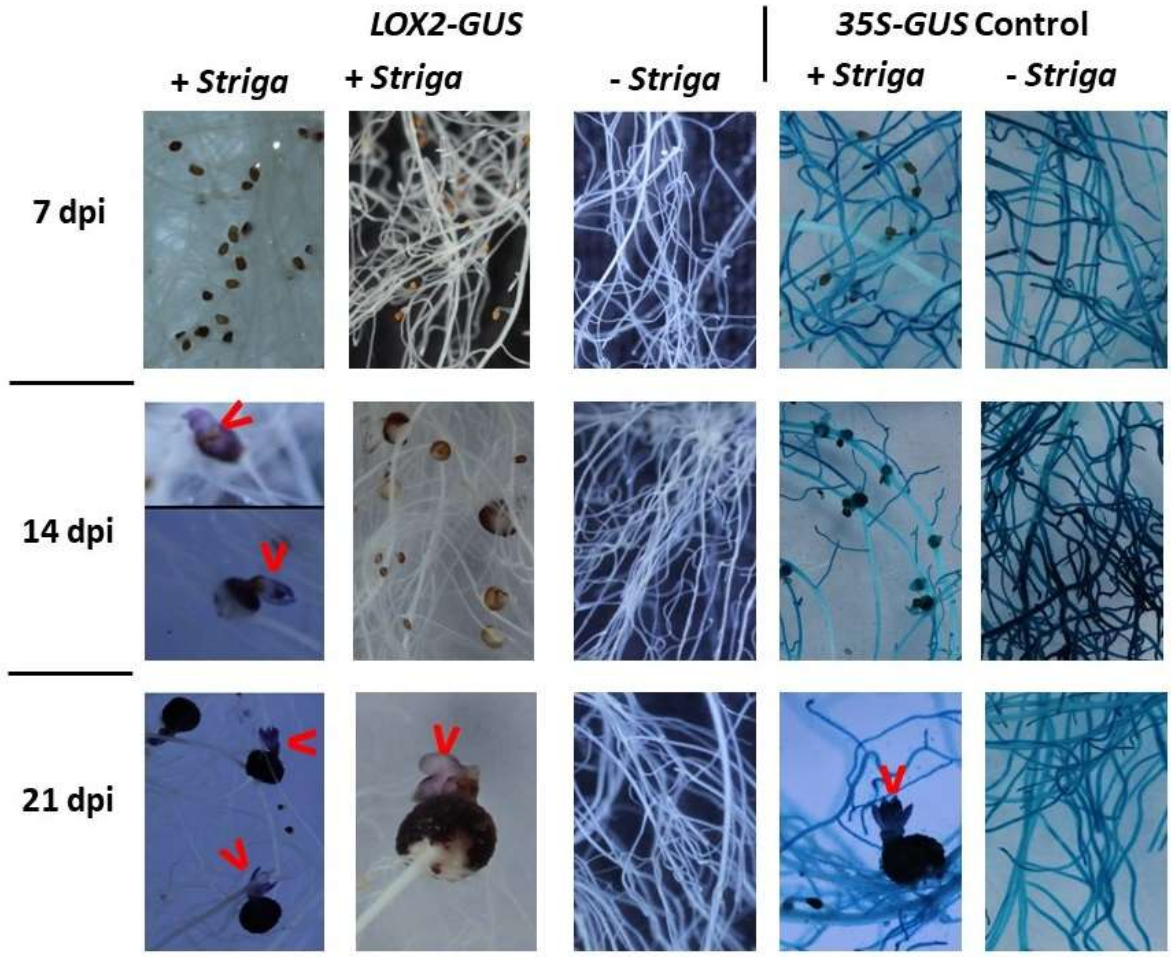


Figure 4.6: Activity of β-glucuronidase (GUS) reporter constructs in transgenic *Arabidopsis* roots, infected with *Striga gesnerioides. LOX2-GUS* is a proxy for jasmonic acid signalling, whilst the *35S-GUS* construct is a constitutive, positive control. Samples were treated with X-Gluc staining solution and incubated overnight at 37°C. Positive activity is visible as a blue precipitate. Red arrows indicate parasite haustoria showing atypical purple colouration.

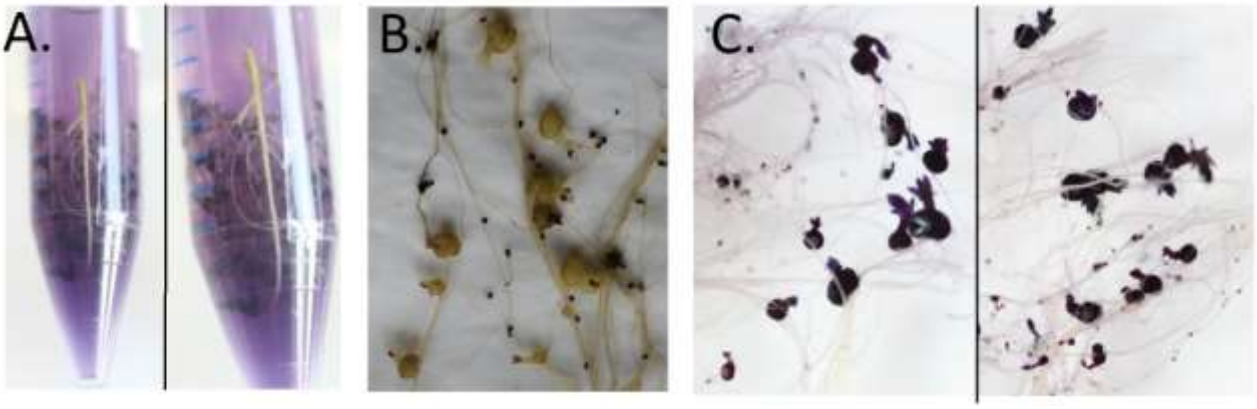


Figure 4.7: Samples of Col-0 *Arabidopsis* root tissue infected with *Striga gesnerioides*, subjected to β-glucuronidase (GUS) staining protocol, without the X-Gluc substrate. Samples were incubated overnight at 37°C. As the host plants did not have any type of GUS transgene, and the solution did not contain the X-Gluc substrate, no coloured reaction was expected. **A.** Appearance of the sample tubes after removal from the 37 °C incubator; note the purple colour of the solution. **B**. Appearance of the parasite haustoria before harvest. The host plants were infected with the parasite 3 weeks previously. **C.** Appearance of the parasite haustoria after removal from the 37°C incubator and following addition of fresh clearing solution.

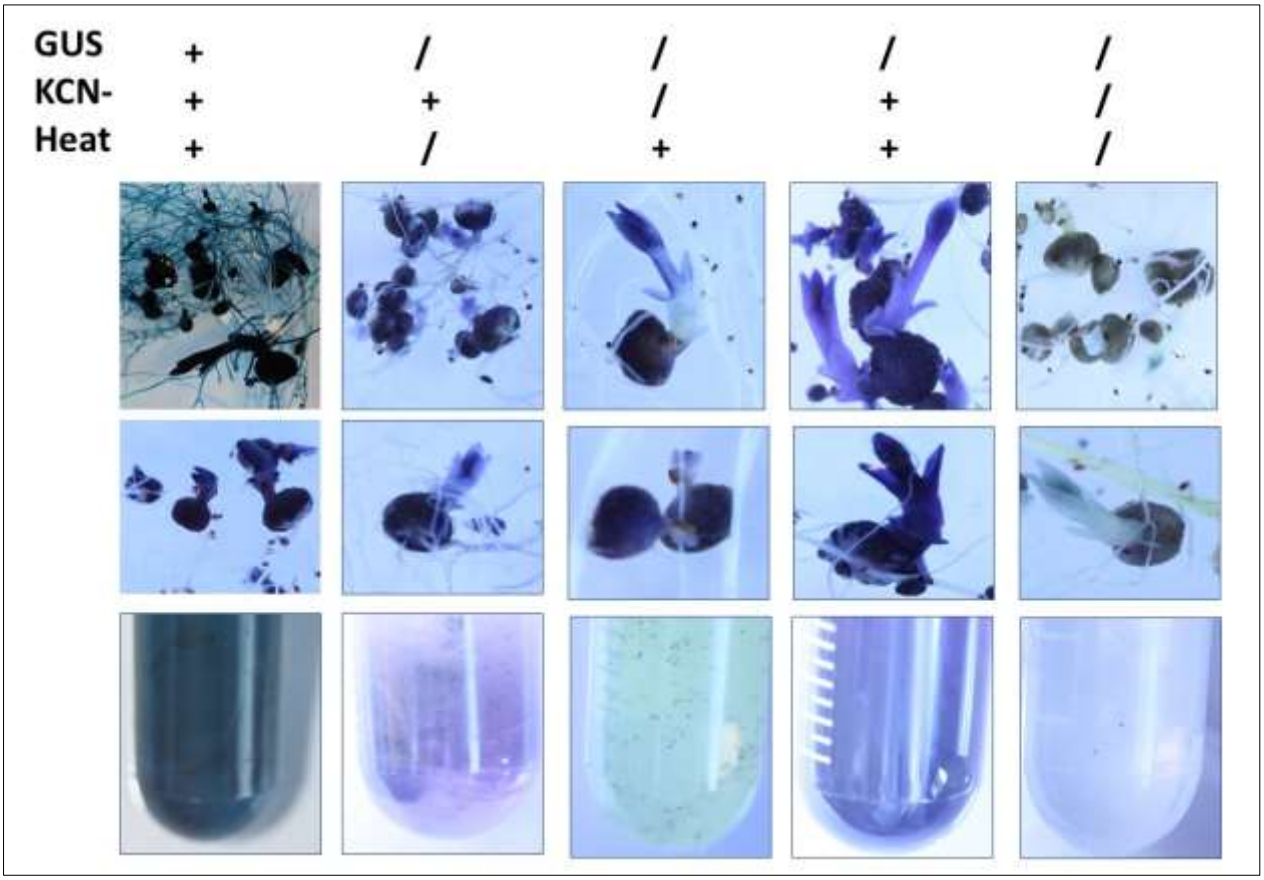


Figure 4.8: Col-0 *Arabidopsis* root tissue infected 4 weeks previously with *Striga gesnerioides*, treated with different combinations of β-glucuronidase staining solution (GUS), 0.5 mM $K_4Fe(CN)_6$ / $K_3Fe(CN)_6$ (KCN-) and overnight incubation at 37°C (Heat). Top two photograph panels show attached *S. gesnerioides* haustoria on the host root. Bottom panel shows residue in the tube after overnight incubation or standing overnight at room temperature.

Because this colour change occurred in the parasite tissue, GUS staining was not suitable to investigate the interaction between *Arabidopsis* and *S. gesnerioides.* Furthermore, the reporter constructs *PR1-GUS* and *LOX2-GUS* showed poor induction in follow-up experiments where roots were treated with SA and JA respectively. This indicates that these transgenes were not expressed potently enough in the roots to indicate significant gene expression changes. Consequently, this line of investigation was not continued further.

3.4.3. 3,3'-Diaminobenzidine (DAB) staining for reactive oxygen species (ROS)

RbohD and *RbohD/RbohF Arabidopsis* mutants showed significantly increased resistance to *S. gesnerioides* (Chapter 3, Figure 3.6), hence the role of extracellular ROS in basal resistance was investigated by 3',3'-Diaminobenzidine (DAB) staining of control and infected root samples. Beyond 10 dpi, the size of the parasite haustoria obscured the host/parasite interface but at 7 dpi, a brown precipitate was clearly visible at the interface where the attached radicle contacts the host root (Figures 4.9 A-B). This appears to originate from the radicle itself and extend into the host root. In the water-treated control root samples, some light brown colouring is visible at the host-parasite interface (Figures 4.9 C-D), but this is not as distinct and appears to be based in the host root, rather than the attached *S. gesnerioides* radicle. Curiously, in germinated *S. gesnerioides* seed that had failed to attach permanently to the root, DAB precipitate can be seen at the very tip of the radicle (Figure 4.10 A-B, arrowed), but this was not present in any unattached *S. gesnerioides* from the water-treated control samples (Figure 4.10 C). This would suggest a high concentration of H_2O_2 at the radicle tip, which contacts the host root during attempted penetration.

By 10 dpi, the brown precipitate was even stronger at the host-parasite interface for the samples treated with DAB stain (Figure 4.11 A-C). Once again, the precipitate appears to originate from the parasite radicle and extend downwards into the host root. For the samples treated with water however, the samples showed no stronger colouration than at 7 dpi (Figure 4.11D).

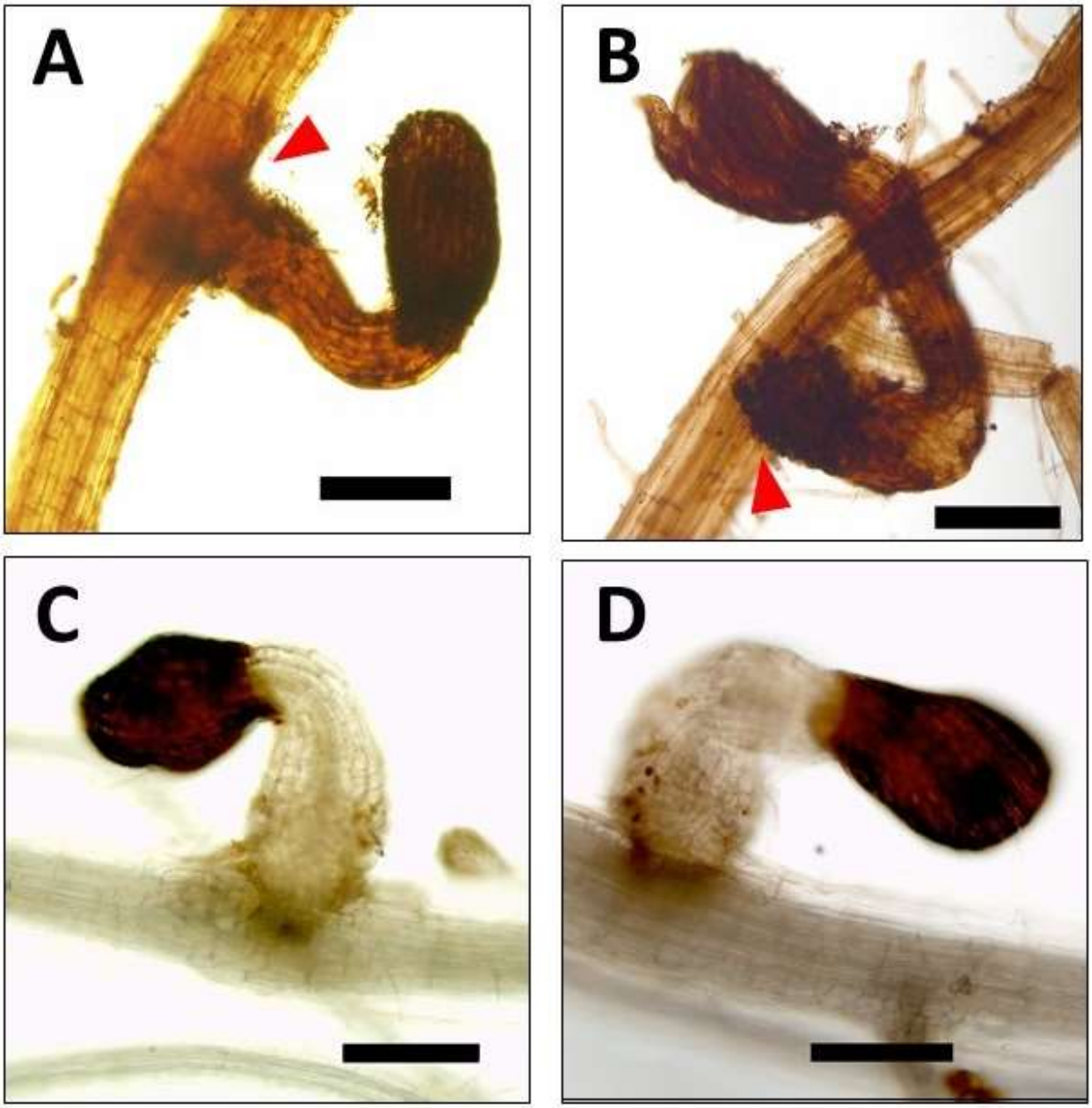


Figure 4.9: Diaminobenzidine (DAB) staining of *Arabidopsis* root sections infected with *S. gesnerioides*, 7 days post-infection. **A-B**: Root sections incubated with DAB solution. Reactive oxygen species activity, particularly through peroxidase enzymes, is visible as a brown precipitate at the host-parasite interface. **C-D:** Root sections incubated with water only. Scale bar: 200 µm. Red arrows indicate DAB stain at the host-parasite interface, indicative of increased H_2O_2 concentration.

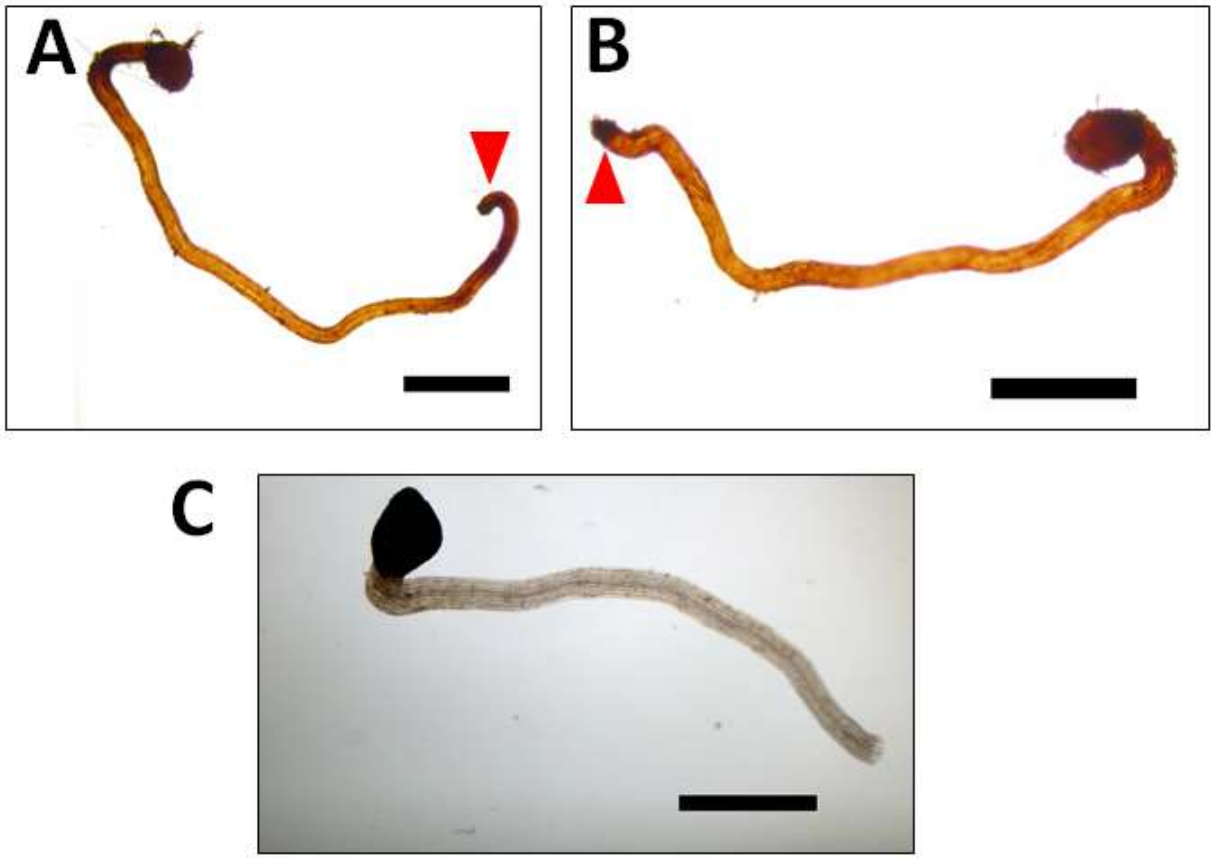


Figure 4.10. Diaminobenzidine (DAB) staining of germinated *S. gesnerioides* that failed to attach to host *Arabidopsis* roots, 7 days post-infection. **A-B:** Samples incubated with DAB solution. Arrows: brown DAB precipitate, indicative of peroxidase enzyme activity. **C.** Sample incubated with water only. Scale bar: 500 µm.

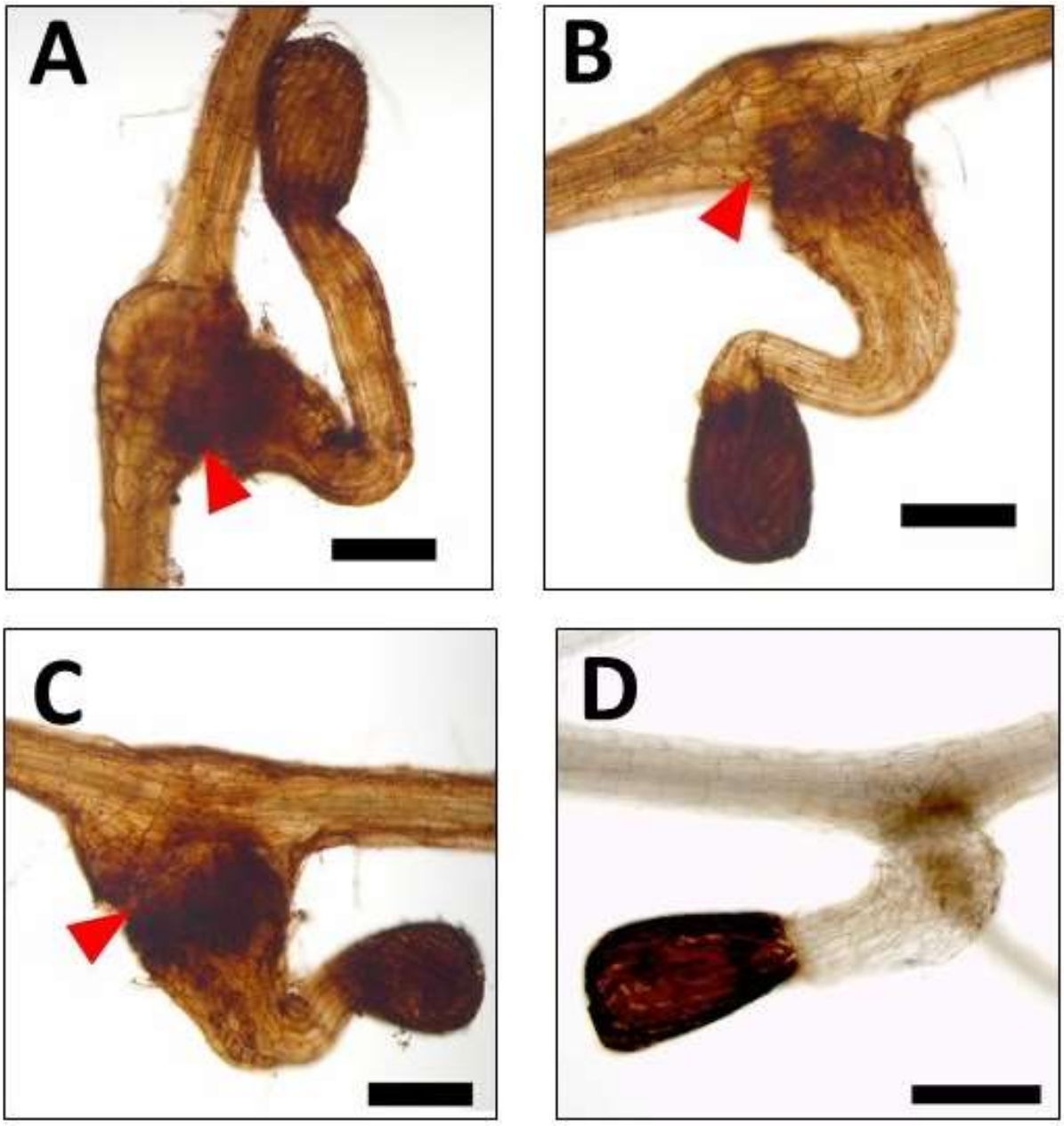


Figure 4.11: Diaminobenzidine (DAB) staining of *Arabidopsis* root sections infected with *S. gesnerioides*,10 days post-infection. **A-C**: Root sections incubated with DAB solution. Reactive oxygen species activity, particularly through peroxidase enzymes, is visible as a brown precipitate at the host-parasite interface. **D:** Root section incubated with water only. Scale bar: 200 µm. Red arrows indicate DAB stain at the host-parasite interface, indicative of increased H_2O_2 concentration.

4.4.4 Quantitative PCR (qPCR) to measure the expression of defence-related genes in *Arabidopsis* infected with *S. gesnerioides*

For each set of samples, fold-change in gene expression change was calculated relative to the control (uninfected) samples for the first time point (7 dpi) to profile changes in basal gene expression over time, as well as gene induction by *S. gesnerioides*. Cluster analysis (Euclidean distance) of the Log_2-transformed fold induction values identified groups of similarly regulated genes that were broadly divided as follows: genes strongly induced by infection; genes slightly induced and genes showing minimal variation between control and infected samples (Figure 4.12). Cluster analysis was also performed on the samples themselves: this verified that the control and infected samples formed two discrete groups (not shown).

Genes strongly induced by *S. gesnerioides* (Clusters 1 and 2):

Five genes were strongly induced by *S. gesnerioides*: these showed over 5-fold upregulation for at least one time point. *PR2* and *PR5* formed a distinct pair (Cluster 1, Figure 4.12) as they were induced most strongly by *S. gesnerioides* (to above 10-fold upregulation) and were upregulated across all time points tested (Figure 4.13). *PR5* upregulation was highest at 7 dpi being 13.3-fold above the controls; this declined slightly at 10 dpi but remained statistically significant. By 12 dpi, *PR5* average fold-induction reduced to 8.9-fold: due to increased variability, this was no longer significant. *PR2* was also significantly induced by *S. gesnerioides* at 7dpi, by 9.2-fold. In contrast to *PR5*, the peak of *PR2* expression occurred at 10 dpi, rising to 17.0-fold. By 12 dpi, however, *PR2* expression reduced and, similar to *PR5,* increased variation across the samples meant this was no longer statistically significant.

PAD3 and *PRX33* formed Cluster 2 (Figure 4.12): these were also significantly induced by S. gesnerioides across all time points (Figure 4.13), but never above 10-fold upregulation. For both genes, fold-induction was highest at 7 dpi, being 6.6-fold for *PAD3* and 8.8-fold for *PRX33.* By 10 dpi, fold-induction decreased slightly for both genes but remained significantly different to the corresponding controls. Fold-induction decreased further for both genes by 12 dpi to 4.4-fold for *PAD3* and 4.2-fold for *PRX33*: at this point the difference between control and infected samples was no longer significant for *PAD3*.

At 7 dpi, *THI2.1* was upregulated by an average of 9.6-fold in the *S. gesnerioides*-infected plants, however due to considerable variability this was not significantly different to the controls (Figure 4.13). By 10 dpi, *THI2.1* induction decreased sharply to 1.7-fold relative to the reference control, however this difference was now significant. By 12 dpi, *THI2.1* expression had decreased further to 1.5-fold and was no longer significantly different.

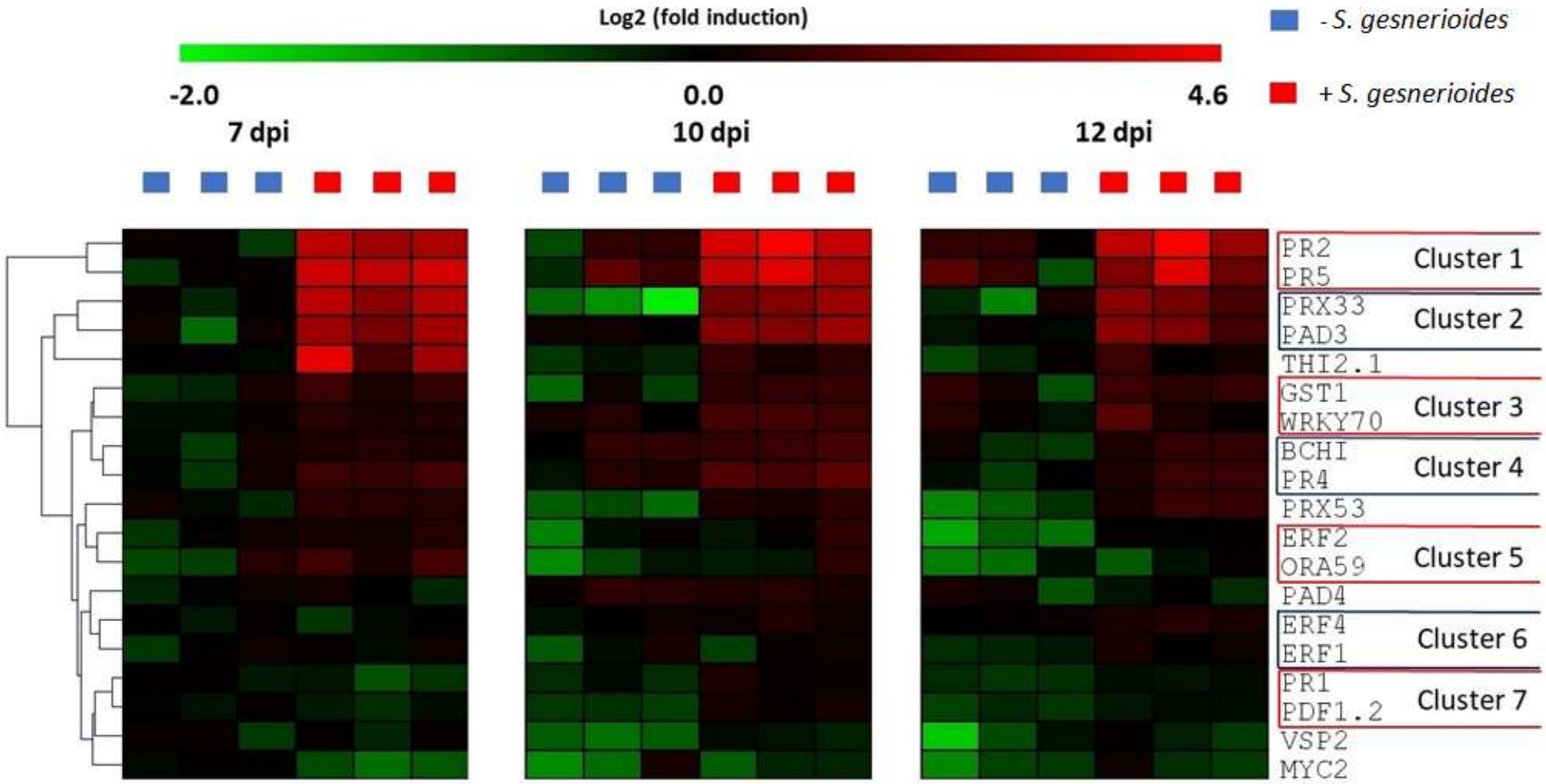

Log2 (fold induction)
-2.0
0.0
4.6
- S. gesnerioides
+ S. gesnerioides
7 dpi
10 dpi
12 dpi
PR2
PR5
Cluster 1
PRX33
PAD3
Cluster 2
THI2.1
GST1
WRKY70
Cluster 3
BCHI
PR4
Cluster 4
PRX53
ERF2
ORA59
Cluster 5
PAD4
ERF4
ERF1
Cluster 6
PR1
PDF1.2
Cluster 7
VSP2
MYC2

Figure 4.12 (overleaf): Fold-induction of defence-associated genes in Col-0 *Arabidopsis* infected with *Striga gesnerioides*, at 7, 10 and 12 days post-infection (dpi) relative to uninfected controls. The expression value of each sample was divided by the mean expression value of the three control samples at 7 dpi. These values were Log_2-transformed and assessed using average linkage clustering (Euclidean distance). The colour intensity of each sample is proportional to the fold induction (red) or repression (green) of each gene.

Genes moderately induced by *S. gesnerioides* (Clusters 3 and 4):

The second broad category of genes showed significant upregulation between 1.5 and 3-fold in response to *S. gesnerioides* for at least one time point. These were *GST1, WRKY70, B-CHI, PR4,* and *PRX53. GST1* and *WRKY70* formed a distinct pair (Cluster 3, Figure 4.12). These were significantly upregulated at 7 and 10 dpi, but not at 12 dpi. The highest fold-induction was 2.0 for *GST1* and 2.4 for *WRKY70. B-CHI* and *PR4* formed another distinct pair (Cluster 4, Figure 4.12). Both these genes were upregulated by *S. gesnerioides* across all time points, with greatest fold-induction occurring at 10 dpi: this was 2.1-fold for *B-CHI* and 2.5-fold for *PR4* (Figure 4.14). At 10 dpi, however, the control samples also showed induction of *B-CHI,* such that the difference between the control and infected plants at this point was not significant. *PRX53* showed significant upregulation at all time points (Figure 4.14): unlike the genes in Clusters 3 and 4, this was highest at 12 dpi, reaching 1.8-fold.

Genes showing minimal response to *S. gesnerioides* (Clusters 5, 6 and 7)*:*

The final broad category contained genes where any significant upregulation in the infected samples did not exceed 1.5-fold. These were *ORA59, ERF2, ERF4, ERF1, PAD4, PR1, PDF1.2, VSP2* and *MYC2.* For *ORA59* and *ERF2* (Cluster 5, Figure 4.12), expression was progressively downregulated in the control plants, being 0.6-fold or less by 12 dpi compared with 7 dpi (Figure 4.15). For both these genes, there was no significant upregulation in response to *S. gesnerioides*, although gene expression was slightly above the control samples at all time points.

For *PAD4*, the gene expression profiles between the control and infected plants were virtually identical, with no noticeable difference at any point (Figure 4.15). However, this gene was unusual in that a significant induction of 1.5-fold occurred at 10 dpi in both the control and infected samples, although expression returned to baseline levels by 12 dpi. *ERF1* and *ERF4* formed a distinct pair (Cluster 6, Figure 4.12). Neither showed any response at 7 or 10 dpi, however both genes were significantly induced at 12 dpi, to 1.2-fold and 1.5-fold respectively (Figure 4.15). For *ERF1* however, this difference may only have been significant because expression decreased in the control plants at this point.

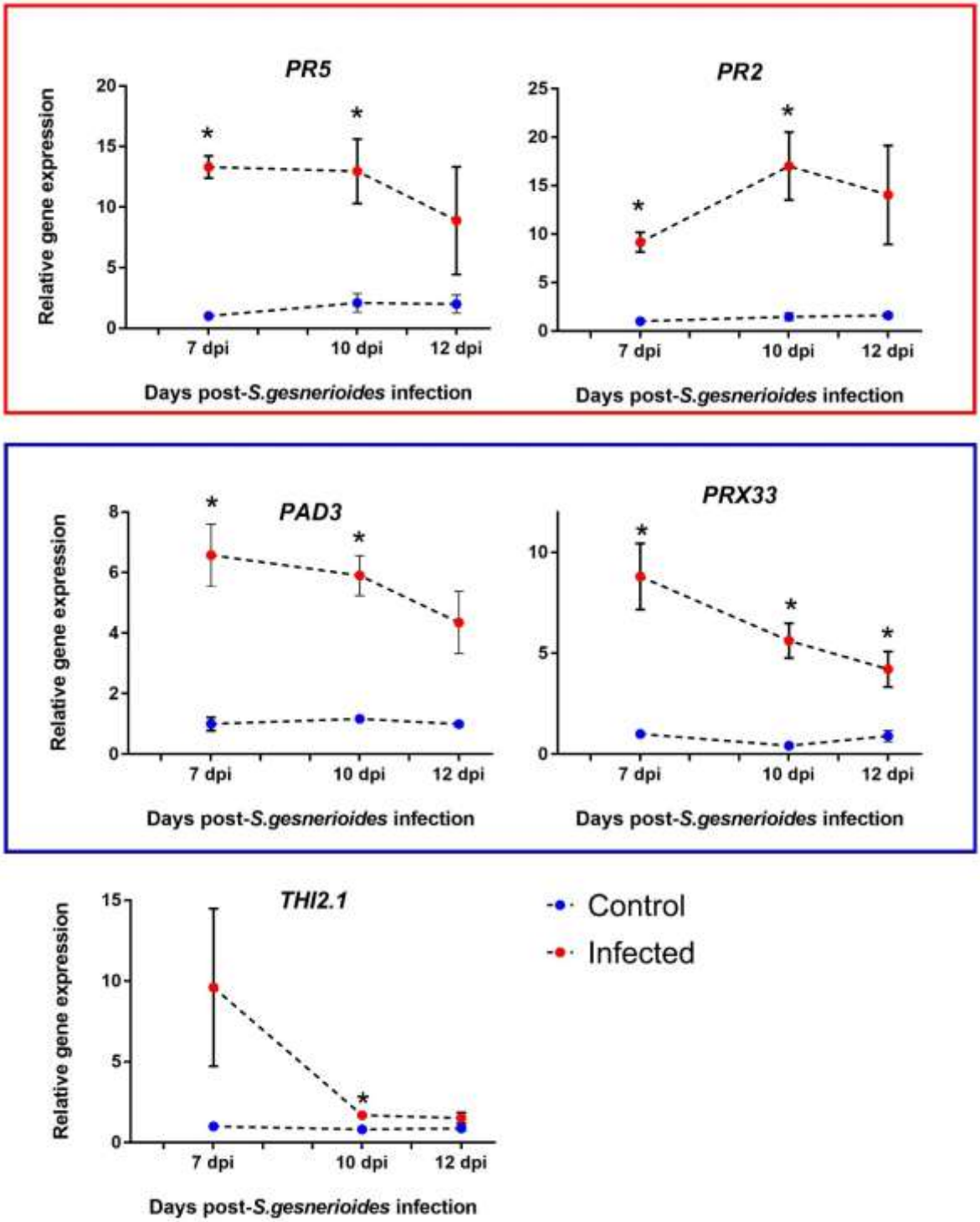


Figure 4.13: qPCR expression analysis of the genes *PR5*, *PR2, PAD3, PRX33* and *THI2.1* in control and *Striga gesnerioides*-infected Col-0 *Arabidopsis* root samples. For each time point, gene expression was tested on three control and three infected root samples. Error bars: Mean +/- standard error. Fold-changes in gene expression were calculated relative to control samples at 7 dpi. Indicated significant differences refer to the difference between the control and infected samples for that time point. * denotes statistical significance at the 5% level. dpi = days post-infection with *S. gesnerioides*. Boxes indicate genes that formed distinct pairs by clustering analysis (Euclidean distance, see Figure 4.12).

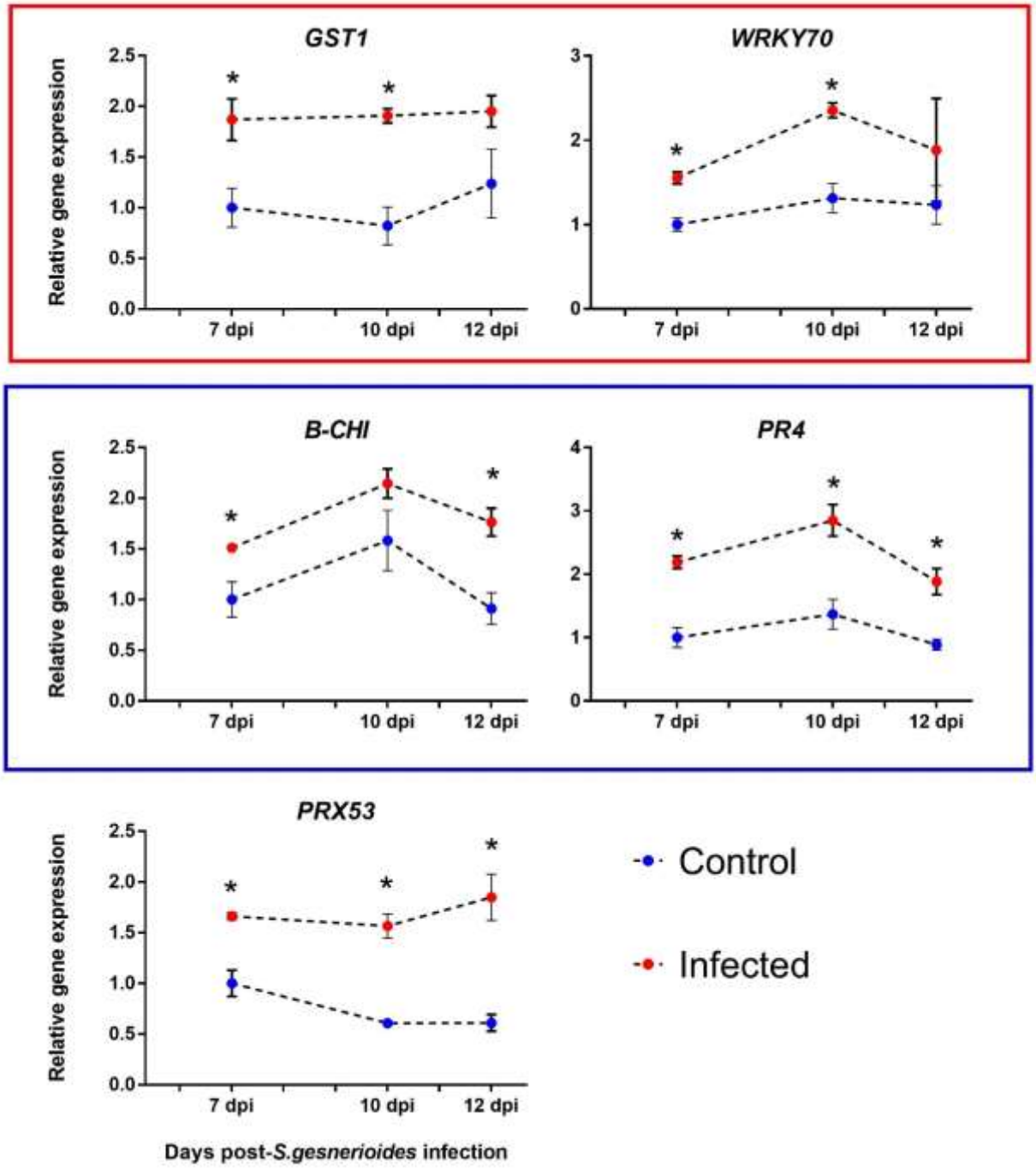


Figure 4.14: qPCR expression analysis of the genes *GST1, WRKY70, B-CHI, PR4* and *PRX53* in control and *Striga gesnerioides*-infected Col-0 *Arabidopsis* root samples. For each time point, gene expression was tested on three control and three infected root samples. Error bars: Mean +/- standard error. Fold-changes in gene expression were calculated relative to control samples at 7 dpi. Indicated significant differences refer to the difference between the control and infected samples for that time point. * denotes statistical significance at the 5% level. dpi = days post-infection with *S. gesnerioides*. Boxes indicate genes that formed distinct pairs by clustering analysis (Euclidean distance, see Figure 4.12).

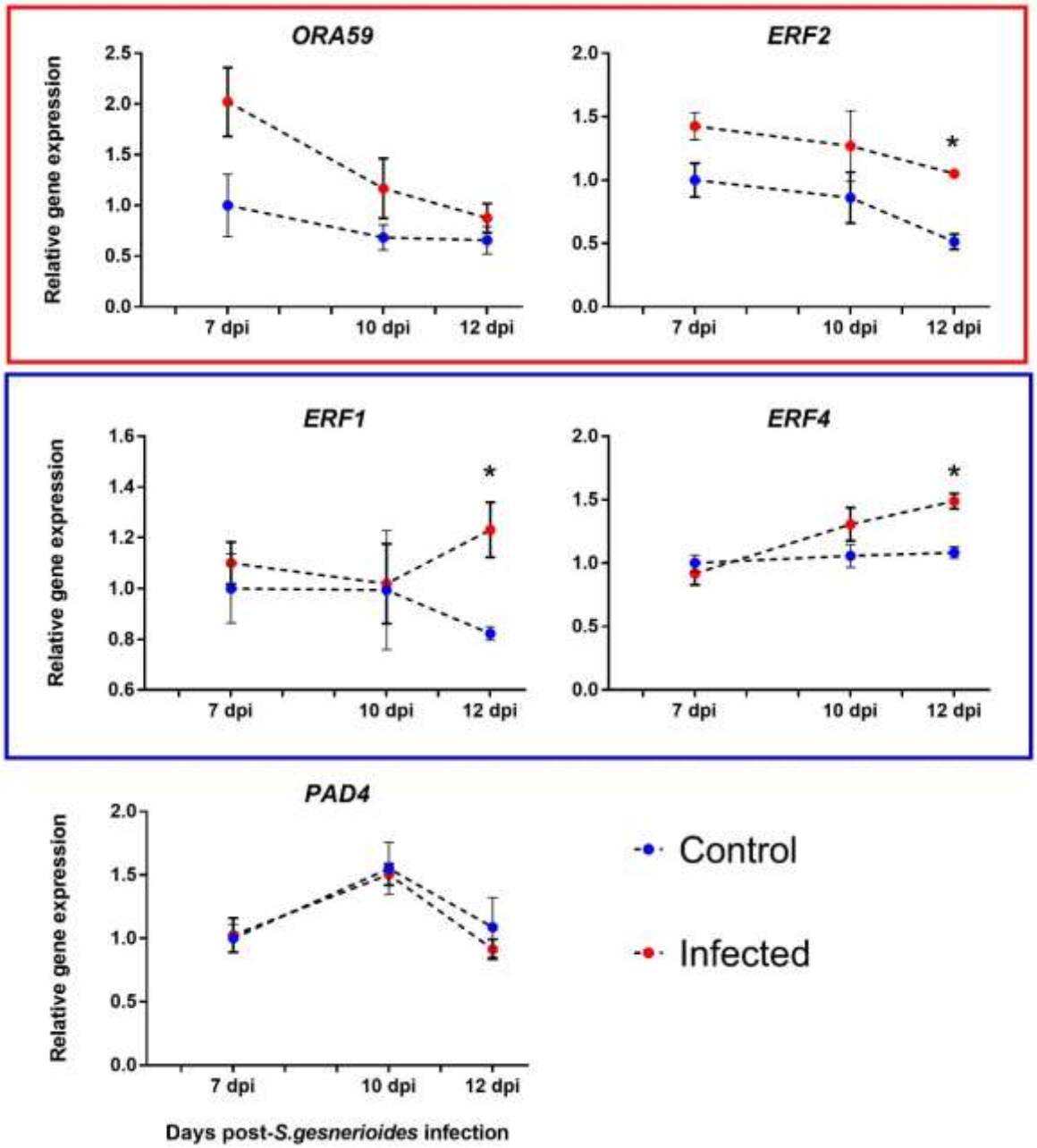


Figure 4.15: qPCR expression analysis of the genes *ORA59, ERF2, ERF1, ERF4* and *PAD4* in control and *Striga gesnerioides*-infected Col-0 *Arabidopsis* root samples. For each time point, gene expression was tested on three control and three infected root samples. Error bars: Mean +/- standard error. Fold-changes in gene expression were calculated relative to control samples at 7 dpi. Indicated significant differences refer to the difference between the control and infected samples for that time point. * denotes statistical significance at the 5% level. dpi = days post-infection with *S. gesnerioides*. Boxes indicate genes that formed distinct pairs by clustering analysis (Euclidean distance, see Figure 4.12).

PR1 and *PDF1.2* showed very similar expression profiles (Figure 4.16) and formed a discrete pair (Cluster 7, Figure 4.12). Both were downregulated in the control samples, so that expression at 12 dpi was only 0.8-fold relative to 7 dpi. In the infected samples, both genes were slightly repressed at 7 dpi, but were induced at 10 dpi to 1.3-fold for *PR1* and 1.2-fold for *PDF1.2,* relative to the reference control. By 12 dpi, expression reduced in the infected samples, but remained slightly higher than the level at 7 dpi. At 10 and 12 dpi, the difference between the control and infected samples for both genes was significant however this appears due to the downregulation that occurred in the control samples.

At 7dpi, *VSP2* expression was almost identical between both sets of samples (Figure 4.16). This remained stable in the infected samples, however in the control samples *VSP2* expression fell at 10 dpi to 0.6-fold the level at 7 dpi, although this recovered slightly by 12 dpi. *MYC2* expression was also downregulated in the control samples, however this decreased progressively being 0.8 and 0.6-fold at 10 and 12 dpi respectively, relative to 7 dpi (Figure 4.16). In the plants infected with *S. gesnerioides*, *MYC2* expression was significantly suppressed at 7 dpi, being 0.6-fold the controls. *MYC2* expression increased slightly at 10 dpi, being almost identical to the level of the 10 dpi controls. At 12 dpi however, *MYC* expression increased again in the infected samples, even as it reduced in the controls. Nevertheless, *MYC2* expression in the infected samples never exceeded that of the 7 dpi controls.

ANOVA analysis

Several genes showed temporal variation in the control samples even if expression was stable or upregulated for the infected plants. In particular, *PR1* and *PDF1.2* were induced at 10 dpi by *S. gesnerioides*, even though gene expression declined in the control samples at this point. To test for significant interaction effects between the treatment of the plants (infected vs control) and temporal gene expression, two-way ANOVA analysis was carried out. *THI2.1* was not included as the data was not normally distributed. The results (Table 4.2) demonstrate that infection by *S. gesnerioides* has a significant ($p < 0.05$) effect on the expression of *PR2, PR5, PR4, WRKY70, B-CHI, PDF1.2, ORA59, VSP2, PRX33, GST1, PRX53, ERF2, ERF4* and *PAD3.* The time point had a significant effect on the expression of *PR4, PAD4, B-CHI, PDF1.2, ORA59* and *ERF4.* A significant interaction effect between treatment and time point was found for *PR1, PDF1.2* and *ERF4.*

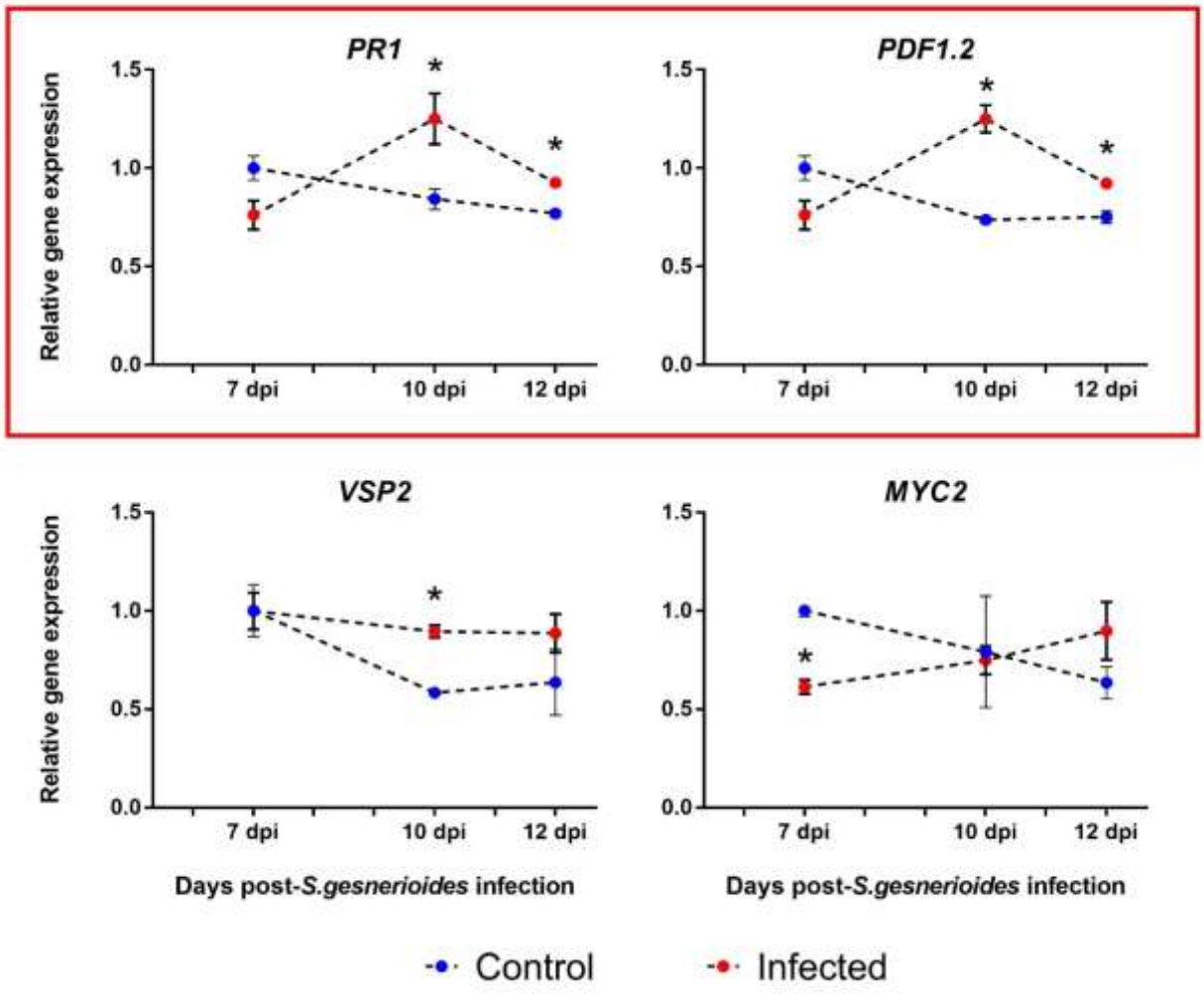


Figure 4.16: qPCR expression analysis of the genes *PR1, PDF1.2, VSP2* and *MYC2* in control and *Striga gesnerioides*-infected Col-0 *Arabidopsis* root samples. For each time point, gene expression was tested on three control and three infected root samples. Error bars: Mean +/- standard error. Fold-changes in gene expression were calculated relative to control samples at 7 dpi. Indicated significant differences refer to the difference between the control and infected samples for that time point. * denotes statistical significance at the 5% level. dpi = days post-infection with *S. gesnerioides*. Boxes indicate genes that formed distinct pairs by clustering analysis (Euclidean distance, see Figure 4.12).

Table 4.2: Two-way ANOVA analysis of defence gene expression in control and *Striga gesnerioides*-infected *Arabidopsis* root samples harvested at 7, 10 and 12 dpi.

Significance values				
Gene	Levene's Test for Equality of Error Variances	Treatment (control v infected)	Time point (7, 10, 12 dpi)	Treatment * Time point
PR1	**0.018**	0.079	0.031	**0.002**
PR2	**0.004**	**0.001**	0.294	0.382
PR4	0.314	**0.001**	**0.006**	0.434
PR5	**0.005**	**0.001**	0.610	0.464
WRKY70	**0.004**	**0.007**	0.181	0.663
PAD4	0.268	0.615	**0.007**	0.808
B-CHI	0.069	**0.001**	**0.009**	0.594
PDF1.2	0.144	**0.001**	**0.014**	**0.001**
ORA59	0.117	**0.013**	**0.023**	0.276
VSP2	0.112	**0.049**	0.052	0.317
MYC2	**0.003**	0.631	0.949	0.104
THI2.1	*Not suitable for ANOVA analysis*			
ERF1	0.184	0.146	0.952	0.379
PRX33	**0.010**	**0.001**	0.039	0.062
GST1	0.461	**0.001**	0.544	0.677
PRX53	**0.032**	**0.001**	0.182	0.104
ERF2	0.054	**0.004**	0.053	0.910
ERF4	0.278	**0.016**	**0.006**	**0.036**
PAD3	**0.005**	**0.001**	0.246	0.273

Significant p values are highlighted in red. Where equal variances can be assumed (i.e. Levene's Test statistic >0.05), p values < 0.05 are regarded as significant in the ANOVA analysis. Where Levene's Test statistic <0.05, p values < 0.01 are regarded as significant.

To what extent are the target genes normally expressed in *Arabidopsis* roots?

For most genes, upregulation never exceeded 3-fold compared with the reference control. To investigate how much these genes are expressed in the roots relative to other regions of the plant, a search was conducted on all 19 genes tested using the Anatomy function on GENEVESTIGATOR (Figure 4.17) (Hruz et al. 2008). This is based on a database of 10,615 samples from Affymetrix *Arabidopsis* ATH1 Genome Arrays. Several genes that showed only minimal upregulation in this assay (*PR1, PDF1.2* and *ERF1*) appeared to be poorly induced in the roots in general. This was not the case for *MYC2* and *ERF4*, which showed a low response to *S. gesnerioides* but have good general expression in roots. In contrast, *PR2* and *PR5* showed weak expression in the roots, relative to other regions; nevertheless, these were strongly upregulated by *S. gesnerioides*.

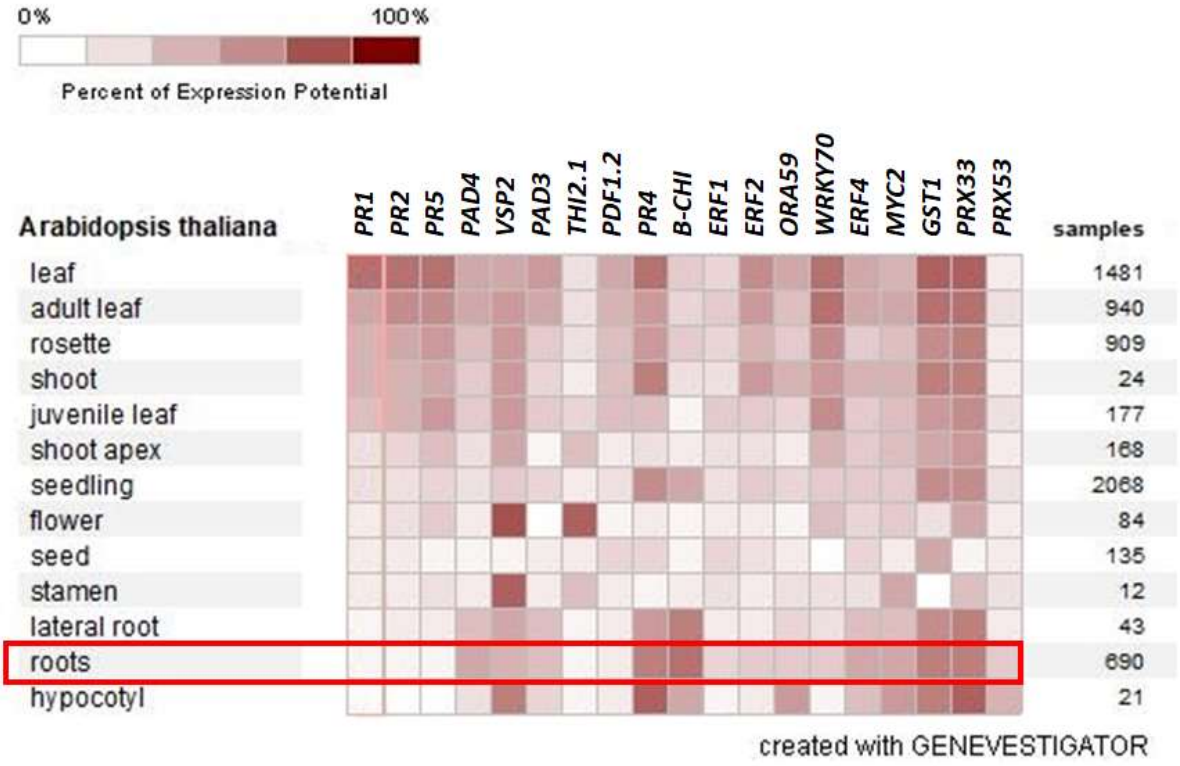


Figure 4.17: Anatomical analysis of target genes performed using the Anatomy function on GENEVESTIGATOR (Hruz et al. 2008). This searches across a database of samples from 10,615 Affymetrix *Arabidopsis* ATH1 Genome Arrays.

Signature similarity search

A signature similarity search was performed on the GENEVESTIGATOR *Arabidopsis* Perturbations database to identify the conditions that induce the most similar gene expression response to infection by *S. gesnerioides*. This searches a database of 10,615 samples from Affymetrix *Arabidopsis* ATH1 Genome Arrays. Each time point was assessed separately, using the gene signatures for the infected

samples. This generated a list of the 50 most similar signatures based on Euclidean distance, each with a 'relative similarity' score. The higher this value, the higher the similarity to the original transcriptional profile.

If the similarity $1/s_i$ is defined as $1/d_i$ where d_i is the distance of category i to the signature, then the relative similarity R of category c is calculated as:

$$R_{s_c} = \frac{s_c}{\frac{1}{N}\sum_{i \in I} s_i}$$

The identified signatures were categorised according to the type of perturbation: these included developmental processes, pathogen attack, abiotic stresses, hormone treatment, chemical treatment (e.g. pesticides) and comparisons between mutant *Arabidopsis* and wildtype. Signatures relating to pathogen attack, abiotic stresses and hormone treatment were investigated further. Between 14 – 20% of similar signatures were responses to pathogens (Figure 4.18). Most of these were biotrophic foliar pathogens (*Hyaloperonospora arabidopsidis, Pseudomonas syringae* and *Erysiphe cichoracearum*), although an arthropod herbivore (Silverleaf whitefly), a necrotrophic fungal root pathogen (*Rhizoctonia solani*; incompatible strain AG8) and an RNA virus (Turnip Mosaic Virus) were also represented. Between 6-10 % of the similar signatures were induced by application of SA or the functional analogue benzothiadiazole (BTH) (Figure 4.18). No studies involving JA or ethylene application were found, although for each time point at least one of the similar signatures was induced by auxin (IAA) (Figure 4.18). Regarding abiotic stresses, there was at least one similar signature induced by hypoxia or drought for each timepoint (Figure 4.18). For 7 and 12 dpi, one of the signatures was induced by heat treatment (Figure 4.18).

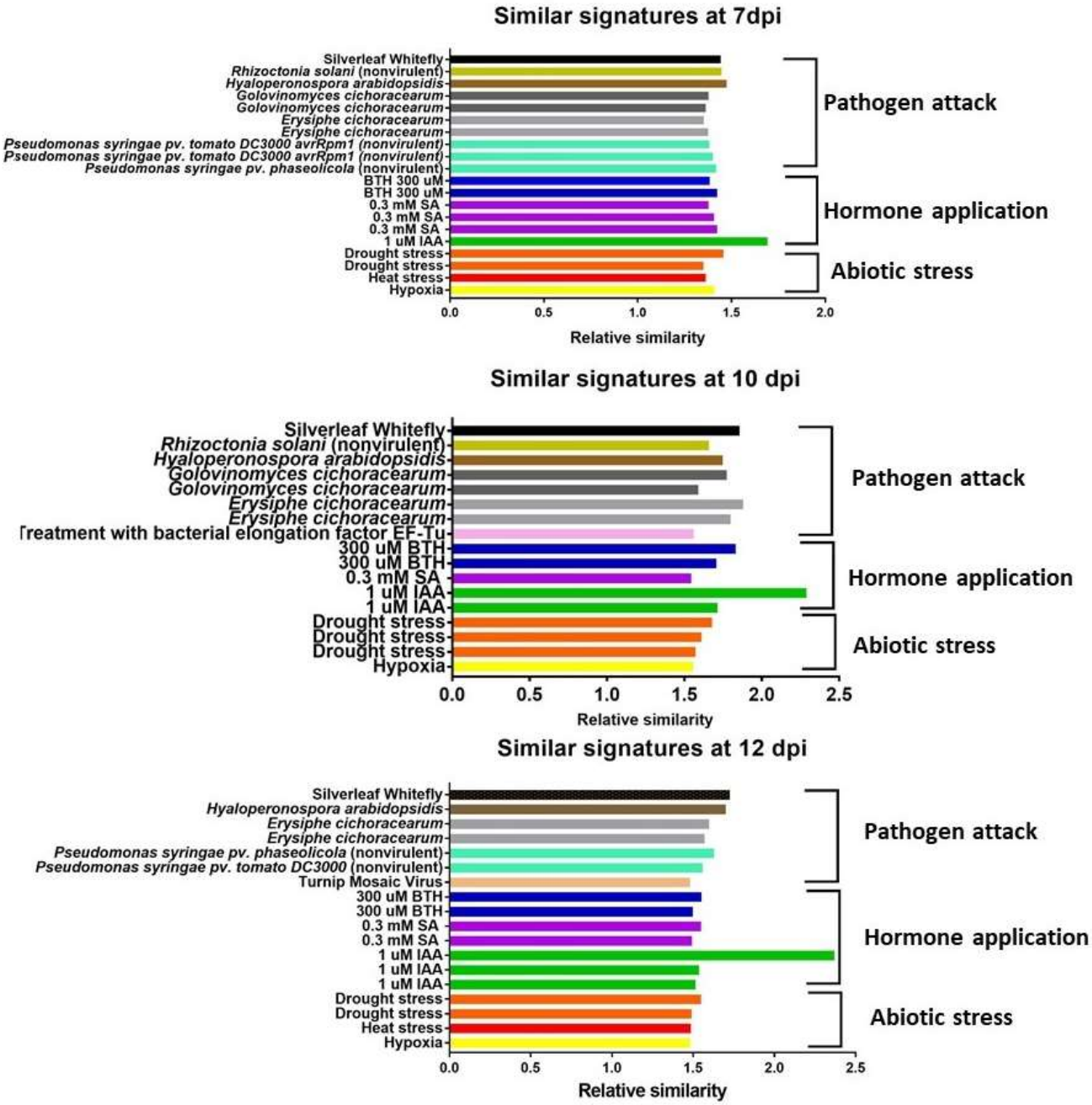


Figure 4.18: Categorical representation of the 50 gene signatures most similar to expression changes induced by *S. gesnerioides* in *Arabidopsis*, for the 19 target genes used in this study. The Signature Similarity search function was used on the GENEVESTIGATOR (Hruz et al. 2008) *Arabidopsis* Perturbations database, which searches across 10,615 samples from Affymetrix *Arabidopsis* ATH1 Genome Arrays. The gene expression signatures recorded at 7, 10 and 12 days post-*S. gesnerioides* infection were investigated separately. Signatures related to pathogen attack, hormone application or abiotic stress were investigated further and represented here. SA = salicylic acid, BTH = benzothiadiazole.

4.5 Discussion

4.5.1 β-GUS reporters are not suitable for assessing changes in genes expression in *S. gesnerioides*

β-glucuronidase (GUS) reporters were not suitable to assess gene expression in *Arabidopsis* hosts since the parasite itself showed a coloured reaction, even when infecting wildtype Col-0 lines. Initially, one possibility was that *S. gesnerioides* has intrinsic GUS activity, as documented for other plant species. Intrinsic GUS activity has indeed been recorded in *Arabidopsis*, rice (*Oryza sativa*), maize (*Zea mays*) and tobacco (*Nicotiana tobacum*) (Sudan et al. 2006), particularly in developing tissues. In this study, staining was most prominent in the developing shoots of attached *S. gesnerioides* haustoria (Figures 4.5-4.6, arrows). Another potential explanation was transfer of the *GUS*-transgene from host to parasite. Nucleic acid transfer has been demonstrated between hosts and parasitic plants and has been proposed as a control strategy. For instance, movement of silencing RNA for the gene *MANNOSE-6-PHOSPHATE REDUCTASE* from transgenic tomato to attached *Orobanche aegyptiaca* haustoria resulted in parasite death (Aly et al. 2009). In another study, movement of a GUS-silencing construct was demonstrated between lettuce and transgenic *Triphysaria versicolor* parasites that constitutively expressed *GUS* (Tomilov et al. 2008). However, unlike other parasitic plant interactions (e.g. *Cuscuta*)(Shahid et al. 2018), movement of small RNAs has not been demonstrated in *Striga* spp. (de Framond et al. 2007), possibly because they only make connections to the host xylem vessels and not the phloem.

In this case, however, *GUS*-transgene transfer from host to parasite were ruled out since the coloured reaction persisted in *S. gesnerioides* infected on wild-type Col-0 *Arabidopsis* that lacked any GUS-transgene (Figure 4.7). Furthermore, it was subsequently found that overnight incubation either at 37°C or with $K_4Fe(CN)_6$ / $K_3Fe(CN)_6$ was sufficient to cause the colour change even without the X-Gluc substrate (Figure 4.8), thus making intrinsic GUS activity in the parasite an unlikely cause. This implies that endogenous compounds within *S. gesnerioides* are responsible for the observed reaction. *Striga* radicles are known to produce high concentrations of H_2O_2 (Keyes et al. 2007, Kim et al. 1998, Wada et al. 2019); potentially the colour change is caused through oxidation of H_2O_2- by the $[Fe(CN)_6]^{3-}$ ions.

4.5.2 DAB staining shows high ROS concentrations of at the host-parasite interface

The results of the DAB staining assays confirm previous reports that germinated *Striga* radicles are sources of ROS, particularly H_2O_2 (Kim et al. 1998, Keyes et al. 2007, Wada et al. 2019). This was particularly concentrated at the host-parasite interface at 7 dpi (Figure 4.9), supporting the model where H_2O_2 extruded from *Striga* radicles liberates polyphenols from host cell walls, which promote the parasite's transition to haustorium development (Keyes et al. 2007). Indeed, a recent study using

a green fluorescent probe for H_2O_2 (carboxy-H2DFFDA) demonstrated intense H_2O_2 accumulation at the tips of the haustorial hairs during the maturation of the haustorium (Wada et al. 2019).

4.5.3 Significant gene expression differences in response to *S. gesnerioides*

These results indicate that *S. gesnerioides* induces host gene expression changes in multiple defensive pathways, including SA signalling, camalexin biosynthesis, the JA/ethylene signalling pathway and ROS-associated activity. This supports previous studies, e.g. (Swarbrick et al. 2008) which concluded that *Striga* induces defences common to a range of plant pathogens. Given this is a susceptible interaction, the level of induction is perhaps surprising; previous studies indicate that susceptible responses to *Striga* are characterised by wide-scale gene repression (Swarbrick et al. 2008, Mellor 2013). In this case, it may be more accurate to view this interaction as partially susceptible, since most pre-germinated *S. gesnerioides* seed applied to host *Arabidopsis* roots fail to attach. The induced genes therefore, are likely to have a role in determining the level of host basal resistance against *S. gesnerioides*.

Although many of the fold-changes were small, it should be borne in mind that several of these genes show low basal expression in the roots relative to other plant regions, e.g. *PR1* (Figure 4.17). Furthermore, samples were composed of whole root systems, and thus contained much tissue that was not in direct contact with the parasite: in studies using foliar pathogens, typically only affected leaf areas are used for gene expression analysis.

S. gesnerioides induces a strong SA-associated defence response

PR2 and *PR5* were the two most strongly induced genes, indicating high SA-associated signalling activity. These genes are also upregulated during the non-host interaction between *Arabidopsis* and *S. hermonthica* (Vasey 2005), but to a much higher extent, for instance over 200-fold for *PR5*, considerably more than that induced by *S. gesnerioides* in this study. This may indicate that SA has a protective effect against *S. gesnerioides*, but SA-associated defences are not realised fully enough in this interaction to prevent every parasite from gaining entry. The results from the mutant assays support this, since the SA-biosynthesis mutant *sid2-1* showed significantly reduced early-stage resistance (Chapter 3, Figure 3.2). In addition, significant upregulation of *PR5* also occured in cowpea during race-specific resistance against *S. gesnerioides*, but not during a compatible interaction with a hypervirulent strain (Li et al. 2009). Various pathogenesis-related genes, including thaumatin-like proteins and β 1,3,-glucanases, were also induced in resistant Nipponbare rice during *S. hermonthica* infection, but not in the susceptible cultivar IAC165 (Swarbrick et al. 2008). Furthermore, as detailed in Section 4.1, enhanced SA-mediated gene induction may be a distinguishing feature of sorghum cultivars resistant to *S. hermonthica* (Hiraoka and Sugimoto 2008).

Lower induction of JA/ethylene co-regulated genes by *S. gesnerioides*

In general, genes co-regulated by JA and ethylene, typically associated with defences against necrotrophic pathogens, showed lower induction compared with SA-associated genes. These included *B-CHI, PR4* and *THI2.1*. It is difficult to assess whether these contribute to basal resistance against *S. gesnerioides* since the fold-changes were low. Furthermore, in the mutant assays the ethylene-insensitive mutant *etr1-1* showed significantly greater early- and late-stage resistance (Chapter 3, Figure 3.5). In addition, the signature similarity search only identified one study relating to a necrotrophic pathogen, the fungal root pathogen *Rhizoctonia solani* (Figure 4.18). The observed upregulation of *B-CHI* is particularly surprising since this encodes chitinase enzyme, which degrades a major component of fungal cell walls (Samac et al. 1990). However, enhanced induction of chitinase enzymes has been associated with resistant responses of sunflower against *O. cumana* (Letousey et al. 2007). Furthermore, chitinase enzymes were induced during an incompatible interaction between cowpea and *S. gesnerioides*, but suppressed during a susceptible interaction (Mellor 2013). In addition, a class I chitinase was induced during the non-host response of *Lotus japonicus* against *S. hermonthica*, but not in the compatible interaction with *O. ramosa* (Hiraoka et al. 2008). Induction of endochitinase was also seen in resistant Nipponbare rice infected with *S. hermonthica*, but not in the susceptible rice cultivar IAC165 (Swarbrick et al. 2008). It remains to be seen whether chitinases have a relevant role in basal resistance against *Striga* parasites, or if their induction results from co-expression with similarly-regulated genes that are effective against parasitic plants. An alternative explanation is that *B-CHI* was induced by degraded pectic fragments (oligogalacturonides, OGs) (Ferrari et al. 2007): it is likely that these are generated as *S. gesnerioides* breaks into the root cortex.

The transcription factors *ORA59, ERF1* and *ERF2* induce genes co-regulated by JA/ethylene and select these pathways over SA-associated pathways (Pré et al. 2008, Solano et al. 1998). *ORA59* and *ERF2* both showed a weakly significant induction at 7 dpi, before being progressively down regulated, whilst *ERF1* was only induced slightly at 12 dpi (Figure 4.15). Overall, *ora59* showed significantly reduced early-stage resistance against *S. gesnerioides* (Chapter 3, Figure 3.4), hence it is surprising that *ORA59* was only induced to a maximum of 2.0-fold by the parasite. Potentially, *ORA59* may have been more highly expressed at preceding time points, since expression declined progressively during the time-course. *ORA59* may also have been inhibited by SA-associated signalling, indicated by the upregulation of *PR5* and *PR2*: SA negatively regulates *ORA59* both at the transcriptional level (Zander et al. 2014) and through protein degradation (Van der Does et al. 2013). Alternatively, the *ora59* phenotype may be due to mis-expression of genes regulated by *ORA59* that contribute to pre-formed defences that normally restrict parasite entry. Since *ora59* was not affected in late-stage resistance to *S. gesnerioides*, this indicates that *ORA59* functions primarily in preventing the parasite from attaching.

At low concentrations, JA can act synergistically with SA to induce SA-associated genes (Mur et al. 2006). In this interaction, low levels of JA activity may therefore support the SA response. Indeed, in the interaction between *S. hermonthica* and resistant Nipponbare rice, JA-regulated responses appear crucial for host defence since the JA-biosynthesis *hebiba* mutant showed greater susceptibility (Mutuku et al. 2015). This phenotype, however, appears to be determined by the strength of SA signalling, since it can be partly rescued by applications of either MeJA or the SA analogue BTH. Nevertheless, since rice has higher intrinsic levels of SA (Raksin et al. 1990) and this is a resistant interaction, rather than a basal resistance response, the relative balance of JA and SA required for effective defence may be different for the *Arabidopsis-S. gesnerioides* interaction. Curiously, the results of the present assay bear great resemblance to gene expression induced in *Arabidopsis* by the green peach aphid (*Myzus persicae*): another invasive pest that targets the host vascular system. This induces genes relating to both SA defences (*PR1, PR2*) and JA signalling (*PDF1.2, LOX2*). As in the results presented here, induction of SA-related genes was greater than JA-related defences. The authors propose that vascular-feeding insects induce a distinct gene expression profile incorporating elements of both signalling pathways (Moran and Thompson 2001).

SA-associated defences may be downregulated at 12 dpi

Notably, *PR5* and *PR2* induction was lowest at 12 dpi and the inter-sample variation is considerably greater for the infected samples at this point, compared with 7 and 10 dpi, and also compared with the controls (Figure 4.13, error bars). At the same time, *ERF1* and *ERF4* are significantly induced at this point by 1.2-fold and 1.5-fold respectively (Figure 4.15). Both these genes are induced by JA/ethylene (Yang et al. 2005, Huang, Catinot and Zimmerli 2015), hence this could indicate that *S. gesnerioides* actively suppresses SA-signalling by manipulating JA signalling once connected to the host vasculature. This is a deliberate strategy of various pathogens including the root necrotroph *Fusarium oxysporum*. Virulent strains of this pathogen excrete JA-leucine and JA-isoleucine conjugates and induce JA-regulated genes downstream of *COI1*. As *Arabidopsis coi1* mutants are significantly more resistant to *F. oxysporum*, this would suggest deliberate exploitation of host signalling pathways by the pathogen (Thatcher et al. 2009, Cole et al. 2014). As reviewed in Chapter 1, there is evidence that *S. gesnerioides* uses effector molecules to target host immunity, since resistance in cowpea is typically based on single genes, of which one has been found similar to R genes against other plant pathogens (Li and Timko 2009). Furthermore, in compatible interactions between cowpea and *S. gesnerioides*, *COI1*, a master-regulator for JA responses, appears suppressed at 3 dpi post infection but is then markedly induced at 5 dpi. This induction occurs to a lesser extent during non-host resistance and not at all in the race-specific response (Li et al. 2009). Since *ERF1* is downstream of *EIN3* (Solano et al. 1998), it is interesting that *ein3-1* was not affected in early-stage resistance to *S. gesnerioides* but showed significantly greater late-stage resistance (specifically; fewer haustoria with shoot development) (Chapter 3, Figure

3.5). This may indicate that EIN3 only has a relevant role in basal resistance after *S. gesnerioides* attaches to the host vascular system.

Nevertheless, if *ERF4* is induced by the parasite to favour infection, this does not explain why the *erf4-1* mutant showed significantly reduced early-stage resistance (Chapter 3, Figure 3.4). An alternative explanation is that host SA defences are downregulated as the parasite connects to the vascular system in order to activate a more appropriate suite of responses. There is evidence that this may occur during interactions with root knot nematodes (RKN), which have an infection strategy similar to *S. gesnerioides*. SA-defences appear to prevent initial RKN penetration and infection, while JA antagonises this function. At later stages of infection however (e.g. root galling), the opposite appears to be the case (Martínez-Medina et al. 2016). Ultimately, a longer time-course would be required to confirm if SA-associated genes are downregulated and *ERF1/ERF4* upregulated beyond 12 dpi.

***S. gesnerioides* strongly induces genes related to reactive oxygen species (ROS) detoxification**

DAB assays revealed strong staining at the host-parasite interface, indicative of increased H_2O_2 concentrations (Figures 4.9 and 4.11). Furthermore, all three genes associated with ROS detoxification, *GST1, PRX33* and *PRX53*, showed significant upregulation, particularly at 7 and 10 dpi (Figures 4.13-4.14). *PRX33* was induced to a much greater extent than *GST1* or *PRX53*, with a maximum average fold-induction of 8.8. Comparison of the Ct values in the control samples however indicate that *PRX53* and *GST1* (Ct ~ 21-22) are more highly expressed than *PRX33* (Ct ~27). Curiously, *PRX33* and *PRX53* have been associated with host-generated ROS during interactions with very different classes of pathogens. *PRX53* is induced by JA, wounding and root penetration by the cyst nematode *Heterodera schachtii* (Jin et al. 2011). *PRX33*, on the other hand, is associated alongside *PRX34* in generating ROS in response to bacterial microbe-associated molecular patterns (MAMPs) (Daudi et al. 2012). This again indicates that *S. gesnerioides* triggers gene expression changes that incorporate multiple defence pathways.

ROS-detoxification genes (*AtGSTF2/F3/F6/F7* and *PEROXIDASE34*) are also induced in *Arabidopsis* during the non-host response against *S. hermonthica* (Vasey 2005). It is not clear, however, whether high levels of oxidative stress are part of host basal resistance or a condition actively induced by the parasite to favour ingression. In race-specific resistance responses of cowpea against *S. gesnerioides*, host-generated ROS facilitate a hypersensitive response (HR) with programmed cell death (PCD) at the host-parasite interface (Lane et al. 1993a). This oxidative burst appears to depend on peroxidases since class III peroxidases showed strong induction in cowpea during race-specific resistance against *S. gesnerioides,* particularly during the HR response at 6dpi (Mellor 2013). These are downregulated by 13 dpi, coinciding with parasite death. Conversely, class III peroxidases are not induced in the susceptible interaction; instead various peroxidase genes appear to be downregulated. Similarly,

Letousey et al. identified two genes using SSH relating to ROS detoxification that are upregulated in sunflower cultivars resistant to *O. cumana* but not in susceptible cultivars (Letousey et al. 2007).

Whilst these studies indicate that a strong oxidative burst is associated with host resistance, ROS-associated genes are also induced during compatible interactions between *Arabidopsis* and *O. ramosa*. In this case, gene upregulation (including *GST1*) is concentrated in the first day of infection, and rapidly declines thereafter (Dos Santos et al. 2003). One interpretation of the present results therefore is that a host-generated oxidative burst is activated against *S. gesnerioides*, however this is not sufficient to prevent infection. *PRX33* expression decreased progressively throughout the time-course, as does the expression of the SA-associated gene *PR5*. Endogenous ROS and SA are thought to form an amplification loop whereby increased intercellular ROS causes SA to accumulate, which maintains the activity of ROS-generating RBOHD and RBOHF by counteracting feed-forward ROS dampening mechanisms (Chaouch, Queval and Noctor 2012). Suppression of SA-signalling by *S. gesnerioides* may thus reduce the host ROS response.

If host-generated ROS are a resistant response, this may be to facilitate HR and PCD at the host-parasite interface. In support of this, the gene signature induced by *S. gesnerioides* is more similar to that induced by biotrophic pathogens, rather than necrotrophic pathogens (Figure 4.18). During incompatible interactions with biotrophic pathogens, host-derived ROS is used to drive HR and PCD to restrict pathogen spread (Torres 2010). This approach is counterproductive against necrotrophic pathogens, however, that feed off dead plant tissue (Glazebrook 2005). Nevertheless, browning of host *Arabidopsis* tissue was not generally observed surrounding failed attempts of *S. gesnerioides* penetration. Similarly, necrosis and PCD do not occur in the non-host response of *Arabidopsis* to *S. hermonthica*, unlike other hosts such as marigold (Vasey 2005). Furthermore, *RbohD/RbohF* double mutants show compromised resistance against virulent *Pseudomonas syringae* pv. tomato DC3000 (*Pst*): this is associated with reduced ROS generation, HR and PCD in response to the pathogen (Torres et al. 2002). Yet this mutant has significantly greater early- and late-stage resistance to *S. gesnerioides* (Chapter 3, Figure 3.6). This may indicate that PCD does not have an intrinsic role in either non-host or basal resistance in *Arabidopsis* against *Striga* parasites. This could be investigated by *Arabidopsis* mutants affected in pathogen-induced PCD (e.g. *sobir1*) for altered basal resistance to *S. gesnerioides.*

Host-derived ROS may stimulate biosynthesis of the phytoalexin camalexin (Glawischnig et al. 2004) rather than PCD, since *cyp79B2/B3* (affected in the production of both glucosinolates and camalexin) showed significantly reduced late-stage resistance against the parasite (Chapter 3, Figure 3.7) although early-stage resistance was not affected. Furthermore *PAD3*, which catalyses the final step of camalexin biosynthesis (Zhou et al. 1999, Schuhegger et al. 2006), was one of the genes most strongly induced by *S. gesnerioides* (Figure 4.13). Potentially, camalexin specifically restricts further development of

attached *S. gesnerioides* haustoria. Nevertheless, *erf4-1 Arabidopsis* show increased basal expression of *PAD3* (Edgar et al. 2006) yet this mutant had signficantly reduced early-stage resistance against *S. gesnerioides* (Chapter 3, Figure 3.4).

Alternatively, *GST1*, *PRX33 and PRX53* may be induced by *S. gesnerioides* itself, since DAB staining demonstrated strong oxidising activity at the radicle tip and host-parasite interface (Figures 4.9-4.11). If excessive ROS favours ingression of the parasite, this would explain the increased early-stage resistance seen in *RbohD/RbohF* and *RbohD* (Chapter 3, Figure 3.6). In *S. asiatica*, H_2O_2 production ceases as part of the developmental commitment to forming a haustorium (Kim et al. 1998). Between 7 and 10 dpi, *S. gesnerioides* breaks into the host vascular system and transitions to haustorium development (Figures 4.1 and 4.2). This may explain why *PRX33* expression declines in the host from 7 dpi onwards, if parasite production of H_2O_2 begins to reduce at this point. *RBOH*-mediated ROS generation may also be activated oligogalacturonide (OG) fragments (Ferrari et al. 2007); as discussed above, these could be generated when *S. gesnerioides* penetrates the root cortex. Furthermore, in the interaction between *Arabidopsis* and *H. schachtii*, *PRX53* upregulation is not thought to facilitate ROS-mediated defence gene activation since PR genes (including *PR2, PAD4* and *PDF1.2*) do not show upregulation in *PRX53* overexpressing lines (Jin et al. 2011), suggesting the main role of *PRX53* induction is to detoxify pathogen-induced ROS.

***S. gesnerioides* may not be perceived as a wound response.**

VSP2 is an acid phosphatase induced by wounding and insect herbivores (Utsugi et al. 1998, Liu et al. 2005) via the JA signalling pathway (Berger, Bell and Mullet 1996). In this assay, *VSP2* expression declined in the control plants during the time-course but was maintained at a steady level in the infected plants. In the non-host interaction between *Arabidopsis* and *S. hermonthica*, however, *VSP2* (and also the related *VSP1*) is induced, to a maximum of 4.5-fold (Vasey 2005). Given that *S. gesnerioides* penetrates further than *S. hermonthica*, using a combination of mechanical pressure and enzymatic digestion of cell walls (Joel et al. 2013a), it is surprising that *VSP2* did not show more significant induction in the infected hosts. Potentially, *S. gesnerioides* uses effector molecules to downregulate the host wound response. Alternatively, greater induction of *VSP2* may be inhibited by the early SA-associated response, since this gene is sensitive to inhibition by SA, including during challenges with pathogens and insects (Leon-Reyes et al. 2009, Koornneef et al. 2008). In the compatible interaction between rice and *S. hermonthica*, a wound-induced gene *WIN2 PRECURSOR* is upregulated immediately after infection at 2pi, but declines thereafter, returning to near baseline levels at 11 dpi (Swarbrick et al. 2008). This indicates that in compatible interactions, *Striga* spp. induce only a transient wound response at most.

WRKY transcription factors may differentiate between compatible/ non-host interactions with *S. gesnerioides*

There was particular interest in assessing *WKRY70*, since *wrky70* had significantly reduced early-stage resistance to *S. gesnerioides* (Chapter 3, Figure 3.4). Furthermore, *WRKY70* is significantly induced during the non-host interaction between *Arabidopsis* and *S. hermonthica*, to approximately 2.4-fold (Vasey 2005). In this assay, *WRKY70* was significantly induced at 7 and 10 dpi (Figure 4.13), yet the maximum fold-change was again 2.4. Since *WKRY70* upregulation is similar in the *Arabidopsis* non-host and susceptible interaction, this may indicate that *WRKY70* upregulation does not have a relevant role in basal host resistance, but is induced as a consequence of SA signalling activity (Wang et al. 2006). Similar to *PR5* and *PR2*, the inter-sample variation in *WRKY70* expression for the infected plants was considerably greater at 12 dpi than for 7 and 10 dpi (Figure 4.13, error bars), which may indicate that *WRKY70* is driven by SA in this interaction. In this case, the phenotype of *wrky70* may be due to altered basal activity of *WRKY*-regulated genes or altered SA levels. *wrky70* mutants are reported to have higher basal activity of both SA-associated genes (*PR1, PR2, PR5, PAD4*) and JA/ethylene-regulated genes (*COR1, PDF1.2*), besides *GST1* and *PAD3* (Ülker et al. 2007, Li et al. 2017).

Alternatively, *WRKY70* may have a relevant role in the basal resistance response and be modified post-transcriptionally through interaction with other WRKY transcription factors. These may play a crucial role in distinguishing between susceptible and resistant interactions with *Striga* spp. The functional activity of *WRKY70* may depend on invading pathogens being successfully perceived as harmful, leading to the induction of appropriate interacting partners. In rice, a suite of *WRKY* transcription factors (including the rice equivalent of *WRKY70*) is induced by *S. hermonthica* in resistant Nipponbare cultivars, but not the susceptible cultivar IAC165 (Swarbrick et al. 2008). WRKY53 and WRKY54 function with WRKY70 to promote basal resistance to *P. syringae*; WRKY53 additionally represses induction of *PDF1.2* by MeJA (Miao and Zentgraf 2007, Hu, Dong and Yu 2012, Li et al. 2017). VQ-motif containing proteins also modify WRKY transcription factors through physically interacting with the DNA-binding WRKY domain and are induced by SA and pathogen challenge (Cheng et al. 2012). Analysis of double or even triple mutants is likely required to genetically dissect the role of individual WRKY transcription factors in this interaction. *wrky54/wrky70* mutants, for instance, show significantly greater resistance to the bacterial necrotroph *Pectobacterium carotovorum,* whereas the individual *wrky54* or *wrky70* mutants do not (Li et al. 2017).

S. gesnerioides induces gene expression changes in _Arabidopsis_ similar to that induced by other pathogens and auxin application

For each time point, the signature similarity search identified several studies relating to SA/BTH application, but none describing gene responses to JA or JA/ethylene (Figure 4.18), suggesting that the parasite is predominantly perceived as a biotrophic pathogen. In support of this, considerably more of the identified studies concerned biotrophic pathogens rather than necrotrophs or insect herbivores. In many cases, this was the fungal biotroph *Golovinomyces cichoracearum* (formerly *Erysiphe cichoracearum*). One of these studies examined the point when *G. cichoracearum* establishes a haustorium for nutrient transfer, which may parallel haustorium formation by *S. gesnerioides* (Fabro et al. 2008). During this process, *G. cichoracearum* induces many genes relating to photosynthetic function and carbon metabolism, besides genes regulated by NPR1 or JAR1, although neither *npr1-1* or *jar1-1 Arabidopsis* mutants show overall increased susceptibility to this pathogen (Fabro et al. 2008). Similarly, neither *npr1-1* or *jar1-1* were affected in early-stage resistance against *S. gesnerioides* in the mutant screen (Chapter 3, Figures 3.2 and 3.3) yet genes regulated by *NPR1* and *JAR1* were induced in the qPCR time-course (e.g. *WRKY70* and *PR4*).

Another highly similar gene signature was that of the *enhanced disease resistance1 (edr1) Arabidopsis* mutant in response to *G. cichoracearum*, relative to Col-0 (Christiansen et al. 2011). *EDR1* encodes a Raf-like MAPK kinase kinase and is a negative regulator of SA-associated defences (Frye, Tang and Innes 2001). The enhanced resistance of *edr1* against *G. cichoracearum* was associated with increased expression of genes relating to both SA- and JA-associated defences (e.g. *PAD4* and *THI2.1* respectively), chitinases and chitin-binding proteins (PR4) and ROS-associated genes. In addition, a number of WRKY transcription factors were more strongly expressed in infected *edr1* plants (relative to infected Col-0), including *WRKY38* and *WRKY59*, known to be induced by NPR1 and BTH (Wang et al. 2006). Given that *THI2.1* and *B-CHI* showed some induction in response to *S. gesnerioides*, it could be informative to test the *edr1* mutant to ascertain whether greater expression of these genes increases basal resistance against *S. gesnerioide*s. Interestingly, another identified gene signature was that of the *pmr4-1* mutant in response to *G. cichoracearum*, relative to Col-0 (Nishimura et al. 2003). *pmr4-1* is more resistant to *G. cichoracearum*, and this is attributed to hyper-induction of SA and pathogen associated genes (e.g. *PR2*, *PR5* and *PR1*) and stronger downregulation of defensins (including *PDF1.2*). In the mutant phenotyping assay, *pmr4-1* showed considerably greater late-stage resistance against *S. gesnerioides*, although early-stage resistance was not affected (Chapter 3, Figures 3.7).

Other biotrophic interactions identified in the signature search included R-gene mediated resistance against the oomycete *Hyaloperonospora arabidopsidis*, which is associated with the hypersensitive

response, rapid PCD and SA-accumulation (Wang et al. 2011). Several studies also concerned *P. syringae;* notably these were all avirulent strains, for instance *P. syringae pv. Tomato* (*Pst*) DC3000 *avrRps4*. In *Arabidopsis*, the qualitative gene expression response is generally similar for virulent and avirulent *Pst* strains, but induced more rapidly and strongly against avirulent strains (Tao et al. 2003). In particular, SA-associated defences are upregulated to a greater extent (Jagadeeswaran, Saini and Sunkar 2009) and ROS generation sustained for longer against avirulent pathogens (Torres, Jones and Dangl 2006). Indeed, in this study SA- and ROS-associated genes still showed upregulation up to 12 days after initial infection (Figures 4.13 and 4.14).

Only one of the identified signatures related to an arthrophod pest, the silverleaf whitefly *Bemisia tabaci* (Kempema et al. 2007). Defences against herbivory and wounding are generally coordinated by JA (Reymond et al. 2000), however this study demonstrates that *B. tabaci* triggers a transcriptomic response distinct to that of other insects, including other phloem feeders such as the green peach aphid *Myzus persicae*. In particular, *B. tabaci* induced strong upregulation of SA accumulation and signalling, and also genes associated with cell wall modification, oxidative stress and glucosinolate/camalexin synthesis. In contrast, JA-biosynthesis and signalling genes were downregulated, leading to the suggestion that *B. tabaci* evades JA/wounding-responses by causing minimal tissue damage during invasion. In this study, *VSP2* was not markedly upregulated by *S. gesnerioides*, indicating that this parasite may similarly avoid triggering a wound response during invasion. Interestingly, *PMR4* was induced by *B. tabaci* and callose deposition observed in the vascular tissue surrounding feeding sites. Since *pmr4-1*, which is defective in callose production, showed greater late-stage resistance to *S. gesnerioides*, this suggests that callose deposition does not play a role in limiting further development of *S. gesnerioides* that have breached the vasculature, unlike *B. tabaci*.

The gene signature at 7 and 10 days post-*S. gesnerioides* infection was highly similar to that induced by the soil-borne necrotrophic pathogen *Rhizoctonia solani*, an economically important pathogen on cereals and legumes (Foley et al. 2013). This was specific, however, to the interaction between *Arabidopsis* and the incompatible *R. solani* strain AG8, rather than the virulent strain AG2-1. Interestingly, this study found that resistance against AG8 is unchanged in various *Arabidopsis* mutants affected in JA, SA, ethylene, ABA and auxin signalling, besides camalexin production. Several of these mutants (*jar1*, *npr1*, *abi1* and *pad4*) also showed no effect on early-acting resistance to *S. gesnerioides* (Chapter 3, Figures 3.2, 3.3 and 3.5). Whilst many genes were commonly induced by AG8 and AG2-1 (including *PR4*, *PR5* and chitinase genes), *PR2* was specifically induced by incompatible AG8, whilst *PR1* was only upregulated by virulent AG2-1. Similarly, *PR2* was strongly induced by *S. gesnerioides* (Figure 4.13), but *PR1* much less so (Figure 4.16). Other genes specifically induced by AG8 included NAC domain and WRKY transcription factors (*WRKY18*, *WRKY22* and *WRKY25*), besides heat shock proteins.

Various genes relating to oxidative stress, including peroxidases and glutathione-S transferases also showed differential regulation: in particular, the NADPH oxidase *RbohD* was only induced by AG8. Loss of function mutations in either *RbohD* or *RbohF* did not affect resistance to AG8, however the double mutant *RbohD/RbohF* was newly susceptible to AG8, with only 7% host survival. Similarly, *RbohD/RbohF* showed a stronger phenotype against *S. gesnerioides* than the individual *Rboh* mutants (Chapter 3, Figure 3.6). In this case, however, *RbohD/RbohF* was more resistant to *S. gesnerioides* at both early and late stages. *RbohD/RbohF* are also more resistant to *H. arabidopsidis* (Torres et al. 2002) but are not affected in basal resistance against avirulent *Pst DC3000* (Torres et al. 2002). These differences have been proposed to be due to the role of RBOHD and RBOHF in regulating cell death around infection sites, and the extent this contributes to basal resistance during different pathogenic interactions (Foley et al. 2013).

For each time point, a number of the results in the signature similarity search concerned gene expression induced by auxin/IAA application (Figure 4.18). Auxin is crucial for xylem differentiation (Fukuda 2004) and may be induced by *S. gesnerioides* via JA/ethylene to achieve vascular continuity with the host. This could explain the induction of *ERF1* and *ERF4* at 12 dpi, and why *ein3-1* mutants were specifically affected in a late stage of basal resistance, with a lower proportion of haustoria showing shoot development. Both symbiotic rhizobia and parasitic nematodes also appear to alter auxin signalling in their hosts, with the hormone being important for establishing nodules and feeding sites respectively (Grunewald et al. 2009). Furthermore, auxin appears necessary for the establishment of haustorial structures for the hemi-parasitic root parasite *Phtheirospermum japonicum* (Ishida et al. 2016). Nevertheless, in the susceptible interaction between rice and *S. hermonthica*, no auxin-responsive genes were identified as being upregulated by the parasite; instead five auxin-responsive genes were downregulated, even at 11 dpi (Swarbrick et al. 2008). It could be informative to test auxin-responsive genes directly using qPCR during the interaction between *Arabidopsis* and *S. hermonthica*.

None of the similar signatures were induced by exogenous ABA, although some were induced by drought (Figure 4.18). Moderate induction of ABA-responsive genes has been reported during the early stages of the compatible interaction between rice and *S. hermonthica* (Swarbrick et al. 2008). Nevertheless, only one ABA-induced gene, *RAB18*, was found to be upregulated during the non-host interaction between *Arabidopsis* and *S. hermonthica*, and not beyond 2 dpi (Vasey 2005). As *S. gesnerioides* appears to induce a minimal wound-response, upregulation of ABA-related genes may not be extensive during the early part of the interaction. Potentially, once the parasite is established on the vasculature and withdrawing water, ABA signalling is more extensive. It could be informative to test the expression of ABA-signalling genes at later time points, to assess the extent to which the parasite imposes abiotic drought/salt stress on the host.

4.6 Conclusion

Whilst the qPCR results revealed some significant changes in gene expression during infection with *S. gesnerioides*, it remains difficult to know the relative contribution of each towards basal resistance. One way to investigate this is to compare expression in a host cultivar with a different level of resistance. This will be a focus of Chapter 5, where gene expression during *S. gesnerioides* infection will be compared between wild-type Col-0 *Arabidopsis* and the significantly more susceptible *erf4-1* mutant.

CHAPTER FIVE

Investigating the role of the *ERF4* gene in basal resistance against *Striga gesnerioides*.

ABSTRACT

The *erf4-1* mutant of *Arabidopsis*, which carries a T-DNA insertion at the 3'-end of the *ERF4* gene, is significantly affected in early-stage resistance against *Striga gesnerioides* (Chapter 3, Figure 3.4). The *ERF4* gene can be alternatively transcribed as a transcriptional activator or repressor (Lyons et al. 2013), which presents multiple explanations for the enhanced susceptibility of the *erf4-1* mutant. Quantitative polymerase chain reaction (qPCR) analysis of control and infected wild-type plants (Col-0) indicated that both *ERF4-Repressor* and *ERF4-Activator* are induced during *S. gesnerioides* infection, although the absolute level of *ERF4-Activator* expression may be too low to be biologically relevant. Epifluorescence microscopy revealed that *S. gesnerioides* establishes a xylem connection earlier in *erf4-1* than in Col-0 plants. Subsequent qPCR analysis of defence genes in control and infected plants demonstrated that *erf4-1* shows impaired induction of *PR5* in response to *S. gesnerioides* and lower overall expression of *PRX33*. Interestingly, qPCR profiling of the different *ERF4* splice variants in Col-0 and *erf4-1* revealed that *erf4-1* retains a residual level of the *ERF4-Repressor* splice variant (10% - 15 % of the wild-type), but does not express the *ERF4-Activator* splice variant at all. Transgenic over-expression of the *ERF4-Activator* variant in a wild-type background (Col-0 *p35S:ERF4-Activator* plants) significantly reduced basal resistance to *S. gesnerioides*, suggesting that the ERF4-Activator variant induces susceptibility. Unexpectedly, an independent T-DNA insertion mutant (*erf4-2*) did not show enhanced disease susceptibility compared to wild-type plants. Further qPCR analysis of all three *ERF4* splice variants in Col-0, *erf4-1* and *erf4-2* plants revealed that *erf4-1*, unlike *erf4-2*, shows near wild-type levels of the intron-retained variant of ERF4 (ERF4-IR). This finding suggests that the increased abundance of ERF4-IR relative to ERF4-Repressor in *erf4-1* causes its enhanced susceptibility. Together, the results of this chapter indicate that the elevated ERF4-Activator : ERF4-Repressor ratio in the *p35S:ERF4-Activator* line and the elevated ERF4-IR : ERF4-Activator ratio in the *erf4-1* mutant both cause enhanced susceptibility to *S. gesnerioides* by interfering with the resistance-inducing activity of the ERF4-Repressor variant.

5.1 Introduction

5.1.1. The *erf4 Arabidopsis* mutant is highly susceptible to *Striga gesnerioides*

Of all the *Arabidopsis* mutants tested, one of the most striking results was seen with *erf4-1*, which showed significantly increased early-stage susceptibility (Chapter 3, Figure 3.4). Therefore, investigating the mechanistic basis of the increased susceptibility of *erf4-1* may reveal defence components contributing to host basal resistance.

5.1.2. *ERF4*: an important regulator of defence signalling pathways

Ethylene-responsive element binding factors (ERFs) are a large family of plant-specific transcription factors that recognise the GCC-box sequence (Ohme-Takagi and Shinshi 1995). ERFs show different expression patterns, indicating that they have specific functions (Fujimoto et al. 2000). Whilst most are transcriptional activators, ERF4, along with ERF3 and at least 6 others, acts as a transcriptional repressor, capable of both directly inhibiting basal gene expression and suppressing the transactivation activity of other factors (Fujimoto et al. 2000). This repressive activity is attributed to a conserved C-terminal DNA motif L/FDLNL/F(x)P, named the ERF-associated amphiphilic repression (EAR) domain: mutations in this eliminate the repressive activity (Ohta et al. 2001). It is thought that ERF4 binds GCC-boxes in the promoters of target genes and recruits histone deacetylase HDA19 via SAP18 to induce chromatin remodelling that prevents gene expression (Song and Galbraith 2006).

In *Arabidopsis, ERF4* is induced in response to ethylene, jasmonic acid (JA) and abscisic acid (ABA) (Yang et al. 2005). It is also induced by abiotic stresses including cold, wounding, high NaCl, and drought stress but not high temperature stress. All of these responses, apart from induction by NaCl or ethylene, are maintained (albeit at a lesser extent) in the *ein2* mutant, indicating that ethylene is only required to induce *ERF4* in response to specific stresses (Fujimoto et al. 2000). *ERF4* has been proposed to be an important regulator of ethylene and JA signalling. Yang et al. (2005) reported that constitutive over-expression of a *ERF4* cDNA in *p35S:ERF4* plants reduced the sensitivity to ethylene but increased the sensitivity to JA, as evidenced by hypocotyl and root growth-inhibition assays, respectively (Yang et al. 2005). These over-expression plants also showed impaired induction of *B-CHI* and *BGL* by ethylene and JA/ethylene, respectively (Yang et al. 2005).

Since defences against necrotrophic pathogens are co-ordinately regulated by JA and ethylene (Glazebrook 2005), it is not surprising that alterations in ERF4 activity affect basal resistance against necrotrophic pathogens. Indeed, *p35S:ERF4 Arabidopsis* have been reported to show increased susceptibility to the fungal pathogen *Fusarium oxysporum* (McGrath et al. 2005), which initially acts as a biotrophic pathogen, but later switches to a necrotrophic mode. Although this over-expression line

did not show altered basal expression of *PDF1.2* (an antimicrobial peptide induced synergistically by JA and ethylene (Penninckx et al. 1996, Ellis and Turner 2001)), *PDF1.2* induction by methyl-jasmonate (MeJA) was impaired. Conversely, the *erf4-1* mutant was reported to express 30-fold higher levels of *PDF1.2* and show increased resistance to *F. oxysporum* (McGrath et al. 2005).

These results strongly indicate that *ERF4* negatively regulates defences against necrotrophic pathogens (Figure 5.1). Yet, *ERF4* is induced in wild-type plants during infection in foliar tissue by *F. oxysporum* (McGrath et al. 2005) and locally and systemically in response to the fungal necrotroph *Alternaria brassicicola* (McGrath et al. 2005, Brown et al. 2003). Curiously, *ERF4* is not induced in the roots during the early biotrophic stages of *F. oxysporum* infection (Edgar et al. 2006). Together, these results suggest that *ERF4* functions in a negative-feedback mechanism to limit costly host defence responses during attacks by necrotrophic pathogens.

It has also been proposed that *ERF4* facilitates SA-induced repression of JA-dependent *PDF1.2* expression by operating downstream of *NPR1* and *WRKY70* (McGrath et al. 2005)(Figure 5.1). This is based on the observation that the *Arabidopsis ERF4* promoter contains five putative W-boxes (recognised by *WRKY* transcription factors; TFs), while none are present in the *PDF1.2* promoter. It is unclear however, whether *ERF4* is induced by SA and SA-induced WRKY TFs, as some studies suggest this is the case (McGrath et al. 2005), whilst others have concluded the opposite (Yang et al. 2005). It is also unresolved whether *ERF4* binds directly to the *PDF1.2* promoter, since in response to MeJA, *ERF4* transcripts are upregulated earlier than those of *PDF1.2* (Brown et al. 2003). Furthermore, according to a later study, the *erf4-1* mutant of *Arabidopsis* shows increased basal expression of SA-dependent *PR5* and *PR1* in the leaves (Edgar et al. 2006), suggesting that *ERF4* may negatively regulate SA-signalling. This study also found that *erf4-1* showed increased basal expression of *PAD3* (Edgar et al. 2006), which catalyses the final step in the biosynthesis of the phytoalexin camalexin and is important for resistance to fungal pathogens (Schuhegger et al. 2006, Ferrari et al. 2007).

In addition to its role in JA, SA and ethylene signalling, *ERF4* has also been implicated as a negative regulator of ABA signalling (Figure 5.1). Based on root growth inhibition assays in *Arabidopsis*, *p35S:ERF4* plants are less sensitive to ABA, but hypersensitive to salt stress (Yang et al. 2005). Furthermore, *ERF4* over-expression reduces the induction of ABA-responsive genes by ABA, such as *ABA INSENSITIVE2 (ABI2)* (Yang et al. 2005). It has been reported that *ERF4* binds to the dehydration-responsive element (DRE, TACCGACAT) (Ohta et al. 2001), which is associated with drought- and cold-induced gene induction (Yamaguchi-Shinozaki and Shinozaki 1994). It has been proposed that *ERF4* is a repressive counterpart to the transcriptional activators CBF1/ DREB, which also recognise this sequence (Yamaguchi-Shinozaki and Shinozaki 1994, Stockinger, Gilmour and Thomashow 1997).

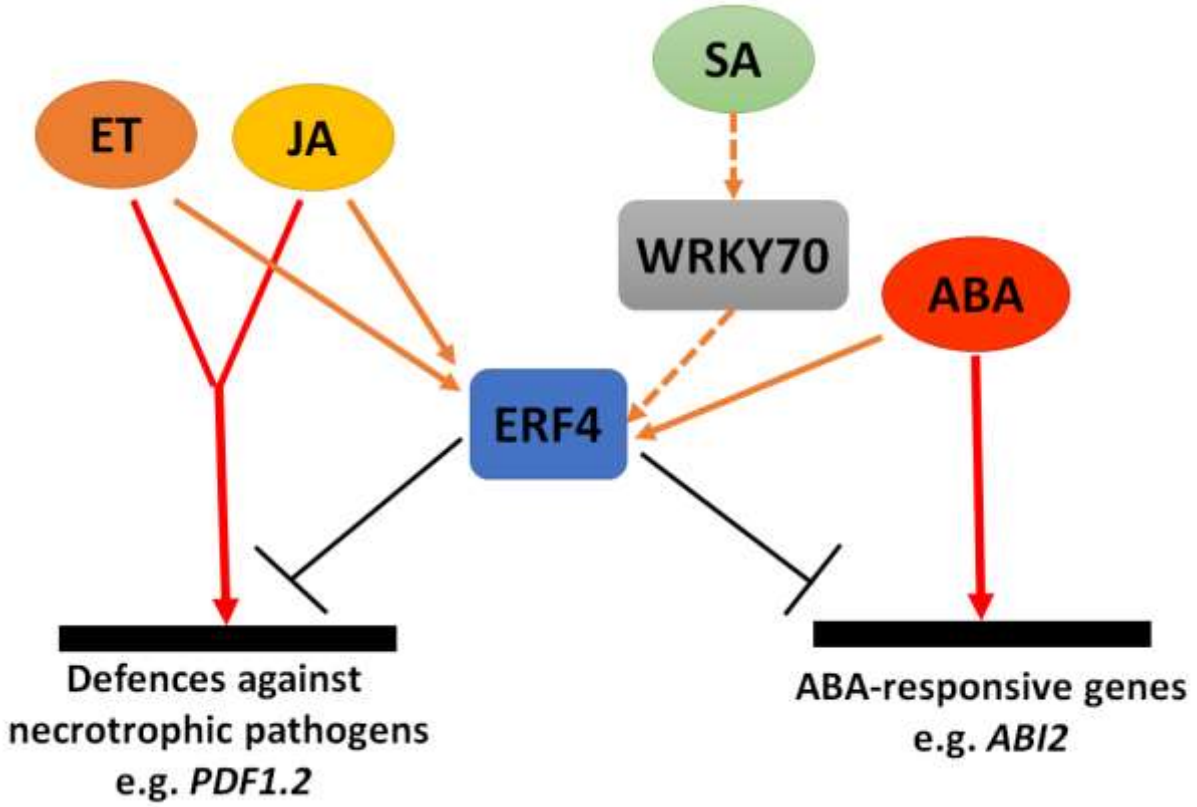


Figure 5.1: Gene model for *ERF4* function, based on over-expression and loss-of-function studies in *Arabidopsis*. *ERF4* is induced by ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) (Yang et al. 2005) and possibly salicylic acid (SA) (McGrath et al. 2005). *ERF4* inhibits genes co-regulated by ethylene and JA that are important in host resistance against necrotrophic pathogens, such as *PDF1.2* (Yang et al. 2005, McGrath et al. 2005). SA has been proposed to antagonise JA/ethylene signalling through induction of *ERF4*, via the transcription factor *WRKY70* (dashed lines) (McGrath et al. 2005). *ERF4* also negatively regulates ABA-responsive genes such as *ABI2* (Yang et al. 2005), and may function as part of a negative-feedback mechanism in the ABA-signalling pathway.

5.1.3. *ERF4*: A multi-faceted regulator?

Recently, it was discovered that the *Arabidopsis ERF4* mRNA transcript can be alternatively spliced, producing a protein variant with different functionality (Lyons et al. 2013). This occurs through selection of a distal polyadenylation site, rather than the more proximal one used under in the absence of pathogen challenge. When the distal polyadenylation site is used, the full-length transcript is spliced such that the repressive EAR domain is removed along with the intervening intron (Figure 5.2). This alternative *ERF4* variant appears to act as a transcriptional activator of GCC box-containing genes. Transgenic over-expression of this *ERF4-Activator* variant increased basal *PDF1.2* expression whilst the normally-transcribed *ERF4-Repressor* variant suppressed *PDF1.2*. In addition, *ERF4-Activator* over-expression suppressed the generation of reactive oxygen species (ROS) in response to bacterial flagellin, while the opposite was the case for the *ERF4-Repressor* variant. Crucially, transcripts of *ERF4-*

Activator were not detected as part of basal gene expression, but only in response to treatment with bacterial flagellin (flg22) to induce Pattern-Triggered Immunity (PTI). According to this study, the RNA binding protein FPA normally selects for the proximal adenylation site, causing the *ERF4-Repressor* variant to be transcribed, since *ERF4-Activator* transcripts accumulate in *fpa Arabidopsis* mutants. FPA thus acts as a negative regulator of PTI-driven responses, such as the flg22-induced ROS burst. Upon recognition of flg22, however, suppression by FPA is lifted, allowing *ERF4-Activator* to be transcribed. Induction of *ERF4-Activator* was not seen in response to treatment with SA or MeJA (Lyons et al. 2013), suggesting that it occurs specifically during the early stage of PTI, rather than being induced later as a result of defence hormone signalling. The authors proposed that induction of *ERF4-Activator* is a host strategy to prevent a run-away ROS-induced defence response, which could incur cell damage and/or death (Lyons et al. 2013).

ERF4 mRNA transcripts are highly unstable with an estimated half-life of less than 60 minutes (Gutiérrez et al. 2002). The ERF4 protein itself is subject to degradation by the proteasome (Koyama et al. 2013). Switching between alternatively-spliced variants could therefore allow host plants to rapidly respond to pathogen attacks and expand the functional repertoire of transcription factors that act as key nodes in the defence signalling network. As reviewed in (Staiger et al. 2013), alternative polyadenylation and splicing of gene transcripts is becoming recognised as an important component of plant immunity, particularly in generating *R* gene products with different functional specificities. Besides alternative polyadenylation/splicing, other post-transcriptional mechanisms may regulate *ERF4*. In *Arabidopsis,* treatment with the protein-synthesis inhibitor cycloheximide (CHX), causes *ERF4* transcripts to significantly accumulate, along with *ERF1*, *ERF2* and *ERF5*. This indicates that other regulatory proteins may modulate the activity of *ERF4,* for instance through mRNA degradation or transcriptional repression (Fujimoto et al. 2000).

Recently, alternative polyadenylation of *ERF4* has been implicated in the *Arabidopsis* leaf senescence program. It had previously been demonstrated that *ERF4* functions together with *ERF8* as a positive regulator of leaf senescence through direct suppression of *EPITHIOSPECIFIER PROTEIN/ EPITHIOSPECIFYING SENESCENCE REGULATOR (ESP/ESR)* (Koyama et al. 2013). *ESP/ESR* in turn suppresses the transcription factor *WKRY53*, which promotes leaf senescence (Miao and Zentgraf 2007). A more recent study, however, found that the different splice variants of *ERF4* have opposing effects on leaf senescence (Riester et al. 2019). This study reported that ERF4-Activator and ERF4-Repressor act antagonistically on *CATALASE3 (CAT3)* expression, functioning as a transcriptional activator and suppressor, respectively. *CAT3* reduces intracellular H_2O_2 levels, which promote leaf senescence. Interestingly, the ratio of ERF4-Activator and ERF4-Repressor in leaves appears to vary across development. At early developmental stages, ERF4-Activator is more prevalent, inducing *CAT3* expression and repressing H_2O_2. At later stages, FPA accumulates, inhibiting the distal polyadenylation

site in *ERF4*. This results in increased production of ERF4-Repressor, which suppresses *CAT3* expression, thereby allowing H_2O_2 to accumulate and trigger the senescence program. As discussed previously, *Striga* radicles are potent sources of H_2O_2, which appear important in generating Haustorial Initiation Factors that promote haustorium formation (Keyes et al. 2007, Wada et al. 2019). The relative abundance of ERF4-Activator and ERF4-Repressor in the roots may therefore also affect the penetration success of *S. gesnerioides.*

The alternative splicing of *ERF4* has interesting repercussions for studies that have used the *erf4-1 Arabidopsis* mutant. The approximate site of the T-DNA insertion in this line occurs at the C-terminal end of the coding sequence for the Repressor variant, after the EAR domain (Figure 5.2). This would likely prevent alternative polyadenylation to form the Activator variant, but may still allow some form of the Repressor variant to be transcribed. Furthermore, previous studies that demonstrated *ERF4* induction in response to hormone treatment (Yang et al. 2005), biotic stress (Fujimoto et al. 2000) and pathogens (McGrath et al. 2005), would likely not have distinguished between the Repressor and Activator variants, thus making it difficult to interpret these results in light of the latest findings.

5.1.4. *ERF4* and basal resistance against *S. gesnerioides*

Comparing the enhanced susceptibility of *erf4-1* to *S. gesnerioides* to the basal resistance phenotypes of the other *Arabidopsis* mutants and gene expression analyses described in Chapters 3 and 4 could point to a potential mechanism by which ERF4 controls basal resistance. Previous literature has established that the normally-transcribed ERF4-Repressor variant is a negative regulator of *PDF1.2* expression (McGrath et al. 2005, Lyons et al. 2013). Accordingly, *erf4-1* has previously been found to show significantly enhanced basal *PDF1.2* expression, besides increased resistance to *F. oxysporum* (McGrath et al. 2005). This suggests that upregulation of necrotrophic defences co-regulated by JA and ethylene could promote susceptibility to *S. gesnerioides*. In support of this, the *wrky70* mutant also showed significantly increased susceptibility to *S. gesnerioides* (Chapter 3, Figure 3.4) and *WRKY70* was significantly induced in wild-type plants at 7- and 10-days post-infection (Chapter 4, Figure 4.14). The WRKY70 transcription factor is believed to promote SA-mediated suppression of JA signalling, as over-expression of *WRKY70* supresses MeJA-induced induction of *PDF1.2* (Li et al. 2004). Nevertheless, suppression of *PDF1.2* by WRKY70 is dependent on functional NPR1 (Li et al. 2004) and the *npr1-1* mutant did not show a significantly altered susceptibility to *S. gesnerioides*. Potentially, other targets of WRKY70 that do not depend on NPR1 are more relevant in basal resistance against *S. gesnerioides*. Finally, the finding that the ethylene-insensitive *etr1-1* is more resistant to the parasite suggests that suppression of JA/ethylene defences may promote basal resistance against *S. gesnerioides* (Chapter 3, Figure 3.5).

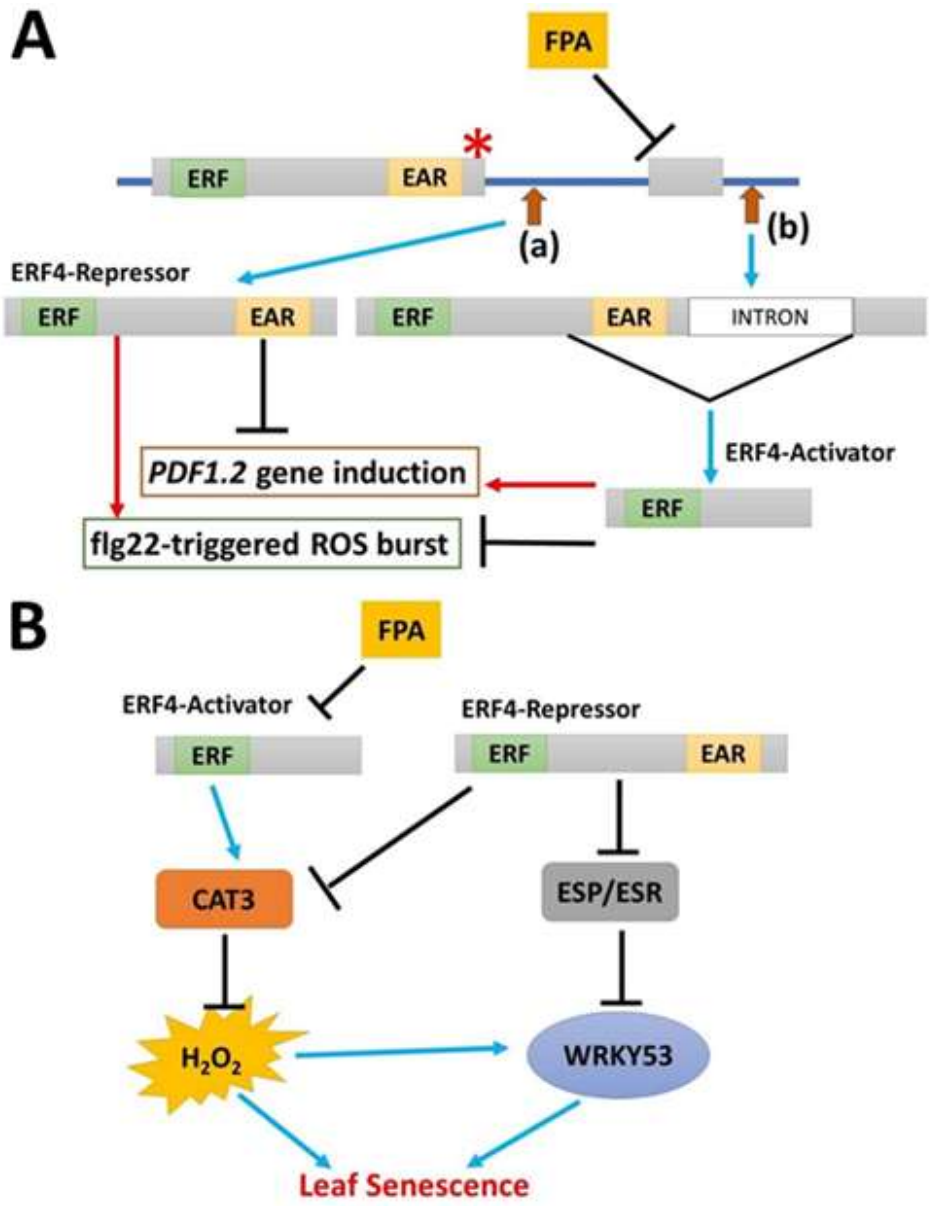


Figure 5.2 Different functions of alternatively generated ERF4 transcription factors. **A.** Model for ERF4 function in pathogen-induced defences. The ERF domain confers sensitivity to ethylene. Selection of the proximal polyadenylation site (a) causes the *ERF-Repressor* variant to be transcribed, containing the repressive ERF-associated amphiphilic repression (EAR) domain. The ERF4-Repressor variant suppresses GCC-box containing genes such as *PDF1.2*, but promotes reactive oxygen species (ROS) generation triggered by bacterial flagellin (flg22). *ERF4-Repressor* transcription is promoted by the RNA binding protein FPA, which inhibits the distal polyadenylation site (b). Under certain conditions, such as recognition of flg22, repression by FPA is lifted and the distal polyadenylation site is selected, resulting in a longer transcript with an intervening intron. Excision of this intron removes the EAR domain, producing the ERF4-Activator variant. This lacks the EAR domain and is a transcriptional activator of *PDF1.2*, yet also suppresses flg22-triggered ROS. It is proposed that the ERF4-Activator variant functions to restrict an otherwise excessive host ROS response during Pattern-Triggered Immunity, (PTI). * denotes the approximate site of the T-DNA insertion in the *Arabidopsis erf4-1* mutant SALK_073394C. Based on published results (Lyons et al. 2013). **B.** Model of ERF4 function in leaf senescence. As plant leaves mature, the ratio of ERFF4-Activator and ERF4-Repressor changes. In early stages, ERF4-Activator dominates and promotes transcription of *CATALASE3 (CAT3)*, which reduces intracellular H_2O_2 (a promoter for leaf senescence). As leaves mature, FPA accumulates and causes ERF4-Repressor to dominate. ERF4-Repressor inhibits CAT3, causing H_2O_2 levels to increase. ERF4-Repressor, together with ERF8, also suppresses *EPITHIOSPECIFIER PROTEIN/ EPITHIOSPECIFYING SENESCENCE REGULATOR (ESP/ESR)*, which in turn suppresses the transcription factor *WKRY53*, a promoter for leaf senescence. The combination of increased H_2O_2 and WRKY53 triggers the onset of leaf senescence. Based on the results of (Koyama et al. 2013, Riester et al. 2019, Miao and Zentgraf 2007).

However, a model where ERF4 modulates basal resistance via its influence on JA/ET signalling does not fit with the basal resistance phenotype reported for the *ora59* mutant (Chapter 3, Figure 3.4). The ORA59 transcription factor acts downstream of COI1, integrating JA and ethylene signals to induce *PDF1.2* and other genes relevant in defence against necrotrophic pathogens, such as *B-CHI* and *PR4* (Pré et al. 2008). It has even been proposed that *ORA59* itself is suppressed by ERF4, since the promoter contains a GCC box that is recognised by the transcriptional activator ERF96 (Catinot et al. 2015). Furthermore, the *ORA59* promoter was reported to interact with ERF4 in a yeast one-hybrid assay (Çevik et al. 2012). Given that the more susceptible *erf4-1* mutant shows increased activity of genes that are positively regulated by ORA59 (McGrath et al. 2005), it follows that the *ora59* mutant should be more resistant to *S. gesnerioides*. This was not the case, since *ora59* also showed overall reduced early-stage resistance (Chapter 3, Figure 3.4). In addition, mutations in neither *JIN1* or *ATAF2* affected basal resistance to *S. gesnerioides*, even though these are proposed to function similarly to ERF4 in repressing JA/ethylene-regulated genes such as *PDF1.2* (Lorenzo et al. 2004, Delessert et al. 2005).

Potentially, the phenotype of the *erf4-1* mutant is related to the proposed role of *ERF4* in restricting ROS during immune responses. The phenotype of the *RbohD* and *RbohD/RbohF* double mutant implies that host-produced ROS favour *S. gesnerioides* invasion, since these showed some significant effects for increased host basal resistance (Chapter 3, Figure 3.6). Furthermore, strong upregulation of the detoxifying genes *GST1 (GLUTATHIONE S-TRANSFERASE 1), PRX33 (PEROXIDASE 33)* and *PRX53 (PEROXIDASE 53)*, occured during the wild-type compatible interaction (Chapter 4, Figures 4.13 and 4.14), indicating that a strong ROS response takes place. Since the ERF4-Activator variant suppresses ROS during PTI, whilst the Repressor variant promotes this (Lyons et al. 2013), the phenotype of *erf4-1* could be caused by an excessive ROS response, due to the inability to induce *ERF4-Activator*.

5.2. Aims and objectives

This chapter describes an in-depth study into the role of the different *ERF4* splice variants in basal resistance against *S. gesnerioides,* including the use of over-expression lines of each splice variant. If the phenotype of *erf4-1* is indeed caused by an inability to induce expression of *ERF4-Activator* during infection with *S. gesnerioides*, it can be expected that *p35S-ERF4 Activator* would show increased resistance. If, however, the phenotype is caused by reduced expression of *ERF4-Repressor*, then the *p35S-ERF4 Repressor* line would show increased resistance, with no apparent difference between Col-0 and the *p35S-ERF4 Activator* line. This chapter also presents results from qPCR assays to determine how the ratio of both splice variants changes during the interaction in the Col-0 wild-type host. In addition, qPCR results are presented to investigate the extent of defence gene mis-regulation in the *erf4-1* mutant under both control conditions and after *S. gesnerioides* infection, as well as the impacts of the *erf4-1* mutation on the expression of ROS-detoxifying enzymes, such as *GST1* and *PRX33*.

Aim:

To investigate the molecular basis of the enhanced susceptibility phenotype of *erf4-1 Arabidopsis* against *S. gesnerioides*.

Objectives:

1. To compare the relative induction of the *ERF4-Activator* and *ERF4-Repressor* variants during infection of wild-type Col-0 *Arabidopsis* with *S. gesnerioides*, over three timepoints.

2. To assess whether the *erf4-1* phenotype is caused by increased penetration of the host vasculature at an early infection stage, using microscopy analysis of infected Col-0 and *erf4-1* root samples.

3. To compare defence gene expression between wild-type Col-0 and *erf4-1* after infection with *S. gesnerioides*.

4. To test basal resistance against *S. gesnerioides* in *Arabidopsis* lines overexpressing either *ERF4-Activator* or *ERF4-Repressor* in the wildtype Col-0 background.

5. To verify the role of ERF4 in basal resistance against *S. gesnerioides* by testing the phenotype of an independent T-DNA insertion mutant of *Arabidopsis* (*erf4-2*).

5.3. Experimental design

5.3.1 qPCR analysis of *ERF4-Activator* and *ERF4-Repressor* in Col-0 *Arabidopsis* during infection with *S. gesnerioides*.

Expression of *ERF4-Activator* and *ERF4-Repressor* was assessed in the Col-0 samples used for the qPCR time-course assay described in Chapter 4. In addition, expression of the full length unspliced *ERF4* transcript was tested: this is denoted as *ERF-IR* for *ERF4-Intron Retention.* Samples from control and *S. gesnerioides*-infected Col-0 *Arabidopsis* harvested at 7, 10 and 12 days post-infection were tested. As before, each run included the housekeeping genes *GADPH* and *20S Proteasome* as reference controls. Primers for *ERF4-Activator*, *ERF4-Repressor* and *ERF4-IR* were based on Lyons et al. 2013 (Table 5.1). Statistical analysis of the qPCR data was performed as described in Chapter 4, Section 4.3.4. Student's T-tests were performed on the relative expression values to identify significant differences between uninfected and infected plants.

5.3.2 Viewing root samples cleared with chloral hydrate under UV light:

Potentially, the increased susceptibility of *erf4-1* is due to the invading *S. gesnerioides* radicles accessing the host vascular system more quickly, enabling them to more effectively supress host defences. To investigate this, root sections were harvested from *S. gesnerioides*-infected *erf4-1* and Col-0 *Arabidopsis* plants at 7, 10 and 12 days post infection (dpi). The *Arabidopsis* plants were germinated, grown in rhizotrons and infected according to the protocol described in Chapter 2, Section 2.3. *S. gesnerioides* seed were also prepared as detailed previously (see Chapter 2, Section 2.2). Four plants from each genotype were harvested at 7, 10 and 12 dpi and samples prepared in chloral hydrate as described previously (see Chapter 2, Section 2.5). These were viewed under UV light using a BX51 Olympus upright fluorescence microscope (Olympus, London) so that lignin-containing elements such as cell walls and xylem could be seen.

5.3.3 qPCR analysis of defence gene expression *erf4-1* and Col-0 *Arabidopsis*

An assay was performed to investigate how the *erf4-1* mutation affects both basal defence gene expression and gene induction in response to *S. gesnerioides*. qPCR was performed on root samples from control and *S. gesnerioides*-infected root samples from Col-0 and *erf4-1 Arabidopsis*. These were prepared in rhizotrons as described previously, and *S. gesnerioides* applied to the roots when the hosts were 24 days old. For each genotype, there were 15 control and 15 infected plants. Root samples were harvested at one timepoint, 7 days post-infection, and as before root sections from three plants were combined to make one biological sample. For each gene, expression was measured on three uninfected biological replicates and three infected biological replicates. Within each qPCR run, each biological replicate was represented twice (i.e. two technical replicates) and an average calculated from these. Given the high susceptibility of this mutant, at later timepoints a significantly greater proportion of the host root would be in contact with the parasite. This would make it difficult to interpret whether differences in expression between Col-0 and *erf4-1* were due to the host genotype or the increased exposure of *erf4-1* to *S. gesnerioides*. RNA extraction, cDNA synthesis and qPCR were performed as described in Chapter 2, Section 2.8. The following target genes were selected *PR4, PR5, ORA59, B-CHI, PR5, WRKY70, GST1* and *PRX33.* As before, each run included the housekeeping genes *GADPH* and *20S Proteasome* as reference controls. Primer sequences can be found in Chapter 2, Table 2.3. In addition, all three *ERF4* gene variants (*Activator, Repressor* and *Intron-Retention*) were tested, along with a primer that would bind to all *ERF4* transcripts (General Primer) (Table 5.1). Statistical analysis of the data was performed as described in Chapter 4, Section 4.3.4. Student's T-tests were performed on the relative expression values to identify significant differences. A two-way ANOVA was also performed on the data to assess interaction effects between treatment and genotype. For both the Student's T-tests and ANOVA analysis, normality checks were carried out. Homogeneity of

variances was verified using Levene's test for Equality of Error variances: where this was not met, significance was taken as $p < 0.01$, rather than $p < 0.05$.

Table 5.1: *ERF4*-related primers used for PCR and qPCR

Variant	Forward/Reverse (5'-3')	Product Length (base pairs)
***ERF4* (General)**	GCGGCTCGTGTTATCAGATC CTAAACGCCGATGTCACAGG	109
ERF4-Activator	GGCTTGTGGTGCCCAAAGCG TCACACCCTCTTATACGTCGTCGT	Unknown
ERF4-Repressor	TTGCCTCCTCCATCGGAACAGG CAAAAAGAAGAAGAAACGCATGCGC	80
ERF4-Intron Retention (IR)	TTCCAGCAGACACGCAGCCG TGTCCGTACTCTGTGAGTGGACCC	118

5.3.4 Analysis of basal resistance in *ERF4-Activator* and *ERF4-Repressor* over-expressing lines

To further investigate the role of ERF4-Activator and ERF4-Repressor in basal resistance against *S. gesnerioides*, seed of an *ERF4-Activator* over-expressing and an *ERF4-Repressor* over-expressing line were obtained. In both these lines, constitutive expression was achieved through introducing a transgene for the *ERF4* variant driven by the *35S* promoter. These were in the background of wildtype Col-0, and could test whether over-expression of either variant is sufficient to alter host basal resistance. The seed was a gift from Dr Rebecca Lyons, CSIRO Agriculture Flagship, Queensland Bioscience Precinct, Brisbane. The intention was to test both transgenic lines however, as previously reported (Lyons et al. 2013), *p35S:ERF4-Repressor* had a severely stunted phenotype which meant no plants were large enough to transplant into rhizotrons for infection with *S. gesnerioides. p35S:ERF4-Activator* was tested in a phenotypic screen with *S. gesnerioides* alongside *erf4-1* and two Col-0 lines: the Col-0 line used in the previous mutant assays (referred to here as Col-01) and a Col-0 line used as the background for the transgenic over-expressing mutants (referred to here as Col-02). *Arabidopsis* seedlings and *S. gesnerioides* seed were prepared as described in Chapter 2. Infection took place when the *Arabidopsis* plants were 28 days old, with 15 plants of each genotype infected. These were scored 3 weeks later by scanning the root systems using an Epsom scanner and quantifying the number of successful *S. gesnerioides* attachments (% infection), the proportion of advanced-stage haustoria (with a developing shoot) and the size of parasite haustoria. For the statistical analysis using Student's T-tests, *erf4-1* was compared against Col-01 as before (Chapter 2), whilst *p35S:ERF4-Activator* was compared against the Col-02 line which was supplied with it.

5.3.5 Comparison of expression of *ERF4* variants between *erf4-1* and *erf4-2*

Given the position of the T-DNA insertion in *erf4-1* (SALK_073394C), it was possible that this mutant was only affected in the expression of the *ERF4-Activator* splice variant. qPCR on *erf4-1* root samples had confirmed that *ERF4-Activator* expression is abolished in this mutant (not shown), and that of *ERF4-Repressor* significantly reduced. To understand whether the phenotype of *erf4-1* is due to reduced expression of the *ERF4-Repressor* variant or the complete absence of *ERF4-Activator* expression, a search was made for alternative T-DNA insertion lines using the T-DNA Express: *Arabidopsis* Gene Mapping Tool hosted by the SALK Institute Genomic Analysis Laboratory (http://signal-genet.salk.edu/cgi-bin/tdnaexpress). This identified the *erf4* mutant SALK_200761C (referred to here as *erf4-2*), which has a T-DNA insertion at least 145 base pairs upstream of *erf4-1* (Figure 5.3). Seed was ordered from the Nottingham Arabidopsis Stock Centre (NASC: http://arabidopsis.info/). Both mutant lines were genotyped to confirm the presence of the T-DNA insertion (see Chapter 2, Section 2.9 for full methods).

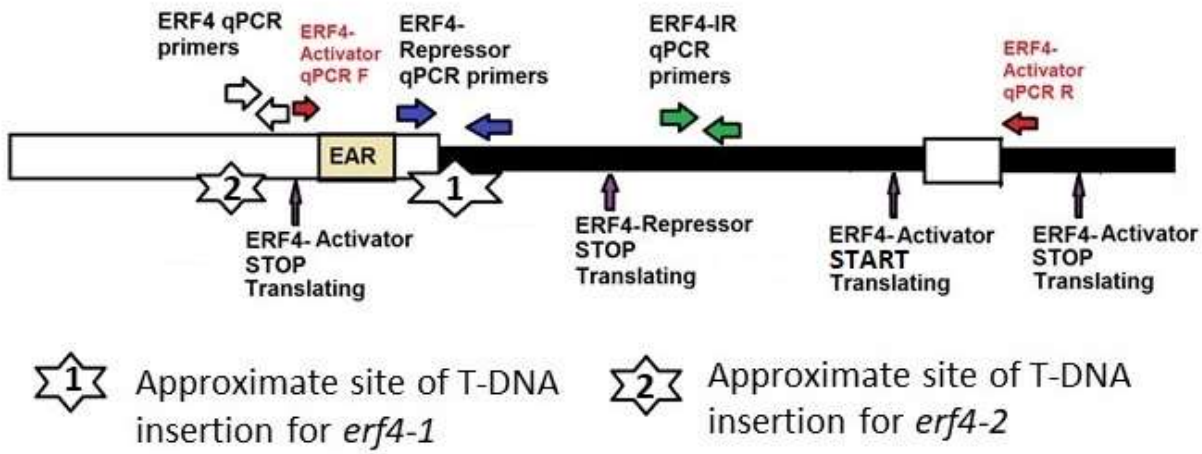


Figure 5.3: Diagram showing the relative locations of the T-DNA insertions in *erf4-1* (SALK_073394C) and *erf4-2* (SALK_200761C), besides the binding positions (horizontal arrows) of the qPCR primers for the *ERF4* (General), *ERF4-Activator, ERF4-Repressor* and *ERF4-Intron Retention* (IR). Vertical arrows indicate the positions of the start and stop translation codons for the *ERF4-Activator* and *ERF4-Repressor* variants.

Both *erf4-1* and *erf4-2* were tested alongside Col-0 in a phenotypic screen for their response to infection with *S. gesnerioides*. *Arabidopsis* seed were sterilised, germinated and grown in rhizotrons as described in Chapter 2, Section 2.3. *S. gesnerioides* seed were also prepared as described previously (Chapter 2, Section 2.2). Ten plants of each genotype were infected when the host plants were 28 days old.

To quantify the difference in expression of *ERF4-Repressor* between the two mutant lines, root samples were harvested in a separate experiment from 6-week-old uninfected Col-0, *erf4-1* and *erf4-2 Arabidopsis* plants grown in rhizotrons. Root harvest, RNA extraction and cDNA synthesis were carried out as described previously (see Chapter 2, Section 2.8) with three plants forming one biological sample. qPCR was performed on three samples of each genotype, using primers for *ERF4*, *ERF4-Activator*, *ERF4-Repressor* and *ERF4-Intron Retention* (Table 5.1). qPCR conditions were the same as described previously (Chapter 2, Section 2.8) with *GADPH* and *20S PROTEASOME* included as reference genes for normalisation. Statistical analysis was performed as described in Chapter 4, Section 4.3.4.

5.4 Results:

5.4.1 *qPCR of ERF4 variant expression during a time-course of Striga gesnerioides infection*

From the qPCR analysis of Chapter 4, it was established that the *ERF4* gene showed a small (1.5 fold) but significant induction at 12 days post-infection with *S. gesnerioides* (Figure 4.15). To investigate whether the ratio of ERF4-Activator and ERF4-Repressor changed during this induction, qPCR was performed on the same samples using primers for the two variants, as well as for *ERF4-Intron Retention* (IR) (Lyons et al. 2013). At 7 dpi, there was no significant difference in the expression of any of these variants between the control and infected samples (Figure 5.4): this was expected since expression of the *ERF4* gene as a whole was unchanged at this point. At 10 dpi, *ERF4-Activator* showed a small but significant increase of approximately 1.5-fold in the infected samples, however expression decreased in the corresponding control samples. By 12 dpi, *ERF4-Activator* expression in both control and infected samples had almost returned to the level at 7 dpi. Similar to *ERF4-Activator, ERF4-Repressor* showed a significant induction in the infected samples at 10 dpi, of 1.3 -fold. Unlike *ERF4-Activator*, there was no noticeable decrease in *ERF4-Repressor* expression in the control samples at this point. At 12 dpi the expression of *ERF4-Repressor* increased further in the infected samples, reaching 1.5-fold. *ERF4-IR* showed an average upregulation of 1.5-fold in the infected samples at 10 dpi, however due to the variability between samples this was not significant. At 12 dpi, *ERF4-IR* expression in both control and infected samples matched that at 7 dpi.

Nevertheless, the degree of upregulation between the *ERF4* variants cannot be directly compared, as the Ct value of *ERF4-Repressor* was always approximately 6 cycles lower than *ERF4-Intron Retention* and 12 cycles lower than *ERF4-Activator*. Given that a cycle difference equates to approximately a doubled amount of product, this indicates that the concentration of ERF4-Repressor was at least 4,000 times greater than ERF4-Activator. This confirms previous work which found the ratio of ERF4-Activator: ERF4-Repressor in *Arabidopsis* leaves to vary between approximately 0.001 and 0.0003 (Riester et al. 2019).

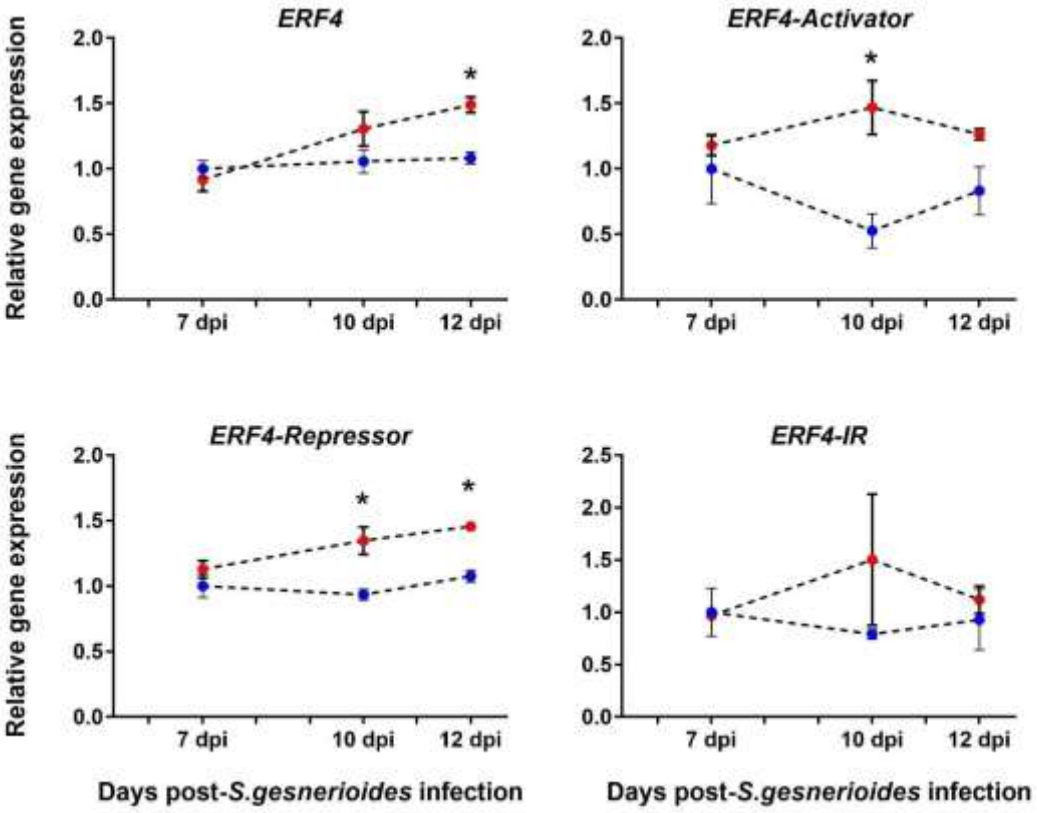


Figure 5.4: qPCR expression analysis of *ERF4* gene and associated variants *ERF4-Activator, ERF4-Repressor* and *ERF4-Intron Retention (IR)* in control and *Striga gesnerioides*-infected Col-0 *Arabidopsis* root samples. For each timepoint, gene expression was tested on three control and three infected root samples. Error bars: Mean +/- standard error. Fold-changes in gene expression were calculated relative to control samples at 7 dpi. Indicated significant differences refer to the difference between the control and infected samples for that timepoint. * denotes statistical significance at the 5% level according to an independent samples Student's T-test. dpi = days post-infection with *S. gesnerioides*.

5.4.2 Viewing root samples cleared with chloral hydrate under UV light:

By 7dpi, *S. gesnerioides* haustoria on Col-0 *Arabidopsis* hosts begin to penetrate through the outer root cortex, but do not begin to form vascular connections with the host (Figure 5.5A). On the *erf4-1* hosts however, some haustoria were seen with distinct 'penetration plugs' at 7 dpi (Figure 5.5B, arrow) or vascular elements between the host and parasite (Figure 5.5C, arrow). By 10 dpi, however, the haustoria were indistinguishable between Col-0 and *erf4-1* hosts (Figure 5.5D and E). This indicates that on *erf4-1* hosts, *S. gesnerioides* breaches the vascular system more quickly, which may enable more effective suppression of host defences.

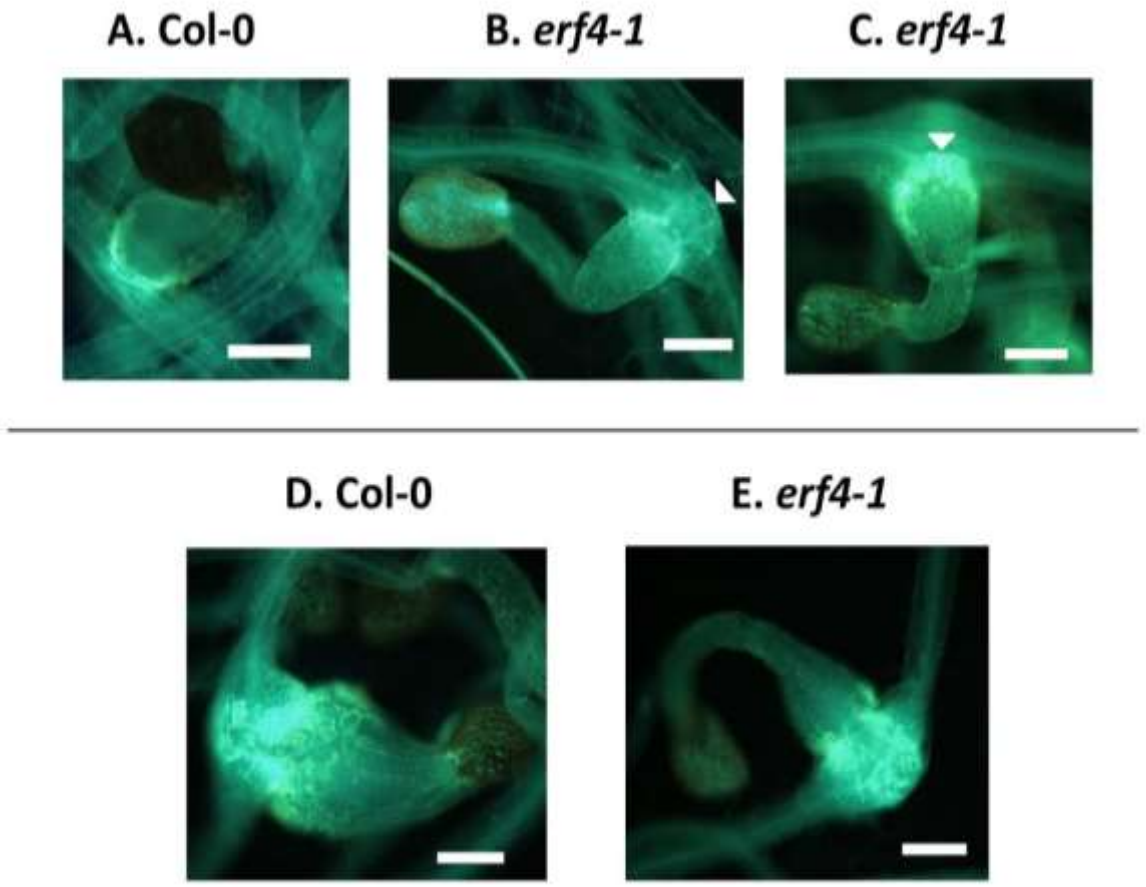


Figure 5.5: Root sections from *Striga gesnerioides*-infected Col-0 and *erf4-1 Arabidopsis* plants, cleared with chloral hydrate and viewed under UV light. Top panel (A-C): root sections harvested 7 days post-infection. Bottom panel (D-E): root sections harvested 10 days post-infection. UV light causes lignin-containing elements to fluoresce, allowing vascular connections between the host and parasite to be seen (arrows). Scale bar: 200 µm.

5.4.3 qPCR comparison of defence gene expression in Col-0 and *erf4-1*

qPCR was used to compare the expression of *PR5, WRKY70, PR4, ORA59, GST1, PRX33* and *B-CHI* in Col-0 and *erf4-1* under basal conditions and in response to *S. gesnerioides* at 7 dpi. These genes were selected because they were all significantly induced in Col-0 by *S. gesnerioides* in the main qPCR assay described in Chapter 4. There was particular interest in assessing the activity of the ROS-associated genes *GST1* and *PRX33*, given the role of *ERF4-Activator* in limiting the PTI-driven oxidative burst (Lyons et al. 2013).There was no significant difference between *erf4-1* and Col-0 in the basal expression of *WRKY70, PR4, ORA59, GST1* and *B-CHI*, or in the induction of these genes in response to *S. gesnerioides* (Figure 5.6). Although there was no significant difference in basal *PR5* expression, induction of this gene by *S. gesnerioides* was significantly reduced in *erf4-1* (Mean fold-induction relative to basal expression in Col-0: Col-0, 10.8-fold; *erf4-1,* 3.4-fold; $p = 0.042$). Of all these genes, *PRX33* was the only gene showing altered basal expression in *erf4-1,* being 0.5-fold the level of Col-0 ($p = 0.02$). In this

assay, induction of *PRX33* in Col-0 by *S. gesnerioides* was considerably lower than that observed previously, being only 1.2-fold induced compared to the 8.8-fold induction at 7dpi in the previous time-course experiment (Figure 4.13). Nevertheless, *PRX33* expression remained significantly reduced in *erf4-1* in response to *S. gesnerioides,* at 0.7-fold the level of the uninfected Col-0 controls.

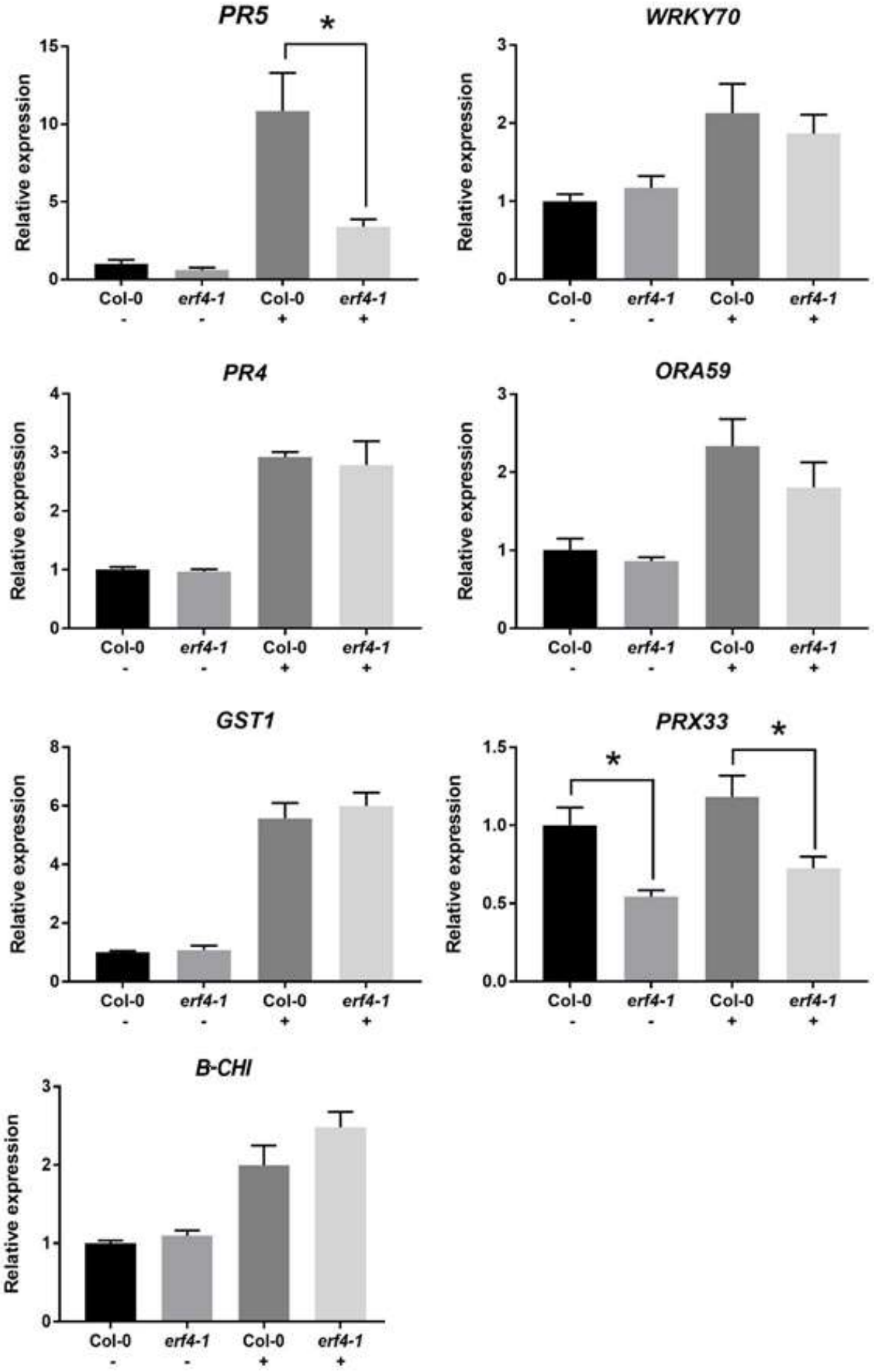
Expression of *ERF4* and its associated variants was also tested in these samples. This demonstrated that basal expression of *ERF4* is similar between *erf4-1* and Col-0 and that *ERF4-Activator* is not expressed at all in *erf4-1* (Figure 5.7). Basal expression of *ERF4-Repressor* was significantly reduced in *erf4-1* to 0.15-fold relative to Col-0. *ERF4-Intron Retention*, however, showed greater basal expression in *erf4-1*, being 1.4-fold the level of Col-0, however this difference was not significant. In response to *S. gesnerioides*, expression of *ERF4, ERF4-Activator, ERF4-Repressor* and *ERF4-Intron Retention* did not change in Col-0. At 7 dpi, *ERF4* was significantly induced by the parasite in *erf4-1*, to 1.5-fold the level relative to uninfected Col-0, however there was no change in *ERF4-Repressor* expression. *ERF4-Intron Retention* was slightly induced by *S. gesnerioides* in *erf4-1*, such that the difference in expression between infected Col-0 and *erf4-1* was significant. This may indicate an overcompensation by the host for the lack of functional *ERF4-Activator* or *ERF4-Repressor*.

Two-way ANOVA analysis was performed to determine any interaction effects between the *erf4-1* mutation and the host response to *S. gesnerioides* (Table 5.2). *ERF4-Activator* was not included in this analysis since it was not expressed at all in the mutant plants. The results (Table 5.2) indicated that infection with *S. gesnerioides* had a significant effect on *PR5, WRKY70, PR4, ORA59, GST1, B-CHI* and *ERF4* expression. Genotype had a significant effect for *PRX33, ERF4, ERF4-Repressor* and *ERF4-Intron Retention*. There was a slight interaction effect between genotype and treatment for *PR5* expression, but this was not significant. The only significant interaction effect found was for *ERF4*.

5.4.4 Phenotypic screen of *ERF4-Activator* over-expressing lines

As in previous assays, the percentage of parasite seed which successfully infected was notably higher for *erf4-1* than on Col-01; in this assay, however, this was significant at the 10% level but not the 5% level ($p = 0.095$) (Figure 5.8A). As before, there was no significant difference between *erf4-1* and Col-01 in either the size of the attached haustoria (Figure 5.8B) or the proportion of *S. gesnerioides* haustoria that transitioned to shoot development (Figure 5.8C).

Figure 5.6 (overleaf): qPCR analysis of defence gene expression for *PR5, WRKY70, PR4, ORA59, GST1 PRX33* and *B-CHI* in Col-0 and *erf4-1* under control conditions and in response to *S. gesnerioides*. (+) and (-) indicates the presence or absence of *S. gesnerioides* respectively. All samples were harvested at 7 days post-infection. Gene expression was tested on three control and three infected root samples. Error bars: Mean +/- standard error. Fold-changes in gene expression were calculated relative to the Col-0 control samples. * denotes statistical significance at the 5% level according to an independent samples Student's T-test. dpi = days post-infection with *S. gesnerioides*.


PR5
*
Relative expression
15
10
5
0
Col-0
erf4-1
Col-0
erf4-1
-
-
+
+
WRKY70
Relative expression
3
2
1
0
Col-0
erf4-1
Col-0
erf4-1
-
-
+
+
PR4
Relative expression
4
3
2
1
0
Col-0
erf4-1
Col-0
erf4-1
-
-
+
+
ORA59
Relative expression
3
2
1
0
Col-0
erf4-1
Col-0
erf4-1
-
-
+
+
GST1
Relative expression
8
6
4
2
0
Col-0
erf4-1
Col-0
erf4-1
-
-
+
+
PRX33
*
*
Relative expression
1.5
1.0
0.5
0.0
Col-0
erf4-1
Col-0
erf4-1
-
-
+
+
B-CHI
Relative expression
3
2
1
0
Col-0
erf4-1
Col-0
erf4-1
-
-
+
+

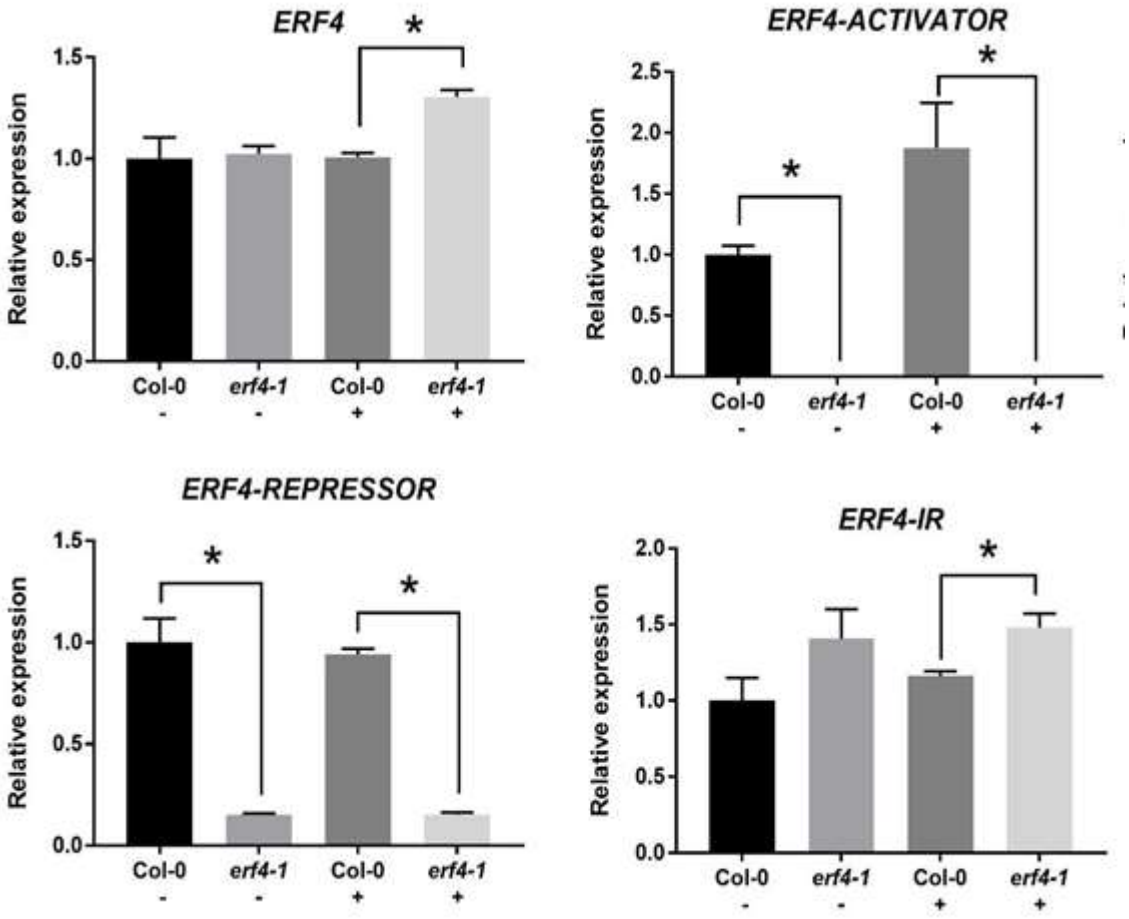


Figure 5.7: qPCR analysis of defence gene expression for *ERF4, ERF4-Activator, ERF4-Repressor* and *ERF4-Intron Retention (IR)* in Col-0 and *erf4-1* under control conditions and in response to *S. gesnerioides*. (+) and (-) indicates the presence or absence of *S. gesnerioides* respectively. All samples were harvested at 7 days post-infection. Gene expression was tested on three control and three infected root samples. Error bars: Mean +/- standard error. Fold-changes in gene expression were calculated relative to the Col-0 control samples. * denotes statistical significance at the 5% level according to Student's T-test. dpi = days post-infection with *S. gesnerioides*.

Over-expression of *ERF4-Activator* in the Col-0 background proved sufficient to induce greater early-stage susceptibility since the % infection was significantly greater for *p35S:ERF4-Activator* (Mean: 26.0%), compared with its control, Col-02 (Mean: 21.8%) ($p < 0.0001$) (Figure 5.8A). In addition, haustorium size was reduced for *p35S:ERF4-Activator* (Mean: 0.013 cm^2) compared with Col-02 (Mean: 0.016 cm^2) (Figure 5.8B): this was significant at the 10% level ($p = 0.074$). There was no difference, however, in the proportion of haustoria showing shoot development between *p35S:ERF4-Activator* and Col-02 (Figure 5.8C).

Table 5.2: Two-way ANOVA analysis of defence gene expression in control and *Striga gesnerioides*-infected Col-0 and *erf4-1 Arabidopsis*.

	Significance values			
Gene	Levene's Test for Equality of Error Variances	Treatment (control v infected)	Genotype (Col-0 vs *erf4-1)*	Treatment * Genotype
PR5	**0.047**	**0.001**	0.015	0.024
WRKY70	0.096	**0.005**	0.852	0.387
PR4	**0.003**	**0.001**	0.694	0.799
ORA59	0.061	**0.002**	0.216	0.453
GST1	0.084	**0.001**	0.501	0.647
PRX33	0.365	0.099	**0.002**	0.996
B-CHI	0.075	**0.001**	0.117	0.274
ERF4	0.066	**0.041**	**0.026**	**0.050**
ERF4-Repressor	**0.003**	0.653	**0.001**	0.638
ERF4-Intron Retention	0.121	0.392	**0.024**	0.728

Significant p values are highlighted in red. Where equal variances can be assumed (i.e. Levene's Test statistic > 0.05), p values < 0.05 are regarded as significant in the ANOVA analysis. Where Levene's Test statistic < 0.05, p values < 0.01 are regarded as significant.

5.4.5 Phenotypic characterization of *erf4-1* and *erf4-2* for basal resistance against *Striga gesnerioides*.

The % of *S. gesnerioides* seed that formed attachments on each host was significantly higher on *erf4-1* than Col-0 (p = 0.012) (Figure 5.10A), confirming the result of the previous mutant screen in Chapter 3 (Figure 3.4). As before, there was no significant difference in either the size of the attached haustoria or the proportion of *S. gesnerioides* haustoria that had transitioned to shoot development between Col-0 and *erf4-1* (Figure 5.10 B and C). Unlike *erf4-1, erf4-2* showed no significant different in the % infection compared with Col-0 (Figure 5.10A). *erf4-2* was also not affected in either haustorium size or the proportion of haustoria with developing shoots (Figure 5.10 B and C).

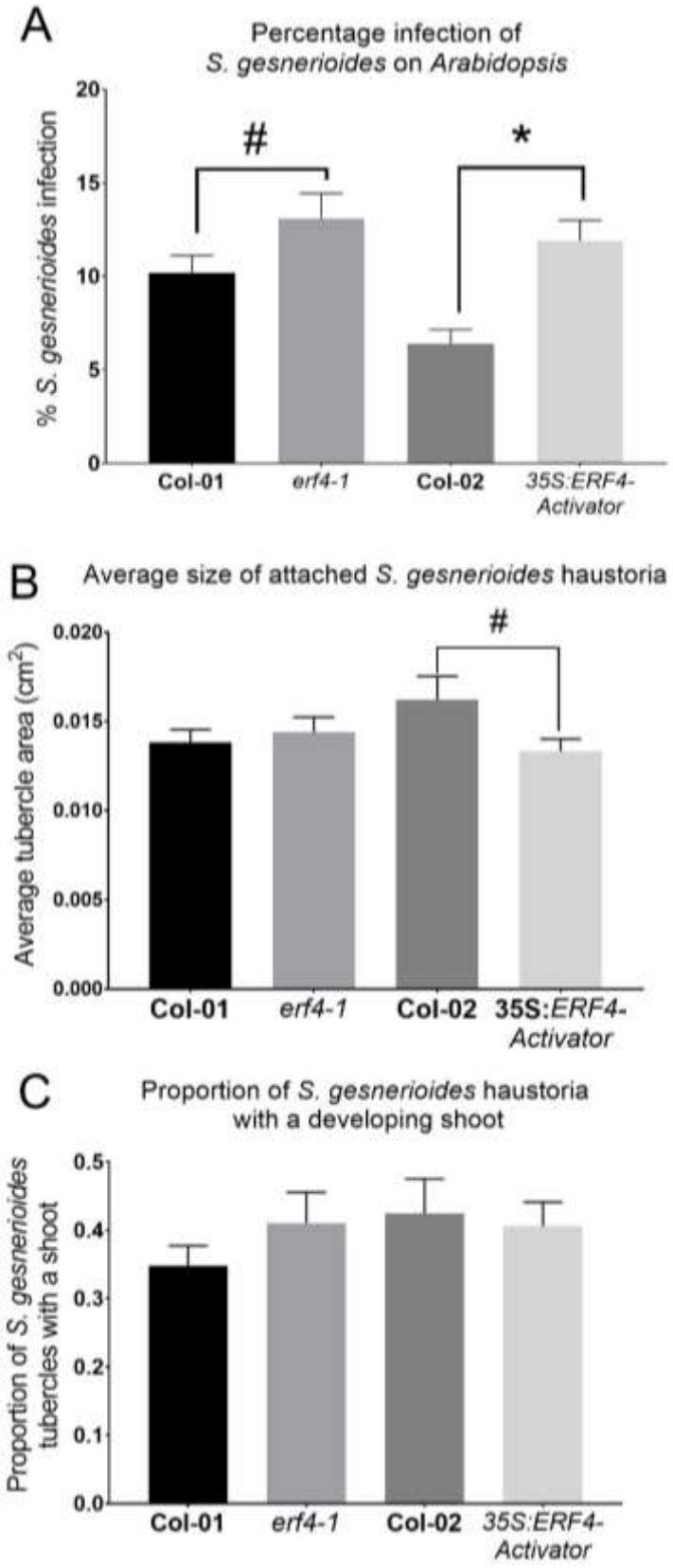


Figure 5.8: Phenotypic comparison of *erf4-1* and *p35S: ERF4-Activator Arabidopsis* mutants infected with *Striga gesnerioides* against two Col-0 (wild-type) lines. Host plants were scored 3 weeks post-infection for the % of applied parasite seed that attached and formed a haustorium (A), haustorium size (B) and proportion of attached haustoria that showed a developing shoot (C). Error bars: mean +/- standard error. Statistical significance was calculated using Student's T-test. * denotes a significant difference at the 5% level, # indicates a significant difference at the 10 % level. Numbers of plants were as follows: Col-01 = 13, *erf4-1* = 15, Col-02 = 15 and *p35S: ERF4-Activator* = 13.

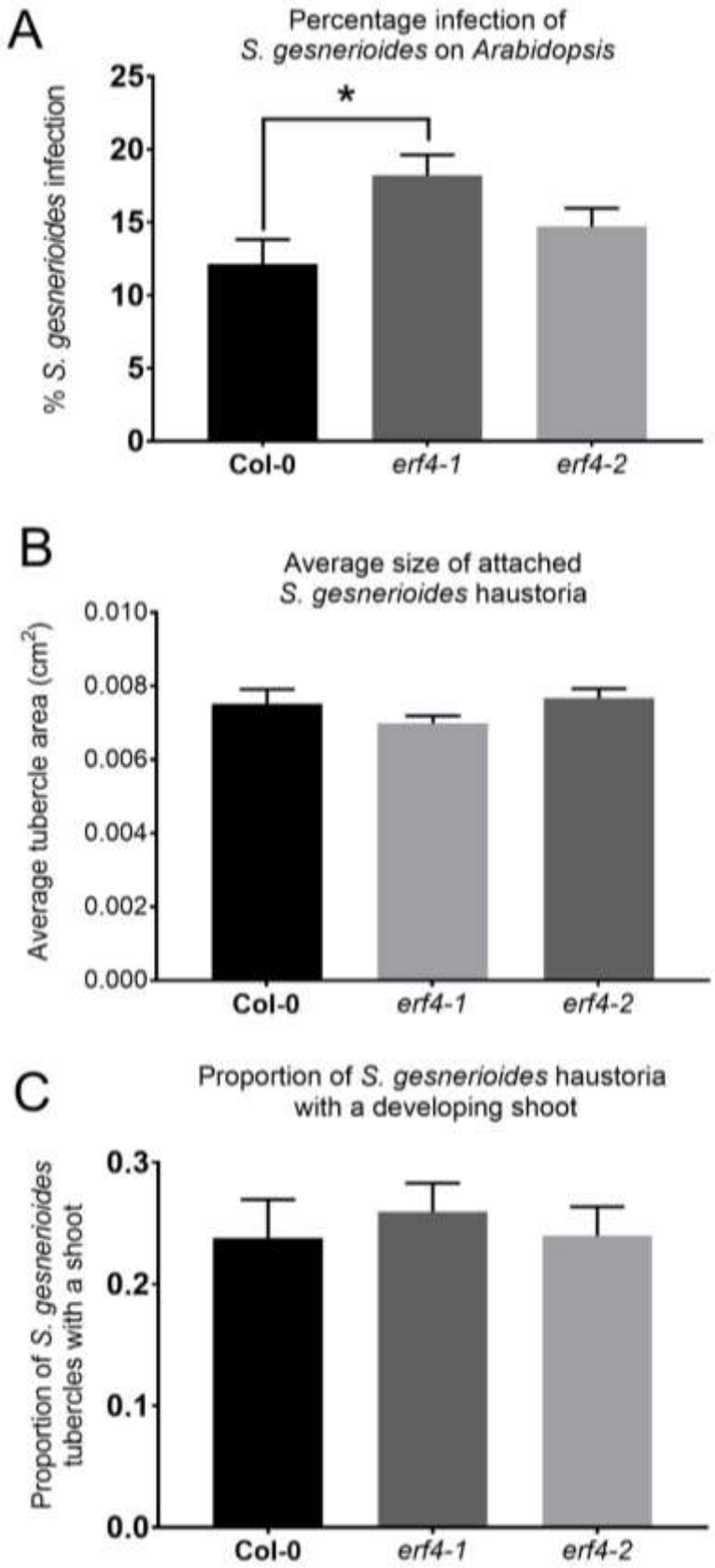


Figure 5.9: Phenotypic comparison of *Arabidopsis* Col-0 (wild-type), *erf4-1* and *erf4-2* infected with *Striga gesnerioides*. Ten plants of each genotype were infected with *S. gesnerioides*. The host plants were scored 3 weeks post-infection for the % of applied parasite seed that attached and formed a haustorium (A), the average size of the attached haustoria (B) and the proportion of attached haustoria that showed a developing shoot (C). Error bars: mean +/- standard error. Statistical significance was calculated using Student's T-test. * denotes a significant difference at the 5% level.

5.4.6 Quantification of *ERF4* gene variant expression between *erf4-1* and *erf4-2*.

qPCR was performed on root cDNA to compare expression of *ERF4, ERF4-Activator, ERF4-Repressor* and *ERF4-Intron Retention* between Col-0, *erf4-1* and *erf4-2.* This analysis confirmed that neither of the mutant lines expressed the *ERF4-Activator* variant. After standardisation to the reference genes *GADPH* and *20S PROTEASOME*, the data indicated that expression of the *ERF4-Repressor* variant relative to Col-0 was approximately 0.1 in *erf4-1* and 0.02 in *erf4-2* (Figure 5.9). Expression of *ERF4-Intron Retention* meanwhile was approximately 0.7 in *erf4-1* and 0.07 in *erf4-2* relative to Col-0 (Figure 5.9).

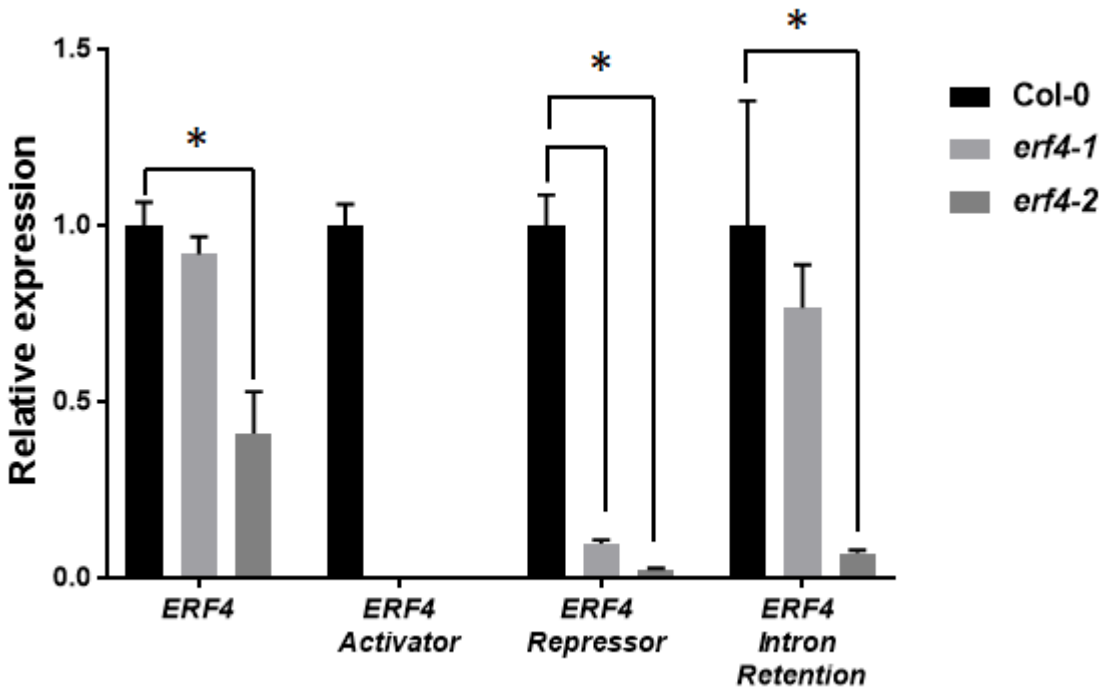


Figure 5.10: Relative expression of *ERF4, ERF4-Activator, ERF4-Repressor* and *ERF4-Intron Retention* in Col-0 *Arabidopsis*, *erf4-1 and erf4-2*. Expression is given as relative to Col-0. Three samples of each genotype were assessed with qPCR with each gene of interest normalised to the reference genes *GADPH* and *20S PROTEASOME* to account for differences in sample cDNA concentration. Error bars: mean +/- standard error. Statistical significance was calculated using Student's T-test. * denotes a significant difference at the 5% level.

5.5 Discussion

Interpreting the results to propose the function of ERF4 in basal resistance against *S. gesnerioides* is far from straightforward. One thing that does seem conclusive is that the over-expression of *ERF4-Activator* is sufficient to induce increased early-stage susceptibility to *S. gesnerioides*, since the % infection was significantly greater for *p35S: ERF4-Activator* than Col-0 (Figure 5.8). Yet, this does not necessarily indicate that ERF4-Activator normally plays a role in suppressing basal resistance, since its expression in wild-type plants is only a fraction of that of the ERF4-Repressor variant. Indeed, the qPCR

results from the Col-0 time-course demonstrate that infected Col-0 plants show a small but significant induction of *ERF4-Activator* at 10 dpi of 1.5-fold (Figure 5.4), but comparison of the Ct cycle scores indicates that the concentration of ERF4-Activator in the roots is only a trace that of ERF4-Repressor. This implies that during the interaction between *Arabidopsis* and *S. gesnerioides, ERF4-Activator* is not induced to biologically relevant levels. Potentially, switching from *ERF4-Repressor* to *ERF4-Activator* requires regulatory elements that are not abundant in root tissues. After all, the two previous studies investigating the role of ERF4-Activator in the flg22-induced ROS response (Lyons et al. 2013) and in leaf senescence (Riester et al. 2019) used whole seedlings or leaves. Indeed, this analysis shows some discrepancies with previous studies assessing ERF4 function in leaf tissues. For instance, in *erf4-1* leaf samples, *PR5* was more highly expressed compared with wildtype, along with *PAD3, PR1* and *PDF1.2* (Edgar et al. 2006). In this assay, however, basal *PR5* expression was significantly lower in the root samples from *erf4-1* and showed a markedly reduced response to *S. gesnerioides* (Figure 5.6).

What then, is the mechanism that can explain the increased susceptibility of both *erf4-1* and the *p35S: ERF4-Activator* line? Potentially, both phenotypes may be caused by compromised function of ERF4-Repressor. The *erf4-1* mutant shows significantly reduced expression of *ERF4-Repressor* (Figure 5.10), whilst over-expression of *ERF4-Activator* in *p35S: ERF4-Activator* may directly interfere with ERF4-Repressor binding activity. However *erf4-2*, which expresses *ERF4-Repressor* at a lower level than *erf4-1* (Figure 5.10), showed no significant change in basal resistance to *S. gesnerioides*. This argues against reduced ERF4-Repressor functionality being the basis of the *erf4-1* and *p35S:ERF4-Activator* phenotype.

Nevertheless, the fundamental difference between *erf4-1* and *erf4-2* is the expression of the *ERF4-Intron Retention (ERF4-IR)* variant, which was close to wild-type levels in *erf4-1*, whilst *erf4-2* showed a >90% reduction in the expression level of this variant (Figure 5.10). Furthermore, *ERF4-IR* was induced more strongly in *erf4-1* than Col-0 after *S. gesnerioides* infection (Figure 5.7). This indicates that the position of the T-DNA insertion in *erf4-1* is such that the full-length mRNA can still be transcribed, but then cannot be spliced to form the *ERF4-Activator* transcript (Figure 5.3). Potentially, the phenotype of *erf4-1* and *p35S: ERF4-Activator* are both caused by abnormal accumulation of a transcript that directly interferes with ERF4-Repressor. *ERF4* mRNA transcripts have been demonstrated to show high turnover, with a half-life of less than 60 minutes (Gutiérrez et al. 2002). In *erf4-1*, *ERF4-IR* may be resistant to this degradation, and may thus be translated into an ERF4-IR protein. Given that the full-length transcript retains the repressive EAR domain, the resulting ERF4-IR protein may still be capable of binding to the target sequences of the ERF4-Repressor variant. However, due to its altered structure, the ERF4-IR protein may disrupt the normal function and binding of the ERF4-Repressor protein to its partners, for instance the histone deacetylase HDA19 (Song and Galbraith 2006), thereby allowing expression of susceptibility-enhancing genes. Previous studies have

demonstrated that *erf4-1* shows significantly increased *PDF1.2* expression in its leaves (Edgar et al. 2006), which is normally suppressed by ERF4-Repressor (Lyons et al. 2013). This would suggest that in *erf4-1*, ERF4-IR does not act as a functional transcriptional repressor, but instead interferes with the resistance-enhancing activity of the ERF4-Repressor variant. One conclusion, therefore, is that the enhanced susceptibility of the *p35S:ERF4-Activator* line and the *erf4-1* mutant is due to disruption of the resistance-enhancing activity of the ERF4-R variant by increased accumulation of the ERF4-Activator and ERF4-IR proteins, respectively.

It is notable that *erf4-1* showed significantly impaired induction of the SA-responsive gene *PR5* in response to *S. gesnerioides* (Figure 5.6). In the qPCR time-course described in Chapter 4, *PR5* was one of the genes most strongly induced by *S. gesnerioides*. Furthermore, the SA-biosynthesis mutant *sid2-1* showed reduced early-stage resistance to *S. gesnerioides*, suggesting that *PR5* induction is relevant in basal resistance. Nevertheless, *WRKY70* expression was similar between Col-0 and *erf4-1* (Figure 5.6) suggesting that only specific components of SA-associated defences may be affected. *PR5* expression was highest at the earliest part of the time-course, which fits a model where SA-associated defences specifically inhibit parasite entry. This is supported by the microscopy analysis of infected Col-0 and *erf4-1* roots where the key distinction is more rapid penetration of the parasite between 7-10 days (Figure 5.5).

In addition, *erf4-1* showed reduced *PRX33* expression under both basal conditions and in response to *S. gesnerioides* (Figure 5.6). Peroxidases are multifunctional enzymes capable of both reducing H_2O_2 to water and generating it via the hydroxylic cycle (Mathé et al. 2010). Given that *Striga* radicles are potent sources of H_2O_2 (Keyes et al. 2007, Wada et al. 2019) and DAB staining revealed ROS activity at the host-parasite interface (Chapter 4, Figure 4.9, 4.11), it is likely that *PRX33* is induced in response to *S. gesnerioides* to increase host detoxification ability. The evidence from the mutant assays (Chapter 3) indicate that ROS may be a pathogenicity factor for *S. gesnerioides* since *RbohD/RbohF* and *RbohD* showed significantly enhanced early- and late-stage resistance (Figure 3.6). This suggests that excess H_2O_2 may be a physiological mechanism behind the *erf4-1* phenotype. Nevertheless, expression of *GST1*, which detoxifies ROS, was unchanged between *erf4-1* and Col-0 (Figure 5.6). Furthermore, the *prx33* mutant was not overtly affected in early- or late-stage resistance against *S. gesnerioides* (Figure 3.6), which would argue against altered *PRX33* expression being the basis of the *erf4-1* phenotype. In addition, *p35S:ERF4-Activator* showed increased susceptibility to *S. gesnerioides*, rather than increased resistance, even though over-expression of this variant has been found to increase *CAT3* expression (Riester et al. 2019), which would reduce intracellular H_2O_2 levels.

5.6. Future directions

Both the *erf4-1* mutation and over-expression of *ERF4-Activator* significantly reduced early-stage resistance to *S. gesnerioides* (Figure 5.8). At this stage, it remains speculative that the elevated accumulation of ERF4-IR and ERF4-Activator protein in *erf4-1* and *p35S:ERF4-Activator*, respectively, interfere with the resistance-inducing activity of the ERF4-Repressor protein to cause enhanced susceptibility to *S. gesnerioides*. Ultimately, this hypothesis could be confirmed by testing whether *p35S:ERF4-Repressor* shows increased resistance to *S. gesnerioides*. Since impaired induction of SA-associated defences and/or ROS detoxification are plausible physiological explanations for the enhanced susceptibility phenotype of *erf4-1*, it would also be informative to test the expression of a wider range of defence- and ROS homeostasis-related genes in both *erf4-1* and *p35S:ERF4-Activator* lines during infection by *S. gesnerioides*. Furthermore, 3,3'-Diaminobenzidine (DAB) staining could also be used to investigate whether ROS activity is more pronounced at the host-parasite interface in *erf4-1* and *p35S:ERF4-Activator* compared to Col-0.

CHAPTER SIX

Are systemic defence responses induced in *Arabidopsis* by *Striga gesnerioides*?

ABSTRACT:

Plants respond to pathogen attacks at both a local and systemic level. Local responses are rapid and designed to destroy the invading pathogen and/or prevent further entry. In addition, immune responses can be induced in distal regions of the plant away from the immediate infection site. This can result in transient activation of defences and also long-term sensitisation that enables a more effective immune response against future pathogens (known as systemic acquired resistance, SAR). The root parasite *Striga gesnerioides* induced significant gene expression changes in the roots of compatible *Arabidopsis* hosts (Chapter 4), however it was unknown whether this translated into a systemic response that altered host basal resistance to foliar pathogens. This was tested by challenging control and *S. gesnerioides*-infected *Arabidopsis* plants with either the foliar pathogens *Hyaloperonospora arabidopsidis* (a biotroph) or *Plectosphaerella cucumerina* (a necrotroph). The results indicated that *S. gesnerioides* weakly affected basal resistance against *H. arabidopsidis*, as the pathogen showed lower levels of sexual reproduction compared to uninfected plants. Basal resistance against *P .cucumerina* was not affected by pre-infection with *S. gesnerioides*. In addition, measurement of gene expression by qPCR was performed on shoot samples harvested throughout a time-course infection of *Arabidopsis* with *S. gesnerioides* and compared with uninfected controls. The results demonstrated that *S. gesnerioides* did not induce significant changes in gene expression in host shoot tissues, in contrast to the roots.

6.1 Introduction

6.1.1 Systemic immunity in plants

The results of the gene expression analysis described in Chapter 4 showed that *S. gesnerioides* induced a strong local response in host *Arabidopsis* roots. This involved upregulation of genes regulated by salicylic acid (SA), jasmonic acid (JA) and ethylene, in addition to defence-associated transcription factors and genes related to reactive oxygen species (ROS) generation/detoxification. This investigation, however, did not examine whether this was a local response restricted to the roots, or if it was also transmitted to above-ground regions. Plant pathogens frequently trigger defence responses in distal, non-infected regions of the host plant: a phenomenon known as systemic acquired resistance (SAR) (Koch and Slusarenko , Fu and Dong 2013, Klessig, Choi and Dempsey 2018). This typically involves the induction of pathogenesis-related (PR) genes that encode defensive molecules / proteins which promote resistance against a broad-spectrum of pathogens. These include chitinases and β-1-3 glucanases that degrade fungal cell walls, proteinase inhibitors, defensins, thionins and peroxidases (Sels et al. 2008). SA appears to be a central requirement for SAR because transgenic *NahG Arabidopsis* and tobacco plants that cannot accumulate SA fail to undergo SAR in response to pathogen attack (Delaney et al. 1994, Gaffney et al. 1993). Furthermore, SAR can be induced by applications of SA (White 1979) or its synthetic analogues 2,6-dichloroisonicotinic acid (INA) (Delaney et al. 1994, Métraux et al. 1991) and benzothiadiazole S-methyl ester (BTH) (Friedrich et al. 1996, Görlach et al. 1996, Lawton et al. 1996) across a wide range of plants. NPR1, a regulatory signalling component downstream of SA, also seems essential to facilitate SAR since *npr1* mutants do not develop SAR in response to chemical or biological inducers (Cao et al. 1994).

Despite its dependence on SA, SAR appears to be unrelated to local SA-mediated responses that form part of effector-triggered immunity (ETI), such as programmed cell death and the hypersensitive response (HR); instead SAR promotes cell survival (Fu and Dong 2013). Furthermore, grafting experiments using *NahG* tobacco rootstock have demonstrated that SA is only required in the distal tissues for SAR to be induced, and not at the site of pathogen invasion (Vernooij et al. 1994). A variety of molecules have been proposed to act as the mobile signal to translocate the trigger for SAR from the site of pathogen attack to uninfected regions. These include reactive oxygen species (ROS), glycerol-3-phospate and the lipid-based protein DIR1 (DEFECTIVE IN INDUCED RESISTANCE 1) (reviewed in Fu and Dong 2013, Durrant and Dong 2004). Girdling experiments imply that the signal for SAR is predominantly transported within the phloem (Guedes, Richmond and Kuć 1980). Nevertheless, in a study on *Arabidopsis*, where single leaves were challenged with the bacteria *Pseudomonas syringae*, the pattern of SAR induction in unchallenged leaves did not align exactly with the flow of radiolabelled sucrose. This indicates that either a portion of the SAR signal is transported

outside the phloem, or that small amounts of 'signal leakage' into leaves of a different orthostichy is sufficient to induce a full SAR response (Kiefer and Slusarenko 2003).

Besides activating transient defence responses against potential imminent invaders, systemic signals can also confer long-term defence against a wide spectrum of pathogens through the distinct mechanism of priming. In contrast to SAR, this does not involve an increase in basal gene activity or the accumulation of defensive compounds in the uninfected tissue. Rather, host tissues are 'primed' or 'sensitised' to respond more rapidly and effectively against subsequent pathogen attacks, similar in principle to a medical vaccine (Conrath 2011, Martinez-Medina et al. 2016). Proposed mechanisms for priming include accumulation of dormant mitogen-activated protein kinases (MAPKs) (Beckers et al. 2009), chromatin remodelling (Jaskiewicz, Conrath and Peterhänsel 2011, Luna et al. 2012) and DNA hypomethylation (Luna et al. 2012). It is widely thought that priming is an adaptive strategy to avoid high fitness costs incurred by constitutive activation of defences (van Hulten et al. 2006).

There is much evidence that these systemic immune responses can have great effect in repelling future pathogen attack, even when they belong to another species or even kingdom. This includes instances where the systemic signal is transmitted between the roots and shoots. For example, *Brassica nigra* (black mustard) plants exposed to root damage from *Delia radicum* (cabbage root fly) showed more rapid induction of defensive glucosinolates in the leaves when challenged with *Pieris rapae* butterfly larvae, compared to plants with undamaged roots (Van Dam, Raaijmakers and Van Der Putten 2005). In another study, root herbivory of cotton plants by click beetles (*Agriotes lineatus*) resulted in reduced susceptibility of the leaves to beet armyworms (*Spodoptera exigua*); this was associated with a greater accumulation of defensive terpenoids in both the roots and leaves (Bezemer et al. 2003). Similarly, below-ground infestations of western corn rootworm *Diabrotica virgifera* on maize increased above-ground resistance to *Setosphaeria turcica (*a necrotrophic pathogen) and *Spodoptera littoralis* (a generalist herbivore) (Erb et al. 2009). In this study, plants with chronic root infestations of *D. virgifera* showed more rapid accumulation of abscisic acid (ABA) and the defence compound chlorogenic acid in the leaves, when challenged with these foliar pathogens (Erb et al. 2009).

SAR is not just induced by pathogens: numerous studies have demonstrated that below-ground inoculation with non-pathogenic rhizobacteria (Van Loon, Bakker and Pieterse 1998, Pieterse et al. 2014) or arbuscular mycorrhizal fungi (Pozo et al. 2009) can also induce systemic immunity in above-ground host tissues (known as induced systemic resistance, ISR).This has been documented for *Arabidopsis*, tobacco and a whole range of crop plants, against bacterial, fungal and viral pathogens. In contrast to SAR, ISR generally appears to be mediated by JA and ethylene, rather than SA (Pieterse et al. 1996, Pieterse et al. 2000, Van der Ent et al. 2018), although it is becoming increasingly clear that individual interactions can have very specific hormone signalling requirements. As an example, ISR

against *S. exigua* is induced by both the rhizosphere-associated *Pseudomonas* sp. WCS417 and SS101. In the former, this requires responsiveness to JA and ethylene, but not SA (Pieterse et al. 1998), however the latter is mediated by SA and independent of JA and ethylene (van de Mortel et al. 2012). ISR is thought to be a priming mechanism, associated with more rapid and stronger gene upregulation during attack (reviewed in (Pieterse et al. 2014)), rather than increased levels of endogenous hormones (Pieterse et al. 2000) or basal gene expression (Van Wees et al. 1999).

Due to the complex inter-regulation between plant defence-signalling pathways (as reviewed in Chapter 1), systemic defence responses induced by one pathogen may result in increased susceptibility, rather than greater resistance, to others. This appears particularly true of biotrophic and necrotrophic pathogens, which have markedly different infection strategies that require distinct host defence responses. Biotrophic pathogens infect and feed off living host cells, hence effective host defence involves the hypersensitive response (HR) and programmed cell death (PCD), coordinated by SA (reviewed by (Glazebrook 2005)). Necrotrophic pathogens, on the other hand, kill plant cells to feed off the dead material and are resisted by defences coordinated by JA and ethylene, including the production of antimicrobial defensins and thionins (Glazebrook 2005). Nevertheless, these categories should more accurately be viewed as a continuum, since certain pathogens incorporate aspects of both lifestyles (e.g. *Fusarium oxysporum*). JA and SA are mutually antagonistic: it is thought this allows the host to tailor the defence response to match the invader, prioritising limited resources and shutting down inappropriate defences (Thaler et al. 2012). Consequently, attacks by one class of pathogen can negatively affect host basal resistance against pathogens of the other type. For instance, infecting *Arabidopsis* with the biotrophic bacterial pathogen *Pseudomonas syringae* increased host susceptibility to subsequent attacks by necrotrophs such as the ascomycete fungus *Alternaria brassicicola*. This effect was abolished in the SA biosynthesis mutant *sid2*, indicating that SA-associated activity induced by *P.syringae* repressed defences effective against necrotrophs (Spoel, Johnson and Dong 2007). There are also reports of non-pathogenic rhizobacteria causing induced systemic susceptibility (ISS). The rhizosphere-associated *Pseudomonas* sp. CH267, for instance, decreased host resistance to the foliar pathogen *Pseudomonas syringae pv. tomato* DC3000 through promoting JA signalling over the SA pathway (Haney et al. 2018). A single organism can thus 're-set' the defence hormone signalling balance, affecting whole-plant fitness against a range of pathogens and herbivores.

Besides altering the balance of hormone signalling, systemic effects on immunity could be caused by disruptions in the flow of defensive metabolites, particularly when these are produced in one region and translocated to distant areas of the host. One example is the anti-herbivore secondary metabolite nicotine, which in tobacco *Nicotiana* species is synthesised in the roots and translocated to the shoots (Shoji, Yamada and Hashimoto 2000). Pathogen attacks in regions important for defensive compound production could thus directly affect the flow of these metabolites to other regions of the host.

Nevertheless, the interactions between root and shoot metabolism are complex and depend on species-specific source and sink relationships, i.e. the relative importance of defending root tissue over shoot tissue. In one study for instance, the shoots of S*enecio jacobaea* (ragwort) were exposed to herbivory from *Mamestra brassicae* caterpillars to investigate the effect on host production of protective pyrrolizidine alkaloids, which are manufactured in the roots and translocated to shoot regions. Shoot herbivory caused pyrrolizidine alkaloids to significantly decrease in the roots, yet this was not caused by reallocation to the damaged shoot regions as the level of pyrrolizidine alkaloids here remained unchanged (Hol et al. 2004).

6.1.2 Effects of parasitic plants on host systemic immunity

Given that root invasion by *Striga gesnerioides* induced local significant induction of a wide range of defence-associated genes (Chapter 4), it is likely that this parasite induces systemic effects within the host. If sufficiently strong, these could even influence basal resistance in above-ground regions to foliar pathogens. There is already some limited evidence that parasitism by *Striga* spp. affects above-ground defences. Two-choice tests have indicated that the stem borer *Chilo partellus* preferentially selects *S. hermonthica*-infected maize for oviposition over non infected plants (Mohamed et al. 2007), suggesting that parasitism alters the balance of volatile chemical cues released by the leaves. Interestingly, tomato plants infected by the stem parasitic plant *Cuscuta pentagona* failed to release several volatile defence compounds if their leaves were then exposed to *S. exigua*. The parasitized tomato plants showed significantly reduced JA accumulation at the onset of herbivory; potentially this was suppressed by SA-mediated defences activated by the parasite (Runyon, Mescher and De Moraes 2008). Nevertheless, any effects of parasitism on insect herbivory may also be caused by reduced nutritional content in the leaves, making the hosts less attractive (Runyon et al. 2008).

6.1.3 Two plant-pathogen interactions to investigate whether *S. gesnerioides* induces systemic immune responses

This chapter describes how two *Arabidopsis* leaf pathogens were used to investigate whether *S. gesnerioides* induces systemic defence responses in the host; these included both a biotroph and a necrotroph. *Hyaloperonospora arabidopsidis* (downy mildew, formerly *Peronospora parasitica*) is a biotrophic oomycete pathogen and is thus resisted by SA-controlled defensive signalling in the host. The pathogen obtains nutrients from the host via hyphae which penetrate living cells in the leaves, fuelling the growth of branched fruiting bodies (conidiophores) bearing spherical asexual conidiospores at the tips. Eventually sexual oospores form in the female oogonium and the male antheridium to complete the cycle (Koch and Slusarenko 1990, Slusarenko and Schlaich 2003) (Figure

6.1). Effective host defence against *H. arabidopsidis* restricts the growth of the pathogen through physical barriers and programmed cell death (Tör et al. 2002).

S. gesnerioides significantly induced certain genes related to SA-signalling in the roots (Chapter 4), including *PR2* and *PR5*. Furthermore, the *sid2 Arabidopsis* mutant was more susceptible to *S. gesnerioides* (Chapter 3, Figures 3.2A), indicating that SA accumulation occurs during the normal host defence response. Given that SA is an essential element of both biotrophic defences and SAR (reviewed above), it could be hypothesised that below-ground infection with *S. gesnerioides* could increase basal resistance against *H. arabidopsidis*. Nevertheless, since the interaction between *Arabidopsis* and *S. gesnerioides* is a susceptible one, the parasite may actively suppress host immunity, potentially through effector molecules. This is supported by the observation that induction of SA-associated genes during infection was transient, peaking at 7 or 10 days post infection (Chapter 4, Figure 4.13). In this case, infection with *S. gesnerioides* may cause enhanced susceptibility to biotrophic foliar pathogens.

The fungal pathogen *Plectosphaerella cucumerina* is the causative agent of black spot disease which is seen as necrotic lesions on the leaves that enlarge over time (Figure 6.2) (Pétriacq, Stassen and Ton 2016). Although *P. cucumerina* is normally classed as a hemibiotroph, when inoculated at high spore densities on *Arabidopsis* this pathogen switches to a necrotrophic mode, causing cell death and necrosis of host leaves (Pétriacq et al. 2016). Host defence responses include the production of glucosinolates, reactive oxygen species (ROS) production (Shimono et al. 2007) and callose deposition around the infection sites (Pétriacq et al. 2016).

According to the gene expression analysis described in Chapter 4, *S. gesnerioides* caused local upregulation of JA/ethylene co-regulated genes related to necrotrophic defence, including *PDF1.2, PR4* and *B-CHI*. For *PR4* and *B-CHI*, expression was highest at the last timepoint tested (12 dpi) indicating that upregulation may be sustained for longer than *PR2* and *PR5*, which showed downregulation at this point. Nevertheless, the increased resistance of the *aos1* and *etr1-1* mutants, deficient in JA and ethylene respectively (Chapter 3, Figures 3.3 and 3.5), indicates that induction of JA/ethylene-related defences is a susceptibility factor, possibly actively triggered by the parasite itself. In any case, transmission of JA/ethylene upregulation to foliar regions could potentially increase host basal resistance against *P. cucumerina*. It is unlikely that pre-infection with *S. gesnerioides* would increase host basal resistance to both *H. arabidopsidis* and *P. cucumerina*: depending on the relative balance of defence hormones in the leaves, one defence pathway would presumably dominate over the other. The pathogens both have the advantage that well-characterised assays to score infection have been developed for *Arabidopsis* leaves. This enables quantitative assessments of disease severity and thus the ability to determine whether basal resistance is affected by *S. gesnerioides.*

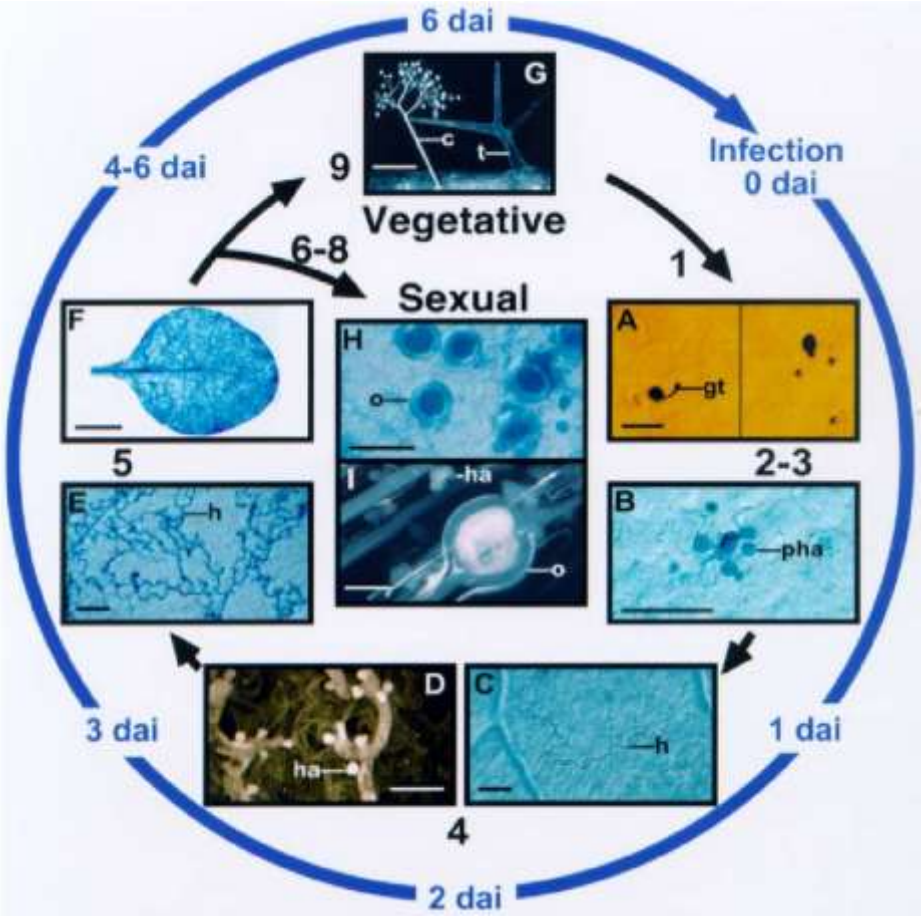


Figure 6.1: Life cycle stages of *Hyaloperonospora arabidopsidis* in wild-type *Arabidopsis*. The outer circle shows the approximate time for each stage after inoculation (dai: days after inoculation). The interior numbers refer to the developmental stage of the pathogen: Stage 1: conidiospores germinate and form germ tubes **(A)**. Stages 2-3: germ tubes penetrate the host epidermis and put out primary haustoria into neighbouring plant cells **(B)**. Stage 4: the hyphae grow vegetatively to form small colonies **(C and D)**. Stage 5: the small colonies of hyphae proliferate to form larger colonies **(E and F)**. Stages 6-8: the antheridia and oogonia differentiate and sexually reproduce diploid oospores **(H and I)**. Stage 9: Conidiophores form and emerge through the stomata to produce vegetative spores or conidia **(G)**. c = conidiophore; gt = germ tube; h = hyphae; ha = haustorium; o = oospore; pha = primary haustorium; t = trichome (plant). Scale bars = 25 µM for A, B, and I ; 50 µM for D and H ; 100 µM for C, E, and G ; 2 mm for F. Figure from (Donofrio and Delaney 2001)

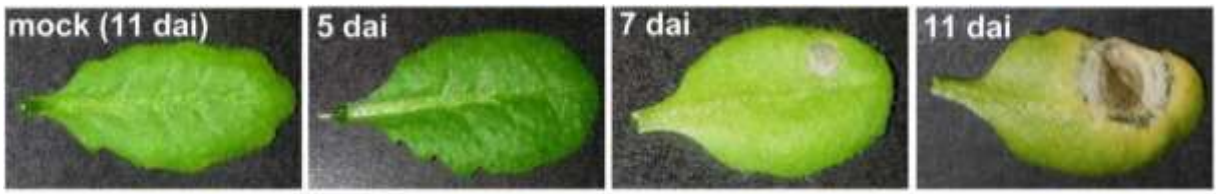


Figure 6.2: Necrotic lesions on *Arabidopsis* leaf caused by the fungal pathogen *Plectosphaerella cucumerina*, when inoculated in droplet form with high spore density (10^6 spores per ml). dai = days after inoculation. Figure from (Pétriacq et al. 2016).

6.2 Aim and objectives

The qPCR analysis in Chapter 4 demonstrated that *S. gesnerioides* induced various defence-related genes in host *Arabidopsis* roots. This may be transmitted to above-ground regions and affect host basal resistance to foliar pathogens. Since the response in the roots against *S. gesnerioides* was mainly associated with SA/biotrophic pathogen-related defence, host resistance against biotrophic foliar pathogens (e.g. *Hyaloperonospora arabidopsidis*) may increase. In contrast, basal resistance to necrotrophic foliar pathogens (e.g. *Plectosphaerella cucumerina*) may decrease as a result of infection by *S. gesnerioides*, given the mutual antagonism between SA and JA.

Aim:

To test the hypothesis that infection with *S. gesnerioides* induces a systemic defence response in the host that altered basal resistance to foliar pathogens.

Objectives:

1. To determine whether pre-infection with *S. gesnerioides* affects host basal resistance against the biotrophic foliar pathogen *Hyaloperonospora arabidopsidis* or the necrotrophic foliar pathogen *Plectosphaerella cucumerina*.

2. To quantify the extent of foliar pathogen infection on *Arabidopsis* hosts with and without infection by *S. gesnerioides*, and determine the relationship between the number of attached parasite haustoria and host susceptibility to foliar pathogens.

3. To perform qPCR analysis of defence gene expression on shoot tissue harvested from control and *S. gesnerioides*-infected *Arabidopsis* hosts, to determine if similar gene expression changes occur as those documented in infected root tissue.

6.3 Experimental design

6.3.1 Investigating systemic effects of *S. gesnerioides* parasitism on foliar susceptibility to *Hyaloperonospora arabidopsidis* (downy mildew)

Two near-identical assays were performed to investigate whether pre-infection with *S. gesnerioides* affected *Arabidopsis* basal resistance against *H. arabidopsidis*. Growth of Col-0 *Arabidopsis* in rhizotrons was carried out following the optimised protocol detailed in Chapter 2, Section 2.3. In experiment 1, 25 *Arabidopsis* plants were established in total. Ten of these were infected with *S. gesnerioides* when the hosts were 26 days old, following the protocol outlined in Chapter 2, Section 2.3. These plants and 10 plants without *S. gesnerioides* infection (controls) were then challenged with

H. arabidopsidis 12 days later. Plants with and without *S. gesnerioides* were randomised between trays. At this point, the parasite had just established vascular connectivity to the host and begun to develop a functional absorptive haustorium (Chapter 4, Section 4.4.1). Five *Arabidopsis* plants acted as negative controls and were sprayed with water only and were not infected with *S. gesnerioides*. Infection with *H. arabidopsidis* was performed according to the protocol detailed in Chapter 2, Section 2.10. Disease symptoms were quantified five days later. For each plant, including controls not infected with *H. arabidopsidis*, 8 leaves were removed and stained with lactophenol-trypan blue solution, according to the protocol outlined in Chapter 2, Section 2.10. These were examined using a compound optical microscope and the extent of *H. arabidopsidis* infection scored using a 10-category system, with four meta-categories (Table 6.1). Following this, the extent of *S. gesnerioides* infection on the host plant was quantified by scanning the root systems of each plant using a Canon 9000F Mark II scanner at resolution 2400 dpi (see Chapter 2, Section 2.3).

Experiment 2 followed the same basic procedure as experiment 1, but used 52 *Arabidopsis* plants in total: 21 of these were infected with *S. gesnerioides* when they were 27 days old. These plants and 21 without *S. gesnerioides* (controls) were infected with *H. arabidopsidis* 11 days later. Ten *Arabidopsis* plants were sprayed with water only and were not infected with *S. gesnerioides,* as a mock treatment control. Five days after *H. arabidopsidis* inoculation, leaves were sampled to quantify disease symptoms. As before, the root systems were scanned to quantify *S. gesnerioide*s infection.

Table 6.1: Ten-category scoring system used to assess *Hyaloperonospora arabidopsidis* infection on *Arabidopsis* leaves

Score value:	Description:	Meta-category
1	Healthy leaf.	Healthy leaf
2	Isolated patches of intracellular hyphae.	Pathogen establishes on the host but does not produce reproductive structures
3	Entire surface of the leaf covered in intracellular hyphae.	
4	Presence of hyphae and isolated patches of conidiophores.	Pathogen produces asexual reproduction structures (conidiophores) but no sexual reproduction structures (oospores)
5	Conidiophores present over large parts of the leaf surface.	
6	Light density of conidiophores over entire leaf surface but no oospores present.	
7	High density of conidiophores over entire leaf surface but no oospores present.	
8	Presence of conidiophores and < 10 oospores.	Sexual reproduction structures present (oospores)
9	Presence of conidiophores and ≥10 oospores (low density).	
10	High density of conidiophores and oospores across the entire surface.	

6.3.2 Investigating systemic effects of *S. gesnerioides* parasitism on foliar susceptibility to *Plectosphaerella cucumerina*

Two initial experiments were performed to investigate whether pre-infection with *S. gesnerioides* affected the susceptibility of *Arabidopsis* to *P. cucumerina*. Growth of Col-0 *Arabidopsis* in rhizotrons was carried out following the optimised protocol detailed in section 2.3.5. In both experiments, 51 *Arabidopsis* plants were used in total. Twenty three of these were infected with *S. gesnerioides* when the *Arabidopsis* plants were 27 days old, following the protocol outlined in section 2.3.5. These plants and 23 others not infected with *S. gesnerioides* were then challenged with *P. cucumerina*: in experiment 1 this took place 11 days after infection with *S. gesnerioides*, and in experiment 2 this took place 12 days later. Five *Arabidopsis* (not infected with *S. gesnerioides*) were treated with distilled water only rather than *P. cucumerina* as a mock-treatment control. Plants with and without *S. gesnerioides* were randomised between trays.

Preparation of and infection with *P. cucumerina* was carried out as described in Chapter 2, Section 2.11. Five leaves were inoculated with the pathogen for each *Arabidopsis* host. *P. cucumerina* infection was quantified by measuring the size of the necrotic lesions using electronic callipers. This was done twice for experiment 2 but only once for experiment 1 (at 10 days post-*P. cucumerina* infection), since there were very few visible signs of infection at 7 dpi. For experiment 2, the lesion diameters were measured twice, at 7- and 11-days post-*P. cucumerina* infection. An average was calculated from the 5 leaves that had been inoculated with the pathogen. For both experiments, the extent of *S. gesnerioides* infection was quantified after the final measurement of *P. cucumerina* disease symptoms. This was done by scanning the rhizotron root systems and counting the number of parasite haustoria using the CellCounter Function on ImageJ dpi (see Chapter 2, Section 2.3). For experiment 2, 4 of the *Arabidopsis* plants showed very poor infection by *S. gesnerioides*, with 3 or fewer attached haustoria, and were removed from the analysis.

To determine whether the time interval between infection with *S. gesnerioides* and challenge with *P. cucumerina* had an effect, a third experiment (experiment 3) was performed with a longer time period (17 days) between infection with *S. gesnerioides* and challenge with *P. cucumerina*. In experiment 3, a total of 49 *Arabidopsis* plants were used. Twenty two were pre-infected with *S. gesnerioides* when the plants were 25 days old. These and 22 others not infected with *S. gesnerioides* were then challenged with *P. cucumerina* 17 days later. Five *Arabidopsis* plants acted as mock treatment controls, treated with water only and not *S. gesnerioides* or *P. cucumerina.* The foliar lesions were measured as before (at 7 days post-infection with *P. cucumerina*) and the root systems were scanned on the same day to assess the level of *S. gesnerioides*.

6.3.3 qPCR analysis of changes in shoot gene expression during infection with *S. gesnerioides*

Previous qPCR analysis of *Arabidopsis* root tissue demonstrated that infection by *S. gesnerioides* induced pronounced defence-associated gene expression changes (Chapter 4). To investigate whether similar expression changes were activated in above-ground regions, qPCR was performed on leaves harvested from Col-0 *Arabidopsis* plants that were either untreated or infected with *S. gesnerioides*. Shoot material was collected at 7, 10- and 12-days post infection (dpi). For each sample, 2 fully-expanded leaves of similar size were removed from 2 different plants with tweezers and collected in 1.5 ml RNase free labelled Eppendorf tubes. These were flash frozen in liquid nitrogen before being stored at -80°C. RNA extraction, cDNA synthesis and qPCR followed the same protocol as described in Chapter 2, Section 2.8. The target genes chosen for qPCR analysis were *THI2.1, B-CHI, PR4, PDF1.2, PR5, WRKY70, ERF4-Activator* and *ERF4-Repressor* (see Chapter 4, Table 4.1 for details). These genes were selected on the basis that they had all shown pronounced changes in expression in *Arabidopsis* root tissue during infection with *S. gesnerioides* (Chapter 4) particularly *PR5*. Primer sequences are contained within Chapter 2, Table 2.3.

6.3.4 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 23. In all cases, normality checks were carried out.

For the *Hyaloperonospora arabidopsidis* assays, the distributions of leaf infection scores of *S. gesnerioide*s-infected and control hosts were compared using a non-parametric Mann Whitney U test and Fisher's Exact test, where significance was taken as $p < 0.05$. For the plants infected with *S. gesnerioides*, linear regression analysis was used to test if there was a significant association between the number of attached parasite haustoria and the average host leaf *H. arabidopsidis* infection score. Normality checks were carried out on residuals and the assumption of normality was met. Significance was taken as $p < 0.05$.

For the assays performed with *P. cucumerina*, independent samples Student's T-tests were used to test for significant differences in the average lesion diameter between *S. gesnerioide*s-infected and control hosts. Levene's test for equality of variances was performed to confirm that equal variances could be assumed. For the plants infected with *S. gesnerioides*, linear regression analysis was used to test if there was a significant association between the number of attached parasite haustoria and the average host lesion diameter. Normality checks were carried out on residuals and the assumption of normality was met. Significance was taken as $p < 0.05$.

qPCR data were analysed with the modified version of the 2-ΔΔCt method used previously (see Chapter 4, Section 4.3.4) (Livak and Schmittgen 2001). As before, fold-gene expression change was calculated relative to the control samples for the first timepoint (7 dpi). Thus, the 7 dpi control samples were the reference against which both basal gene expression over time and change induced by *S. gesnerioides* was quantified. The final normalised values for the samples from control and *S. gesnerioides*-infected *Arabidopsis* plants were compared using Student's T-test to test for significant differences in gene expression. Levene's test for equality of variances was performed to verify whether equal variances could be assumed. Where this was not the case, significance was taken as $p < 0.01$, rather than $p < 0.05$.

6.4 Results

6.4.1 Investigating systemic effects of *S. gesnerioides* parasitism on foliar susceptibility to *H. arabidopsidis* (downy mildew)

In both experiments *H. arabidopsidis* disease symptoms varied from healthy leaves to the presence of sexual reproduction structures (oospores) (Figure 6.3). For experiment 1, the Mann-Whitney U test showed that there was no significant difference ($U = 3048$, $p = 0.279$) in the downy mildew infection scores (categories 1-10) between control *Arabidopsis* plants and those pre-infected with *S. gesnerioides*. The results were also analysed using a Fisher's Exact Test on the four meta-categories of disease scores (Table 6.1). Once again, this indicated that experiment 1 showed no significant differences in the distribution of *H. arabidopsidis* disease scores between *Arabidopsis* plants infected with *H. arabidopsidis* only and those infected with both *H. arabidopsidis* and *S. gesnerioides*: $\chi^2 = 1.500$, $p = 0.687$. In experiment 2, however, the Mann-Whitney U test indicated a significant difference ($U = 10{,}756$, $p = 0.012$) in the downy mildew infection scores between control host plants and those pre-infected with *S. gesnerioides*. The Fisher's Exact Test also indicated a significant difference in the distribution of *H. arabidopsidis* disease scores between the populations of *Arabidopsis* plants: $\chi^2 = 7.962$, $p = 0.034$. This difference was caused by a greater proportion of leaves from *Arabidopsis* plants infected with *H. arabidopsidis* only having higher disease scores (≥ 8: oospores present) (Figure 6.3). In contrast, a higher proportion of *Arabidopsis* plants infected with both *H. arabidopsidis* and *S. gesnerioides* showed the presence of conidiophores (categories 5-7) but no sexual oospores.

Linear regression analysis indicated that there was no significant relationship between the number of *S. gesnerioides* haustoria and the average host *H. arabidopsidis* disease score for both experiment 1 ($R^2 = 0.082$, $p = 0.424$) and experiment 2 ($R^2 = 0.116$, $p = 0.131$) (Figure 6.4).

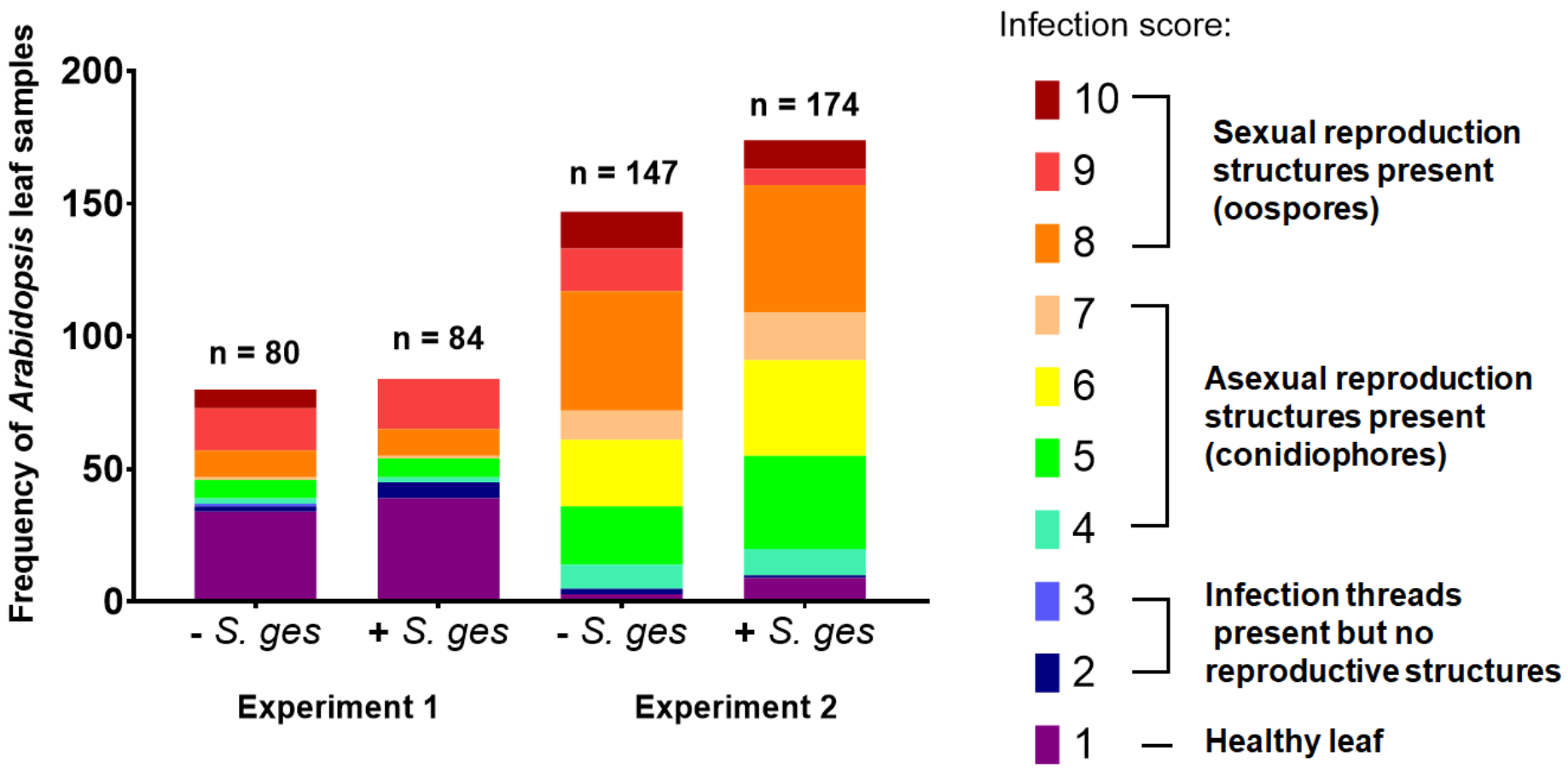


Figure 6.3: Frequency distribution of *Hyaloperonospora arabidopsidis* (downy mildew) infection scores for leaves from *Arabidopsis* hosts either pre-infected with the root parasite *Striga gesnerioides* or infected with *H. arabidopsidis* only. For descriptions of infection scores see Table 6.1. n = total number of host *Arabidopsis* plants.- *S. ges* = *Arabidopsis* plants not pre-infected with *S. gesnerioides*. + *S. ges* = *Arabidopsis* plants pre-infected with *S. gesnerioides* 12 (experiment 1) or 11 (experiment 2) days before challenge with *H. arabidopsidis*.

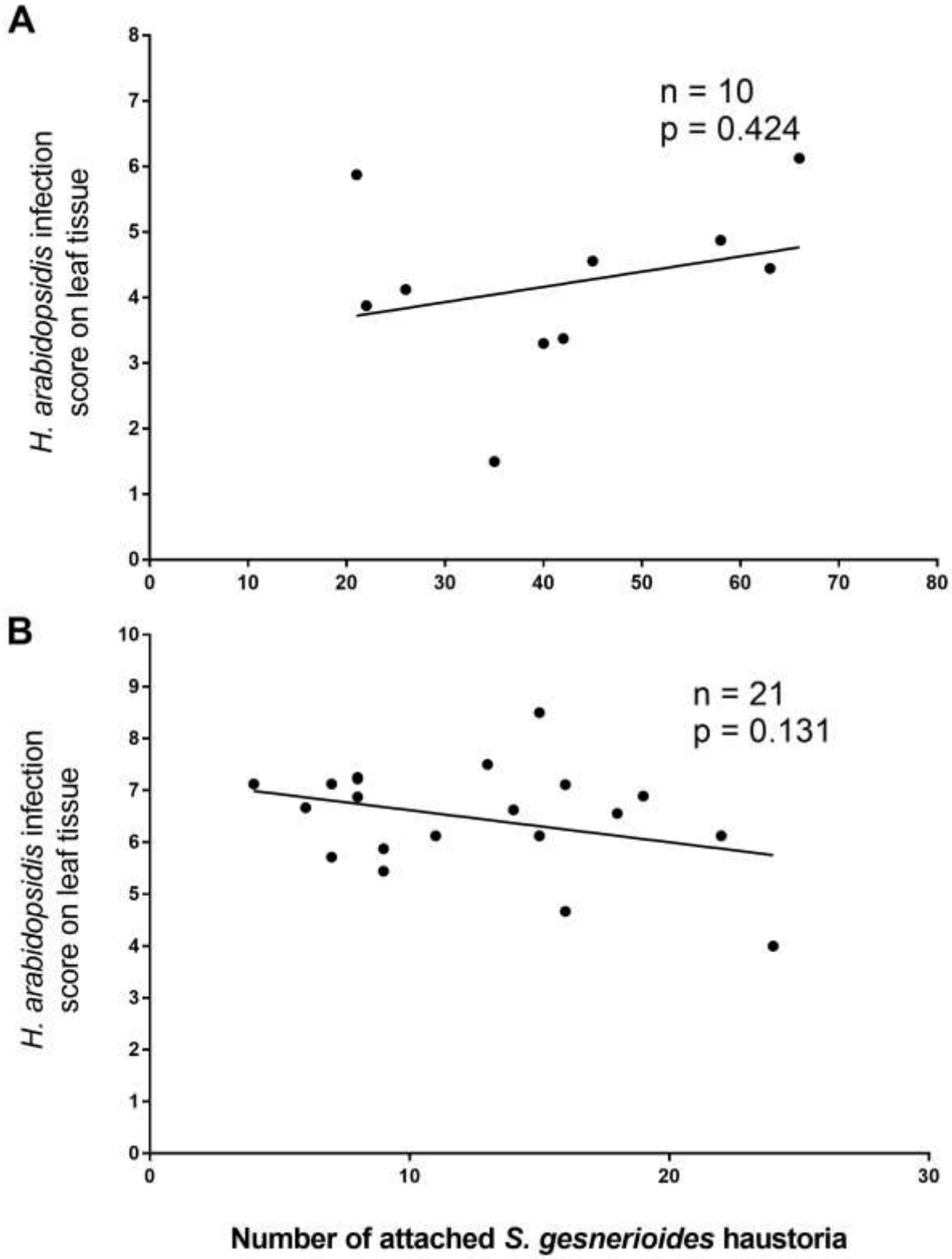


Figure 6.4: The relationship between the number of attached *Striga gesnerioides* haustoria and host *Arabidopsis* susceptibility to *Hyaloperonospora arabidopsidis* for experiment 1 (**A**) and experiment 2 (**B**). *H. arabidopsidis* disease scores are the average calculated from 8 leaves from each host. For descriptions of infection scores see Table 6.1. n = total number of host *Arabidopsis* plants. p = p value for significance from linear regression analysis.

6.4.2 Investigating systemic effects of *S. gesnerioides* parasitism on foliar susceptibility to *P. cucumerina*

For experiment 1, host *Arabidopsis* leaves showed few *P. cucumerina* disease symptoms at 7 dpi although necrotic lesions were clearly visible by 10 dpi (Figure 6.5). At this point, there was no significant difference in the average lesion diameter between *Arabidopsis* infected with *S. gesnerioides* and *P. cucumerina* (2.797 mm) and those infected with *P. cucumerina* only (2.648 mm) ($t = -0.848$, $df = 43$, $p = 0.401$) (Figure 6.6).

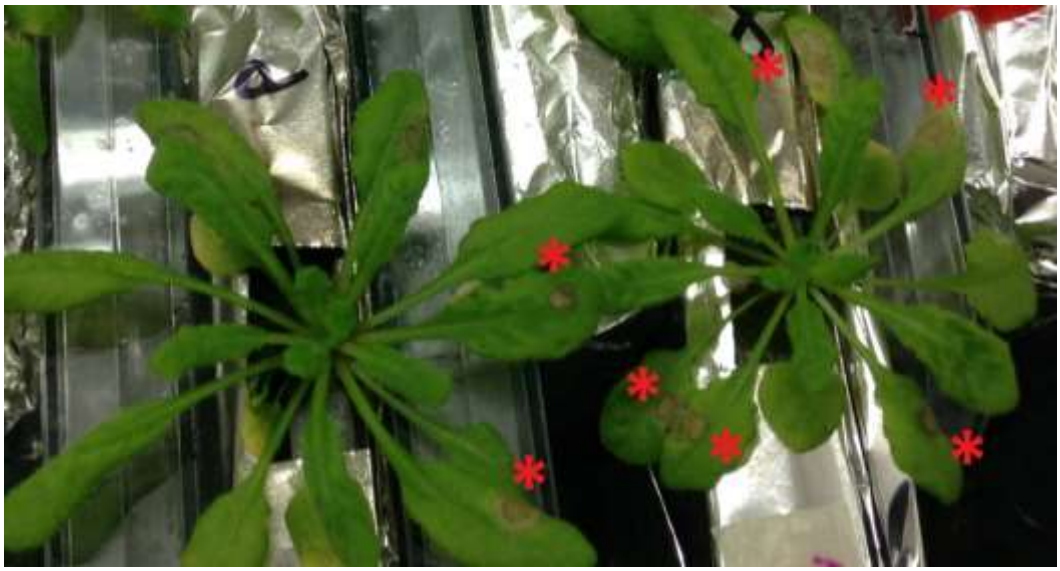


Figure 6.5: Necrotic lesions (*) on host *Arabidopsis* plants caused by the necrotrophic pathogen *Plectosphaerella cucumerina*. This image shows 10 days post-infection, experiment 1. The pathogen was prepared as a droplet inoculum with a spore concentration of 5×10^6 per ml: 5 µl was applied using a multipipette to the surface of 5 leaves for each host *Arabidopsis*.

P. cucumerina infection was much stronger in experiment 2, hence measurements were taken at both 7 and 11 dpi. Similar to experiment 1, at 7dpi there was no significant difference in the average lesion diameter for *Arabidopsis* infected with *P. cucumerina* only (3.94 mm) and *Arabidopsis* infected with *S. gesnerioides* and *P. cucumerina* (4.10 mm) ($t = -0.841$, $df = 37$, $p = 0.406$). At 11 dpi, there was also no significant difference in the average lesion diameter for *Arabidopsis* infected with *P. cucumerina* only (5.84 mm) and *Arabidopsis* infected with *S. gesnerioides* and *P. cucumerina* (5.97 mm) ($t = 0.484$, $df = 37$, $p = 0.631$) (Figure 6.6). This indicates that below ground parasitism with *S. gesnerioides* does not affect host basal resistance to the foliar pathogen *P. cucumerina*.

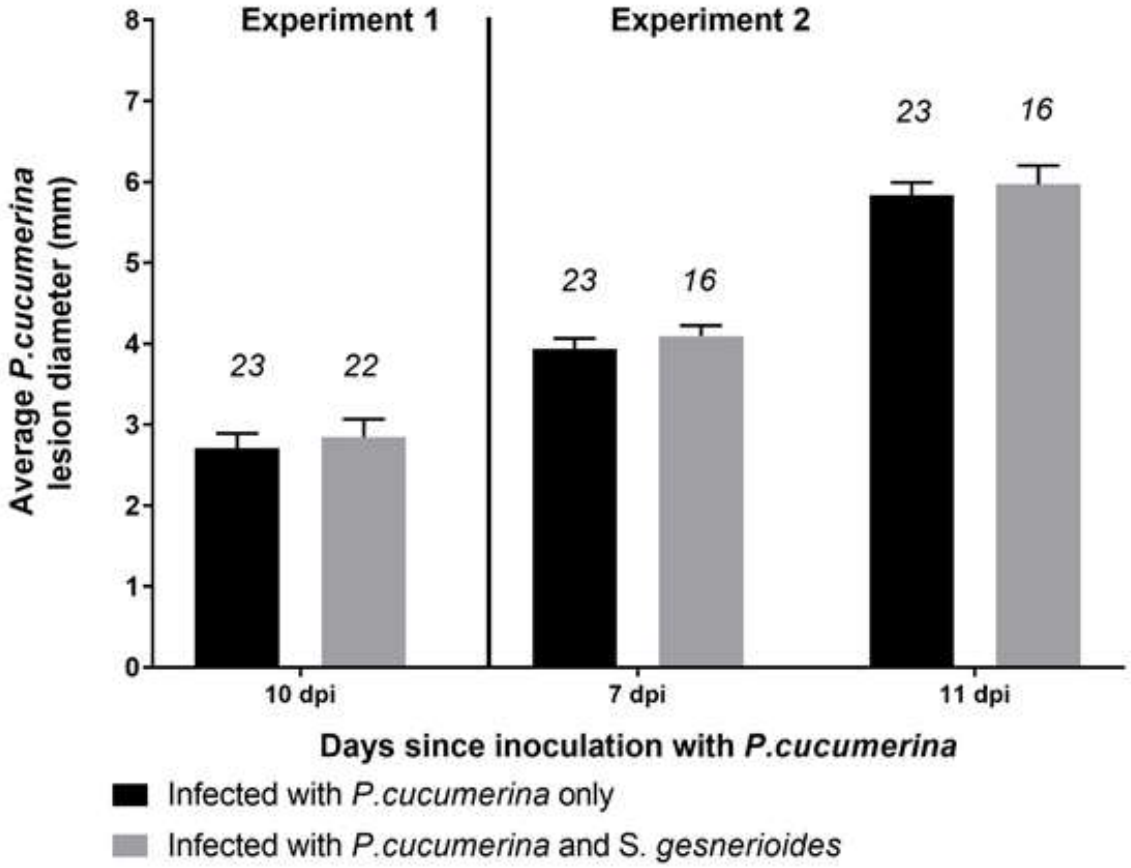


Figure 6.6: The effect of *Striga gesnerioides* root parasitism on host *Arabidopsis* basal resistance to the foliar pathogen *Plectosphaerella cucumerina*. Average *P. cucumerina* lesion diameters were calculated for each host *Arabidopsis*, using the scores of 5 infected leaves. For experiment 1, lesion size was measured at 7 days post infection (dpi); for experiment 2, lesions were measured at 7 and 11 dpi. Error bars: mean +/- standard error. Numbers in italics denote host *Arabidopsis* sample sizes.

The number of attached *S. gesnerioides* haustoria varied considerably, ranging from 13 - 86 in experiment 1, and 5 - 26 in experiment 2. This would reduce the ability to detect any subtle effect caused by *S. gesnerioides* on basal resistance against *P. cucumerina*. To take this into account, the relationship between the average *P. cucumerina* lesion diameter for each *Arabidopsis* host and the number of attached *S. gesnerioides* haustoria was tested using linear regression (Figure 6.7). Linear Regression analysis indicated that there was no significant relationship between the number of attached *S. gesnerioides* haustoria and the average *P. cucumerina* lesion size either for experiment 1 ($R^2 = 0.042$, $p = 0.371$) or experiment 2 ($R^2 = 0.0097$, $p = 0.728$).

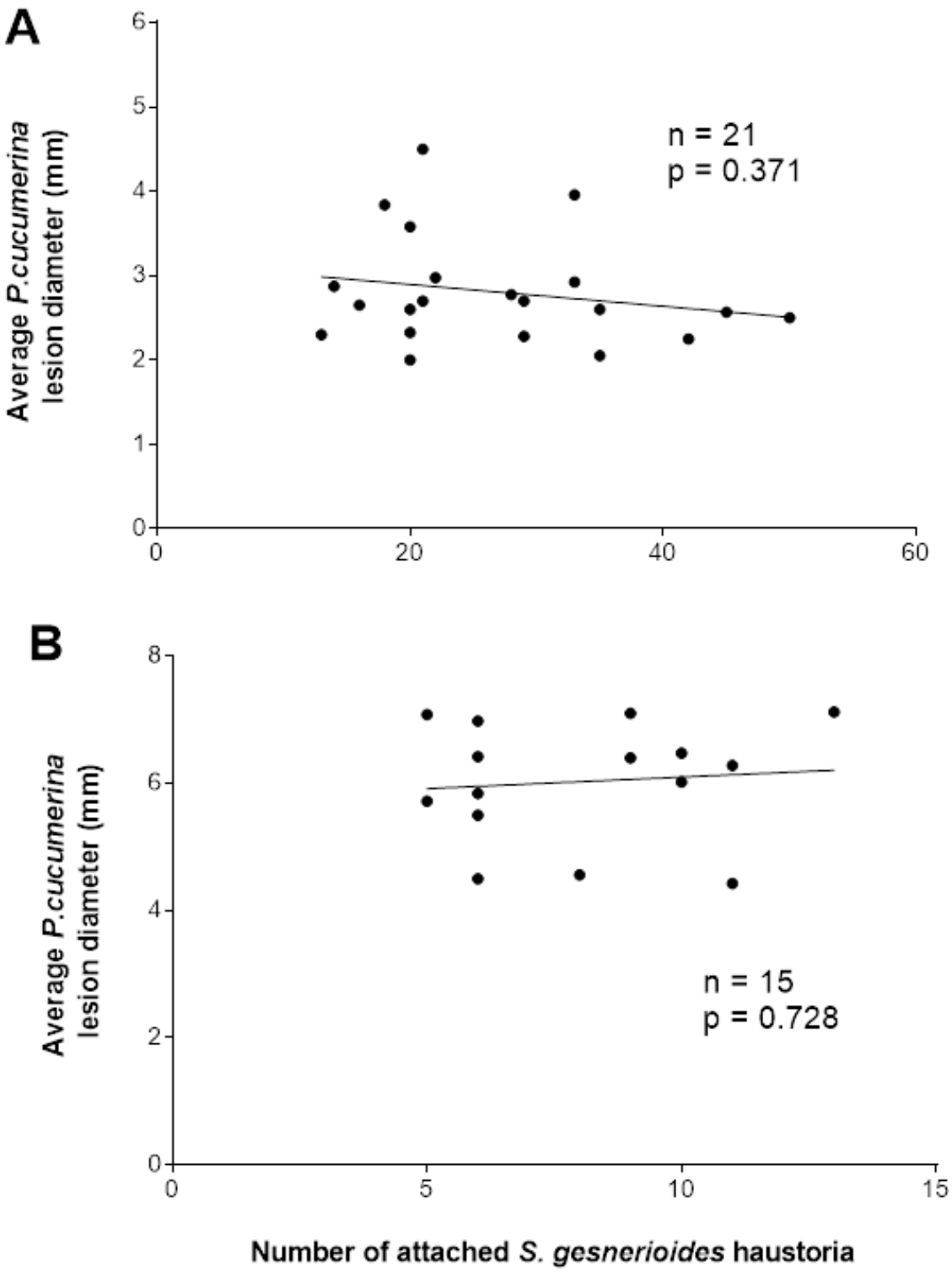


Figure 6.7: Relationship between the number of attached *Striga gesnerioides* haustoria and the average *Plectosphaerella cucumerina* lesion size for host *Arabidopsis* plants. A: Results from experiment 1, where lesions were measured 10 days post- *P. cucumerina* infection. B. Results from experiment 2, where lesions were measured 11 days post- *P. cucumerina* infection. For each host *Arabidopsis*, the average lesion size was calculated from 5 infected leaves. n = total number of host *Arabidopsis* plants. p = p value for significance from linear regression analysis.

In experiment 3, there was a 17-day interval between infection with *S. gesnerioides* and challenge with *P. cucumerina* to investigate whether the time interval between infections with *S. gesnerioides* and challenge with *P. cucumerina* had an effect. The size of the lesions was measured once, 7 days after *P. cucumerina* infection. There was no observable difference in the average *P. cucumerina* lesion diameter between *Arabidopsis* infected with *P. cucumerina* only (4.56 mm) and those infected with both *S. gesnerioides* and *P. cucumerina* (4.81 mm) ($t = 1.014$, $df = 41$, $p = 0.316$) (Figure 6.8A). Linear regression analysis indicated that there was no significant correlation between the number of attached *S. gesnerioides* haustoria and the average *P. cucumerina* lesion size for each *Arabidopsis* host (Figure 6.8B) ($R^2 = 0.011$, $p = 0.644$). Taken together, these experiments show that parasitism by *S. gesnerioides* does not significantly affect host basal resistance to *P. cucumerina.*

6.4.3 qPCR analysis of changes in shoot gene expression during infection with *S. gesnerioides*

There were no significant differences in the expression of any tested defence genes in the leaves of *Arabidopsis* plants infected with *S. gesnerioides* compared to uninfected plants (Figure 6.9). This indicates that below-ground infection with *S. gesnerioides* does not induce systemic changes that affect gene expression in the leaves.

6.5 Discussion

6.5.1 Infection with *S. gesnerioides* does not induce a systemic resistance response against foliar pathogens

Overall pre-infection of *Arabidopsis* roots with *S. gesnerioides* caused no significant difference to host basal resistance in the leaves against either of the foliar pathogens tested. In the assays with the biotrophic pathogen *H. arabidopsidis,* there was no significant difference in the proportion of healthy leaves between control- and *S. gesnerioides*-infected *Arabidopsis* (Figure 6.3). Similarly, the average lesion size caused by the necrotrophic pathogen *P. cucumerina* did not significantly differ between control- and *S. gesnerioides*-infected *Arabidopsis* (Figures 6.6 and 6.8). Furthermore, for both pathogens, the severity of infection did not correlate with the number of attached *S. gesnerioides* haustoria (Figures 6.4, 6.7 and 6.8). This suggests that the defence response induced by *S. gesnerioides* in the roots is not transmitted to foliar regions. Consistent with this, defence gene expression in host leaves (when unchallenged with foliar pathogens) showed no significant difference between control- and *S. gesnerioides*-infected *Arabidopsis* plants.

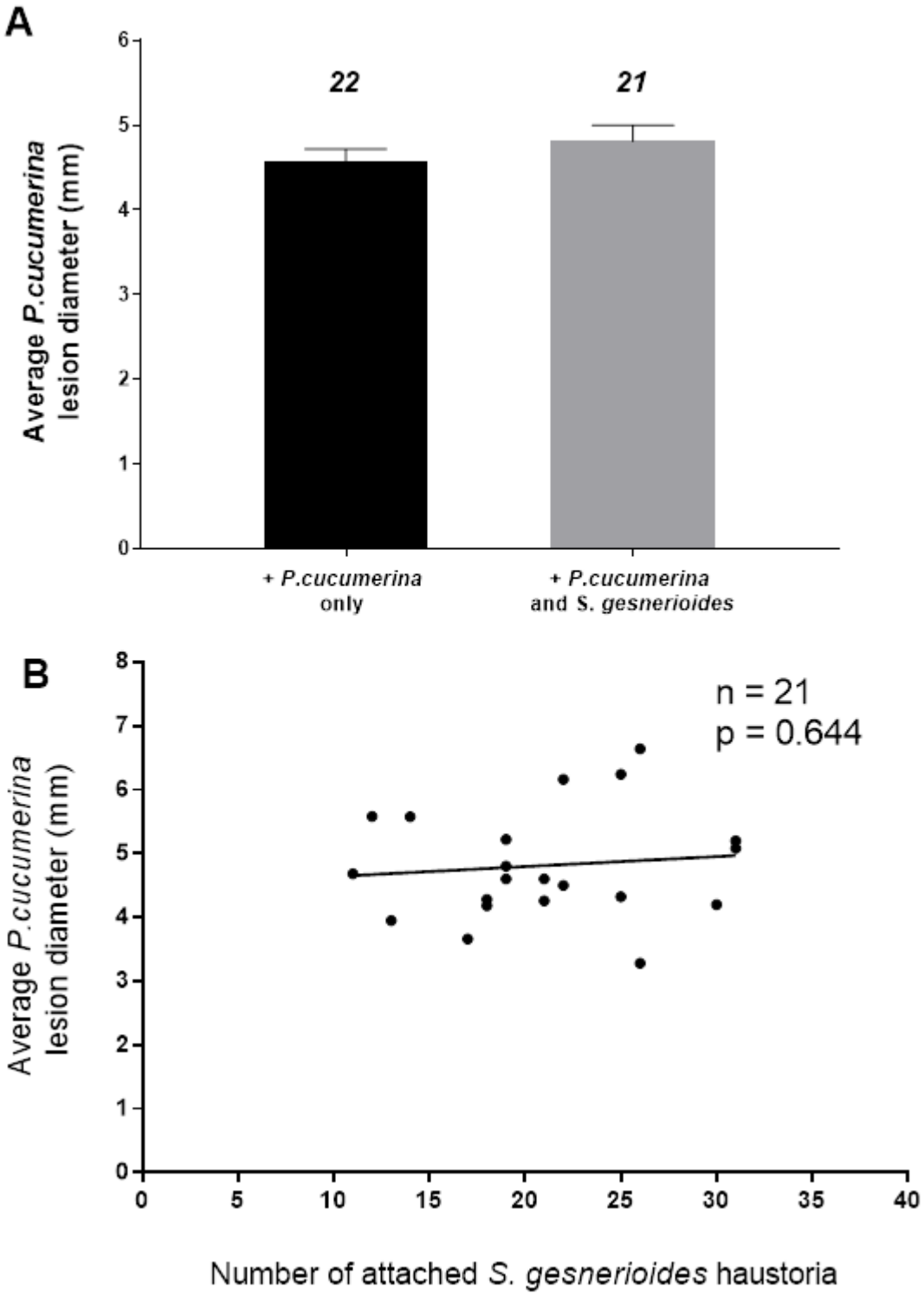


Figure 6.8: The effect of *Striga gesnerioides* root parasitism on host *Arabidopsis* basal resistance to the foliar pathogen *Plectosphaerella cucumerina*. **A**. Average *P. cucumerina* lesion diameter for host *Arabidopsis* at 7 days post-infection (dpi), averaged over 5 infected leaves. Error bars: mean +/- standard error. Numbers in italics denote total number of host *Arabidopsis* plants. **B.** Relationship between the number of *S. gesnerioides* haustoria and the average *P. cucumerina* lesion diameter for each *Arabidopsis* host. n = total number of host *Arabidopsis* plants. p = p value for significance from linear regression analysis.

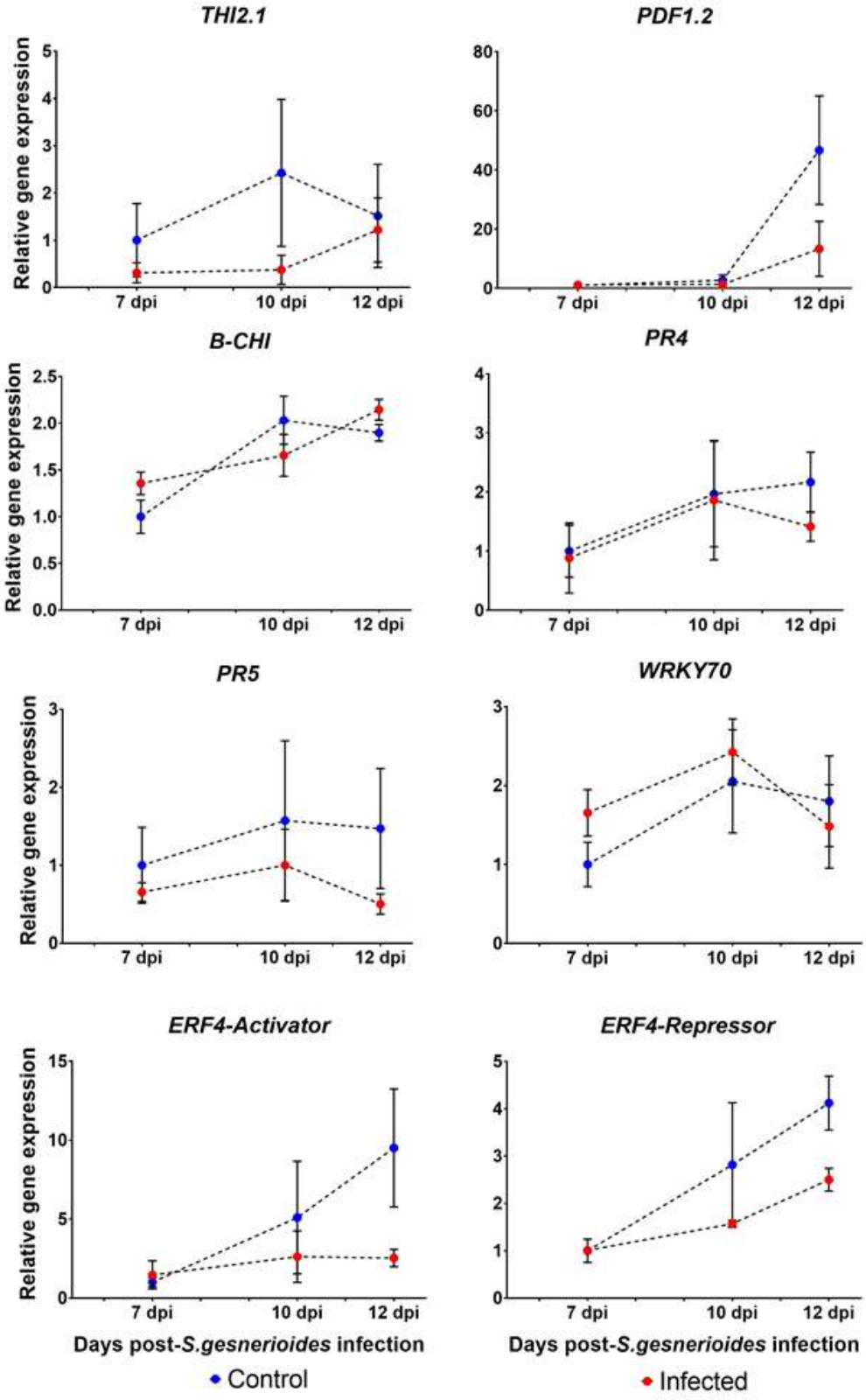


Figure 6.9: qPCR analysis of the expression of defence-associated genes *THI2.1, PDF1.2, B-CHI*, *PR4, PR5, WRKY70, ERF4-Activator* and *ERF4-Repressor* in control and *Striga gesnerioides*-infected *Arabidopsis* shoot samples. For each timepoint, gene expression was tested on three samples from each group of *Arabidopsis* plants. Average expression values were calculated relative to the control samples at 7 dpi. Error bars: Mean +/- standard error. Independent samples Student's T-tests were used to calculate significant differences between control and infected plants at each timepoint. * denotes statistical significance at the 5% level. dpi = days post-infection with *S. gesnerioides*.

Other studies have also found little evidence of systemic induction of defence pathways in the leaves of plants infected with root-invading parasitic plants. Using suppression subtractive hybridization (SSH), Hiraoka and Sugimoto identified sixteen genes upregulated by more than 8-fold in the roots of *Lotus Japonicus* roots parasitized by *O. aegyptiaca* (a compatible interaction). These included genes related to nodulation, JA biosynthesis and pathogenesis. Of these genes, only four were induced systemically by *O. aegyptiaca* throughout the roots, and only one showed systemic upregulation in leaf tissue: *LjOa9*, a pathogen-associated miraculin-like protein. Meanwhile, in the incompatible interaction between *S. hermonthica* and *L. japonicus*, none of the 14 tested genes that showed strong local induction were induced systemically in the roots (Hiraoka et al. 2008). A similar study found 30 genes differentially induced in the roots of susceptible sorghum infected with *S. hermonthica*, yet only two of these showed induction in the leaves, of less than 3.5 fold (Hiraoka and Sugimoto 2008). Other root pathogens also seem capable of supressing systemic gene expression changes. The root-knot nematode *Meloidogyne incognita* for instance, induced the SA-responsive genes *PR1, PR2* and *PR5* in host *Arabidopsis* roots, but repressed these in the leaves. In contrast, infection with beet-cyst nematode *Heterodera schachtii*, caused upregulation of these genes in both the roots and shoots (Hamamouch et al. 2011). Nevertheless, these results do not preclude the possibility that below-ground infection with *S. gesnerioides* has a priming effect for defence gene induction in the leaves if subsequently challenged with foliar pathogens. This could ultimately be investigated using qPCR to test whether pre-infection with *S. gesnerioides* causes more rapid and/or stronger defence gene induction in the leaves of *Arabidopsis* plants when challenged with foliar pathogens.

These lack of systemic defence-related activity in the leaves suggest that *S. gesnerioide*s, and potentially other root parasites, may actively suppress SAR, possibly through the secretion of effector molecules into the host plant (Saucet and Shirasu 2016) or through rapid downregulation of the local root response before a SAR signal can fully develop. The latter case could be supported by the sharp decrease in *PR5* expression in the host roots between 7 and 12 dpi (Chapter 4, Figure 4.9). As detailed in Chapter 1, the race-specific and monogenic nature of *S. gesnerioides* resistance in cowpea indicates that the parasite supresses host immunity using specific effector molecules. Furthermore, the discovery of the cowpea resistance gene *RSG-301*, a nucleotide-binding domain and leucine-rich repeat-containing (NLR) receptor, indicates that the host can recognise molecular compounds associated with *S. gesnerioides* infection, which may be effector molecules (Li and Timko 2009, Saucet and Shirasu 2016). In the stem parasite *Cuscuta reflexa*, meanwhile, a peptide secreted by the parasite has been isolated that triggers the HR in resistant tomatoes ('Cuscuta factor') (Hegenauer et al. 2016). This study also identified a leucine-rich repeat receptor like protein (LRR-RLP) in tomato capable of responding to Cuscuta factor, subsequently named CuRe1 (Cuscuta receptor 1). Expression of *CuRe1* in tobacco *N. benthamiana* rendered the plants newly responsive to Cuscuta factor and increased

resistance against *Cuscuta* infection. This adds compelling evidence that parasitic plants specifically deploy molecular agents to target host immunity.

Striga parasites have also been reported to increase concentrations of ABA in the leaves of their hosts (Frost et al. 1997), which may be a mechanism for SAR inhibition as indicated by certain studies. In *Arabidopsis*, chemically induced SAR effective against virulent *P. syringae* pv. tomato (*Pst*) was abolished with pre-treatment of 500 µm ABA. This is apparently through suppression of SA-mediated signalling since the ABA pre-treated plants showed reduced SA accumulation and induction of *PR1, PR2* and *PR5* (Yasuda et al. 2008). Hence it is plausible that high concentrations of ABA in foliar tissues inhibit the induction of SAR by *S. gesnerioides*. This could be investigated by testing systemic responses between Col-0 *Arabidopsis* and mutants impaired in ABA biosynthesis or signalling. Furthermore, by increasing ABA concentrations, *Striga* spp. cause the stomata of their hosts to close (Frost et al. 1997). The invasive hyphae of *H. arabidopsidis* tend to target wounds in the leaf epidermis and the gaps between pavement cells rather than the stomata (Faulkner and Robatzek 2012). Pre-infection by *Striga* spp. may therefore still have a significant protective effect against different biotrophic pathogens that enter via stomatal pores (e.g. *P. syringae pv*. Tomato (Melotto et al. 2017)).

If *S. gesnerioides* does indeed actively suppress SAR in above-ground regions, it would be interesting to investigate whether this also the case for the roots, or if systemic defence signals can still be relayed across the host root system. This could potentially be investigated using a split root system. Indeed, this approach was used in the aforementioned study investigating systemic gene induction in *L. japonicus* parasitized by either *S. hermonthica* or *O. aegyptiaca*, with the host roots grown in two separate rhizotrons (Hiraoka et al. 2008). Since *Arabidopsis* is considerably smaller than *L. japonicus*, it may be more feasible to assess whether, in a single rhizotron system, pre-infection of one half of the root system affects basal resistance to *S. gesnerioides* in the remaining half.

Despite the overall result, the *H. arabidopsidis* experiment 2 indicated that infection with *S. gesnerioides* was associated with reduced sexual reproduction of the foliar pathogen, with fewer sexual oospores present (Figure 6.3). This could indicate a weak systemic response against biotrophic pathogens, albeit only effective at the end of the *H. arabidopsidis* lifecycle. Interestingly, the *Arabidopsis comt1 (caffeic acid O-methyltransferase)* mutant, defective in lignin biosynthesis, showed a significantly elevated number of sexual oospores during *H. arabidopsidis* infection, despite having similar levels of hyphal growth to wild-type plants (Quentin et al. 2009). This was attributed to accumulation of the monolignol precursor hydroxyferuloyl malate (OH-FM), which stimulates oospore formation in vitro. Lignification has been associated with basal resistance against root parasites, particularly for preventing penetration of the vascular system (Pérez-De-Luque et al. 2005, Pérez-de-Luque et al. 2007, Haussmann et al. 2004, Maiti et al. 1984). It may be that localized lignin production

in response to *S. gesnerioides* depleted monolignol precursors that promote sexual reproduction in *H. arabidopsidis*. Since this result was not seen in experiment 1, further assays are required to investigate this.

6.5.2 Future directions

Assays involving multiple plant pathogens are always challenging, with the potential for high experimental variation; hence the conclusions of these assays may have been limited by the experimental design. For the assays with *H. arabidopsidis*, for instance, a considerably higher proportion of leaves were healthy in experiment 1 compared with experiment 2 (Figure 6.3). This may be due to the pathogen reaching a higher virulence level on the susceptible Col::*NahG* host (see Chapter 2, Section 2.10) in experiment 2, where the pathogen was transplanted to the experimental hosts after 6 days, compared with only 5 days for experiment 1. Similarly, during the *P. cucumerina* assays, infection was very poor initially for experiment 1, with few necrotic lesions on the infected plants after 7 days. Furthermore, the number of attached *S. gesnerioides* haustoria varied considerably between host *Arabidopsis*, making it difficult to infer if parasitism affected foliar defences. This was particularly the case for the *H. arabidopsidis* experiment 1, where the range of *S. gesnerioides* haustoria per host was 45. This may explain why no significant result was found for experiment 1, in contrast to experiment 2 where the range of attached haustoria was only 20. It is likely that the variability in *S. gesnerioides* infection was exacerbated by the conditions of high humidity which both *H. arabidopsidis* and *P. cucumerina* require. Possibly, more revealing experiments could be done using pathogens adapted to less humid conditions, similar to the climate where *S. gesnerioides* is endemic.

In addition, the rhizotron system is highly artificial and excludes many factors normally abundant in the rhizosphere surrounding the host roots. This includes non-pathogenic rhizobacteria which, as reviewed in this chapter's introduction, can induce systemic resistance or susceptibility in host plants (Van Loon et al. 1998). Volatile organic compounds have also been proposed to play a role in the transmission of systemic signals (Erb et al. 2008). If these traveled externally, rather than through the vasculature, the rhizotron covers may physically prevent these from being transmitted. In its gaseous form, ethylene can also modify plant defense. One study, for instance, found that exposing tobacco plants to ethylene gas decreased the size of lesions caused by tobacco mosaic virus (TMV) (Knoester et al. 2001). If any systemic host signals induced by *S. gesnerioides* were gaseous in nature, they may have been unable to access above-ground regions in these assays. Nevertheless, the specific lifestyle of *S. gesnerioides* makes this a difficult limitation to overcome. In the meantime, the notion that *S. gesnerioides* may use effector molecules to suppress SAR in the host is worthy of exploring, for instance using genome analysis to identify candidate effector genes.

CHAPTER SEVEN

General Discussion.

The aim of this thesis was to investigate the molecular basis of basal resistance in *Arabidopsis thaliana* against the parasitic weed *Striga gesnerioides*, in order to generate fundamental knowledge that may be relevant for controlling this parasite on economic crops.

The core objectives were:

1. To test a range of *Arabidopsis* mutants impaired in different components of plant defence for altered basal resistance against *S. gesnerioides* (**Chapter 3**).
2. To profile changes in host gene expression throughout the course of infection by *S. gesnerioide*s (**Chapter 4**).
3. To study the mechanisms by which the *ERF4* gene controls both resistance and susceptibility responses to *S. gesnerioides* (**Chapter 5**).
4. To investigate whether infection by *S. gesnerioides* induces systemic changes in host defence, such that basal resistance to foliar pathogens is affected (**Chapter 6**).

The mutant phenotyping assay (Chapter 3) was successful in identifying defence-associated pathways that appear to contribute to host basal resistance against *S. gesnerioides*. Broadly speaking, these indicated that salicylic acid (SA) has a protective effect against the parasite whilst jasmonic acid (JA) promotes infection. This was not always consistent however: for instance, the JA-signalling mutant *jar1-1* appeared more susceptible than the Col-0 control (Figure 3.3). Interestingly, certain mutations specifically affected either early- or late-stages of resistance. This indicates that the host defence mechanisms that resist parasite invasion are distinct from those that act later to inhibit further development of *S. gesnerioides* once it has established vascular connectivity. For example, the *pmr4-1* mutant showed significantly enhanced late-stage resistance, but no difference in early-stage resistance (Figure 3.7) whereas *erf4-1* showed greater early-stage susceptibility but was not affected in late-stage susceptibility (Figure 3.4). The results also suggest that the overall host basal resistance response depends on the activation of highly specific factors downstream of general defence hormones. For instance, the SA-deficient mutant *sid2-1* showed greater early-stage susceptibility (Figure 3.2), yet *npr1-1* showed no difference even though many responses downstream of SA are coordinated by NPR1 (Figure 3.2). Yet *wrky70* showed increased early-stage susceptibility (Figure 3.4), despite WRKY70 being considered a downstream factor of NPR1 (Wang et al. 2006). This may indicate that WRKY70 is activated by non-canonical pathways during this interaction. Similarly, the *Arabidopsis* mutant of the JA/ethylene-regulated transcription factor ORA59 showed increased early-stage susceptibility (Figure 3.4), despite the phenotypes of *aos1, etr1-1* and *ein3-1* suggesting that JA and ethylene favour *S. gesnerioides* infection. It may be that the increased resistance of *aos1, etr1-1* and *ein3-1* are fundamentally caused through interaction effects between JA/ethylene and SA, rather than genes directly downstream.

The gene expression analysis of infected host root tissues (Chapter 4) meanwhile was successful in identifying a suite of genes showing altered regulation in response to *S. gesnerioides*. Without phenotyping the corresponding mutant *Arabidopsis* lines, however, it is not clear whether these all contribute to host basal resistance, or whether their activation results from coregulation with other target genes that do have a relevant role. It may also be the case that certain gene pathways are deliberately triggered by the parasite to aid infection (as has been demonstrated with the fungal root pathogen *Fusarium oxysporum* on *Arabidopsis* (Cole et al. 2014)). Nevertheless, high induction of certain SA-associated genes (e.g. *PR5*) and the result of the signature similarity search give strong evidence that *S. gesnerioides* is perceived by the host as a biotrophic pathogen in this interaction. This fundamental knowledge could be invaluable in developing effective control measures.

Both the mutant phenotyping and gene expression studies indicate that the overall balance of reactive oxygen species (ROS) in the host root has a critical effect on basal resistance. This evidence includes the intense DAB staining at the host-root interface (Figures 4.9 and 4.11): the increased resistance of *RbohD* and *RbohD/RbohF* mutants (Figure 3.6) and the induction of ROS-detoxifying genes (e.g. *GST1, PRX33*) during infection (Figures 4.13 and 4.14). ROS generation, however, may either be a host-defence response, or induced by the parasite to aid infection. The greater resistance of *RbohD* and *RbohD/RbohF* to *S. gesnerioides* (which are impaired in ROS-production) would suggest the latter. This has parallels with the interaction between *Arabidopsis* and certain parasitic nematode worms which, like *S. gesnerioides*, switch from a destructive invasive phase to establishing a long-term feeding relationship with the host. For instance, *RbohD* and *RbohD/RbohF Arabidopsis* show enhanced early- and late-stage resistance to the beet cyst nematode *Heterodera schachtii.* This study found that nematode-induced ROS generation depends on host *RbohD/RbohF*, and that the corresponding mutants showed greater host cell-death around the infection site. The authors propose that during initial infection, nematode migration causes cellular damage that triggers cell death, but these signals are disrupted by ROS produced by *RbohD/RbohF*, favouring infection. At later stages of infection, as the nematodes establish a feeding syncytium, RBOH-generated ROS inhibit SA-mediated defence responses, allowing greater nematode growth (Siddique et al. 2014). Interestingly, the effector 10A06 secreted by *H. schachtii* increases susceptibility of *Arabidopsis* and is associated with repressing SA-signalling activity. This may be via disrupting ROS balance, since 10A06 stimulates the enzyme SPERMIDINE SYNTHASE 2, the product of which is catabolised by POLYAMINE OXIDASE to produce H_2O_2 (Hewezi et al. 2010). Since SA appears to have a protective role against *S. gesnerioides*, the phenotype of the *RbohD* and *RbohD/RbohF* mutant may plausibly be caused by reduced suppression of SA defences from ROS. Furthermore, the *erf4-1* mutant showed impaired induction of the ROS-detoxifying gene *PRX33* during infection (Figure 5.6), again indicating that ROS are a pathogenicity factor in this interaction.

On the other hand, ROS may instead support an effective host defence response, with ROS-detoxifying genes being expressed as a consequence of this, rather than as a reaction to pathogen-induced ROS. The resistance of cowpea cultivar B301 to multiple races of *S. gesnerioides*, for instance, is based on a swift, ROS-driven hypersensitive response that causes cell death at the host-parasite interface (Figure 1.3). Similarly, host-derived ROS appear crucial for basal resistance against root-knot nematodes (RKN) of the genus *Melodoigyne*. The effector molecule MjTTL5 from *Meloidogyne javanica* appears to induce susceptibility in *Arabidopsis* by increasing host antioxidant activity via the ferredoxin : thioredoxin reductase catalytic subunit. Thus MjTTL5 would promote ROS-scavenging in the host (Lin et al. 2016). *Melodoigyne incognita* induces ROS in tomato roots yet a comparison of susceptible and resistant hosts indicated that resistance is associated with a greater initial ROS burst, and a second ROS-production phase not seen in susceptible lines (Melillo et al. 2006). This second burst was accompanied by host suppression of antioxidant activity, leading to the hypersensitive response and cell death which prevented further nematode invasion. In the susceptible interactions, however, the short ROS burst induced antioxidant enzymes and suppressed host cell death. These authors suggest that the specific defence responses elicited by ROS during pathogenic interactions may vary depending on their subcellular localisation, temporal dynamics and concentration (Melillo et al. 2006). In rice, meanwhile, application of thiamine to the roots of the Nipponbare cultivar was found to enhance resistance to the RKN *Meloidogyne graminicola*. This was apparently due to a priming mechanism that increased host H_2O_2 generation on infection, leading to greater lignin deposition via the phenylpropanoid pathway (Huang et al. 2016).

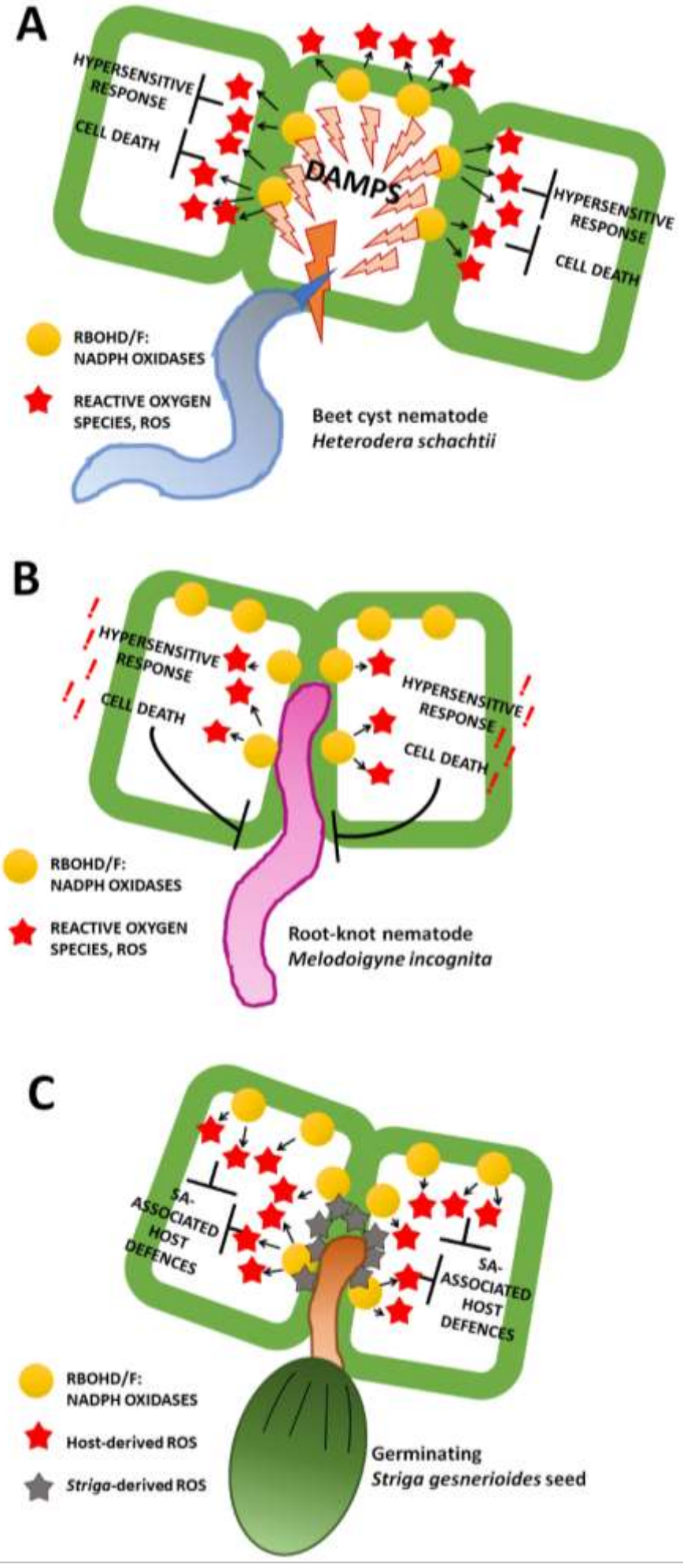
This discrepancy between root-knot nematodes (RKN) and beet cyst nematodes concerning the influence of ROS may well result from the contrasting infection processes of these parasites. Beet cyst nematodes penetrate the roots by piercing cell walls with their stylets, disrupting cells in the process (Wyss and Zunke 1986). RKN, on the other hand, migrate intercellularly and therefore generate less damage (Williamson and Gleason 2003). It may be that greater Damage Associated Molecular Patterns (DAMPs) induced by beet cyst nematodes cause excessive host ROS-generation that overwhelms the appropriate defence response (Figure 7.1, A). Whereas during infection by RKN, enhanced host ROS generation supports a more effective defence response (Figure 7.1, B). It is difficult to ascertain whether the *S. gesnerioides* infection strategy is more similar to that of beet cyst nematodes or RKN. On the one hand, the apparent low induction of wound response genes (e.g. *VSP2*) during infection would suggest that minimal damage to the host occurs during penetration (Figure 4.16). Nevertheless, there is much evidence that parasitic plants secrete cell wall modifying enzymes during infection, which may generate DAMPs (Hood et al. 1998, Reiss and Bailey 1998, Dörr 1997, Veronesi et al. 2007). Furthermore, *Striga* radicles are themselves sources of ROS (Keyes et al. 2007, Kim et al. 1998, Wada et al. 2019), which may thus induce uncontrolled ROS-generation in the host and suppression of

appropriate defences (Figure 7.1, C), similar to beet cyst nematodes. This raises the possibility that *S. gesnerioides* may have evolved to manipulate host ROS signalling to favour infection.

The mechanistic basis of the increased susceptibility of *erf4-1*

Although the investigation regarding the increased susceptibility of *erf4-1 Arabidopsis* was unable to give a conclusive explanation, this successfully demonstrated that loss of the ERF4-Activator variant *per se* is not the basis of the phenotype. Rather, the conclusion of Chapter 5 was that basal host resistance is significantly affected if the function of ERF4-Repressor is disrupted. In *p35S:ERF4-Activator*, this occurs through over-expression of *p35S:ERF4-Activator*, whilst in *erf4-1*, ERF4-Intron Retention accumulates to excess (Figure 7.2). However, ERF4-Repressor has been demonstrated to have multiple roles, making it difficult to ascertain the precise contribution it makes to basal resistance against *S. gesnerioides*. ERF4-Repressor suppresses *PDF1.2* expression, a marker of JA/ethylene co-regulated defences (Lyons et al. 2013). Ethylene and JA-associated *Arabidopsis* mutants (e.g. *etr1-1*, *ein3-1*, *aos1*) generally showed enhanced resistance to *S. gesnerioides*, suggesting that activation of this pathway promotes host susceptibility. Hence, the phenotype of *erf4-1* may be explained by increased *PDF1.2* expression, due to decreased suppression by ERF4-Repressor. Nevertheless, the JA/ethylene-regulated gene *B-CHI* did not show higher expression in *erf4-1* compared with Col-0 under both basal and infected conditions (Figure 5.6). Furthermore, mutants in *ORA59*, a positive regulator of *PDF1.2* expression, showed increased early-stage susceptibility (Figure 3.4). This argues against the prime function of ERF4-Repressor in this interaction as being to supress JA/ethylene co-regulated genes.

Figure 7.1 (overleaf): Model for the influence of reactive oxygen species (ROS) on host defence for parasitic nematodes and *Striga gesnerioides*. **A.** Beet cyst nematodes such as *Heterodera schachtii* invade by piercing host cell walls with their stylets, generating Damage Associated Molecular Patterns (DAMPs). This may cause excessive ROS generation by host NADPH oxidases such as RBOHD and RBOHF, leading to suppression of the host hypersensitive response and cell death that normally restricts nematode entry. **B.** Root-knot nematodes, for instance *Melodoigyne incognita*, generate less damage during penetration since they migrate intercellularly. In this case, host generated ROS appears to potentiate appropriate defence responses. **C.** ROS appear to be a pathogenicity factor for *S. gesnerioides*, since *RbohD/RbohF* mutants show enhanced resistance. *Striga* radicles are potent sources of H_2O_2: this may trigger ROS generation from host RBOHD/RBOHF NADPH oxidases, suppressing salicylic acid (SA) regulated defences.


A
HYPERSENSITIVE RESPONSE
CELL DEATH
DAMPS
HYPERSENSITIVE RESPONSE
CELL DEATH
RBOHD/F: NADPH OXIDASES
REACTIVE OXYGEN SPECIES, ROS
Beet cyst nematode
Heterodera schachtii
B
HYPERSENSITIVE RESPONSE
CELL DEATH
HYPERSENSITIVE RESPONSE
CELL DEATH
RBOHD/F: NADPH OXIDASES
REACTIVE OXYGEN SPECIES, ROS
Root-knot nematode
Melodoigyne incognita
C
SA-ASSOCIATED HOST DEFENCES
SA-ASSOCIATED HOST DEFENCES
RBOHD/F: NADPH OXIDASES
Host-derived ROS
Striga-derived ROS
Germinating
Striga gesnerioides seed

As detailed above, ROS homeostasis appears to have a crucial role in basal resistance against *S. gesnerioides*, hence the role of ERF4-Repressor as a stimulant for host-generated ROS may be more relevant. So far, this has been demonstrated in response to bacterial flagellin (Lyons et al. 2013) and during leaf senescence (Koyama et al. 2013, Riester et al. 2019, Miao and Zentgraf 2007). *erf4-1* showed reduced expression of *PRX33*, both under basal and infected conditions (Figure 5.6), suggesting altered ROS homeostasis (although *GST1* expression was unaffected). Nevertheless, for the reasons outlined above, it is unclear whether ROS would promote or resist infection in this scenario. Reduced *PRX33* expression could reflect reduced detoxification ability against *S. gesnerioides*-induced ROS; alternatively, it may limit the host's ability to generate ROS for an effective hypersensitive response against the parasite. Without further experiments, no further conclusions can be drawn at this stage, although it is noteworthy that *erf4-1* also showed reduced expression of the SA-reporter gene *PR5*, particularly during infected conditions (Figure 5.6). Intercellular ROS and SA are thought to form a mutual amplification loop (Chaouch et al. 2012); this may be inhibited in *erf4-1* due to the loss of ERF4-Repressor function, which promotes ROS. Yet the enhanced resistance of *RbohD* and *RbohD/RbohF* make it clear that ROS elsewhere may be a pathogenicity factor. This fits the growing consensus that ROS have multiple roles in plant-pathogen interactions, with the effects highly dependent on spatio-temporal dynamics (Qi et al. 2017). Dissecting these levels further is the next stage towards understanding host basal resistance against *S. gesnerioides*. This could be done, for instance, using transgenic ROS-reporter genes localised to specific cell compartments or using ROS-inhibitors such as diphenyleneiodonium. It is worth bearing in mind, however, that previous work into the functions of ERF4-Repressor and ERF4-Activator focused on leaf tissues: potentially ERF4 may function very differently in root tissues. One study, for instance, concluded that *erf4-1* had increased basal expression of *PR5* in leaf tissues (Edgar et al. 2006), whilst this study found reduced basal *PR5* expression in the roots.

***S. gesnerioides* may suppress systemic defence responses**

The investigations on systemic resistance of Chapter 6 gave strong evidence that a systemic signal is not transmitted between infected host roots and above-ground tissues. One interpretation of this is that host activation of defences against *S. gesnerioides* are restricted to the roots. However, since root : shoot transmission of defence signals has been demonstrated on other systems, an alternative explanation is that *S. gesnerioides* inhibits the development of a systemic signal. This may be through direct suppression via effector molecules, or through alterations in the level of hormones such as ABA. As reviewed in Chapter 1, there is evidence that *S. gesnerioides* may use effector molecules to target host defence (Li and Timko 2009).

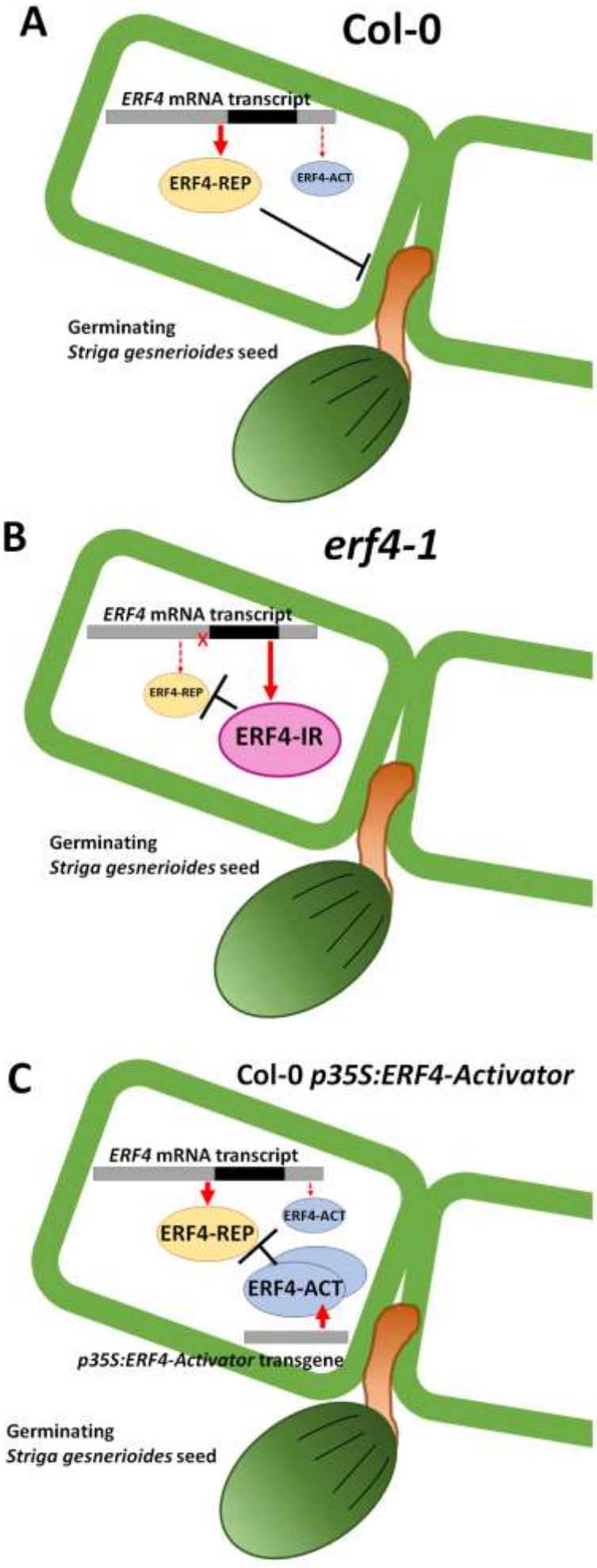

A
Col-0
ERF4 mRNA transcript
ERF4-REP
ERF4-ACT
Germinating
Striga gesnerioides seed
B
erf4-1
ERF4 mRNA transcript
ERF4-REP
ERF4-IR
Germinating
Striga gesnerioides seed
C
Col-0 p35S:ERF4-Activator
ERF4 mRNA transcript
ERF4-REP
ERF4-ACT
ERF4-ACT
p35S:ERF4-Activator transgene
Germinating
Striga gesnerioides seed

Figure 7.2 (overleaf): Model for the increased susceptibility of *erf4-1* and Col-0 *p35S:ERF4-Activator Arabidopsis* to *Striga gesnerioides.* A. Scheme for Col-0 (wild-type) plants, where the *ERF4* mRNA transcript can be alternatively spliced to produce either a Repressor (ERF4-Rep) or Activator (ERF4-Act) variant. The ERF4-Repressor variant promotes defence against invading *S. gesnerioides* and is dominantly transcribed: the Activator variant is only produced at a minimal level, so does not compromise ERF4-Repressor function. B. Scheme for *erf4-1*, which has a mutation (X) in the *ERF4* gene that prevents transcription of *ERF4-Activator* and significantly reduces *ERF4-Repressor* expression. The full-length mRNA transcript cannot be spliced and is transcribed instead to form ERF4-Intron Retention (ERF4-IR). This accumulates and inhibits ERF4-Repressor function. C. Scheme for Col-0 *p35S:ERF4-Activator* which constitutively expresses *ERF4-Activator*. Accumulation of ERF4-Activator inhibits ERF4-Repressor, preventing an effective defence response against *S. gesnerioides.*

Directions for future study:

- It would be informative to assess the induction of a wider range of genes associated with defences against biotrophic pathogens, during infection with S*. gesnerioides*. This may help identify the downstream targets of SA that affect basal host resistance in this interaction. The corresponding *Arabidopsis* knock-out mutants could then be phenotyped for their susceptibility against *S. gesnerioides*, to confirm that these genes have a relevant role in basal resistance.
- The gene expression and mutant phenotyping analysis indicate that *WRKY70* and *ORA59* may have a role in host basal resistance: this could be investigated further through assessing whether transgenic over-expression lines show altered susceptibility, or if transgenic expression can rescue the increased susceptibility of the knock-out lines.
- To understand whether ROS act principally as a pathogenicity factor or defence response (or both, depending on the spatio-temporal dynamics) it could be informative to phenotype *Arabidopsis* mutants with reduced or enhanced antioxidant capacity. Alternatively, host *Arabidopsis* could be treated with ROS-inhibitors, such as diphenyliodonium (DPI), which inhibits RBOH NADPH oxidases (Siddique et al. 2014).
- The relevance of ROS on downstream defences could also be investigated through assessing gene expression in the *RbohD* and *RbohD/RbohF* mutants during infection *with S. gesnerioides.*
- The model concerning the increased susceptibility of *erf4-1* could be confirmed by phenotyping Col-0 *p35S:ERF4-Repressor* and Col-0 *p35S:ERF4-Intron Retention* for decreased and increased susceptibility respectively. It would also be informative to test whether transgenic expression of *ERF4-Repressor* rescues the *erf4-1* phenotype (*erf4-1 p35S:ERF4-Repressor*).
- It would be interesting to determine whether *S. gesnerioides* suppresses systemic defence signals for other hosts and foliar pathogen systems, for instance cowpea and powdery mildew (*Erysiphe polygoni*).

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