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Plant-mediated interactions between the entomopathogenic fungus *Beauveria bassiana*, insect herbivores and a plant pathogen

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Maya Raad

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Philosophy in Plant-Microbe Interactions

Plant-mediated interactions between the entomopathogenic fungus *Beauveria bassiana*, insect herbivores and a plant pathogen

By

Maya Raad

The entomopathogenic fungus *Beauveria bassiana* can adopt an endophytic lifestyle by colonising a wide array of plant species. Several studies have reported enhanced resistance against insects and plant pathogens from colonised plants. However, little is known about the molecular and physiological mechanisms that govern such interactions. Elucidating the responses of the plant is therefore needed and will help to better understand this recently discovered aspect in the ecology of entomopathogens. The *B. bassiana* strains FRh2 and BG11 were used in this study. Their antagonistic effects, as endophytes, on the necrotic fungus *Sclerotinia sclerotiorum* and the two herbivore species *Myzus persicae* and *Plutella xylostella* were assessed. Treatment of *Arabidopsis thaliana* roots with *B. bassiana* FRh2 and BG11 significantly decreased leaf lesion size caused by *S. sclerotiorum* but did not affect *M. persicae* population growth and *P. xylostella* body mass. Genome expression analysis of *A. thaliana* leaves 15 days post infestation provided evidence for transcriptional reprogramming and induction of plant defence pathways following colonisation by both *B. bassiana* strains. The transcriptional responses aligned with reports on *A. thaliana* interaction with other plant growth promoting fungi such as the known root endophytes *Trichoderma* spp. and *Piriformospora indica*. The results also showed that *B. bassiana* strains FRh2 and BG11 colonisation evoked microbe-associated molecular pattern triggered immunity and the induction of several jasmonic (JA) and salicylic acid (SA) signalling pathway genes. Furthermore, systemic colonisation of plant tissue by FRh2 and BG11 resulted in the induction of genes involved in the biosynthesis of the antimicrobial phytoalexin camalexin and genes encoding multiple reactive oxygen species scavengers such as peroxidases and glutathione transferases. However, JA levels measured in FRh2 and BG11 colonised plants were only induced by *P. xylostella* caterpillar feeding but were not influenced by the presence

of the fungus. Neither caterpillar feeding nor *B. bassiana* presence had an effect on endogenous SA levels. Also, *B. bassiana* colonisation by either strain did not result in major changes in leaf glucosinolate profiles, although a lower content of total aliphatic glucosinolates was recorded in FRh2 colonised *Arabidopsis* only. Based on this, it can be speculated that camalexin-induction by *B. bassiana,* and possibly priming of SA or JA mediated defences, could have resulted in enhanced resistance against *S. sclerotiorum*. However, since *B. bassiana* colonised both roots and shoots, direct effects on the pathogenic fungus cannot be ruled out entirely. Further investigations are required to determine the mechanisms underlying the protective effects of *B. bassiana* against *S. sclerotiorum* infection. This is one of the first studies to assess the metabolic and transcriptomic responses of a plant colonised by the entomopathogen *B. bassiana* and showed induction in the expression of host defence-related genes.

Keywords: *Beauveria bassiana*, *Arabidopsis thaliana*, endophyte, *Sclerotinia sclerotiorum*, induced resistance, plant-microbe interaction, plant defence, biotic stress, salicylic and jasmonic signalling, priming, microarrays, MapMan.

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List of Acronyms

ABA Abscisic Acid

AG Aliphatic Glucosinolate

BP Biological Process

BSM Beauveria Selective Medium

CC Cellular Componment

DEG Differentailly Expressed Gene

EF Entomopathogenic Fungi

ER-QC Endoplasmic Reticulum-Quality Control

ET Ethylene

ETI Effector-Triggered Immunity

GO Gene Ontology

GLS Glucosinolate

GST Glutathione-S-Transferases

IG Indole Glucosinolate

ISR Induced Systemic Resistance

JA Jasmonic Acid

MP Molecular Function

MS Murashige and Skoog

MTI MAMPs-Triggered ImmunityMAMPs- triggered immunity (MTI

effector-triggered immunity (ETI).

OA Oxalic Acid

PAMP-MAMP Pathogen or Microbial-Associated Molecular Pattern

PGPF Plant Growth-Promoting Fungi

PGPR Plant Growth-Promoting Rhizobacteria

PR Pathogenesis-Related

PRRs Pattern Recognition Receptors

SA Salicylic Acid

SAR Systemic Acquired Resistance

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Chapter 1

General introduction

Worldwide there is an increased interest in finding natural and environmentally safe alternatives to the chemical fertilisers and pesticides that have been essential for modern agriculture in the past decades. For this reason many scientists are now exploring the world of endophytic microorganisms, i.e., microorganisms that live inside plants without causing disease, in the hope that they can be the key to solving some of the pest and disease issues of modern agriculture. Boosting resistance, improving stress-drought tolerance and resistance to plant pathogens are all important functions in which endophytes have been found to play a part as they interact at a cellular and biochemical level with their hosts plants.

The study of endophytism began in 1884 when the German scientist Heinrich Anton De Bary put forth the concept of "endophyte"(De Bary, 1884) . The term is used to define microorganisms that occur inside plants without causing symptoms of disease (Wilson, 1995; Roy et al., 2006). However, the discipline received limited attention until the recent recognition of their pharmaceutical and ecological significance (Gunatilaka, 2006). Along with mycorrhizal fungi, epiphytes, saprophytes and pathogens, endophytes are an important component of plant microbiomes with which they interact and overlap in function (Porras-Alfaro & Bayman, 2011).

The topic of entomopathogenic fungi (EF) as endophytes has been a fast moving field of research in recent years. Entomopathogenic fungi are better known for their ability to infect insects, and have been widely evaluated as biological control agents. Many commercial products for microbial control of insects pests are primarily based on pathogenic fungi such as *Beauveria* spp., *Metarhizium* spp., *Isaria fumosorosea* and *Lecanicillium* spp. (Lacey et al., 2015). Recently, it was found that many EF belonging to the Ascomycota can live as an endophyte by colonising plant tissues without causing disease symptoms (Roy et al., 2006). This is a new paradigm which has opened many new avenues of research into the ecology of insect-killing fungi.

1.1 *Beauveria bassiana*

1.1.1 *Beauveria bassiana* as an entomopathogen

The fungus *Beauveria bassiana* is the anamorph stage of *Cordyceps bassiana* (Ascomycota, Clavicipitaceae) and best known as an entomopathogen that attacks a broad range of insects (Sung et al., 2007). It can produce a range of biologically active metabolites (Vey & Hoagland, 2001).

Agostino Bassi di Lodi (1773-1856), an Italian entomologist, first described *Beauveria* as the causal agent of mal del segno also known as calcinaccio or cannellino in Italy and white muscardino in France, which caused economically devastating epizootics of domestic larval silkworms in southern Europe during the 18th and 19th centuries. From further experiments, Bassi demonstrated that microbes can act as contagious pathogens of animals, providing a proof of the germ theory of disease (Ainsworth, 1956). The Italian naturalist Giuseppe Gabriel Balsamo-Crivelli proposed the first taxonomic recognition of the muscardino fungus and acknowledged Bassi's discoveries by naming this pathogen *Botrytis bassiana*. However, the genus *Beauveria* was formally described by Vuillemin (1912), who designated the existing species, *Botrytis bassiana* Bals.-Criv. as the new type species.

Being a ubiquitous entomopathogenic fungus, *B. bassiana* has been isolated from a wide variety of insects from different orders such as Lepidoptera, Coleoptera, Hemiptera and Diptera (Macleod, 1954; Leatherdale, 1970). Like other entomopathogenic fungi, *B. bassiana* attack their host insects by penetrating the cuticle. The infection pathway consists of the following steps: (1) attachment of the spore to the cuticle due to the hydrophobicity of both the conidia and the cuticular surfaces, (2) germination, (3) penetration through the cuticle by enzymatic and mechanical action, (4) overcoming the host response and immune defence reactions, (5) proliferation within the host by formation of hyphal bodies/blastospores, i.e. yeast-like cells, (6) saprophytic outgrowth from the dead host and production of new conidia (Tanada & Kaya, 1993; Hajek & St. Leger, 1994)

The genus *Beauveria* is characterised morphologically by its clusters of short-globose to flask-shaped conidiogenous cells, from which one-celled conidia are produced in sympodial succession on an indeterminate, denticulate rachis. Species identification in *Beauveria* is difficult because of its structural simplicity and the lack of distinctive phenotypic variation. Conidia have been the principal morphological feature used for species identification in *Beauveria,* although recently molecular approaches have been used to refine the genus (Rehner et al., 2011). In shape, conidia may be globose, ellipsoidal, cylindrical and range in size from 1.7 to 5.5 µm (Rehner & Buckley, 2005; Rehner et al., 2011). Currently, 15 species are recognised mainly distinguishable by sequence analysis of conserved regions (Rehner et al., 2011; Zhang et al., 2012; Agrawal et al., 2014; Imoulan et al., 2016).

B. bassiana has long been targeted as a potential biocontrol agent. However, the inability to produce and formulate fungal propagules that are stable and provide consistent mortality rates under constantly changing biotic and abiotic has restrained large-scale development of the insect pathogen (Vega et al., 2009).

1.1.2 *Beauveria bassiana* as an endophyte

Bing and Lewis (1991) were the first to demonstrate tha *B. bassiana* was able to grow endophytically. The authors injected suspensions of *B. bassiana* spores into maize (*Zea mays*) to obtain season-long resistance against caterpillars of *Ostrinia nubilalis* and concluded that this was due to *B. bassiana* establishment as an endophyte in the plant. Following this study, research has been directed towards the endophytic capabilities of *B. bassiana* and the fungus has been reported as an endophyte in cocoa (Posada & Vega, 2005; Posada et al., 2010; Amin et al., 2014), coffee seedlings (Posada & Vega, 2006; Posada et al., 2007), opium poppy (Quesada-Moraga et al., 2006; Quesada-Moraga et al., 2009; Landa et al., 2013), tomato (Ownley et al., 2008; Powell et al., 2009; El-Deeb et al., 2012), banana (Akello et al., 2007; Akello et al., 2008b; Prabhavathi et al., 2013), faba beans (Gurulingappa et al., 2010; Akello & Sikora, 2012; Akutse et al., 2013; Parsa et al., 2013; Akutse et al., 2014; Behie et al., 2015), sorgum (Tefera & Vidal, 2009; Reddy et al., 2009; Mantzoukas et al., 2015), strawberry (Dara et al., 2013), brassica (McKinnon, 2011) and onion (Muvea et al., 2014). Endophytic colonisation of woody species, such as *Carpinus caroliniana* (Bills & Polishook, 1991), date palm (Gomez-Vidal et al., 2006), elm (Doberski & Tribe, 1980), radiata pine (Brownbridge et al., 2012) and western white pine (Ganley & Newcombe, 2006) by *B. bassiana* have also been reported.

A number of studies have looked into the mechanisms of plant colonisation but further investigations are required. Using scanning electron microscopy, penetration of epithelial cells without formation of specialized structures was observed (Griffin, 2007). The fungus is capable of spreading systemically throughout the plant and growth through the xylem vessels was reported in corn and opium poppy (Wagner & Lewis, 2000; Quesada-Moraga et al., 2006). In an attempt to localise *B. bassiana* colonisation *in planta*, Behie et al. (2015) used a green fluorescent protein (GFP) tagged *Beauveria* strain and reported the localisation of *B. bassiana* throughout *Phaseolus vulgaris* beans, whereas another entomopathogenic fungus, a *Metarhizium* sp., was restricted to roots.

Endophytic *B. bassiana* was reported in the literature to negatively affect herbivores and pathogens. Akello et al. (2008a) showed that banana weevil (*Cosmopolites sordidus*) (Germar) populations were decreased by more than 50% when roots of banana plants had been dipped in a *B. bassiana* spore suspension. In addition, Akello and Sikora (2012) showed that *B. bassiana* treated fava beans had significantly lower numbers of the aphid *Acyrthosiphon pisum* when compared to the untreated

controls. The birth rates of all offspring arising from females of *A. pisum* and another aphid, *Aphis fabae* fed on endophyte-treated plants for two generations were significantly lower than those arising from females fed on control plants. Reddy et al. (2009) observed reduced tunneling by larvae of *Chilo partellus* and lower aphid infestations in *B. bassiana* inoculated sorghum plants. Feeding by *Aphis gossypii* on cotton leaves colonised by *B. bassiana* slowed aphid reproduction, and consumption of wheat leaves colonised by *B. bassiana* slowed the growth of the plague locust *Chortoicetes terminifera* nymphs (Gurulingappa et al., 2010). Cherry et al. (2004) reported suppression of the stem-borer *Sesamia calamistis* in maize following seed dressing, topical application and stem injection with African isolates of *B. bassiana*. Ownley et al. (2008) reported a protective role of endophytic *B. bassiana* against the plant pathogenic fungi *Rhizoctonia solani* and *Pythium myriotylum.* Furthermore, systemic protection of *Papaver somniferum* against the poppy gall wasp, *Iraella luteipes*, by endophytic *B. bassiana* was reported by Quesada-Moraga et al. (2009). Biswas et al. (2013) showed that endophytic colonisation of white jute (*Corchorus capsularis*) plants by different *B. bassiana* strains can control stem weevil (*Apion corchori*). *Liriomyza huidobrensis* survival was reduced following exposure to *B. basiana* colonised *Vicia faba* and a reduction in the number of pupae produced by *Liriomyza huidobrensis* was reported by Akutse et al. (2013).

Although the above mentioned studies suggest that endophytic *B. bassiana* negatively affects herbivores and plant pathogens, the mechanisms underlying this effect remain elusive. *B. bassiana* mycosis on the insect herbivore was reported in some studies (Powell et al., 2007; Akello et al., 2008b) but it wasn't clear if this was caused by the endophytic form of the fungus. Moreover, very little is known on how the host plant response to the presence of *B. bassiana*. So far, there is only one study that has addressed this question. Gomez-Vidal et al. (2009) reported the induction of proteins related to plant defence and stress response in *Phoenix dactylifera* leaves following *B. bassiana* colonisation. This suggested that *B. bassiana* presence induced molecular and/or chemical changes in the plant which might explain the deterrent or detrimental effect.

1.2 *Beauveria bassiana*–plant interaction: what to expect?

In 1991, studies started to provide evidence that selected plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF) that often grow endophytically inside the roots can enhance plant health through stimulation of the plant immune system. Van Peer et al. (1991) showed that following the colonisation of carnation roots by *Pseudomonas fluorescens* WCS417r, the above-ground parts of the plant acquired an enhanced level of resistance against infection by the fungal pathogen *Fusarium oxysporum*. In addition, *P. fluorescens* WCS417r–treated plants produced significantly more antimicrobial phytoalexins at the site of infection upon pathogen challenge. In cucumber, Wei et al. (1991) demonstrated that colonisation of roots by different beneficial *Pseudomonas* and *Serratia*

PGPR strains resulted in a significant reduction of disease symptoms after inoculating leaves with *Colletotrichum orbiculare*. Thus, it was concluded that the enhanced level of disease resistance was caused by a plant-mediated immune response called rhizobacteria-induced systemic resistance (ISR).

Following these seminal studies on rhizobacteria (Van Loon et al., 1998; Kloepper et al., 2004), fungus-mediated ISR was reported from non-pathogenic *Trichoderma* (Shoresh & Harman, 2008; Shoresh et al., 2010) and *Piriformospora indica* (Franken, 2012) PGPF strains .

1.2.1 Plant immune system

Plants have developed strategies to perceive their attackers and to translate this perception into effective defence responses. Transmembrane pattern recognition receptors (PRRs) (Yang et al., 1997) have evolved to recognise common microbial compounds such as chitin, flagellin, glycoproteins and lipopolysaccharides. These receptors are called pathogen or microbial-associated molecular patterns (PAMPs-MAMPs). Pattern recognition leads to the activation of so-called basal resistance, innate immune response or MAMPs- triggered immunity (MTI). Many pathogens secret effector molecules that are transported into the host cell to suppress MTI. In turn, plants acquired a secondary immune response called effector-triggered immunity (ETI). ETI is driven in plants by the polymorphic nucleotide-binding site leucine-rich repeat (NBS-LRR) disease resistance proteins (major *R* gene products) that recognise directly or indirectly specific pathogen-derived effectors and is accompanied by programmed cell death. MAMPs- triggered immunity and effector-triggered immunity often induce resistance through long distance signals that propagate in the undamaged parts of plants. The translation of these early induced signalling events into an effective defence response is governed by the action of phytohormones and, depending on the type of attacker, two mechanisms are recognised: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Chisholm et al., 2006; Jones & Dangl, 2006; Pieterse et al., 2009; Newman et al., 2013).

1.2.1.1 Pathogen-induced resistance

Pathogen triggered induced resistance is commonly called systemic acquired resistance (SAR) (Ross, 1961) and is characterised by the accumulation of the hormone salicylic acid (SA) (Vlot et al., 2009) and by the activation of a specific set of pathogenesis-related (PR) genes, many of which are known to produce antimicrobial proteins. *PR1* is among the best characterised of the *PR* genes and is often used as a marker for SAR (Ryals et al., 1996). Despite its accumulation, SA itself is not the translocated SAR signial (Vernooij et al., 1994). A lipid-transfer protein, Defective in Induced Resistance1 (DIR1), is likely to act as a chaperone for the mobile SAR signal(s) and is crucial for SAR initiation in distal organs (Maldonado et al., 2002). Metabolites such as methyl ester of SA (MeSA) glycerol-3-phosphate (G3P)-dependent factor are putatively involved in long-distance SAR signaling

(Dempsey & Klessig, 2012; Kachroo & Robin, 2013; Shah & Zeier, 2013). Flavin-dependent MonoOxygenase 1 (FMO1) is required for the onset of SAR in distal tissues, probably to amplify long-distance signals originating from primary leaves (Mishina & Zeier, 2006). The redox-regulated protein non-expressor of *PR* gene 1 (NPR1) which, upon activation by SA, acts as a transcriptional coactivator of a large set of *PR* genes. SA mediates a change in the cellular redox potential facilitating, therefore, the monomerization of NPR1, after which it translocates into the nucleus. NPR1 then interacts with members of the TGA family of transcription factors that, together with WRKY transcription factors, bind to the promoters of SA responsive defence genes, resulting in their activation (Figure 1-1) (Dong, 2004; Pieterse et al., 2012; Spoel & Dong, 2012; Pajerowska-Mukhtar et al., 2013).

1.2.1.2 Herbivore-induced resistance

Plants have evolved *R* genes (resistance genes) against herbivore effectors such as damage-associated molecular pattern (DAMPs) and elicitors from insect oral secretions. An example is the *Mi* gene that confers resistance against aphid feeding (Rossi et al., 1998). Plant detection of herbivory-related elicitors results in rapid release of oxylipins from membrane lipids. The jasmonate (JA) family of oxylipins emerged as key signals, as JA biosynthesis and signaling mutants are impaired in herbivore-induced resistance (Howe & Jander, 2008; Wasternack & Hause, 2013). Jasmonoyl-isoleucine (JA-Ile) was identified as the biologically active signal in the brassica *Arabidopsis thaliana* (Staswick & Tiryaki, 2004). JA-Ile binds to the F-box protein Coronatine Intensitive 1 (COI1) in the SCF^{COI1} complex, after which the jasmonate ZIM-domain (JAZ) proteins are ubiquitinated and subsequently degraded through the 26S proteasome. This results in the activation of JA-responsive genes through the action of transcription factors such as MYC2, ERF1 and ORA59 (Figure 1-1) (Memelink, 2009).

1.2.1.3 Beneficial microbe-induced resistance

Since the discovery of rhizobacteria-induced systemic resistance (ISR) (Van Peer et al., 1991; Wei et al., 1991) studies have aimed to characterise the molecular mechanism behind this type of resistance.

Studies on *P. fluorescens* WCS417r in radish and *A. thaliana* provided evidence that ISR developed without accumulation of the PR proteins that are characteristic for SAR (Hoffland et al., 1995; Pieterse et al., 1996). Testing transgenic *A. thaliana* NahG plants that are unable to accumulate SA provided genetic evidence that *P. fluorescens* WCS417r-ISR is mediated by an SA-independent signaling pathway and does not coincide with enhanced SA levels (Pieterse et al., 1996). Van Loon and Bakker (2006) concluded that the ability to activate an SA-independent ISR pathway is common

for beneficial microbes and that rhizobacteria-mediated ISR and SA-dependent SAR are regulated by different signaling pathways.

However, several PGPR have been reported to trigger an SA-dependent type of ISR. Examples are *Paenibacillus alvei* K165 (Tjamos et al., 2005) and *P. fluorescens* SS101 (Van de Mortel et al., 2012). Also, a role for SA in the induction of systemic resistance has been established for several *Trichoderma* PGPF (Contreras-Cornejo et al., 2011; Mathys et al., 2012; Martinez-Medina et al., 2013). Evidence showing that the role of NPR1 in ISR seems to be different from that in SAR. In SA signaling, NPR1 is clearly connected to a function in the nucleus (Dong, 2004) whereas evidence is accumuling for a cytosolic function of NPR1 in JA/ET signaling and ISR (Pieterse et al., 2000; Stein et al., 2008; Spoel & Dong, 2012).

Along with SA, JA and ethylen (ET) are central players in the regulation of rhizobacteria-mediated ISR. Mutants in JA signaling and ET signaling pathways were shown to be defective in *P. fluorescens* WCS417r–ISR (Pieterse et al., 1998). Many other PGPR and PGPF, such as *Trichoderma harzianum* T39 and *P. indica,* pointed to a role for JA and/or ET in the regulation of ISR in *A. thaliana* (Ahn et al., 2007; Stein et al., 2008).

1.2.1.4 Priming: characteristic of ISR induced by beneficial microbes

Plants are constantly exposed to stressful situations due to changing environmental conditions or through their contact with numerous pests and pathogenic microorganisms. To be more efficient in countering such situations, plants often switch to a potentiated or primed state of enhanced defence. Primed plants respond to biotic and abiotic stress with faster and stronger activation of defence (Conrath et al., 2015; Balmer et al., 2015).

The first evidence that potentiation of plant defence responses is involved in PGPR-mediated ISR came from experiments with ISR-expressing carnation (*Dianthus caryophyllus*) in which inoculation with *F. oxysporum* f. sp. *dianthi* caused a faster increase in phytoalexin levels than in uninoculated control plants (Van Peer et al., 1991).

Analysis of the *A. thaliana* transcriptome revealed that ISR-inducing WCS417r bacteria elicited a substantial change in the expression of almost 100 genes locally in the roots but not in leaves (Verhagen et al., 2004; Leon-Kloosterziel et al., 2005). Upon challenge with *Pseudomonas syringae* pv. tomato DC3000 the transcriptome of *A. thaliana* leaves during ISR revealed 81 genes with augmented expression indicating that the plants were primed to respond faster and more strongly to pathogen attack (Verhagen et al., 2004).

Upon the oomycete plant root pathogen *Phytophthora parasitica* attack, mycorrhized tomato accumulates significantly more PR1a than non-mycorrhized plants (Cordier et al., 1998; Pozo et al., 2002). Similarly, challenge infection with the leaf pathogen *P. syringae* pv. *lachrymans* of cucumber plants that had been pre-inoculated with the PGPF *Trichoderma asperellum* T203 led to potentiated *PR* gene expression (Shoresh et al., 2005). Recently Mathys et al. (2012) showed that the establishment of ISR during the priming phase in *Trichoderma* T382–*A. thaliana* interaction wasn't dependent on JA-ET pathway. However, the post-challenge primed state upon *Botrytis cinerea* challenge was characterised by an increased response to JA and wounding pathways. Furthermore, the defence related process "response microbial phytotoxin" and the biosynthesis of secondary metabolites e.g. anthocyanins and galactolipids were reported. Barley (*Hordeum vulgare*) plants inoculated with *P. indica* show faster induction of 22 defence-related genes when infected with *Blumeria graminis* compared with control plants (Molitor et al., 2011). These major changes in the expression of defence genes were only visible in plants inoculated with ISR-inducing beneficial microorganisms and upon herbivore or pathogen challenge probably because this would lead to heavy investments in resources and reduced fitness of the host (Heil & Bostock, 2002; Van Hulten et al., 2006).

1.2.2 Overview of key signalling nodes in SA-JA-ET network

Most plant cells appear able to produce diverse chemical messengers or hormones. With their pleiotropic effects, hormones affect all phases of the plant lifecycle from seed to seed, and their responses to both biotic and abiotic stress. Once synthesised, plant hormones move throughout the plant body via the xylem or phloem transport stream or move short distances between cells by regulated transport proteins. Hormone perception is achieved through the binding to receptor proteins in target cells. This perception initiates a course of action leading to the alteration in the expression patterns of many genes whose activities respond to hormonal signalling (Davies, 2010).

Under stress situations, the precise plant response is not activated only by a single hormone but is the result of a network of interactions between different signalling pathways (Figure 1-1). Several examples of cross talk between different hormonal pathways, such as JA, ET, SA, auxin, or abscisic acid (ABA), have been reported (Reymond & Farmer, 1998; Turner et al., 2002; Farmer et al., 2003; Rojo et al., 2003; Lorenzo et al., 2004). This interplay between hormonal signalling pathways provides plants with a powerful regulatory tool to protect them from the complex environment they live in.

1.2.2.1 SA-JA crosstalk

The defence regulatory protein NPR1, the glutaredoxin GRX480 and WRKY transcription factor proteins such as WRKY70 represent some of the most prominent molecular players for SA-JA crosstalk.

The regulatory protein NPR1 plays a central role in SA signal transduction. Mutations in the *NPR1* gene render the plant largely unresponsive to pathogen-induced SA production (Dong, 2004). In wild-type *A. thaliana* cells, SA-mediated changes in the redox status regulating the nucleocytoplasmic localisation of NPR1. Upon localisation to the nucleus, NPR1 interacts with TGA transcription factors, resulting in the activation of SA-responsive pathogenesis related *PR* genes (Dong, 2004). In addition SA-mediated suppression of JA-inducible gene expression is blocked in mutant *npr1* plants, demonstrating a crucial role for NPR1 in the cross talk between SA and JA signalling (Spoel et al., 2007). Spoel et al. (2003) showed nuclear localisation of NPR1 is not required for SA-mediated suppression of the JA response and suppression of the JA response is controlled by a novel function of NPR1 in the cytosol. Interestingly, *NPR1*-silenced wild tobacco (*Nicotiana attenuata*) plants demonstrated that these transgenic plants accumulated increased levels of SA upon insect herbivory and were highly susceptible to herbivore attack (Rayapuram & Baldwin, 2007). It was proposed that in wild-type plants NPR1 is required to negatively regulate SA production during herbivore attack and thus suppress SA/JA cross talk to allow induction of JA-mediated defences against herbivores.

Glutaredoxin GRX480 was identified by Ndamukong et al. (2007) in a two-hybrid screen for interactors with TGA transcription factors. Expression of *GRX480* was found to be inducible by SA and dependent on NPR1 and overexpression of *GRX480* completely abolished MeJA-induced plant defensin 1.2 (*PDF1.2*) expression, known to be sensitive to SA-mediated suppression, but hardly affected the induction of the JA-responsive genes Lipoxygenase 2 (*LOX2*) and Vegetative Storage Protein (*VSP2*). These results suggest a model in which SA-activated NPR1 induces GRX480, which in turn interacts with TGA transcription factors to suppress JA-responsive gene induction.

WRKY70 transcription factor acts as a positive regulator of the SA-mediated defences while repressing the JA response. (Li et al., 2004) showed that overexpression of *WRKY70* caused enhanced expression of SA-responsive *PR* genes and concomitantly suppressed methyl jasmonate (MeJA)-induced expression of the JA-responsive marker gene *PDF1.2*. WRKY62 was added to the list of WRKY transcription factors with a putative role in SA/JA cross talk. Mao et al. (2007) reported that the expression of *WRKY62* was synergistically induced by SA and JA in wild-type *A. thaliana*, but not in mutant *npr1-3*. Furthermore, transposon-tagged *wrky62* plants showed enhanced MeJA induced transcription of the JA-responsive *LOX2* and *VSP2*, whereas overexpression of *WRKY62* resulted in

suppression of these genes. These findings point to a repressive effect of WRKY62 on the JA response.

1.2.2.2JA-ET crosstalk

JA and ET signalling is known to be synergistic. Ethylene and jasmonate signalling pathways need to be triggered concomitantly, and not sequentially, to activate the *A. thaliana* plant defensin gene *PDF1.2* upon pathogen infection (Penninckx et al., 1998).

Two members of the Apetala2/Ethylene Response Factor (AP2/ERF) superfamily of transcription factors ERF1 and Octadecanoid Responsive *Arabidopsis* AP2/ERF 59 (ORA 59) emerged as principal integrators of the JA and ET signalling pathway (Lorenzo et al., 2003; Pre et al., 2008). JA and ET activated synergistically the expression of both *ERF1* and *ORA59*. Overexpression of *ERF1* or *ORA59* in the JA-insensitive mutant *coi1*, or *ERF1* in the ET-insensitive mutant *ein2* constitutively activated the *PDF1.2* gene, which indicates that these transcription factors are important nodes of convergence of JA and ET signalling.

MYC2 (originally called JIN1, for Jasmonate Insensitive 1) is a nuclear-localised basic helix-loop-helix-leucine zipper transcription factor, whose expression is upregulated by JA, in a COI1 dependent manner. MYC2 differentially regulates the expression of two groups of JA-induced genes. MYC2 functions as a positive regulator of JA-responsive genes such as *VSP2* and *LOX2*, whereas it acts as a negative regulator of JA/ET-responsive genes such as *PDF1.2* that are activated by ERFs (Lorenzo et al., 2004). Hence, when the JA response is activated in combination with ET, the ERF branch of the JA response is activated, while the MYC2 branch of the JA response is activated when ET is absent. The interplay between the ERFs and MYC2 may allow the plant to activate the set of JA-responsive genes that is required for optimal defence against the attacker encountered. Nickstadt et al. (2004) showed that *A. thaliana* mutant *jin1/myc2* is resistance against *B. cinerea*. Given that the inhibitory effect of MYC2 on the ERF branch of the JA response is relieved in the *jin1/myc2* mutant, the enhanced resistance against *B. cinerea* may be caused by a potentiated expression of ERF-dependent defences in this mutant. Interestingly, Pozo et al. (2008) showed that MYC2 is also involved in the regulation of genes that show a primed expression pattern after pathogen infection in plants expressing JA/ET-dependent rhizobacteria-mediated ISR.

1.2.2.3 ET-SA crosstalk

ET was shown to enhance the response of *A. thaliana* to SA, resulting in a potentiated expression of the SA-responsive marker gene *PR-1* (Lawton et al., 1994). This synergistic effect of ET on SA-induced *PR-1* expression was blocked in the ET-insensitive mutant *ein2* (De Vos et al., 2006), which indicates

that the modulation of the SA pathway by ET is EIN2 dependent and thus functions through the ET signalling pathway.

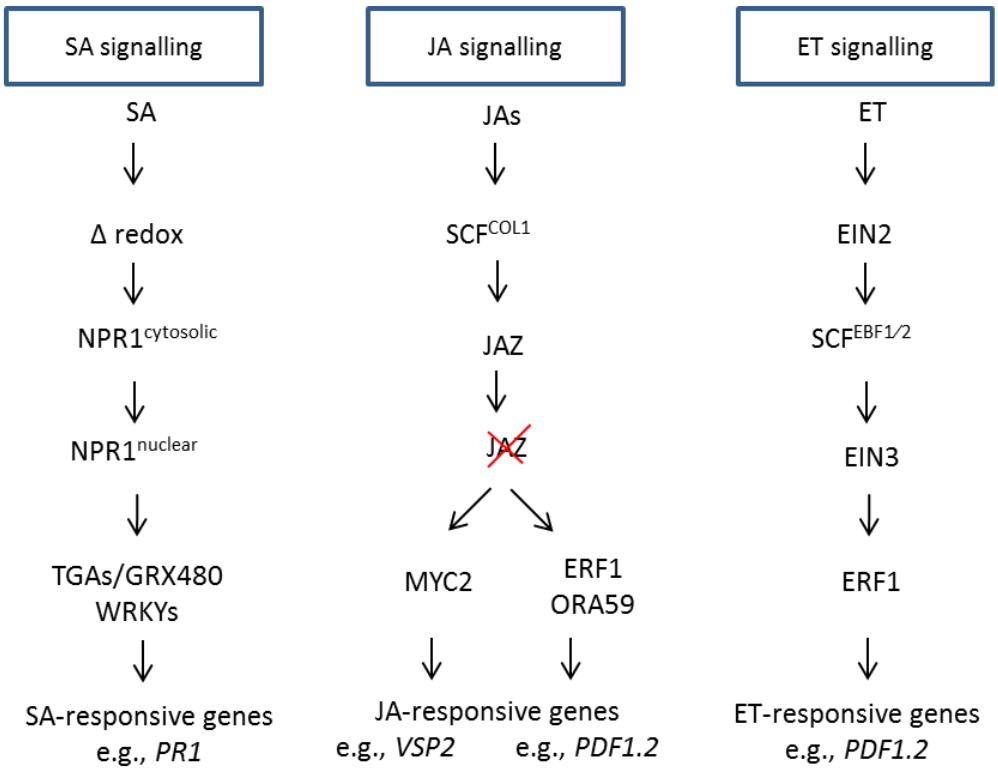


Figure 1-1 Simplified diagram representing the salicylic acid (SA), jasmonic (JA) and ethylene (ET) signalling pathways

Abbreviations are: NPR1, non-expressor of PR genes 1; GRX480, glutaredoxin 480; *PR1*, pathogenesis-related gene; SCF^{COI1}, E3 ubiquitin ligase; JAZ, jasmonate ZIM-domain proteins; ERF1, ethylene response factor 1; ORA59, octadecanoid responsive Arabidopsis 59; *VSP1*, vegetative storage protein 1; *PDF1.2,* plant defensin gene 1.2; EIN2, ethylene-insensitive2; EIN3: ethylene-insensitive 3. Adapted from (Pieterse et al., 2009).

1.3 Aim and outline of this study

Beauveria bassiana has now been demonstrated as a commonly occurring endophyte in plants. Several studies have reported enhanced resistance against insects and plant pathogens from colonised plants but little is known of the plant responses to *B. bassiana* colonisation and how this could play a role in conferring resistance against insects and diseases.

The present study aimed at elucidating the interactions of this entomopathogenic fungus with the model plant *Arabidopsis thaliana* at the molecular, physiological and ecological level. The hypothesis that *B. bassiana* colonisation can enhance plant defence mechanisms, which confers resistance against insects and a plant pathogen, was investigated. *Arabidopsis thaliana* was chosen because it is characterised by a wealth of available genome-scale data resulting from numerous transcriptomic, proteomic and metabolomic studies. This genome-scale information gives a comprehensive systems-level understanding of the model plant (Liberman et al., 2012) and makes it highly suited for the purpose of this work.

In chapter 2 the question was investigated whether *B. bassiana* can endophytically colonise *A. thaliana* and whether this colonisation confers resistance against two herbivore species and a plant pathogen. *B. bassiana* was found to colonise a wide range of plant species but so far there were no reports on the ability of *B. bassiana* to colonise and become established in *A. thaliana* over a sustained period of time. Phenotypic assessment of any indirect antagonism between *B. bassiana* and herbivore/pathogen through the plant would allow the use of this system for a wide transcriptomic analysis for further investigation.

Chapter 3 reports on *A. thaliana* responses to *B. bassiana* colonisation at the molecular level. Transcriptomic analyses of *A. thaliana* colonised by the endophytic *B. bassiana* strains FRh2 and BG11 were performed to assess the ability of *B. bassiana* to induce *A. thaliana* defence. The use of two *B. bassiana* strains was to assess strain-specific effects on the plant transcriptome. In addition, transcriptomic data were validated through quantitative real time polymerase chain reaction (RT-qPCR). Moreover, parallels and projections from different fungus-plant systems studied so far were highlighted for a better interpretation of the generated transcriptomic data.

Experiments reported in chapter 4 addressed the question whether *B. bassiana* colonisation is accompanied by detectable increases in defence related metabolites such as glucosinolates and the phytohormones jasmonic acid and salicylic acid. Furthermore, it was investigated whether subsequent challenge of colonised plant with an herbivorous insect would affect the production of such defence molecules.

To gain a comprehensive picture of the association of *B. bassiana* with *A. thailana*, knowledge gained from this study is discussed in Chapter 5. The molecular and physiologial mechanisms were correlated with the ecological findings involved in this interaction which could explain any enhanced resistance from colonised plants.

Chapter 2

Arabidopsis thaliana colonisation by *Beauveria bassiana* and its effects on herbivorous insects and a plant pathogen

2.1 Introduction

The entomopathogenic fungus *B. bassiana* was reported to endophytically colonise a broad plant host range and by adopting this endophytic lifestyle, *B. bassiana* has been reported to confer resistance against herbivores from four different taxa: aphids (Gurulingappa et al., 2011; Akello & Sikora, 2012; Castillo Lopez et al., 2014), moths (Bing & Lewis, 1993; Cherry et al., 2004; Powell et al., 2009; Mantzoukas et al., 2015), weevils (Akello et al., 2008b; Biswas et al., 2013), gall wasps (Quesada-Moraga et al., 2009) and pathogens such as *R. solani* and *Xanthomonas axonopodis* (Ownley et al., 2008). This motivated the exploration of *B. bassiana'*s capacity to endophytically colonise the non-mycorrhizal model *A. thaliana* (hereafter called *Arabidopsis*) and to assess this association effect on the chewing insect *Plutella xylostella* (Lepidoptera, Plutellidae), the sucking insect *Myzus persicae* (Hemiptera: Aphididae) and on the necrotrophic plant pathogen *Sclerotinia sclerotiorum* (Ascomycetes).

Arabidopis is a member of the mustard (Brassicaceae) family, which includes cultivated species such as cabbage and radish. Characterised by the almost completely sequenced genome (Arabidopsis Genome Initiative, 2000), a large number of characterised mutants and the relatively easy, rapid growth under laboratory conditions, *Arabidopis* has developed as a model plant for plant biology research at the genomic, transcriptomic, proteomic and metabolomic levels (Liberman et al., 2012). Many studies have used *Arabidopsis* to unravel the mechanisms underlying the plant-microbe interaction leading to disease, mutualism or symbiosis (Bakker et al., 2007; Zamioudis & Pieterse, 2012; Bulgarelli et al., 2013). In addition, *Arabidopsis* has provided valuable information on plant-insect interactions including those involving insects in the orders Coleoptera (Nielsen et al., 2001), Diptera (Whiteman & Jander, 2010), Hemiptera (Mewis et al., 2006; Kim & Jander, 2007), Lepidoptera (Stotz et al., 2000; Caputo et al., 2006) and Thysanoptera (De Vos et al., 2005).

The specialist herbivore *Plutella xylostella* is a well-known destructive pest of brassica crops. It has shown significant resistance to almost every synthetic insecticide applied in the field. This resistance has prompted evaluation of alternative pest management strategies mainly biological and cultural control (Sarfraz et al., 2006). The herbivore is attracted to its brassicaceous host plant by olfactory, gustatory and tactile stimuli (Badenes-Perez et al., 2004; Bukovinszky et al., 2005). In general, *P.*

xylostella females do not oviposit on non-hosts and both intact plant glucosinolate (GLS) and volatile isothiocyanates derived from aliphatic GLS stimulate *P. xylostella* oviposition (Renwick et al., 2006). The larvae are reliant on their mothers for host selection and are biochemically adapted to the intake of large amounts of GLS and myrosinase. In their gut, they possess a GLS sulfatase that converts GLS into desulfoglucosinolates which are not substrates for myrosinases and are excreted with the faeces (Ratzka et al., 2002). The generalist *Myzus persicae* feeds on a wide array of plant species and is considered polyphagous. As a generalist, it cues in on a combination of plant primary and secondary metabolites to make its host selection (Powell et al., 2006). *M. persicae* exhibits an anholocyclic life cycle and reproduces parthenogenically (offspring produced without fertilisation) (Blackman, 1974). The destructive ascomycete *Sclerotinia sclerotiorum* is closely related to *B. cinerea* and attacks more than 400 plant species including *Arabidopsis* and important crops such as canola, tomato, lettuce, sunflower, and legumes (Boland & Hall, 1994). Early results showed that the oxalic acid (OA)-deficient mutant, A2 strain, of *S. sclerotiorum* is non-pathogenic on hosts *Phaseolus vulgaris* and tomato (Godoy et al., 1990), suggesting that OA is a necessary pathogenicity factor of this fungus.

Therefore, this chapter assessed first the ability of *B. bassiana* to endophytically colonise *Arabidopsis* and evaluated the effects of this colonisation on the three above-mentioned plant antagonists.

2.2 Materials and Methods

2.2.1 Plant material

Arabidopsis thaliana seeds (wild type ecotype Colombia Col-0) were surface sterilised in 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min and then rinsed five times in sterile double distilled water (ddH2O). Seeds were then plated on Murashige and Skoog salt agar medium (MS) (see A1 Appendix A) at pH 7 in a row 1.5 cm from the edge of the petri dish. Petri dishes were sealed with parafilm to prevent moisture loss and kept in darkness at 4 ^{o}C for 3 days in order to break dormancy and synchronise germination. Sterilised and stratified *Arabidopsis* seeds plated on agar Petri dishes were transferred into a growth chamber at 20-22 ^{o}C, 60-70% RH under a 12L: 12D day/night cycle and placed in a vertical position to allow root growth down along the agar surface and upward shoot growth.

2.2.2 Determining *Beauveria* levels in untreated sterilised plants

Arabidopsis thaliana seeds and seedlings were checked for background presence of *B. bassiana*. Seeds were washed with 0.01% Tween 80 in sterile water and washing was plated on *Beauveria* semi-selective agar medium (BSM) (see A3 Appendix A). Genomic DNA (gDNA) was extracted from three aliquots of *Arabidopsis* seedlings growing on MS agar. Each aliquot contained ten one-week-old seedlings (four leaves growth stage) using the Plant Genomic DNA Extraction Miniprep System Kit (VIOGENE). Presence or absence of *B. bassiana* was confirmed for each aliquot by polymerase chain reaction (PCR) protocol using primers from a segment of *B. bassiana* Co-Acetyl gene designed by Prof Travis Glare (Bio-protection research Centre) (see Table B1 Appendix B) and the FastStart™ Taq DNA Polymerase, dNTPack -Roche. PCR programme and reaction components can be viewed in Tables B2 and B3 Appendix B.

2.2.3 Fungal isolates

Two *B. bassiana* genotypes, strains FRh2 and BG11, were used in this study (see Table B4 Appendix B). *B. bassiana* strain BG11 was recovered from *Bellis perennis* (Asterales: Asteraceae) by Annabel Clouston (Bio-Protection Research Centre, Lincoln University) and *B. bassiana* strain FRh2 was recovered from *Hylastes ater* (Coleoptera: Curculionidae) (Reay et al., 2010).

Isolate identity was confirmed for each culture by PCR using primers from a segment of the elongation factor gene (*EF1-α* gene) designed by Prof Travis Glare (Bio-protection research Centre) (see Table B1 Appendix B). PCR programme and reaction components can be viewed in Tables B2 and B3 Appendix B.

2.2.4 Conidia suspensions

Suspensions were prepared from *B. bassiana* strains FRh2 and BG11 cultured on potato dextrose agar (PDA) (see A2 Appendix A) for three weeks at 20 ^{o}C in darkness. Approximately 10 ml of sterile 0.05% Tween 80 in sterile water was added to each plate per isolate, mixed gently with a sterile cell spreader to dislodge and blend conidia and then poured into a sterile bottle to make a 10 ml suspension (see Appendix C). A concentration of 1 x 10^{8} conidia per ml was used for inoculation. The concentration of conidia per ml was calculated from 100 µl of a 10^{-3} dilution of the initial (10^{-1}) suspension using a Neubauer haemocytometer counting chamber.

2.2.5 Plant root inoculation

One-week-old *Arabidopsis* seedlings (four leaves growth stage) growing in a gnotobiotic environment as described in 2.2.1, were removed from MS medium and washed. Seedling roots were dipped in *B. bassiana* conidia suspension at a final concentration of 1 x 10^{8} conidia per ml as described in 2.2.4. Seedlings were transferred into containers (9 x 9 x 10 cm) filled with gamma irradiated potting mix for growth. Control seedlings were mock-inoculated in the same manner with 0.05% Tween 80 in sterile water. Inoculated plants and control plants were kept in separate propagation boxes (mini propagator Hortlink New Zealand Ltd) to prevent cross contamination. Propagation boxes were incubated for four weeks in a growth chamber at 20-22 ^{o}C, 60-70% RH under a 12L: 12D day/night cycle.

To test for endophytic colonisation, an experiment was carried out in a randomised block design with three treatments: control plants, FRh2 inoculated plants and BG11 inoculated plants. Each treatment consisted of 24 to 26 independent replicates.

2.2.6 Determination of *Beauveria bassiana* endophytic colonisation

Arabidopsis plants were evaluated for *B. bassiana* colonisation using standard isolation techniques on culture medium.

Five-week-old inoculated and mock-inoculated plants were divided into three parts: leaves, rosette and inflorescence. Plant parts were surface sterilised for 1 min in 70% ethanol, 2 min in 4.2% NaOCl and then rinsed 3 x 3 min in 0.01% Triton X-100 in sterile water. The sterilisation method was tested on aliquots of fungal conidia to check the effectiveness of the combination time vs percentage of NaOCl on the viability of conidia. After sterilisation, an imprint of the leaf and inflorescence was made on BSM to check the effectiveness of the sterilisation procedure. Subsequently, the same leaf tissue and inflorescence was cut into segments of 0.5 to 1 cm, plated on BSM, and cultivated for up

to three weeks in 20 ^{o}C dark incubator. Emerging mycelia were isolated and identified on the basis of colony and conidia morphology. In addition, a sample of the recovered fungus was cultured on BSM plate for subsequent DNA extraction using the Plant Genomic DNA Extraction Miniprep System Kit (VIOGENE). Following DNA extraction confirmation of *B. bassiana* was achieved by conducting a PCR reaction using the elongation factor gene (*EF1-α* gene) primers as described in 2.2.3. The PCR product was sequenced and blasted against *B. bassiana* genome using BLAST (NCBI) http://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.2.7 Insects

Two herbivore species were used to test the effect of endophytic *B. bassiana* on insect performance and behaviour: *P. xylostella*, a specialist leaf-chewer of Brassicaeae commonly known as diamondback moth (DBM), and *M. persicae*, a generalist phloem feeder commonly known as green peach aphid (GPA).

Larvae of *P. xylostella* were reared on *Brassica oleracea* plants in constant temperature (CT) rooms with 16L: 8D day/night cycle at a temperature of 22 ^{o}C.

M. persicae were reared on *B. oleracea* plant in CT rooms with 16L: 8D day/night cycle at a temperature of 22 ^{o}C.

2.2.8 Plant pathogen

The plant necrotic fungus *S. sclerotiorum* was used to test the effect of *B. bassiana* colonisation on resistance against a plant pathogen.

Sclerotinia sclerotiorum strain SsOSR was supplied by Michael Kuchar (Bio-Protection Research Centre-Lincoln University). The fungal pathogen was cultured on PDA for 3 weeks at 20 ^{o}C under a 12L: 12D day/night cycle.

2.2.9 Feeding bioassay with *Plutella xylostella*

One-week-old *Arabidopsis* seedlings were inoculated with *B. bassiana* strain FRh2 and BG11 as described in 2.2.5.

A single third instar caterpillar was transferred to each of five-week-old inoculated or mock-inoculated *Arabidopsis* plants using a fine camel hair brush. Caterpillar body mass was measured before the experiment and after a feeding period of 48, 72 and 96 hours. The experiment consisted of two treatments: FRh2 inoculated plant, BG11 inoculated plants and their corresponding controls

organised in a completely randomised design with 24-26 plants per treatment maintained at 20-22 ^{o}C, 60-70% RH under a 12L: 12D day/night cycle. Following the feeding bioassay each plant was checked for *B. bassiana* colonisation using the standard isolation technique as described in 2.2.6.

2.2.10 Population growth of *Myzus persicae*

One-week-old *Arabidopsis* seedlings were inoculated with *B. bassiana* strain FRh2 and BG11 as described in 2.2.5.

Five-week-old inoculated and mock-inoculated *Arabidopsis* plants were infested with 5 nymphs of *M. persicae*. Nymphs or immature stages of *M. persicae*, characterised by their wide cauda, were inspected under a microscope and transferred carefully onto a young leaf of an *Arabidopsis* plant using a fine brush. Nymphs were caged onto leaves by using clip cages of 44 mm diameter. The cages were removed 24 hours post infestation and only one single nymph was left on an *Arabidopsis* leaf. After 5 days the number of next generation nymphs was recorded daily till the 10th day post infestation. The experiment consisted of three treatments: control plants, FRh2 inoculated plant and BG11 inoculated plants organised in a completely randomised design with 12 to 14 plants per treatment maintained at 20-22 ^{o}C, 60-70% RH under a 12L: 12D day/night cycle. Following the population growth bioassay each plant was checked for *B. bassiana* colonisation using the standard isolation technique as described in 2.2.6.

2.2.11 *Sclerotinia* assay

One-week-old *Arabidopsis* seedlings were inoculated with *B. bassiana* strain FRh2 and BG11 as described in 2.2.5.

Five-week-old *B. bassiana* inoculated and mock-inoculated *Arabidopsis* were infected by *S. sclerotiorum* using an agar plug method. With a cork borer, a 3 mm agar disk with fungal hyphae was taken 1 cm from the edge of the petri dish. The agar disk was placed on a single leaf surface. All selected leaves for infection were standardised for size. During the infection, and to maintain a high humidity level, the disk with the leaf was wrapped together with a transparent cover for three days after which the cover was discarded. Plants were incubated in propagation boxes (mini propagator Hortlink New Zealand Ltd) in a growth chamber at 20-22 ^{o}C, 60-70% RH under a 12L: 12D day/night cycle.

The lesion sizes were measured five days post infection according to the method of Rostas et al. (2006). The lesions were scanned and the area of each lesion was calculated using the software Surface (freeware developed by Carsten Thiemann - Free University of Berlin - Germany).

2.2.12 Statistical analyses

Data were analysed using *t*-tests for independent samples and repeated measures (ANOVA) for feeding bioassay with *P. xylostella* and population growth of *M. persicae* respectively. Count data for reproduction bioassay with *M. persicae* were squareroot transformed and homogeneity of variance was tested using Cochran's test. *S. sclerotiorum* assay data was analysed using one-way analysis of variance (ANOVA) followed by Tukey HSD and Fisher's least significant difference (LSD) post hoc tests. *S. sclerotiorum* data was log transformed to meet assumption of normality and homogeneity of variance. Statistical analyses were performed using IBM ® SPSS statistics 22 and Statistica 13 software. Graphs were generated using SigmaPlot 13.0.

2.3 Results

2.3.1 *Arabidopsis thaliana* colonisation by *Beauveria bassiana*

Beauveria bassiana was found to endophytically colonise the non-mycorrhizal model plant *A. thaliana*. The percentage of colonisation varied between strains. The lowest rate of colonisation recorded was 61% and 40% of plants inoculated for each of FRh2 and BG11-treated respectively (Figure 2-1). The use of both molecular detection and the standard isolation techniques on culture medium failed to detect any background of *B. bassiana* in *Arabidopsis* seeds and seedlings.

Following the standard isolation techniques on culture medium, *B. bassiana* was recovered from rosettes/leaves and inflorescences suggesting systemic colonisation of the endophyte when roots were dipped in conidia suspension. This colonisation persisted until the flowering stage of the *Arabidopsis* life cycle. None of the control plants were colonised by the fungus. All control plant tissue imprints and cuts had no *Beauveria* present. Recovered *B. bassiana* was identified on the basis of colony and conidial morphology. In addition *B. bassiana* identity was confirmed by the PCR and sequencing of a fragment of the elongation factor gene as described in 2.2.3.

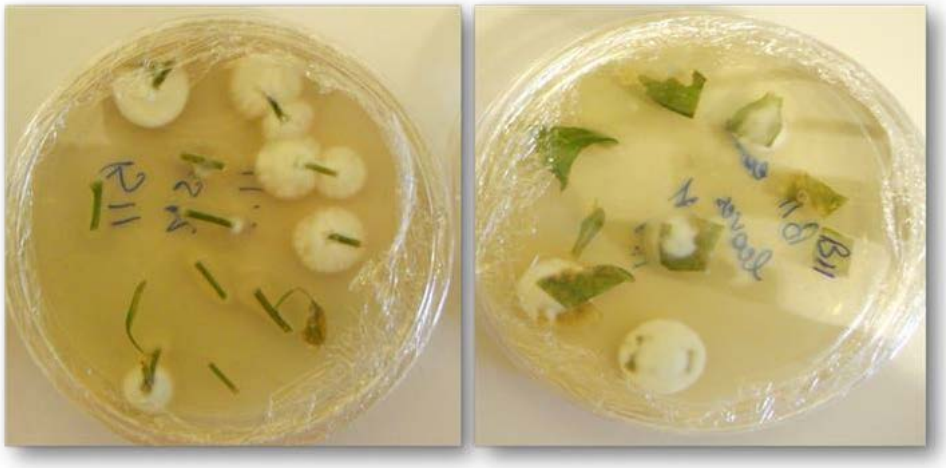


Figure 2-1 *Beauveria bassiana* recovered from a surface sterilised five-week old *Arabidopsis thaliana* inflorescence and leaves.

2.3.2 Effects of *Beauveria bassiana* colonisation on herbivorous insects and a plant pathogen

2.3.2.1 Feeding bioassay with *Plutella xylostella*

No significant differences ($P > 0.05$) in *P. xylostella* caterpillar body mass were observed after 48, 72 and 96 hours of feeding on *B. bassiana* inoculated plants when compared to caterpillars that fed on control plants (*t*-test for FRh2 at 0 hours; $t = 0.39$, $P = 0.969$, $n = 24\text{-}26$; 48 hours; $t = 0.502$, $P = 0.618$, $n = 22\text{-}24$; 72 hours; $t = -1.228$, $P = 0.227$, $n = 20\text{-}19$; 96 hours; $t = -1.959$, $P = 0.059$, $n = 17\text{-}18$; *t*-test for BG11 at 0 hours; $t = -0.607$, $P = 0.547$, $n = 23\text{-}24$; 48 hours; $t = 0.459$, $P = 0.649$, $n = 20\text{-}22$; 72 hours; $t = 0.377$, $P = 0.709$, $n = 20$; and 96 hours; $t = -0.345$, $P = 0.732$, $n = 15\text{-}17$, Figure 2-2). *B. bassiana* had no detrimental effect on *P. xylostella* feeding. A non-significant trend showed more weight gain in larvae feeding on *B. bassiana* colonised plants.

In a preliminary experiment another *B. bassiana* strain, E17-p, did not lose its pathogenicity during plant colonisation since infection of *P. xylostella* with re-isolated *B. bassiana* caused similar mortality to before plant inoculation. Pathogenicity of recovered FRh2 and BG11 is therefore expected but was not tested.

Caterpillar body mass after feeding on each treatment is shown in Table D1 Appendix D and the statistical analyses results in E1 Appendix E.

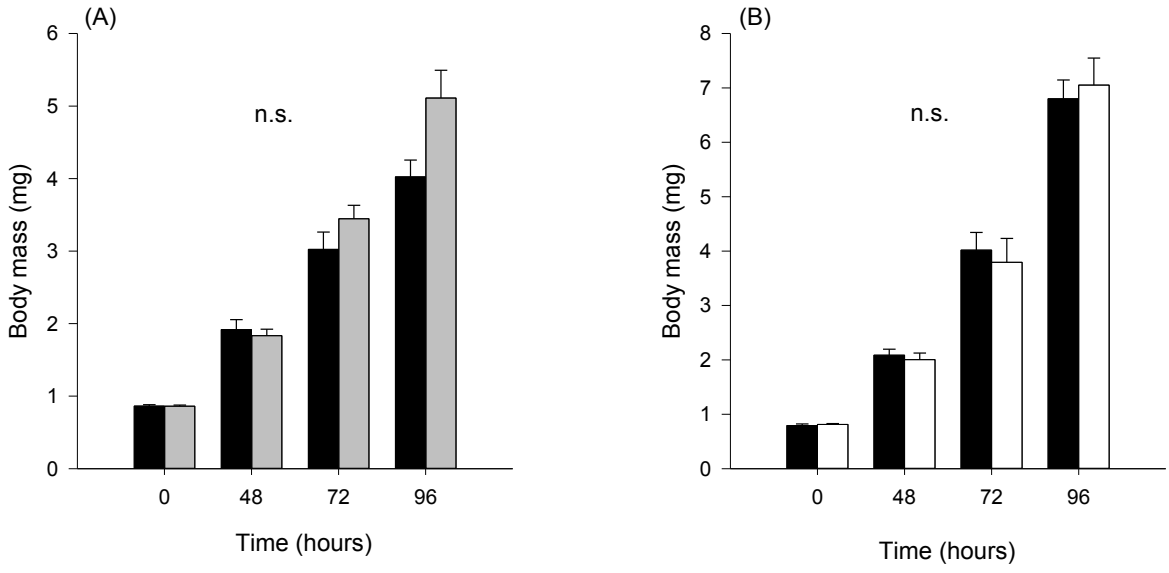


Figure 2-2 *Plutella xylostella* body mass (means ±SE) after 48, 72 and 96 hours feeding on *Beauveria bassiana* colonised *Arabidopsis thaliana*.
Black bars represent control plants, grey bars represent FRh2 inoculated plants (A) and white bars represent BG11 inoculated plants (B). Error bars represent the standard error of the mean (N = 24-26). No significant differences between treatments were found (*t*-test $P \leq 0.05$), n.s. = not significant.

2.3.2.2 Population growth of *Myzus persicae*

B. bassiana colonisation did not influence aphid population growth. After 10 days of infestation no significant differences in aphid populations were observed between treated and control plants (repeated measures, ANOVA, d.f. = 2, $F = 0.37$, $P = 0.692$, Figure 2-3).

Aphid number rearing on each treatment is shown in Table D2 Appendix D and the statistical analyses results in E2 Appendix E.

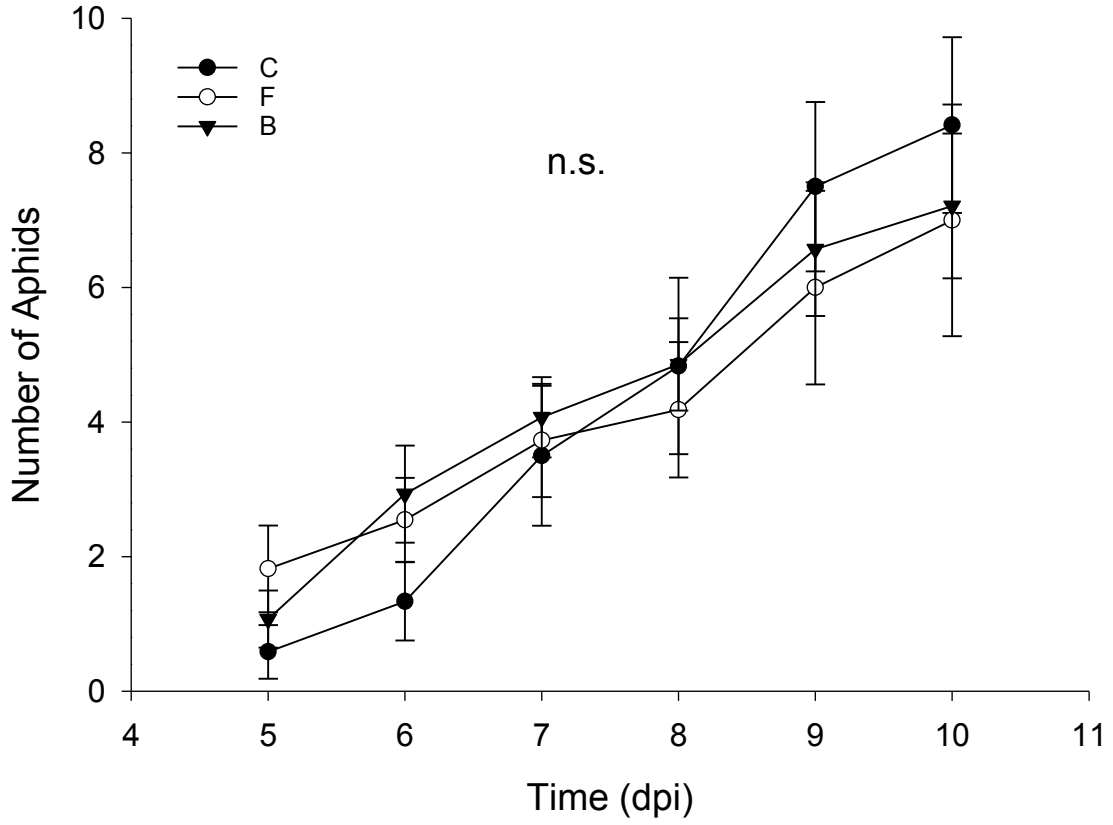


Figure 2-3 Effect of endophytic *Beauveria bassiana* colonisation on *Myzus persicae* populations. Symbols represent mean number of *Myzus persicae* on control plants (C), FRh2 colonised plants (F) and BG11 colonised plants (B). Error bars represent the standard error of the mean (N = 12-14). No significant differences between treatments were found (repeated measures, ANOVA, $P = 0.692$), n.s. = not significant.

2.3.2.3Effect of *Beauveria bassiana* colonisation on the necrotrophic fungus *Sclerotinia sclerotiorum*

B. bassiana colonisation reduced the symptoms of *S. sclerotiorum* infection. By comparing the average of lesion area, *Arabidopsis* treatment with *B. bassiana* resulted in a significant reduction in disease symptoms (one-way ANOVA, d.f. = 2, F = 22.062, P < 0.001, Figure 2-4). The effect was significant for both fungi compared to the controls.

Lesion area of each treatment can be viewed in Table D3 Appendix D and the statistical analyses results in E3 Appendix E.

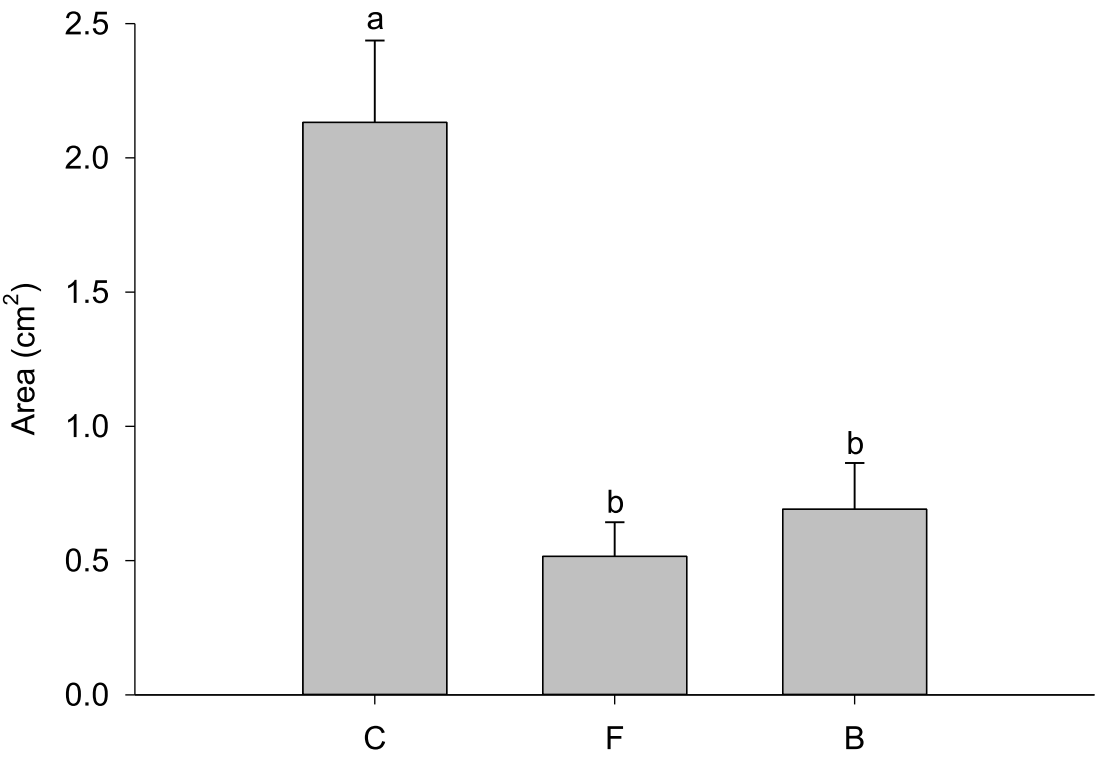


Figure 2-4 Area of leaf infected with *Sclerotinia sclerotiorum* measured in *Beauveria bassiana* colonised (F = FRh2 and B = BG11) and control (C) *Arabidopsis thaliana* plants 5 days post infection. Disease intensity was calculated as average lesion area. Error bars represent the standard error of the mean (N = 19-22). Different letters above bars indicate significant differences (one-way ANOVA, $P \leq 0.05$ followed by Tukey HSD and Fisher's least significant difference)

2.4 Discussion

This study reports, for the first time, that the entomopathogen *B. bassiana* is able to endophytically colonise the non-mycorrhizal model plant *Arabidopsis* following root inoculation treatment. The colonisation of different parts indicated that the fungus can grow systemically throughout the plant and can persist until the flowering stage of the *Arabidopsis* life cycle. This is in agreement with many studies in which *B. bassiana* was reported as an endophyte (Chapter 1). Root dipping in a conidia suspension was found to be a suitable inoculation method. Many inoculation methods, including seed coating, soil drenches, stem injection and foliar sprays, have been used to introduce *B. bassiana* to different crops (Gomez-Vidal et al., 2006; Posada et al., 2007; Akello & Sikora, 2012; Parsa et al., 2013; Akutse et al., 2014). Tefera and Vidal (2009) showed that *B. bassiana* endophytically colonised sorghum leaves, stems, and roots regardless of the inoculation method (leaf, seed, or soil inoculation). However, plant growth medium (sterile soil, non-sterile soil, or vermiculite) apparently influenced colonisation rates. In the Tefera and Vidal (2009) study, seed inoculation with conidia caused no stem or leaf colonisation by the fungus in non-sterile soil but did result in substantial endophytic colonisation in vermiculite and sterile soil. Hence, the use of gamma irradiated soil in this study.

Colonisation success varied between *B. bassiana* strains FRh2 and BG11. The highest percentage of colonisation was recorded for the insect-derived strain FRh2. In addition, *Beauveria* outgrowth from plant sections was random, which could indicate uneven colonisation of the plant. The isolation technique of plating on selective media is known to be an error prone method as it relies on the efficacy of the sterilisation, size of the plant pieces and the time given to allow the outgrowth of fungal hyphae from plant parts. PCR based detection methods are an alternative and have been reported in many studies. However, detection of fungi in mixtures of plant and fungal DNA is problematic when using primers like ITS1 and ITS4 that amplify the internal transcribed spacer (ITS) region of the nuclear rDNA repeat because they can also amplify plant ITS sequences. Landa et al. (2013) developed species-specific primers by combining the ITS region of the nuclear rDNA of *B. bassiana* with the universal ITS1/ITS4 primer set in a two-step nested PCR protocol. This newly developed protocol would facilitate the detection of endophytic *B. bassiana* in *planta* but was not used in the current study as colonisation was able to be confirmed through direct plating.

Although the accumulated reports show that entomopathogenic fungi, especially species like *B. bassiana*, *Metarhizium anisopliae* and *Lecanicillium lecanii* (Vidal & Jaber, 2015), can colonise plants endophytically, there is a need for an efficient, reliable and rapid detection method. The use of microscopic verification of endophytic colonisation by using florescence tagged isolates has become

more common to detect endophytic colonisation in different tissues and plants hosts (Behie et al., 2015).

The antagonistic activity of endophytic *B. bassiana* against pest insects and a plant pathogen of Brassicaceae were tested by studying the performance of *P. xylostella*, *M. persicae* and the necrotic fungus *S. sclerotiorum. B. bassiana* had no effect on *P. xylostella* performance and did not influence aphid population growth. However, *B. bassiana* colonisation reduced the symptom severity of *S. sclerotiorum* infection.

Several studies indicated that endophytic *B. bassiana* has a negative effect on insects (Chapter 1). However, neutral and positive effects have been reported, too. Gurulingappa et al. (2010) showed that *Chortoicetes terminifera* locusts were not deterred by *B. bassiana* in wheat while Lopez et al. (2014) reported a positive effect on *Aphis gossypii* reproduction after seven days of exposure to endophytic *B. bassiana.* However, after 14 days of exposure *B. bassiana* presence reduced insect reproduction. Akello et al. (2008a) showed that five days after plant infestation with the banana weevil *Cosmopolites sordidus* there was no effect of *B. bassiana* on oviposition rate and egg hatchability. However, the presence of *B. bassiana* as an endophyte in banana tissues greatly reduced banana weevil populations and their damage to plants after 15 weeks. These studies suggest that while no effect was detected in a short term laboratory assay, effects may be seen over a longer time period with crop plants.

In addition, evidence has been presented indicating endophytic *B. bassiana* has potential as a microbial control organism against plant pathogens such as *R. solani,* and *P. myriotylum* (Clark et al., 2006; Ownley et al., 2008). Reduction of *S. sclerotiorum* infection symptom following *B. bassiana* colonisation adds to this evidence.

Although the presence of entomopathogens as endophytes can negatively affect insects and pathogens, determining the mechanisms underlying this negative effect require further investigation. Some studies attributed this effect to the toxicity of metabolites produced by endophytic *B. bassiana* (Cherry et al., 2004; Vega, 2008). *B. bassiana* is known to produce toxic metabolites such as bassacridin, oosporein, beauvericin and bassianolide (Strasser et al., 2000; Quesada-Moraga & Vey, 2004; Xu et al., 2009; Wang & Xu, 2012). Thus it remains to detect these metabolites within plants and at concentrations that can influence insects or pathogens *in vivo*. Interestingly the phytotoxic potential of *Beauveria brongniartii* and its main metabolite oosporein were evaluated against seed potatoes (*Solanum tuberosum*). The weight of haulm and tubers was unaffected by *B. brongniartii* and no oosporein was detected in the potatoes (Abendstein et al., 2000; Strasser et al., 2000; Seger et al., 2005). Moretti et al. (2002) showed that beauvericin from *Fusarium* species did not cause any

symptoms on roots of melon, tomato, wheat and barley; however, it showed high toxicity towards the protoplasts of these plants.

It has been suggested that entomopathogenic endophytes can trigger an induced systemic resistance in plants that might contribute to resistance against insects and pathogens (Ownley et al., 2008; 2010). However, little is known of the plant response to *B. bassiana* colonisation and whether any plant responses plays a role in plant defence against insects and disease. Thus, *Arabidopsis* response to *B. bassiana* colonisation at the molecular level is discussed in the following chapter.

Chapter 3

Transcriptomic analysis of *Arabidopsis thaliana* colonised by the entomopathogen *Beauveria bassiana*

3.1 Introduction

Large-scale gene expression microarray analyses have been used to elucidate the molecular mechanisms underlying plant-endophyte associations. These analyses concerned plant interactions with the fungi *Trichoderma* spp. (Moran-Diez et al., 2012; Mathys et al., 2012) and *Piriformospora indica* (Molitor et al., 2011).

DNA microarray is a commonly used technique for expression profiling, i.e., monitoring expression levels of thousands of genes simultaneously in different cell types, under different experimental conditions and at different developmental stages or disease states The DNA array (also called chip) is an arrayed series of microscopic spots of single stranded DNA fragments (probes) immobilised on a solid substrate such as glass or silicon and used to probe a labelled solution of nucleic acids. Microarray is in principle an extension of the colony hybridisation method of Li et al. (2013). The hybridisation of this labelled solution of nucleic acids to the probes on the array is used to measure the relative concentration of the nucleic acids in solution. Array hybridisation produces a large amount of complex data. Transforming this data into knowledge to obtain biological insights requires the use of multiple bioinformatic and computational tools and techniques. In the last decades microarray technology progressed rapidly with new methods of production, labelling and data analyses. In addition, the increased knowledge of DNA sequences of multiple genomes provided the necessary information to assure that arrays are fully representing the genes in a genome and all the sequence in a genome (Bumgarner, 2013; Grewal & Das, 2013). Therefore, microarrays have become the primary tool for expression analysis.

Thus, with an array analysis of *Arabidopsis* rosettes colonised by *B. bassiana* 15 days post root inoculation, this study provides a first comprehensive analysis of the molecular mechanisms that govern *B. bassiana* interaction with *Arabidopsis* and attempts to explain *B. bassiana* effects on the plant defence system. In addition, this study assessed strain-specific effects on the plant transcriptome by using two different strains of *B. bassiana*: FRh2 an insect-derived strain and BG11 a plant-derived strain.

To validate the array data results, a quantitative real time polymerase chain reaction (RT-qPCR) was performed on *B. bassiana* strain FRh2 colonised *Arabidopsis* rosettes to assess the expression pattern of specific genes related to different defence responses and identified as differentially expressed in the transcriptomic data.

3.2 Materials and Methods

3.2.1 Plant inoculation

Arabidopsis thaliana seeds (ecotype Colombia Col-0) were surface sterilised and stratified as described in 2.2.1. Seeds were sown in 500 ml containers (plastic containers (PC) natural polypropylene (PP) screw cap, Labserv, Thermo Fischer Scientific) filled with MS agar at pH 7. Containers were incubated in a growth chamber at 20-22 °C, 60-70% RH under a 12L: 12D day/night cycle.

Five-week-old *Arabidopsis* plants were removed from MS medium and washed. Roots were dipped in *B. bassiana* conidia suspension at a final concentration of 1×10^8 conidia per ml as described in 2.2.4. Control plants were mock-inoculated with 0.05% Tween 80 in sterile water. Plants were transferred into gamma irradiated 250 ml containers (polypropylene, Labserv, Thermo Fischer Scientific) filled with twice autoclaved vermiculite.

The first experiment consisted of two treatments: *B. bassiana* strain FRh2-inoculated *Arabidopsis* (AtFRh2) and mock-inoculated control plants (AtCO) organised in a completely randomised design with four independent biological replicates per treatment. Inoculated and control plants were kept in separate autoclaved 4.2 L cereal containers (Sistema Klip It Cereal Storer) and were incubated for another 15 days in a growth chamber at 20-22 °C, 60-70% RH under a 12L: 12D day/night cycle.

The second experiment assessed the transcriptome of plants inoculated with *B. bassiana* strain BG11 (AtBG11) and of mock-inoculated control plants (AtCO). The above mentioned experimental design was used.

3.2.2 Determination of *Beauveria bassiana* colonisation

Inoculated and control plants were divided into two parts: rosette and inflorescences. The rosettes were immediately frozen and pulverised in liquid N_2 for gDNA extraction and subsequently for total RNA extraction. The inflorescences were surface sterilised and plated on the semi selective BSM as described in 2.2.6. *B. bassiana* presence in the *Arabidopsis* rosette was further verified by PCR using SCARS primers developed by Castrillo et al. (2003) for *Beauveria* detection. Among the three developed SCARS primers, $SCA15_{441}$ primer was the only primer set that amplified only *B. bassiana,*

generating a band of 400 bp without also generating any amplicon for *Arabidopsis*. PCR programme and reaction components using the FastStart™ Taq DNA Polymerase, dNTPack-Roche can be viewed in Tables B5 and B6 Appendix B.

3.2.3 RNA isolation

Total RNA was extracted from inoculated and control *Arabidopsis* rosettes of four independent biological replicates per treatment after 15 days of inoculation using the RNeasy Plant Mini Kit (QIAGEN). One-column DNase digestion treatment using the RNase-Free DNase Set (QIAGEN) was incorporated in the extraction protocol to eliminate any DNA contamination in downstream experiments. RNA quality check was performed by electrophoresis and by electrophoretic analysis via the 2100 Bioanalyzer (Agilent Technologies) and photometrical measurement with the Nanodrop 2000 spectrophotometer (Thermo Scientific) for determination of the RNA integrity number (RIN) and detection of potential contamination.

3.2.4 cRNA synthesis, microarray hybridisation

The cRNA synthesis and microarray hybridisation were performed by OakLabs, Hennigsdorf, Germany. The Low Input QuickAmp Labeling Kit (Agilent Technologies) was used for generating fluorescent cRNA (complementary RNA). By default cRNA is amplified by using oligo-dT primer for eukaryotic samples and a random primer for prokaryotic samples and labelled with cyanine 3-CTP following the manufacturer's protocol.

For the hybridisation the Agilent Gene Expression Hybridisation Kit (Agilent Technologies) was used following the manufacturer's protocol. Six hundred ng of cRNA was hybridised on an 8 x 60K microarray using the manufacturer's recommendations at 65 ^{o}C for 17 hours. Finally the microarray was washed once with the Agilent Gene Expression Wash Buffer 1 for one minute at ambient temperature followed by a second wash with preheated (37 ^{o}C) Gene Expression Wash Buffer 2 for one minute. Fluorescent signals on the microarray were detected by the SureScan Microarray Scanner (Agilent Technologies) at a resolution of 3 micron for SurePrint G3 Gene Expression Microarrays and 5 micron for HD Microarray formats, generating a 20 bit TIFF file respectively.

Agilent's Feature Extraction software version 11 was used to read and process the TIFF files. The software accurately determines feature intensities, flags outlier features, and calculates statistical confidences.

The 8 x 60K microarray used was exclusively available at OakLabs with 32072 target IDs representing 30541 gene loci where annotation is based on *Arabidopsis thaliana* Genome, TAIR10 (https://www.arabidopsis.org).

3.2.5 Data Normalisation

Microarray data were normalised prior to statistical analyses using the ranked median quantiles according to Bolstad et al. (2003). Quantile normalisation of the signals was performed by OakLabs, Hennigsdorf, Germany using DirectArray software.

Briefly, the mean signal of each target is ranked relative to all other targets. The ranked signal value is replaced with the median quantile value of the same rank. So the highest value in all samples becomes the mean of the highest values, the second highest value becomes the mean of the second highest values, and so on.

Boxplots of data distribution were used before and after normalisation to evaluate if normalisation was effective and to identify potential problematic samples. Box plots before and after normalisation can be viewed in Figure F1 Appendix F.

3.2.6 Data analysis

To assess the variability in the expression patterns of plant replicates, hierarchical clustering analysis (HCA) and principal component analysis (PCA) were performed by OakLabs on microarray data using R package version 3.2.0 (April, 2015), a free software environment for statistical computing and graphics.

Hierarchical clustering analysis groups samples with similar expression profiles. Thus HCA aligns the two most-related replicates to each other in order to produce the first cluster. It then aligns the next most related replicate to this cluster or the next two most-related replicates to each other in order to produce another cluster. The objective of PCA is to reduce the dimensionality of the data set and to identify the two highest variabilities of the data. The data is visualised in a two dimensional coordinate system, where both axes represent the two highest variabilities of the data. The percentage of variance the 1st and the 2nd principal components account for is denoted on the axes. Similar to a HCA, a PCA plot shows whether or not the distance of samples within one group is bigger than the distance between samples of different groups (Guide to Agilent Gene Expression Data-OakLabs, Hennigsdorf, Germany).

Expression profiles of all the significantly differentially expressed genes and the top 50 and 100 DEGs were displayed as heat maps where rows represent genes and columns represent samples illustrating

the normalised gene expression level of each gene in each sample. Z-scores were used for the colour representation. The z-score is the difference of a gene's normalised signal of one sample and the gene's mean signal of all samples divided by the standard deviation. The absolute value of z represents the distance between a sample's gene signal and the gene's mean signal of all samples in units of the standard deviation. Z is negative (blue) if the sample's gene signal is below the mean, positive (red) if above (Guide to Agilent Gene Expression Data- OakLabs, Hennigsdorf, Germany).

In a first step to identify differentially expressed genes (DEGs), log transformation was applied to all four replicates in each treatment. Two-sample *t*-test with unequal variance, also known as Welch's *t*-test, was applied to assess significant differences with a p-value of 5% using DirectArray software (OakLabs, Hennigsdorf, Germany). Furthermore, all log2-fold change values between -1 and 1 were rejected and only targets with p-values ≤ 0.05 and a log2-fold change < -1 or > 1 were considered. Data were further subjected to a false discovery rate correction with a threshold of 5% (Schweizer et al., 2013; Appel et al., 2014; Staats et al., 2014; Hill et al., 2015). For practical reasons the codes AtFRh2 and AtBG11 will be used to indicate the DEGs resulting from the comparative transcriptome study to present the comparison of control (AtCO) vs AtFRh2 and control (AtCO) vs AtBG11 respectively.

3.2.7 Gene ontology, enrichment analysis and MapMan analysis

Transcriptome data for AtFRh2 and AtBG11 were divided into upregulated and downregulated DEGs. Gene ontology (GO) and enrichment analysis for each group of up and downregulated DEGs were performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID: https://david.ncifcrf.gov/home.jsp) v6.7 and the *Arabidopsis* Information Resource (*Arabidopsis thaliana* Genome, TAIR, https://www.arabidopsis.org/) database as identifier for the target genes.

Gene ontology (GO) is a bioinformatics tool allowing a consistent description of a gene role and product in any organism (Smith et al., 2003). GO is divided into three domains: the cellular component, the molecular function, and the biological process. Cellular component (CC) refers to the component of the cell where the gene product is active. Molecular function (MF) describes the biochemical activity of this product at the molecular level such as catalytic or binding activities. Biological process (BP) describes the biological aim of the gene product which is accomplished by a series of molecular events or steps (Ashburner et al., 2000). Thus the main focus of the GO analysis was on the domain biological process.

Gene expression data were visualised in the context of metabolic pathways using MapMan 3.1.0 software (http://mapman.gabipd.org). MapMan is a tool that displays large datasets such as gene expression experiments data onto diagrams of metabolic pathways. The SCAVENGER module in the

software contributes each of the measured parameters to a pathway or bin. There are a total of 36 major bins. The IMAGENNATATOR module converts these groupings onto metabolic diagrams (Thimm et al., 2004).

In addition, a comparison between AtFRh2 and AtBG11 was performed to identify shared DEGs and their expression patterns.

3.2.8 Microarray validation and effects of *Beauveria bassiana* on *Arabidopsis thaliana* defence signalling pathways

The expression of specific defence-related genes (*AXR5, ASC4, MYB122, ARR11, GLIP1, WRKY63,* chitinase – Table G1 Appendix G) was monitored in FRh2-inoculated and mock-inoculated *Arabidopsis* rosettes of three additional independent biological replicates after 15 days of inoculation (as described in 3.2.1) by quantitative Real-Time PCR (RT-qPCR) using PE Applied Biosystems and StepOne Software v2.2.2. Actin 2 (*Act-2*; At3g18780), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; At1g13440) and elongation factor (*EF1α*; AT5G60390) genes were used as reference genes to normalise the RT-qPCR data.

AXR5 (Hardtke et al., 2007), *ASC4* (Abel et al., 1995), *MYB122* (Frerigmann & Gigolashvili, 2014) and *ARR11* (Kieber & Schaller, 2014) are involved in auxin, ethylene, cytokinin and glucosinolates pathway. *GLIP1* and chitinase are known to be involved in resistance against bacteria and fungi (Oh et al., 2005; Lee et al., 2009; Hermosa et al., 2012). *WRKY63* involved in abiotic stress resistance and mediates plant responses to drought tolerance (Ren et al., 2010; Bakshi & Oelmuller, 2014).

3.2.8.1 RNA Extraction

Total RNA was extracted from FRh2-inoculated and mock-inoculated *Arabidopsis* rosettes using the RNeasy Plant Mini Kit (QIAGEN) as described in 3.2.3. RNA quality check was performed by electrophoresis and photometrical measurement with the Nanodrop 2000 spectrophotometer (Thermo Scientific).

3.2.8.2 Reverse transcriptase RT-PCR (cDNA synthesis)

A total of 2 µg (700-800 ng μl^{-1}) of total RNA was reverse-transcribed into the first-strand cDNA using SuperScript® III First-Strand Synthesis System - Invitrogen. cDNA synthesis was performed according to the manufacturer's instructions, using Oligo dB 12-18 primer and including an RNase H digestion as a last step to remove RNA template from the cDNA:RNA hybrid molecule.

3.2.8.3 Primer design

Primers (Table G1 Appendix G) were designed to amplify short cDNA fragments using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were designed to span an Exon/Exon junction with a product size between 70-100 bp using the RefSeq accession database. The concentration of primers was optimised for maximum yield using conventional PCR.

3.2.8.4 Quantitative real time PCR condition

The RT-qPCR was conducted with a reaction mixture contained gene specific primers, cDNA template with a dilution value of 1:10, SYBR Green reagent, ROX Reference Dye to normalise the fluorescent reporter signal and the FastStart™ Taq DNA Polymerase, dNTPack –Roche using triplet technical replicates for each of the three independent biological replicates. The reaction component can be viewed in Table G2 Appendix G.

The thermal cycling conditions were 95 °C for 10 min followed by 95 °C for 15”, 60 °C for 45” and 72 °C for 45” for 40 cycles, followed by melting curve step at 95 °C for 15”, 60 °C for 1’ and 95 °C for 15”.

The relative expression levels were analysed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and are presented as $\log_2$ relative levels of gene expression.

3.3 Results

3.3.1 Detection of *Beauveria bassiana* in plant tissues

Using standard isolation technique on a culture medium, *B. bassiana* was recovered from all inflorescences of plants that had been root-inoculated with FRh2 and BG11, respectively. None of the control plants revealed any colonisation by the entomopathogen.

The molecular detection of *B. bassiana* colonisation using $SCA15_{441}$ primer was able to detect *B. bassiana* inoculated plants. No amplification was found for control plants (Figure 3-1).

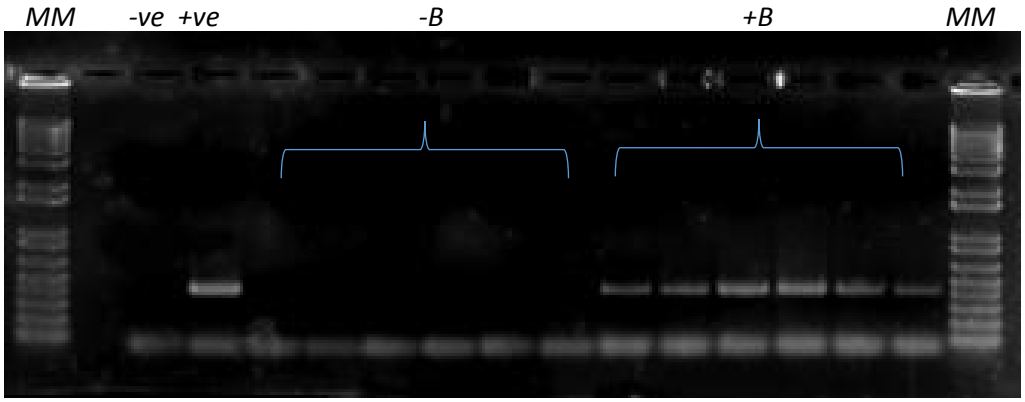


Figure 3-1 Agarose gel electrophoresis for the amplification of *Beauveria bassiana* gDNA extracted from *B. bassiana* (+B) and mock (-B) inoculated *Arabidopsis* rosette growing in a sterile closed system (MM) 1Kb Plus DNA Ladder (-ve) Negative control (+ve) Positive control.

3.3.2 Analysis of microarray data

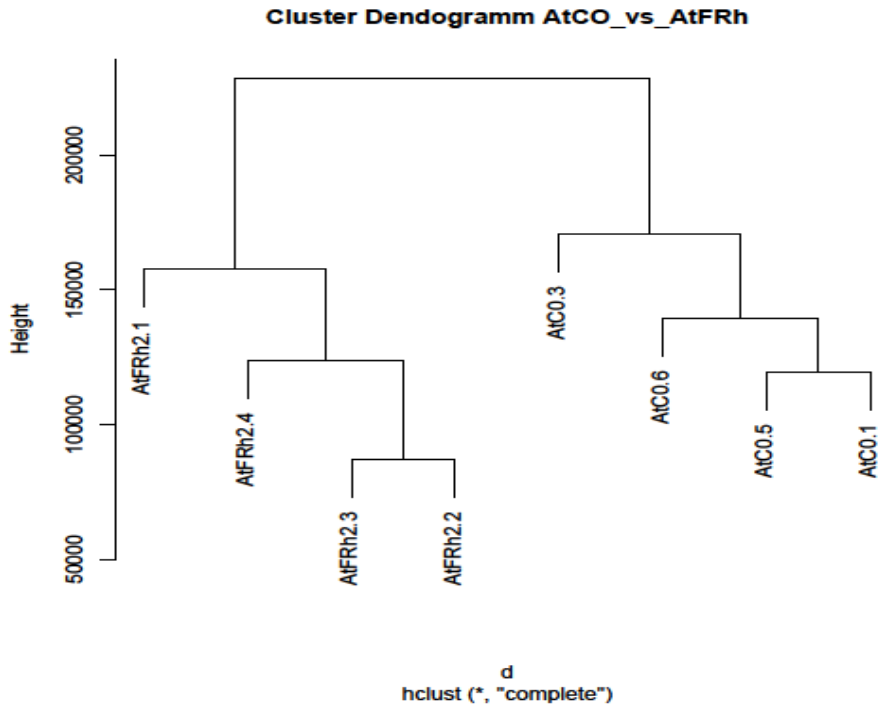
Microarray data were generated from inoculated (AtFRh2, AtBG11) and mock-inoculated (AtC0) *Arabidopsis* rosettes of four independent biological replicates per treatment to identify genes that were differentially expressed during *B. bassiana* interaction with *Arabidopsis*.

Both hierarchical clustering analysis (HCA) and principal component analysis (PCA) separated *B. bassiana* inoculated plants (AtFRh2 and AtBG11) from control plants (AtCO) showing that the expression pattern of inoculated plants differed from that of the control plants (Figure 3-2 and Figure 3-3). Heat maps for all top 100 and top 50 DEGs for AtFRh2 and AtBG11 assessed the expression profile of each DEG in each replicate and divided the gene expression patterns into two clusters: *B. bassiana* inoculated plants and control plants. Heat maps can be viewed in Figures F2 – F7 Appendix F.

The comparative transcriptome study revealed a total of 1166 and 552 differentially expressed genes (DEGs) compared to control for AtFRh2 and AtBG11, respectively. The resulting DEGs are listed in Tables F1 and F2 Appendix F and q-values following FDR correction are shown in Tables F27 and F28 Appendix F.

The ratio of upregulated to downregulated *Arabidopsis* transcript was 58:42 % in AtFRh2 and 52:48 % in AtBG11 (Figure 3-4). A total of 58 DEGs were shared between the AtFRh2 and AtBG11. Thirty-eight of the 58 shared DEGs had a similar expression pattern in the presence of FRh2 or BG11 while the remaining showed diverging expression patterns (Figure 3-5). Detailed information on the shared DEGs can be viewed in Table 3-1.

(A)



(B)

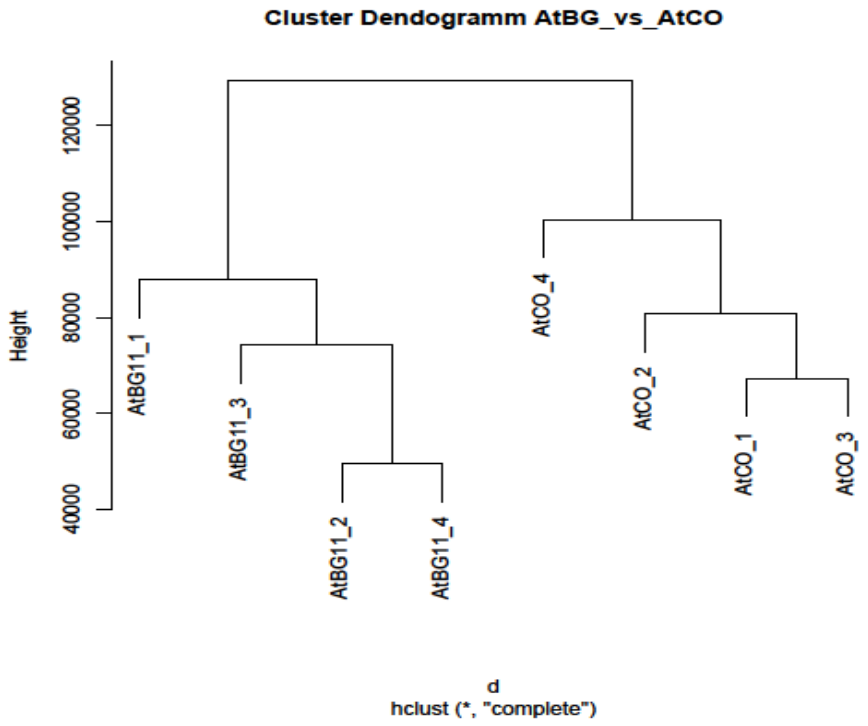
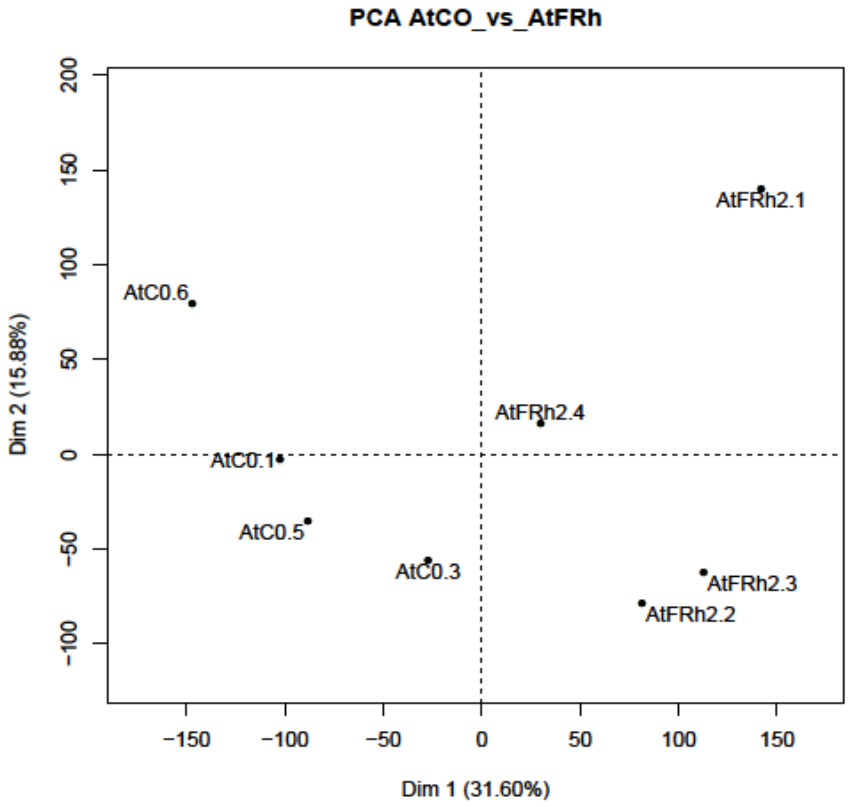


Figure 3-2 Hierarchical Clustering (HC) Analysis.
HC dendrograms of AtCO_vs_AtFRh2 (A) and AtCO_vs_AtBG11 (B) cluster the samples with similar expression patterns close to each other separating control (AtCO) from inoculated replicates (AtFRh2 = plants colonised by *B. bassiana* FRh2, AtBG11 = plants colonised by *B. bassiana* BG11).

(A)



(B)

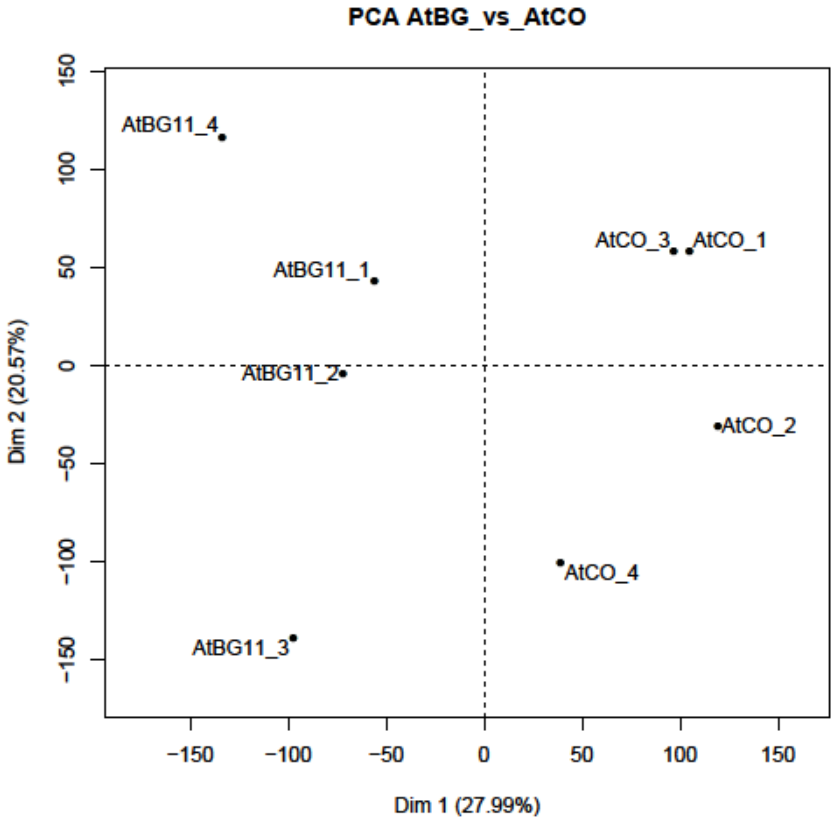


Figure 3-3 Principal Component Analysis (PCA).
PCA plots of AtCO_vs_AtFRh2 (A) and AtCO_vs_AtBG11 (B) separate control (AtCO) from inoculated replicates (AtFRh2 = plants colonised by *B. bassiana* FRh2, AtBG11 = plants colonised by *B. bassiana* BG11).

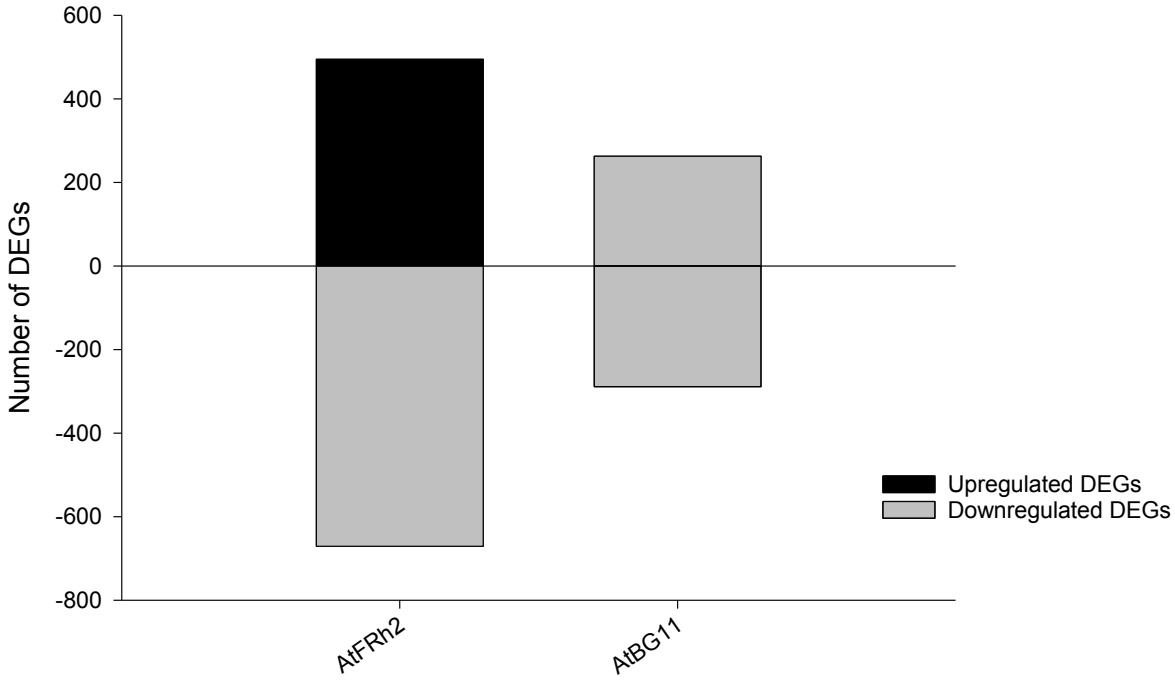


Figure 3-4 Numbers of up and downregulated differentially expressed genes (DEGs) in *Arabidopsis thaliana* colonised by *Beauveria bassiana.*
AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

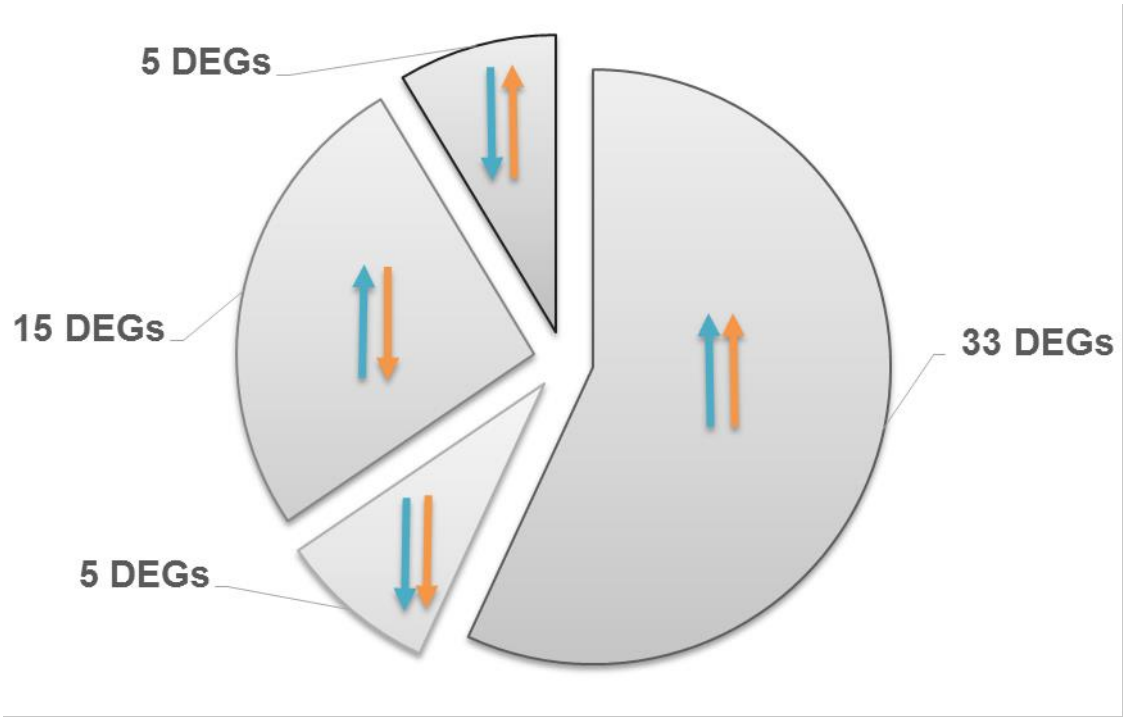


Figure 3-5 Number of shared differentially expressed genes (DEGs) between AtFRh2 and AtBG11 and their expression patterns.
Blue and orange arrows indicate FRh2 and BG11 colonisation respectively. Upward and downward pointing arrows indicate upregulated and downregulated DEGs respectively. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

Table 3-1 Shared differentially expressed genes (DEGs) between AtFRh2 and AtBG11 and their expression pattern.
Red and blue boxes show up and downregulated DEGs, respectively. Expression levels for each DEG shown as LogFC= log2 fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

		LogFC	
ID	**Symbols/Description**	**AtFRh2**	**AtBG11**
at1g61560	MLO6	1.017	1.041
at4g18430	RABA1e	1.101	1.059
at1g33030	O-methyltransferase family 2 protein	1.121	1.001
at1g61800	GPT2	1.125	1.53
at2g18660	EXLB3	1.166	1.178
at2g25440	AtRLP20	1.206	1.178
at1g69930	GSTU11	1.232	1.226
at1g04980	PDIL2-2	1.253	1.007
at4g25110	AtMC2	1.297	1.008
at3g61390	U-box domain-containing protein	1.314	1.351
at5g62150	LysM domain-containing protein	1.331	1.141
at4g09300	unknown protein	1.386	1.053
at5g13080	WRKY75	1.403	1.663
at5g22530	unknown protein	1.475	1.004
at4g25070	unknown protein	1.501	1.006
at1g36640	unknown protein	1.633	1.335
at5g67310	CYP81G1	1.64	1.453
at1g47890	RLP7	1.678	1.331
at1g21310	EXT3,	1.694	1.312
at1g66960	lupeol synthase, putative	1.765	1.054
at2g13810	ALD1	1.801	1.785
at1g21240	WAK3	1.807	1.178
at3g28580	AAA-type ATPase family protein	1.842	1.034
at5g38900	DSBA oxidoreductase family protein	1.896	1.002
at3g22600	lipid transfer protein (LTP) family protein	1.995	1.248
at2g43570	chitinase, putative	1.998	1.374
at3g63380	calcium-transporting ATPase,putative	2.004	1.661
at4g11170	disease resistance protein, putative	2.2	1.124
at5g11210	GLR2.5	2.371	1.06
at4g23700	CHX17	2.409	1.002
at1g44130	nucellin protein, putative	2.51	1.429
at3g11340	UDP-glucoronosyl family protein	2.595	1.801
at1g19250	SFMO1	2.982	1.648
at5g45920	carboxylesterase/ hydrolase	-1.321	-1.32
at5g63160	BT1	-1.141	-1.711
at3g48115	other RNA	-1.201	-1.073
at4g02810	unknown protein	-1.018	-1.248
at5g35525	unknown protein	-2.467	-1.485

Table 3-1 Shared differentially expressed genes (DEGs) between AtFRh2 and AtBG11 and their expression pattern (continued).
Red and blue show up and downregulated DEGs, respectively. Expression levels for each DEG shown as LogFC= log_2 fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

		LogFC	
ID	**Symbols/Description**	**AtFRh2**	**AtBG11**
at3g28180	CSLC04	-1.064	1.084
at1g32540	LOL1	-1.433	1.055
at4g13564	MIR841A	-1.382	1.216
at5g62280	unknown protein	-1.333	1.444
at5g53410	unknown protein	-1.394	1.049
at1g40089	putative fructose-2,6-bisphosphatase	1.375	-1.039
at2g36750	UGT73C1	1.815	-1.826
at1g05680	UDP-glucosyl transferase family protein	1.793	-1.413
at2g36760	UGT73C2	1.336	-1.321
at1g05530	UGT75B2	1.329	-1.282
at5g42760	unknown protein	1.132	-1.887
at3g21890	zinc finger (B-box type) family protein	1.151	-1.782
at5g08070	TCP17	1.083	-1.396
at1g68150	WRKY9	1.338	-1.162
at4g15248	zinc ion binding	1.145	-1.803
at5g15500	ankyrin repeat family protein	2.62	-1.08
at1g04570	membrane transporter family protein	1.305	-1.498
at5g58770	DEDOL-PP synthase, putative	1.539	-1.379
at4g29770	Target of trans acting-siR480/255	1.003	-1.543
at1g31300	unknown protein	1.568	-1.086

3.3.3 Gene ontology and enrichment analysis

With respect to the upregulated processes, a similarity between AtFRh2 and AtBG11 was observed in defence related processes (Table 3-6) such as “response to innate immune defence” and “response to chitin” (Table 3-2, 3-3). Interestingly, only AtBG11 was associated with response to wound and cell death (Table 3-3). Although both showed induction in genes involved in oxidative stress process, AtFRh2 was associated with many additional abiotic stress related process that include response to heat, temperature, radiation and light while this wasn’t observed in AtBG11. In addition only AtFRh2 was involved in BP related to triterpenoid metabolism (Table 3-2).

The striking difference between AtFRh2 and AtBG11 was observed in BP related to plant hormones (Table 3-6): the upregulated AtBG11 DEGs were related to response to jasmonic acid, ethylene and salicylic acid and systemic acquired resistance (SAR) (Table 3-3) whereas the downregulated AtFRh2 DEGs were associated with response to ethylene, gibberellin and auxin stimulus (Table 3-4).

While both AtFRh2 and AtBG11 downregulated DEGs were involved in BP related to regulation of transcription, AtFRh2 downregulated DEGs were associated with cell cycle and division process (Table 3-4) whereas AtBG11 downregulated DEGs were related to flavonoid metabolism (Table 3-5). Detailed gene ontology and enrichment analyses are given in Tables F3- F26 Appendix F.

Table 3-2 Upregulated AtFRh2 differentially expressed genes (DEGs) biological process enriched. (p- value ≤ 0.05)

Term	p-value
Response to heat	1.80E-17
Response to oxidative stress	1.50E-09
Response to temperature stimulus	2.11E-08
Response to hydrogen peroxide	5.25E-08
Response to reactive oxygen species	7.79E-08
Protein folding	1.72E-06
Response to inorganic substance	1.81E-06
Defence response	6.37E-06
Response to high light intensity	1.87E-05
Response to abiotic stimulus	2.08E-05
Toxin catabolic process	2.34E-04
Toxin metabolic process	2.34E-04
Response to light stimulus	2.66E-04
Response to bacterium	2.71E-04
Response to radiation	4.18E-04
Response to light intensity	4.94E-04
Defence response to bacterium	8.66E-04
Innate immune response	8.90E-04
Secondary metabolic process	1.40E-03
Immune response	1.63E-03
Defence response, incompatible interaction	2.49E-03
Chitin metabolic process	1.18E-02
Chitin catabolic process	1.18E-02
Aminoglycan catabolic process	1.18E-02
Calcium ion homeostasis	1.32E-02
Cellular calcium ion homeostasis	1.32E-02
Pentacyclic triterpenoid biosynthetic process	1.50E-02
Pentacyclic triterpenoid metabolic process	1.50E-02
Aminoglycan metabolic process	1.61E-02

Table 3-2 Upregulated AtFRh2 differentially expressed genes (DEGs) biological process enriched. (p-value ≤ 0.05) (continued)

Term	p-value
Inorganic anion transport	1.75E-02
Positive regulation of immune response	1.77E-02
Activation of immune response	1.77E-02
Positive regulation of innate immune response	1.77E-02
Activation of innate immune response	1.77E-02
Positive regulation of immune system process	1.77E-02
Anion transport	1.80E-02
Positive regulation of response to stimulus	2.11E-02
Positive regulation of defence response	2.12E-02
Triterpenoid biosynthetic process	2.14E-02
Triterpenoid metabolic process	2.88E-02
Response to endoplasmic reticulum stress	2.88E-02
Regulation of innate immune response	2.92E-02
Cellular response to stress	3.61E-02
Heat acclimation	3.70E-02
Metal ion homeostasis	4.93E-02
Cellular metal ion homeostasis	4.93E-02

Table 3-3 Upregulated AtBG11 differentially expressed genes (DEGs) biological process enriched. (p- value ≤ 0.05)

Term	p-value
Response to jasmonic acid stimulus	6.80E-09
Defence response	1.34E-07
Response to organic substance	7.15E-07
Response to endogenous stimulus	1.66E-05
Response to ethylene stimulus	2.98E-04
Defence response, incompatible interaction	4.46E-04
Innate immune response	5.24E-04
Response to wounding	7.79E-04
Immune response	8.54E-04
Response to salicylic acid stimulus	8.75E-04
Response to hormone stimulus	5.21E-03
Response to chitin	9.75E-03
Response to bacterium	1.27E-02
Cell wall organization	2.37E-02
Polysaccharide metabolic process	2.50E-02
Ethylene mediated signalling pathway	3.03E-02
Cellular response to stress	3.08E-02
External encapsulating structure organization	3.08E-02
Response to water deprivation	3.45E-02
Activation of immune response	3.51E-02
Positive regulation of innate immune response	3.51E-02
Activation of innate immune response	3.51E-02
Positive regulation of immune system process	3.51E-02
Positive regulation of immune response	3.51E-02
Positive regulation of defence response	3.97E-02
Response to water	4.13E-02
Systemic acquired resistance	4.21E-02
Cell wall macromolecule catabolic process	4.21E-02
Death	4.62E-02
Cell death	4.62E-02
Regulation of innate immune response	4.95E-02
Jasmonic acid mediated signalling pathway	4.95E-02

Table 3-4 Downregulated AtFRh2 differentially expressed genes (DEGs) biological process enriched. (p-value ≤ 0.05)

Term	p-value
Response to hormone stimulus	2.36E-11
Response to endogenous stimulus	3.25E-10
Response to organic substance	5.24E-09
Response to auxin stimulus	8.50E-09
rRNA modification	8.64E-07
Regulation of transcription	3.44E-06
Cellular response to hormone stimulus	1.27E-05
Hormone-mediated signalling	1.27E-05
Regulation of transcription, DNA-dependent	3.67E-05
Regulation of RNA metabolic process	4.17E-05
Transcription	5.10E-05
rRNA metabolic process	9.22E-05
rRNA processing	9.22E-05
RNA modification	1.85E-04
Response to gibberellin stimulus	3.18E-04
DNA replication	4.15E-04
Gibberellic acid mediated signalling	6.45E-04
Gibberellin-mediated signalling	6.45E-04
Regulation of cell cycle	9.28E-04
RNA processing	9.73E-04
two-component signal transduction system (phosphorelay)	1.19E-03
Response to carbohydrate stimulus	1.66E-03
intracellular signalling cascade	2.11E-03
DNA-dependent DNA replication	2.93E-03
Ethylene mediated signalling pathway	4.11E-03
Response to ethylene stimulus	4.72E-03
Cell cycle	4.81E-03
Cellular glucan metabolic process	6.15E-03
Cellular polysaccharide metabolic process	6.45E-03
DNA endoreduplication	7.50E-03
RNA metabolic process	8.08E-03
Cell division	1.32E-02
Response to red or far red light	1.38E-02
External encapsulating structure organization	1.38E-02
Ribosome biogenesis	1.40E-02
Glucan metabolic process	1.95E-02

Table 3-4 Downregulated AtFRh2 differentially expressed genes (DEGs) biological process enriched. (p-value ≤ 0.05) (continued)

Term	p-value
Cell wall organization	2.11E-02
Terpenoid metabolic process	2.20E-02
Lipid biosynthetic process	2.33E-02
Ribonucleoprotein complex biogenesis	2.35E-02
DNA metabolic process	2.50E-02
Cell fate commitment	2.59E-02
Cell cycle process	2.90E-02
Terpenoid biosynthetic process	2.97E-02
Cellular component morphogenesis	3.37E-02
Epidermal cell differentiation	4.02E-02
Cell morphogenesis	4.27E-02
Ectoderm development	4.32E-02
Epidermis development	4.32E-02
Gibberellin biosynthetic process	4.81E-02
Cell growth	4.96E-02

Table 3-5 Downregulated AtBG11 differentially expressed genes (DEGs) biological process enriched. (p-value ≤ 0.05)

Term	p-value
Cellular amino acid derivative biosynthetic process	5.27E-04
Response to UV-B	1.01E-03
Flavonoid biosynthetic process	1.59E-03
Flavonoid metabolic process	2.08E-03
Secondary metabolic process	2.34E-03
Flavonol metabolic process	2.42E-03
Flavonol biosynthetic process	2.42E-03
Flavone metabolic process	2.42E-03
Flavone biosynthetic process	2.42E-03
Pigment metabolic process	2.75E-03
Response to UV	4.87E-03
Cellular amino acid derivative metabolic process	5.28E-03
Anion transport	6.76E-03
Phenylpropanoid biosynthetic process	7.49E-03
Pigment biosynthetic process	9.44E-03
Cell redox homeostasis	1.21E-02
Inorganic anion transport	1.32E-02
Response to light stimulus	1.34E-02
Regulation of nitrogen utilization	1.38E-02
Regulation of transcription	1.40E-02
Regulation of transcription, DNA-dependent	1.48E-02
Regulation of RNA metabolic process	1.55E-02
Response to radiation	1.66E-02
Phenylpropanoid metabolic process	2.11E-02
Peptide transport	2.89E-02
Oligopeptide transport	2.89E-02
Response to abiotic stimulus	3.43E-02
Sulfate reduction	3.75E-02
Amine biosynthetic process	4.52E-02
Nicotianamine metabolic process	4.66E-02
Nicotianamine biosynthetic process	4.66E-02

Table 3-6 Shared GO terms between AtFRh2 and AtBG11.
Blue and red boxes show up and downregulated biological process enriched at p-value < 0.05.
AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

	p-value	
Term	**AtFRh2**	**AtBG11**
Defence response	6.37E-06	1.34E-07
Response to abiotic stimulus	2.08E-05	3.43E-02
Response to light stimulus	2.66E-04	1.34E-02
Response to bacterium	2.71E-04	1.27E-02
Response to radiation	4.18E-04	1.66E-02
Innate immune response	8.90E-04	5.24E-04
Secondary metabolic process	1.40E-03	2.34E-03
Immune response	1.63E-03	8.54E-04
Defence response, incompatible interaction	2.49E-03	4.46E-04
Inorganic anion transport	1.75E-02	1.32E-02
Positive regulation of immune response	1.77E-02	3.51E-02
Activation of immune response	1.77E-02	3.51E-02
Positive regulation of innate immune response	1.77E-02	3.51E-02
Activation of innate immune response	1.77E-02	3.51E-02
Positive regulation of immune system process	1.77E-02	3.51E-02
Anion transport	1.80E-02	6.76E-03
Positive regulation of defence response	2.12E-02	3.97E-01
Regulation of innate immune response	2.92E-02	4.95E-02
Cellular response to stress	3.61E-02	3.08E-02
Response to organic substance	5.24E-09	7.15E-07
Response to endogenous stimulus	3.25E-10	1.66E-05
Response to ethylene stimulus	4.81E-03	2.98E-04
Response to hormone stimulus	2.36E-11	5.21E-03
Cell wall organization	2.11E-02	2.37E-02
Ethylene mediated signalling pathway	4.72E-03	3.03E-02
External encapsulating structure organization	1.40E-02	3.08E-02
Regulation of transcription	3.44E-06	1.40E-02
Regulation of transcription, DNA-dependent	3.67E-05	1.48E-02
Regulation of RNA metabolic process	4.17E-05	1.55E-02

3.3.4 *Arabidopsis thaliana* responses to *Beauveria bassiana* colonisation and pathways analysis

Gene ontology analysis was complemented by mapping the transcriptomic data using MapMan. An overview of the plant metabolic pathways that were affected by *B. bassiana* colonisation was generated.

MapMan mapped 1163 and 548 DEGs into the 35 major bins for AtFRh2 and AtBG11 respectively. Bins 7, 12, 14, (OPP; oxidative pentose phosphate, N-metabolism, S-assimilation, polyamine metabolism) and bins 8, 9, 18 (Tricarboxylic acid (TCA) cycle / organic acid (org) transformation, Mitochondrial electron transport / ATP synthesis, Co-factor and vitamin metabolism) were the only non-represented bins for AtFRh2 and AtBG11 respectively (Figure 3-6). A total of 367 DEGs (142 upregulated and 225 downregulated) and 159 DEGs (61 upregulated and 98 downregulated) for AtFRh2 and AtBG11 respectively were unassigned (bin 35) DEGs (Figure 3-6).

The biotic stress overview pathway generated by Mapman highlighted the involvement of both AtFRh2 and AtBG11 in defence-related responses and gave a visual assessment of how *B. bassiana* shapes major parts of the plant immune response. MapMan identified 374 and 175 DEGs associated with the biotic stress overview pathway for AtFRh2 and AtBG11, respectively (Figure 3-7, Table 3-7). Among these, only 38 AtFRh2 and 13 AtBG11 associated DEGs were related to abiotic stress (bin 20.2) whereas the remaining were related directly or indirectly to biotic stress. An additional 6 DEGs were identified as related to abiotic stress in AtFRh2 (Nakashima et al., 2000; Devaiah et al., 2007; Suzuki et al., 2008; Ren et al., 2010; Krishnaswamy et al., 2011; Fu et al., 2014). Detailed information on AtFRh2 and AtBG11 DEGs mapped to MapMan biotic stress overview is given in Tables F29 and F30 in Appendix F.

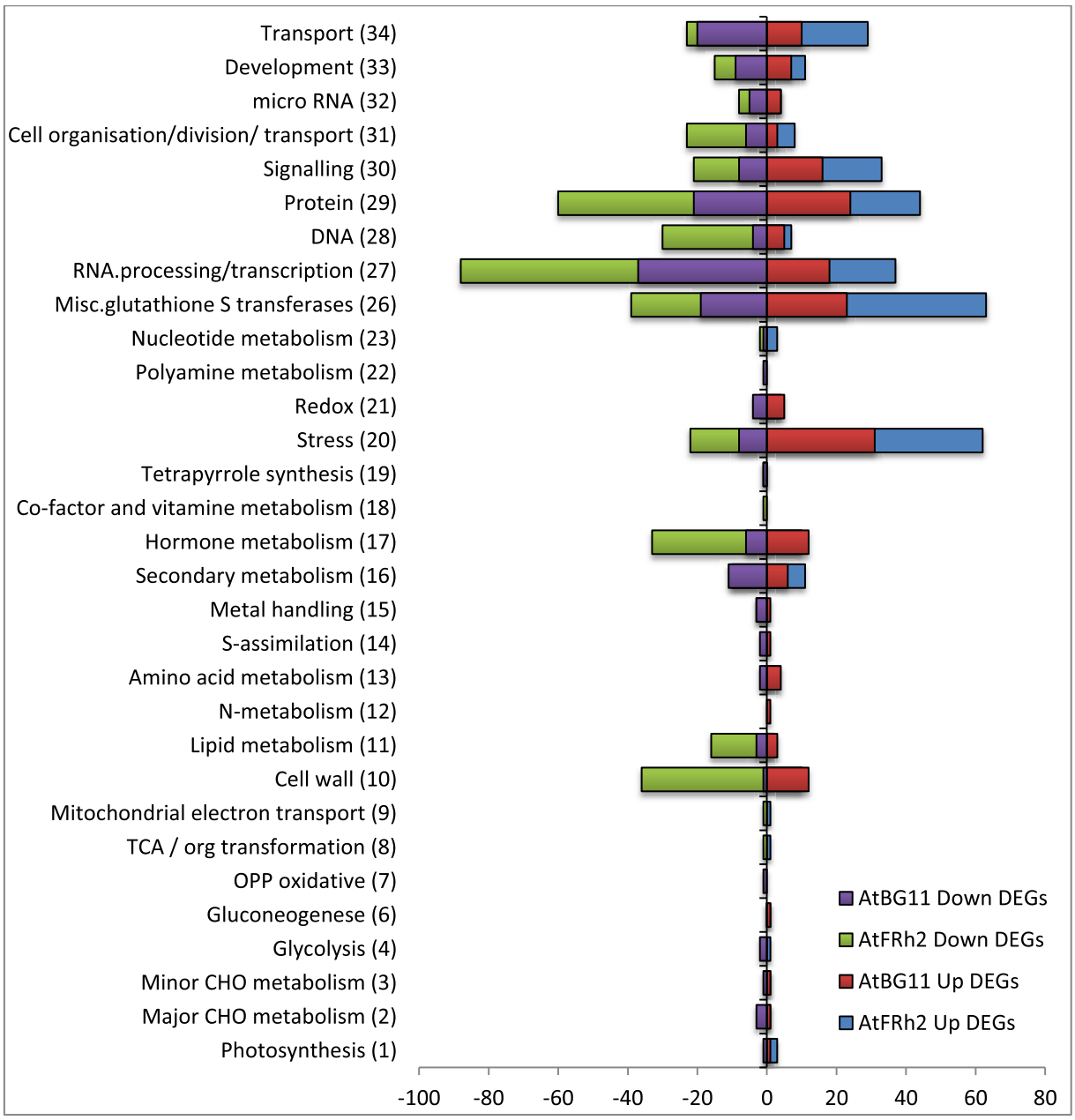
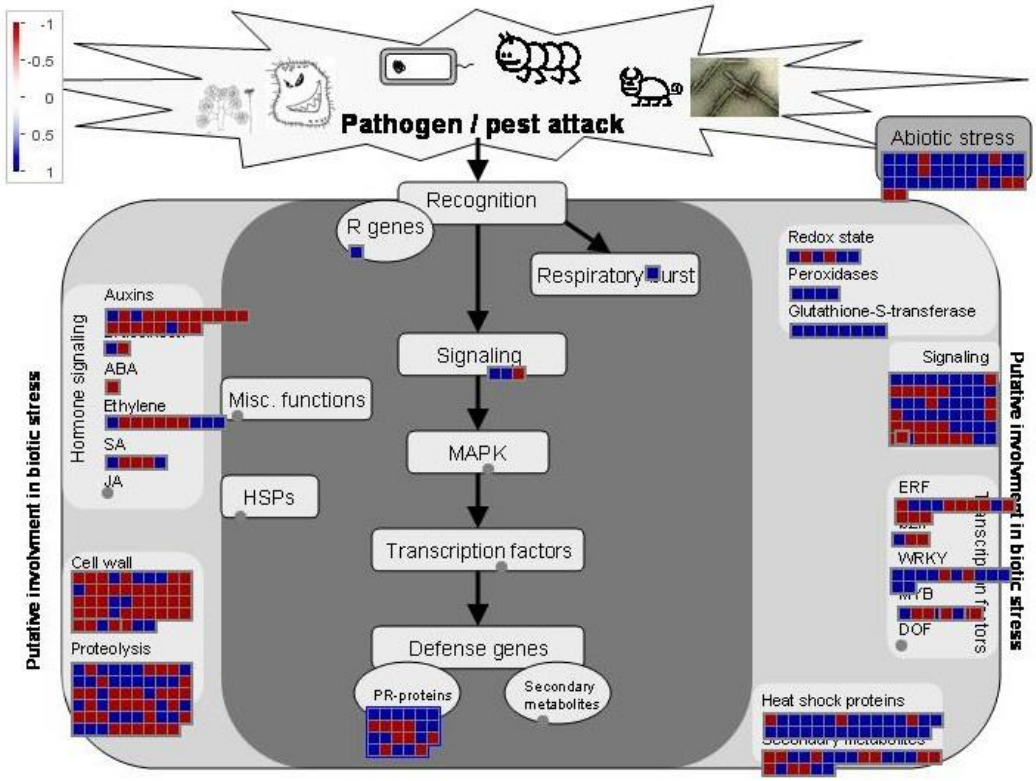


Figure 3-6 Numbers of differentially expressed genes (DEGs) assigned into MapMan bins (bin number in brackets) from AtFRh2 and AtBG11.
Downregulated DEGs are negative. Unassigned DEGs were 142 , -225 and 61, -98 for AtFRh2 and AtBG11 respectively. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

(A)



(B)

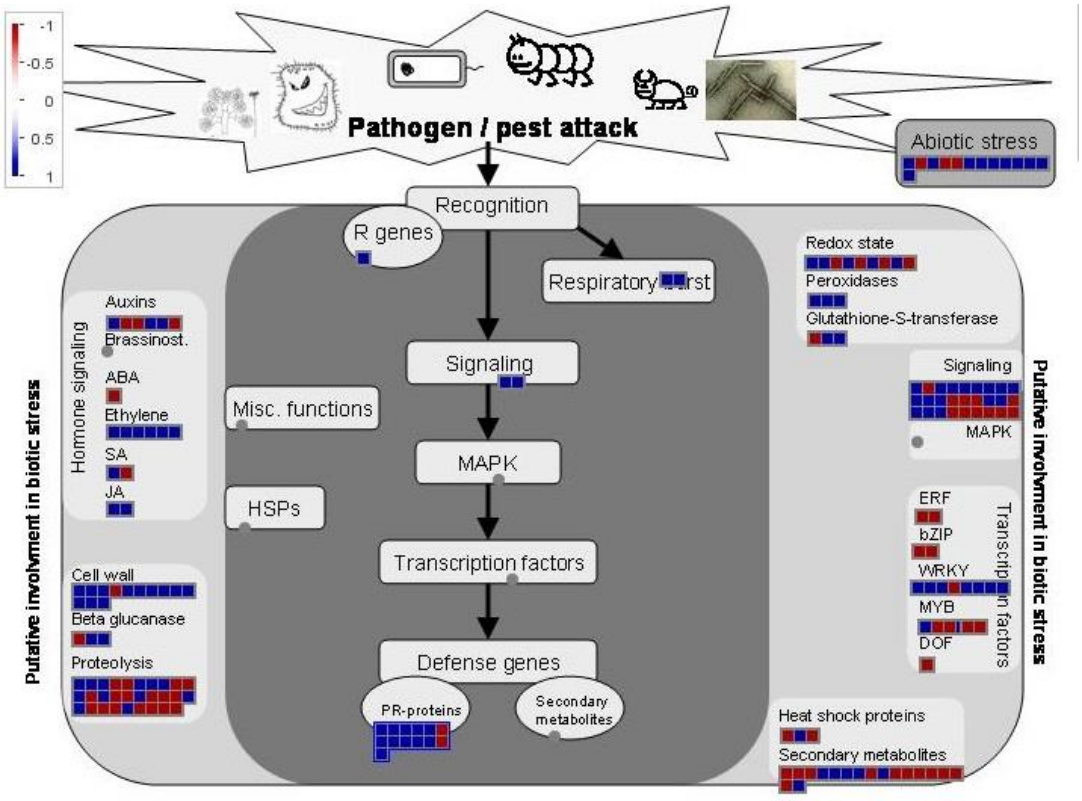


Figure 3-7 Biotic stress overview pathway for AtFRh2 (A) and AtBG11 (B) DEGs as generated by MapMan software.

Blue and red boxes represent up and downregulated genes respectively. Colour intensity represents the degree of expression given as log fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

Table 3-7 Numbers of differentially expressed genes (DEGs) mapped to the biotic stress overview pathway for AtFRh2 and AtBG11.
DEGs grouped by the first two sub-bins of MapMan bin codes and first three sub-bins for stress-related pathway (bin 20). AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

		AtFRh2		AtBG11	
Bin code	Bin name	Up	Down	Up	Down
10.1	cell wall.precursor synthesis	0	1	1	0
10.2	cell wall.cellulose synthesis	3	3	1	0
10.3	cell wall.hemicellulose synthesis	1	1	0	0
10.5	cell wall.cell wall proteins	2	13	2	1
10.6	cell wall.degradation	1	6	2	0
10.7	cell wall.modification	1	10	4	0
10.8	cell wall.pectin*esterases	0	3	2	0
16.1	secondary metabolism.isoprenoids	4	3	4	1
16.2	secondary metabolism.phenylpropanoids	2	2	2	2
16.4	secondary metabolism.N misc	1	0	1	0
16.5	secondary metabolism.sulfur-containing	1	0	1	0
16.7	secondary metabolism.wax	0	1	0	1
16.8	secondary metabolism.flavonoids	2	4	2	4
17.2	hormone metabolism.auxin	3	15	3	3
17.3	hormone metabolism.brassinosteroid	1	1	0	0
17.1	hormone metabolism.abscisic acid	0	1	0	1
17.5	hormone metabolism.ethylene	4	6	6	0
17.8	hormone metabolism.salicylic acid	2	3	1	1
17.7	hormone metabolism.jasmonate	0	0	2	0
20.1.1	stress.biotic.respiratory burst	1	0	2	0
20.1.2	stress.biotic.receptors	1	0	1	0
20.1.3	stress.biotic.signalling	2	1	2	0
20.1.7	stress.biotic.PR-proteins	14	9	11	2
20.2	stress.abiotic	3	0	1	0
20.2.1	stress.abiotic.heat	26	3	1	2
20.2.3	stress.abiotic.drought/salt	1	2	0	1
20.2.99	stress.abiotic.unspecified	0	3	8	0
21.1	redox.thioredoxin	1	0	2	1
21.5	redox.peroxiredoxin	2	0	0	0
21.4	redox.glutaredoxins	1	2	2	3
21.2	redox.ascorbate and glutathione	0	0	1	0
26.4	misc.beta 1,3 glucan hydrolases	1	3	2	1
26.9	misc.glutathione S transferases	8	0	2	1
26.12	misc.peroxidases	4	0	3	0
27.3	RNA.regulation of transcription	19	18	10	10
29.5	protein.degradation	28	31	12	16
30.1	signalling.in sugar and nutrient physiology	3	0	1	0
30.2	signalling.receptor kinases	23	10	10	2
30.3	signalling.calcium	2	5	3	3
30.4	signalling.phosphinositides	0	0	1	0
30.5	signalling.G-proteins	3	2	1	1
30.6	signalling.MAP kinases	0	1	0	0
30.8	signalling.misc	0	2	0	0
30.11	signalling.light	1	1	0	5

3.3.4.1 Biotic stress-related DEGs

Several subsets of DEGs were identified as associated directly or indirectly to biotic stress responses including pathogenesis-related proteins, protein kinases, *R* genes, transcription factors, phytohormone signalling-related genes, oxidative stress-related, secondary metabolism-related genes and cell-wall modification genes.

Genes encoding pathogenesis-related proteins

AtFRh2 showed upregulation in three chitinase-related genes; two belonging to class IV chitinase and one belonging to class III chitinase whereas AtBG11 was characterised by the upregulation of two class IV chitinase genes and by the upregulation of both *PR-1* gene and ß-1, 3 glucanase gene, which belongs to the PR-2 family of pathogenesis related proteins (Van Loon & Van Strien, 1999).

Genes encoding receptor like kinases/proteins

Receptor-like kinases/proteins (RLKs/RLPs) are cell surface receptors characterised by a crucial role in disease resistance. They perceive signature molecules from the invading pathogen and trigger a basal resistance to pathogen (Yang et al., 2012).

A LysM receptor-like kinase gene was upregulated in both AtFRh2 and AtBG11. LysM Receptor-Like Kinase was identified to play a role in chitin signalling and fungal resistance in *Arabidopsis* (Wan et al., 2008).

In addition, AtFRh2 was characterised by the upregulation of nine RLPs genes (*RLP7, RLP20, RLP21, RLP22, RLP28, RLP30, RLP35, RLP43,* and *RLP50*). RLP30 confers partial resistance to necrotrophic fungi in *Arabidopsis* (Zhang et al., 2013). Moreover, the Flg22-induced receptor-like kinase 1 (*FRK1*) was upregulated in AtFRh2. FRK1 is known to be involved in the early defence response against the 22-amino-acid peptide Flg22, corresponding to the most conserved domain of eubacterial flagellin (Asai et al., 2002).

AtBG11 showed upregulation in five *RLPs* genes (*RLP7, RLP18, RLP20, RLP23,* and *RLP53*). Recently, AtRLP23 was found to mediate immune activation in *Arabidopsis* by being a receptor for the nlp20 conserved motif in Nep1-like proteins (NLPs) produced by differing microorganisms including bacteria, fungi and oomycetes. Expression of *RLP23* in potato (*Solanum tuberosum*) confers nlp20 pattern recognition and enhanced immunity against plant pathogens, such as *Phytophthora infestans* and *Sclerotinia sclerotiorum* (Albert et al., 2015).

Both AtFRh2 and AtBG11 were characterised by the upregulation of wall associated kinase 3 gene (*WAK3*) whereas *WAK4* was upregulated in AtFRh2 only. Wall associated kinases bind with pectin in

the cell wall, induced upon pathogen attack, and serve a role in cell elongation (Wagner & Kohorn, 2001; Lally et al., 2001; Ringli, 2010).

Genes encoding mitogen-activated protein kinase

Activation of mitogen-activated protein kinase genes (*MAPK*) depends on calmodulin binding in a Ca^{2+} dependent manner (Rodriguez et al., 2010; Takahashi et al., 2011). Only AtBG11 was characterised by the upregulation of two calmodulin (CaM)-binding proteins and a single calcium-transporting ATPase genes. None of the *MAPK* genes were affected in AtBG11 whereas AtFRh2 showed a downregulation in a single MAPKKK-related gene.

***R*-genes**

AtFRh2 and AtBG11 were characterised by diverging expression patterns of many disease resistance protein coding genes characterised by nucleotide-binding sites leucine-rich repeat NBS-LRR domains. NB-LRR proteins act as plant immune receptors responsible for the initiation of ETI and are encoded by diverse genes and divided into the Toll/interleukin-1 receptor(TIR) domain-containing (TNL) and the coiled-coil (CC)-domain-containing (CNL) subfamilies (McHale et al., 2006).

HR genes are homologues of Resistance to Powdery Mildew 8 (*RPW8*) present in *Arabidopsis* Ms-0 ecotype and known to control resistance to powdery mildew pathogens (Xiao et al., 2001; Sáenz-Mata & Jiménez-Bremont, 2012). AtBG11 showed diverging expression patterns in three *HR* genes: *HR*4, *HR2* and *HR1*.

The phloem-based defence mechanism (PBD) includes two types of the most abundant proteins in the phloem sap: the phloem protein 1 (PP1) and phloem protein 2 (PP2). The PBD is activated by phloem-feeding insects, wounding and by oxidative stress (Zhang et al., 2011). Only AtFRh2 was characterised by the upregulation of *AtPP2 -A5*.

Genes encoding transcription factors

WRKY Transcription factors: Characterised by the WRKY domain with the almost invariant WRKY amino acid sequence, WRKY TFs act in a complex defence response network as both positive and negative regulators. With their redundancy and dual functionality they are involved in many plant process related to biotic and abiotic stress (Rushton et al., 2010).

Eleven and eight WRKY factors genes were differentially expressed in AtFRh2 and AtBG11 respectively. AtFRh2 was characterised by the upregulation of ten WRKY genes (*WRKY8*, *WRKY9*, *WRKY29*, *WRKY31*, *WRKY41*, *WRKY58*, *WRKY61*, *WRKY63*, *WRKY71* and *WRKY75*) and with the downregulation of only *WRKY44* whereas seven WRKY genes (*WRKY6*, *WRKY26*, *WRKY38*, *WRKY 40, WRKY55*, *WRKY72* and *WRKY75*) were upregulated and only *WRKY9* was downregulated in AtBG11.

Loss and gain of function studies in *Arabidopsis* revealed many WRKY protein functions. Overexpression of *WRKY41* showed enhanced resistance against virulent *P. syringae* but decreased resistance to *Erwinia carotovora* (Higashi et al., 2008). WRKY38 contributed negatively to basal resistance against *P. syringae* (Kim et al., 2008b) but along with WRKY6, WRKY26, WRKY40 and WRKY75 induced resistance against the cabbage aphid *Brevicoryne brassicae* (Kusnierczyk et al., 2008). WRKY6 positively regulated *PR1* promoter activity, most likely involving NPR1 function (Robatzek & Somssich, 2002). Similarly to WRKY38, WRKY58 acted downstream of NPR1 and negatively affected SAR (Wang et al., 2006). WRKY40 is closely related to WRKY18 and 60 and has a redundant function in negatively regulating resistance to *P. syringae* (Xu et al., 2006). In addition, *WRKY18* and *WRKY40* are induced in response to herbivory by the generalist caterpillar *Spodoptera littoralis* (Schweizer et al., 2013). Ren et al. (2010) showed that stomatal closure in *wrky63* mutant was less sensitive to abscisic acid (ABA) and the mutant was less drought tolerant than the wild type. *WRKY75*, induced in both FRh2 and BG11 colonised *Arabidopsis,* enhanced resistance to *Sclerotinia sclerotiorum* (Chen et al., 2013) and acted as a modulator of phosphate starvation (Devaiah et al., 2007). WRKY29 along with WRKY22 were reported to function downstream of the flagellin receptor FLS2 enhancing resistance to both bacterial and fungal pathogens (Asai et al., 2002).

MYB Transcription factors: MYB proteins are key factors in regulatory networks controlling development, metabolism and responses to biotic and abiotic stresses. Seven and five MYB factors genes were differentially expressed in AtFRh2 and AtBG11 respectively. *MYB7, MYB93*, *MYB122* were upregulated and *MYB0, MYB66, MYB73, MYB77* were downregulated in AtFRh2 whereas AtBG11 was characterised by the upregulation of *MYB15, MYB50, MYB80* and by the downregulation of *MYB11* and *MYB111*.

MYB122 along with MYB34 and MYB51 are known to regulate indole glucosinolate (IG) biosynthesis. However, knockout analysis revealed that *MYB122* plays only a minor role in JA/ET-induced glucosinolate biosynthesis (Frerigmann & Gigolashvili, 2014). *MYB73* and *MYB77* were transiently upregulated by cold stress (Fowler & Thomashow, 2002). In addition MYB77 regulated lateral root formation by modulating the expression of auxin-inducible genes (Shin et al., 2007). In *myb77* mutants, auxin-responsive genes expression were greatly attenuated and lateral root density was lower than in the wild type. MYB15 was involved in cold stress tolerance and the *myb15* mutant plants showed increased tolerance to freezing stress whereas its overexpression reduced freezing tolerance (Agarwal et al., 2006). MYB80, formerly known as MYB103, was found to regulate tapetum development, callose dissolution and exine formation in *Arabidopsis* anthers (Zhang et al., 2007). MYB11/PFG1 and AMYB111/PFG3 acted in an additive manner to control flavonol biosynthesis through targeting several genes of flavonoid biosynthesis, including chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase and flavonol synthase 1 (Stracke et al., 2007).

Ethylene response factors AP2/ERF: The AP2/ERF is a superfamily of transcription factors defined by the AP2/ERF domain, which consists of about 60 to 70 amino acids and is involved in DNA binding. This superfamily contains the ERF family, the AP2 and RAV families (Riechmann et al., 2000).

AtFRh2 was characterised by diverging expression patterns of thirteen AP2/ERF transcription factors genes know to be involved in abiotic stress and hormones regulation responses. *DREB2A, TEM1* and *Rap2.6L* were upregulated whereas *RAV2, DDF1/CBF1, DREB1B, ERF5, ERF6* and *ERF104* were downregulated. *ERF38* and *TINY2* were the only two downregulated AP2/ERF in AtBG11.

Overexpression of *RAP2.6L* enhanced performance under salt and drought (Krishnaswamy et al., 2011). A transgenic plant overexpressing *RAV2* were more susceptible to drought and salt-induced inhibition of germination (Fu et al., 2014). TEM1, a homolog of TEM2/RAV2, repressed growth by direct binding to the GA biosynthetic genes, *GA3OX1* and *GA3OX2*, leading to a reduction in GA content (Osnato et al., 2012). Expression of *DREB1B* was strongly induced by low-temperature stress, whereas that of *DREB2A* was induced by dehydration and high-salt stress (Nakashima et al., 2000) *CBF1* or *DDF1* is identical to *DREB1B* (Stockinger et al., 1997) and as well was induced by cold (Medina et al., 2011). *ERF38* was considered as a regulator of secondary wall metabolism (Lasserre et al., 2008) and *TINY2* was induced in respond to cold, wound, NaCl and drought treatment (Wei et al., 2005). ERF5 as activators of jasmonate (JA)-ethylene (ET)-responsive defence gene (Fujimoto et al., 2000; Ohta et al., 2001) and ERF6, as an ET-independent TF, along with ERF104 activated the expression of *PR* genes such as *PDF1.2* (Son et al., 2012; Meng et al., 2013; Wang et al., 2013).

Aux/IAA Transcription factors: The plant hormone, auxin, regulates many aspects of growth and development. Auxin signals are mediated by a master set of diverse transcriptional regulators. The Auxin/Indole-3-Acetic Acid (Aux/IAA) family function as transcriptional regulators and are generally thought to act as repressors of auxin-induced gene expression by interacting with members of the Auxin Response Factor protein family (Abel et al., 1995; Ulmasov et al., 1997).

Six Aux/IAA factor genes were differentially expressed in AtFRh2 whereas none were affected in AtBG11. In AtFRh2, *IAA 1, IAA3, IAA19, IAA29, IAA30* were all downregulated except *IAA6*. This could indicate a low pool of Aux/IAA proteins which is likely to affect positively the transcriptional response of auxin response genes (Ljung, 2013).

Along with the Aux/IAA and the Small Auxin Up RNA (*SAUR*), Gretchenhagen-3 (*GH3)* gene family were rapidly and transiently induced in response to auxin. *GH3* genes encode IAA-amido synthetase which helps to maintain auxin homeostasis by conjugating excess IAA to amino acids (Staswick et al., 2005). Only AtBG11 was characterised by the upregulation of *GH3.2* and *GH 3.3*.

NAC domain transcription factor family: *NAC* gene family have been suggested to play important roles in the regulation of the transcriptional reprogramming associated with plant stress responses.

NAC factor genes were only differentially expressed in AtBG11; NAC domain containing protein 055 coding gene (*ANAC055*) was upregulated whereas the *ANAC074* was downregulated.

The role of ANAC055 in JA signalling was examined along with the ANAC019. Transgenic plants overexpressing the two *NAC* genes showed enhanced JA-induced Vegetative Storage Protein1 (*VSP1*) and Lipoxygenase2 (*LOX2*) expression. Furthermore, the response of the *anac019* and *anac055* double mutant to a necrotrophic fungus showed high similarity to that of the *atmyc2-2* mutant (Tran et al., 2004; Bu et al., 2008). Zheng et al. (2012) showed that, along with ANAC019 and ANAC072, ANAC055 inhibited salicylic acid accumulation. In addition Schweizer et al. (2013) showed that *ANAC055* was involved in the resistance against the caterpillar *Spodoptera littoralis*. *ANAC074* was shown to be upregulated during senescence (Podzimska-Sroka et al., 2015)

Phytohormone signalling-related genes

Involvement of JA, ET and SA in plant-microbe interaction is often characterised by the involvement of specific marker genes. Many genes reported to be related to JA-ET and SA pathways were differentially expressed in AtFRh2 and AtBG11 (Table 3-8 and 3-9). However, only AtBG11 showed upregulation of marker genes of those pathways.

JA-ET pathway-related genes: AtBG11 was characterised with the upregulation of *PDF1.1* and *PDF1.2*. *PDF1.2* is globally used as a marker gene to study JA-ET-mediated defence responses. PDF1.1 has an antifungal activity against a broad range of fungi (Terras et al., 1993) and yeasts (Sels et al., 2007). Overexpression of *PDF1.1* in *Arabidopsis* resulted in an enhanced resistance against the non-host *Cercospora beticola* (De Coninck et al., 2010) In addition, *ORA59*, *ERF1* and *ERF2,* known to integrate JA and ethylene signals to regulate the expression of *PDF1.2* were upregulated in AtBG11 (Pre et al., 2008) whereas *ERF5*, *ERF6* and *ERF104* that induce the expression of *PR* genes (Son et al., 2012; Meng et al., 2013; Wang et al., 2013) were downregulated in AtFRh2. This might explain the no change in the expression of *PDF1.2* in AtFRh2.

In addition, two putative 12-oxophytodienoate (*OPDA*) reductase-encoding genes required for jasmonic acid (JA) biosynthesis (Stintzi et al., 2001), were upregulated in AtBG11. AtFRh2 was characterised by the upregulation of *CYP82C4, CYP82C3* and *CYP82C2*. The *CYP82C2*-overexpressing plants showed enhanced resistance to the fungus *B. cinerea*. This resistance was accompanied by increased expression of JA-induced defence genes and elevated levels of JA-induced IGs (Liu et al., 2010).

There is growing evidence that Glutamate Receptor-Like (*GRL*) genes can stimulate the expression of jasmonate-regulated genes (Kang et al., 2006; Mousavi et al., 2013). Three GLRs (*GLR2.5*, *GLR1.2, GLR1.3*) were upregulated in AtFRh2 whereas only *GLR2.5* was upregulated in AtBG11. *GLR1.2* and *GLR1.3* were shown to be involved in resistance against *Pseudomonas syringe* (Barah et al., 2013) and *GLR2.5* was induced in *Arabidopsis* cell cultures after wounding (Guan & Nothnagel, 2004).

1-Aminocyclopropane-1-carboxylic acid (ACC) synthase is the key regulatory enzyme in the biosynthetic pathway of the plant hormone ethylene. Only AtFRh2 was characterised by the downregulation of *ACS4*, one of the five divergent gene family members encoding this enzyme (Abel et al., 1995), while no change was seen in AtBG11. Ethylene has important roles in plant growth development and regulating plant defence, as described in Chapter 1.

Table 3-8 Overview of expression of differentially expressed genes (DEGs) identified as related to jasmonic-ethylene (JA-ET) pathway related genes in AtFRh2 and AtBG11.
Up and downregulated genes are shown in blue and red respectively. Log FC= log fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

			Log FC	
	ID	Symbols/Description	AtFRh2	AtBG11
JA-ET pathway related genes	at3g28740	CYP81D1		-2.809
	at2g29500		1.937	
	at4g31970	CYP82C2	3.056	
	at3g12580	HSP70	2.385	
	at1g32350	AOX1D	1.725	
	at5g13080	WRKY75	1.403	1.663
	at3g63380		2.004	1.661
	at2g43510	ATTI	1.289	
	at1g17380	JAZ5		1.048
	at5g13220	JAZ10		1.340
	at1g30135	JAZ8		1.898
	at1g80840	WRKY40		1.162
	at5g05410	DREB2A	1.381	
	at1g51760	IAR3		1.092
	at5g11210	GLR2.5	2.371	1.060
	at5g48400	GLR1.2	2.585	
	at5g48410	GLR1.3	1.839	
	at1g18020	12-oxophytodienoate reductase, putative		1.116
	at1g17990	12-oxophytodienoate reductase, putative		1.103
	at4g17500	ERF1		1.234
	at1g06160	ORA59		1.906
	at5g47220	ERF2		1.269
	at5g47230	ERF5	-1.412	
	at4g17490	ERF6	-1.516	
	at5g61600	ERF104	-1.233	
	at1g75830	PDF1.1		1.866
	at5g44420	PDF1.2		1.778
	at2g22810	ASC4	-1.416	
	at3g15500	ANAC055		1.736
	at3g26830	PAD3		1.870
	at2g24850	TAT3		1.790
	at5g05730	ASA1		1.440
	at1g05560	UGT75B2	1.106	
	at1g74590	GST putative	2.042	
	at2g29460	GST putative	1.431	
	at1g15520	PDR12		1.987
	at2g29500	HSP17.6	1.937	
	at1g32350	AOX3	1.725	
	at1g75280	Isoflavone reductase putative		-1.006
	at3g05360	LRR family protein	1.086	
	at3g13610	Oxidoreductase		1.860
	at2g23130	AGP17	-1.033	
	at5g57560	TCH4	-1.812	
	at4g12730	FLA2	-1.626	
	at3g02120	Hyp rich glycoprotein	-1.112	
	at4g38400	EXPL2	-1.132	
	at3g06770	Glycoside hydrolase family 28	-1.192	
	at4g32280	AUX/IAA	-2.120	
	at1g04240	IAA3	-1.408	
	at3g62100	auxin response protein putative	-1.093	

SA pathway-related genes: The SA pathway is a key player in SAR, which depends on the accumulation of SA and the activation via NPR1 and WRKY TFs of *PR* genes such as *PR1* and *PR2* (Robatzek & Somssich, 2002). In addition, Maleck et al. (2000) identified a cluster group of 26 genes termed *PR-1* regulon genes as SAR marker genes. AtBG11 was characterised by the upregulation of seven of this 26 gene regulon including *PR1* and *PR2* marker genes of the SA pathway (Table 3-9). In addition *WRKY6* and *GRX480* (Ndamukong et al., 2007; Jaskiewicz et al., 2011) were upregulated only in AtBG11.

Table 3-9 Overview of expression of differentially expressed genes (DEGs) identified as related to *PR1* regulon (A) and salicylic (SA) pathway (B) in AtFRh2 and AtBG11.
Up and downregulated genes are shown in blue and red respectively. Log FC= log fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

(A)

			Log FC	
	ID	Symbols	AtFRh2	AtBG11
PR1 regulon	at1g02930	ERD11	1.269	
	at2g14560	82C1T7		1.163
	at2g24850	245M18T7		1.790
	at2g26560	111G9T7		1.723
	at3g48100	186J13T7	-1.481	
	at3g49120	PRXCB		1.124
	at2g14610	PR1		1.230
	at3g57260	PR2		1.154
	at4g02380	SAG21		1.132

(B)

			Log FC	
	ID	Symbols	AtFRh2	AtBG11
SA pathway related genes	at3g25882	NIMIN2		1.146
	at1g28480	GRX480		1.018
	at5g22570	WRKY38		1.087
	at1g62300	WRKY6		1.172
	at2g14610	PR1		1.230
	at3g57260	PR2		1.154
	at3g26830	PAD3		1.870
	at4g17500	ERF1		1.234
	at2g23560	MES7	1.490	
	at3g01080	WRKY58	1.110	
	at5g40990	GLIP1	2.970	
	at1g53940	GLIP2	2.110	

Gibberellin pathway-related genes: AtFRh2 was characterised by the downregulation of GA-20-oxidase and GA-3-oxidase coding genes, both of which are known to catalyse the conversion of precursor GAs to their bioactive forms, therefore playing a direct role in plant growth and development (Coles et al., 1999; Mitchum et al., 2006). This wasn't the case in AtBG11.

Referring back to the TFs, AtFRh2 showed induction in TEM1 coding gene. TEM1, homologue of TEM2/RAV2, represses growth by direct binding to the GA biosynthetic genes, *GA3OX1* and *GA3OX2*, leading to a reduction in GA content. Navarro et al. (2008) demonstrated an enhanced resistance against the necrotrophic fungus *Alternaria brassicicola* in *Arabidopsis* mutants blocked in GA signalling. However, *DELLA* mutants, characterised by a constitutive GA signalling, were susceptible.

Oxidative stress-related genes

Oxidative stress is characterised by a rapid generation of reactive oxygen species (ROS) and accumulation of hydrogen peroxide (H_2O_2). Through the production of ROS scavengers such as peroxidase and glutathione-S-transferases plants can cope better under both biotic and abiotic stress.

AtFRh2 was characterised by the upregulation of four peroxidase-related genes, seven glutathione-S-transferases (GST) encoding genes, the inhibitor of apoptosis *TPX2* and α-dioxygenase (*α -DOX*) genes (Table 3-10). It was shown that following pathogen and herbivore attack the expression of *α-DOX* gene is induced. De Leon et al. (2002) suggested that plant α-dioxygenases generate lipid-derived molecules involved in the protection of plant tissues from oxidative stress and cell death.

AtBG11 showed induction in three peroxidase related genes including the *PERX34* involved in PTI (Daudi et al., 2012), two glutathione S transferases (GST) related genes, the *MDHAR* gene, the *OXI1* gene, a *GRX480* gene, a RbohE/ NADPH oxidase coding gene and many germin-like protein (GLP) coding genes (Table 3-10). The germin protein has been identified as an oxalate oxidase and GLPs may be involved in plant defence through the production of H_2O_2 (Carter et al., 1998). The *MDHAR* gene, coding a mono-dehydro-ascorbate reductase, is known as a key regulator gene of the ascorbate-glutathione pathway for ROS detoxification (Guan & Nothnagel, 2004). GRX480, a member of glutaredoxins, mediated redox regulation of proteins by catalysing disulphide transitions and is known as a potential regulator of JA/SA crosstalk. RbohE is a NADPH oxidase know to be the major source of ROS (Bolwell, 1999). Oxidative Signal Inducible 1 (OXI1) is a serine/ threonine kinase induced by H_2O_2 and mediated signalling in *Arabidopsis* roots (Camehl et al., 2011).

Table 3-10 Overview of expression of differentially expressed genes (DEGs) identified as related to oxidative stress in AtFRh2 and AtBG11.
Up and downregulated genes are shown in blue and red respectively. Log FC= log fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

		LogFC	
ID	**Symbol/Description**	**AtFRh2**	**AtBG11**
at3g09940	MDHAR		2.071
at1g28480	GRX480		1.018
at3g49120	PERX34		1.124
at4g36430	peroxidase, putative		2.003
at1g68850	peroxidase, putative		1.142
at5g14130	peroxidase, putative	1.801	
at5g64120	peroxidase, putative	2.187	
at5g24070	peroxidase family protein	1.476	
at1g33660	peroxidase family protein	1.046	
at1g10370	GST30		-1.002
at1g69930	GSTU11	1.232	1.226
at1g69920	GSTU12		1.212
at1g02930	GST1	1.269	
at1g02920	GSTF7	1.047	
at1g74590	GSTU10	2.042	
at2g29450	GSTU5	1.057	
at2g29440	GST24	1.153	
at2g29460	GST22	1.431	
at1g60740	peroxiredoxin type 2, putative	2.464	
at1g65970	TPX2	1.208	
at1g19230	RbohE / NADPH oxidase		1.268
at5g51060	RBOHC NAD(P)H oxidase		1.22
at5g39160	(GLP2a) (GLP5a)		1.488
at5g39190	GER2		1.469
at5g39100	GLP6		1.933
at5g39110	germin-like protein, putative		2.105
at1g18970	GLP4		2.509
at5g39120	germin-like protein, putative		2.889
at5g39180	germin-like protein, putative		2.552
at5g39130	germin-like protein, putative		1.364

Secondary metabolism-related genes

Camalexin pathway: The phytoalexin 3-thiazol-2'-yl-indole produced by *Arabidopsis* can be induced by a variety of biotic factors (Ahuja et al., 2012). The camalexin pathway was induced in both FRh2 and BG11 colonised plants. In AtFRh2, the induction was through the upregulation of *CYP71A12* and *CYP71A13* whereas in AtBG11 camalexin induction was through the upregulation of *CYP71B15* or *PAD3* (Phytoalexin Deficient 3) (Figure 3-8). CYP71A13 catalysed the conversion of indole-3-acetaldoxime to indole-3-acetonitrile (IAN) in the synthesis of the brassicaceae-specific phytoalexin (Nafisi et al., 2007). Phytoalexins are known to be important for resistance to necrotrophic fungal pathogens, such as *A. brassicicola* and *B. cinerea. cyp71A12* mutant *Arabidopsis* plants were more susceptible to *A. brassicicola* and produced low amounts of camalexin after infection by *A. brassicicola or P. syringae* (Millet et al., 2010). CYP71B15 follows CYP71A13 in the camalexin pathway and converts cysteine-indole-3-acetonitrile to camalexin (Böttcher et al., 2009). *pad3* mutants showed enhanced susceptibility to *A. brassicicola* (Thomma et al., 1999) and *B. cinerea* (Ferrari et al., 2003).

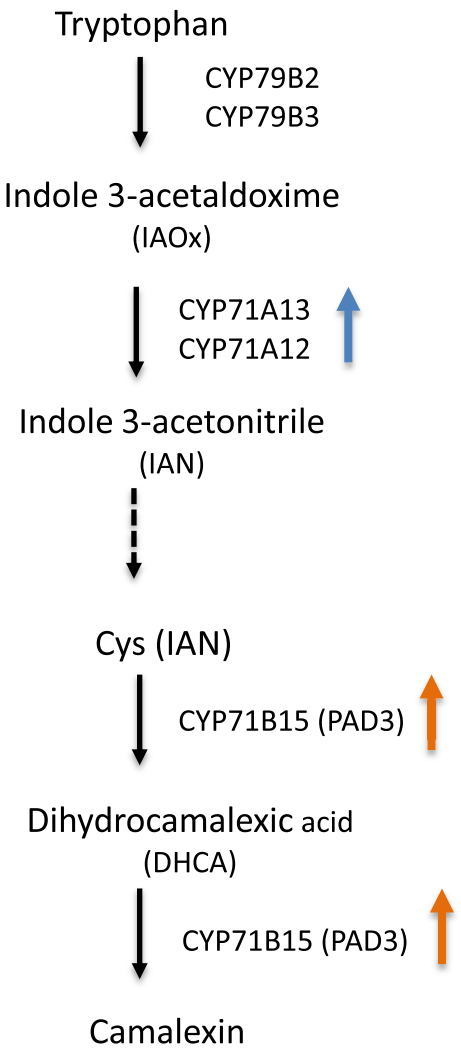


Figure 3-8 Overview of modulation of expression of camalexin pathway-related genes in *Arabidopsis thaliana* following *Beauveria bassiana* colonisation.
Blue and orange arrows indicate FRh2 and BG11 colonisation respectively. Upward pointing arrow indicates upregulated genes. Pathway construction is based on http://biocyc.org/.

Phenylpropanoid pathway: This pathway serves as a starting point for the production of many important compounds, such as lignin, flavonoids and anthocyanin (Fraser & Chapple, 2011). Both FRh2 and BG11 had no effect on the phenylalanine ammonia-lyase (PAL) coding gene that catalysis the first step of the phenylpropanoid pathway. However, the fungi affected many enzymes-coding genes working downstream of PAL (Figure 3-9).

The flavonoid branch: AtFRh2 was characterised by the downregulation of two chalcone-flavanone isomerase (*CHI*) genes whereas the flavanone 3-hydroxylase (*F3H*) and the flavonol synthase (*FLS*) genes were found to be downregulated in AtBG11. Reflecting back to the TFs genes, *MYB11* and *MYB111,* known to control flavonol biosynthesis, were downregulated in AtBG11.

The lignin branch: Only FRh2-colonised plants showed a diverging expression pattern through the upregulation of *CCoAOMT* (Caffeoyl-CoA 3-O-methyltransferase) gene and by the downregulation of *4CL* (4-coumarate: coenzyme A ligase) gene. CCoAOMT is a key enzyme involved in the lignin biosynthesis and lignin reduction was reported in transgenic plants downregulating *CCoAOMT* gene (Li et al., 2013).

Glucosinolates: Only a single gene *CYP79F1* affecting the GS was downregulated in AtBG11. A knock-out of *CYP79F1* known by the *bus1-1* mutation was depleted only in short chain methionine-derived glucosinolates (Reintanz et al., 2001).

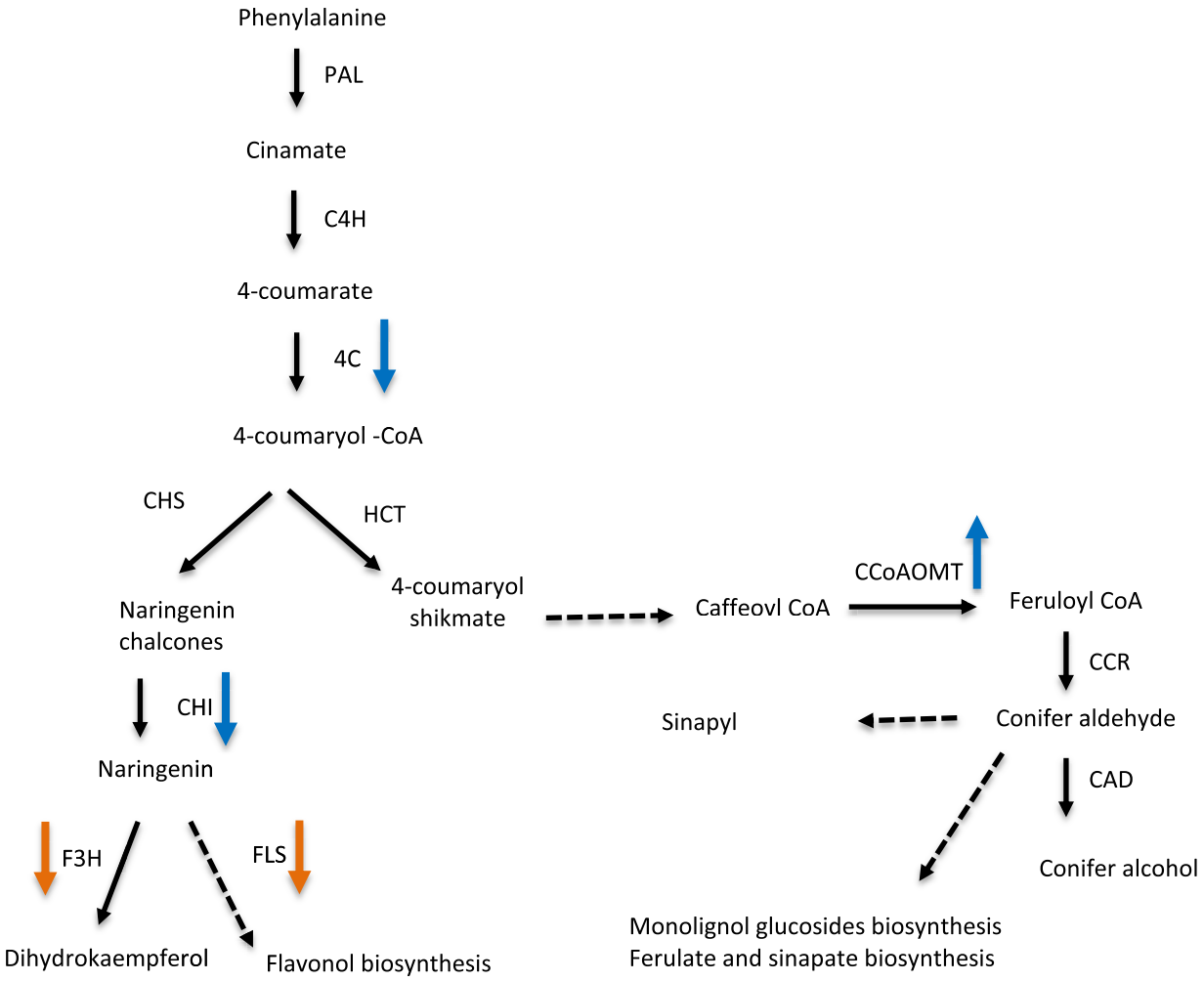


Figure 3-9 Overview of modulation of expression of phenylpropanoid pathway-related genes in *Arabidopsis thaliana* following *Beauveria bassiana* colonisation.
Blue and orange arrows indicate FRh2 and BG11 colonisation respectively. Upward and downward pointing arrow indicates up and downregulated genes respectively. Enzymes in each step: PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4C, ρ-coumaroyl-CoA synthase; CHS, chalcone synthase; CHI, chalcone-flavanone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; HCT, hydroxycinnamoyl-CoA:skimimate/quinate hydroxycinnamoyltransferase; CCoAOMT, caffeoyl-CoA 3-O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase. Pathway construction is based on http://biocyc.org/.

Terpene biosynthesis: Terpene synthases (TPSs) are responsible for the synthesis of the various terpene molecules from the isomeric 5-carbon building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) leading to 5-carbon isoprene, 10-carbon monoterpenes, 15-carbon sesquiterpenes and 20-carbon diterpenes (Chen et al., 2011; Tholl & Lee, 2011).

The terpene synthase 04 gene (*AtTPS04*) was upregulated in AtBG11 whereas the *AtTPS13* was downregulated in AtFRh2 (see Tables F29 and F30 Appendix F). *AtTPS04* expressed in leaves catalyses the formation of (E, E)-geranyllinalool in the biosynthesis of the insect-induced volatile C16-homoterpene (E, E)-4, 8, 12-trimethyltrideca-1, 3, 7, 11-tetraene (TMTT) (Herde et al., 2008; Lee et al., 2010). It was also induced by the bacterial pathogen *P. syringae* (Attaran et al., 2008). *AtTPS13* expressed in roots encode sesquiterpene synthases catalysing the conversion of farnesyl diphosphate to (Z)-γ-bisabolene; a wound inducible compound (Ro et al., 2006).

Cell wall modification genes

Plants cell walls are composed of a variety of polymers, which typically include cellulose, hemicelluloses, pectin and structural proteins. In addition, some cell walls contain lignin, which is a polymer of monolignols derived from the phenylpropanoid pathway.

Cell wall associated genes were downregulated in FRh2 colonised plants. This includes genes encoding proteins involved in hemicellulose biosynthesis, cell wall fortification and cell wall proteins. However, this downregulation wasn't observed with plants colonised with BG11. In addition, two pectin methylesterase (*PME*) genes involved in the crosslinking of fungal and host cell walls (Pelloux et al., 2007) were downregulated in AtFRh2 and a single *PME* was upregulated in AtBG11 (Table 3-11).

AtFRh2 was characterised by the downregulation of arabinogalactan proteins (AGPS) coding genes. In addition many hemicellulose related genes such as UDP-D-glucose, cellulose like synthase (CSL) and xyloglucan endotransglucosylase/hydrolases (XTHs) had diverging expression patterns (Table 3-11). *CYP77A6*, *CYP86A4* and *CYP86A2* were downregulated in AtFRh2. These three P450 type enzyme coding genes were shown to be involved in cutin biosynthesis. Insertional knock-outs *cyp77a6-1* and *cyp77a6-2* were shown to have a strong reduction in cutin content, with no detectable 10, 16-dihydroxypalmitate: major monomer in the cutin of *Arabidopsis* flowers. A T-DNA insertion in *CYP86A4* resulted in a 45-58% reduction of 16-hydroxypalmitate, 10, 16-dihydroxypalmitate, and 1, 16-hexadecanedioicacid in flower cutin (Li-Beisson et al., 2009). The *cyp86A2* mutant showed a reduction in total cutin content and an enhanced expression of *P. syringae* type III genes (Xiao et al., 2004).

Table 3-11 Overview of expression of differentially expressed genes (DEGs) identified as related to cell wall modification genes in AtFRh2 and AtBG11.
Up and downregulated genes are shown in blue and red respectively. Log FC= log fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

		Log FC	
ID	**Symbols/Description**	**AtFRh2**	**AtBG11**
at2g26440	PME		1.131
at4g19420	pectinacetylesterase family protein		1.086
at3g10720	PME	-1.51	
at5g04960	PME	-1.283	
at5g45280	pectinacetylesterase, putative	-1.448	
at5g44480	UDP-glucose 4-epimerase/		1.148
at4g10960	UDP-glucose 4-epimerase	-1.146	
at3g28180	ATCSLC04		1.084
at4g07960	ATCSLC12	-1.273	
at3g28180	ATCSLC04	-1.064	
at4g16590	ATCSLA01	1.981	
at1g23480	ATCSLA03	-1.102	
at4g15290	ATCSLB05	1.132	
at4g15320	ATCSLB06	1.031	
at2g03210	FUT2	1.556	
at5g22940	F8H	-1.334	
at1g35230	AGP5		1.045
at2g23130	AGP17	-1.033	
at2g22470	AGP2	1.316	
at1g03870	FLA9	-1.252	
at2g47930	AGP26	-1.239	
at4g37450	AGP18	-1.246	
at2g04780	FLA7	-1.242	
at1g55330	AGP21	-1.186	
at5g65390	AGP7	-1.383	
at2g14890	AGP9	-1.046	
at5g53250	AGP22	-1.219	
at4g12730	FLA2	-1.626	
at5g10430	AGP4	-1.657	
at4g29240	leucine-rich repeat family protein		-1.104
at4g13340	leucine-rich repeat family protein	-1.356	
at4g18670	protein binding	-1.429	
at1g21310	ATEXT3	1.694	1.312
at5g62150	LysM domain-containing protein	1.331	1.141

Table 3-11 Overview of expression of differentially expressed genes (DEGs) identified as related to cell wall modification genes in AtFRh2 and AtBG11 (continued).
Up- and downregulated genes are shown in blue and red respectively. Log FC= log fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.Continued.

		Log FC	
ID	**Symbols/Description**	**AtFRh2**	**AtBG11**
at5g49360	BXL1		1.02
at1g02640	BXL2	-1.5	
at4g33830	catalytic/ cation binding / hydrolase	-1.442	
at5g48900	pectate lyase family protein	-1.366	
at3g07010	pectate lyase family protein	-1.229	
at3g06770	glycoside hydrolase family 28 protein	-1.192	
at4g23820	glycoside hydrolase family 28 protein	-1.298	
at5g57540	xyloglucosyl transferase, putative	-1.175	
at5g48070	ATXTH20	-1.214	
at2g18660	EXLB3	1.166	1.178
at2g14620	xyloglucosyl transferase, putative		1.834
at5g57530	xyloglucosyl transferase, putative		1.61
at4g18990	xyloglucosyl transferase, putative		1.33
at1g11545	xyloglucosyl transferase, putative	-1.692	
at2g06850	EXGT-A1, EXT	-1.926	
at1g20190	ATEXPA11	-1.636	
at5g57560	TCH4, XTH22	-1.812	
at4g38400	ATEXLA2	-1.132	
at4g30280	ATXTH18	-2.036	
at4g30290	ATXTH19	-2.098	
at1g65310	ATXTH17	-2.215	

3.3.4.2 Abiotic stress-related DEGs

FRh2 colonisation showed a high impact on the expression of different abiotic stress associated genes highlighting a potential protective role of FRh2 against major abiotic stress such as drought/salt, heat and phosphate homeostasis stresses (Table 3-12).

Drought/salt tolerance-related genes: Among the differentially expressed P450 enzyme-coding genes, *CYP707A3* was downregulated in AtFRh2. Mutant analyses have shown that disruption in *CYP707A3* resulted in more drought tolerance, whereas overexpression resulted in an increased transpiration rate and reduced drought tolerance (Umezawa et al., 2006).

In addition, FRh2 colonisation potentially enhanced drought tolerance by the upregulation of *WRKY63*, *RAP2.6L* and by the downregulation of *RAV2* known to be involved in defence against abiotic stress.

Heat tolerance-related genes: Functioning as molecular chaperones, heat shock proteins (HSPs) are responsible for protein folding, assembly, translocation and degradation under stress conditions including pattern recognition receptors (PRRs) and resistance (R) proteins involved in plant immune system (Park & Seo, 2015). Seventeen heat shock protein (HSP) coding genes related to HSP100, HSP90, HSP70, and sHSP families were upregulated in AtFRh2. Among AtFRh2 upregulated genes, the upregulated *HSP101, HSP81-1, HSP81-2* and *HSP70* have been found to play a role in thermotolerance in *Arabidopsis* (Takahashi et al., 1992; Queitsch et al., 2000). In addition the *MBF1c* gene, a key regulator of thermotolerance in *Arabidopsis*, was upregulated in AtFRh2 (Suzuki et al., 2008).

In addition, AtFRh2 and AtBG11 were characterised by the upregulation of *BiP 1, BiP2* and *BiP3* respectively. BiP are ER members of the Hsp70 family of chaperones (Park & Seo, 2015). Pattern recognition receptors (PRRs) that can initiate PTI are known to undergo the so-called endoplasmic reticulum quality control (ER-QC). Although ER-QC isn't fully understood in plants its initiation relies on the activity of BiP (Li et al., 2009).

Phosphate (Pi) homeostasis-related genes: AtFRh2 was characterised by the upregulation of a single SPX domain gene (*AtSPX3*). Proteins containing the SPX domain control a set of processes involved in Pi homeostasis. Duan et al. (2008) reported that the repression of *AtSPX3* alters the response of Pi-starvation induced (*PSI*) genes to Pi starvation and aggravate phosphate-deficiency symptoms. In addition, *WRKY75*, upregulated in AtFRh2 and AtBG11, modulates Pi starvation. Suppression of *WRKY75* expression resulted in plants more susceptible to Pi stress (Devaiah et al., 2007).

Table 3-12 Overview of expression of differentially expressed genes (DEGs) identified as related to abiotic stress in AtFRh2 and AtBG11.
Up and downregulated genes are shown in blue and red respectively. Log FC= log fold change >1 and <-1. AtFRh2 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtFRh2 and AtBG11 = DEGs resulting from the comparative transcriptome study for AtCO_vs_AtBG11.

		Log FC	
ID	**Symbol/Description**	**AtFRh2**	**AtBG11**
at5g45340	CYP707A3	-2.455	
at2g45130	SPX3	1.613	
at3g01420	ALPHA-DOX1	3.756	
at4g11650	ATOSM34	1.572	
at1g09080	BIP3		1.205
at5g42020	BIP2	1.045	
at5g28540	BIP1	1.03	
at5g51440	HSP23.5-M	3.004	
at1g07400	HSP17.8-CI	2.433	
at5g37670	HSP15.7-CI	1.098	
at3g09440	HSP70-3	1.527	
at3g12580	HSP70	2.385	
at2g32120	HSP70T-2	1.001	
at2g29500	HSP17.6B-CI	1.937	
at5g56030	HSP81-2	1.431	
at1g59860	HSP17.6A-CI	1.992	
at5g56010	HSP81-3	1.329	
at5g56000	HSP81-4	1.201	
at5g12030	HSP17.6A	2.619	
at1g74310	HSP101	2.098	
at4g25200	HSP23.6-MITO	3.377	
at5g09590	HSC70-5	1.152	
at5g52640	HSP81-1	2.543	
at2g25140	HSP98.7	1.198	
at1g66600	WRKY63	1.568	
at5g13080	WRKY75	1.403	
at1g68840	RAV2, RAP2.8, TEM2	-1.48	
at5g05410	DREB2A, DREB2	1.381	
at5g13330	Rap2.6L	1.456	
at3g24500	MBF1C	2.16	
at5g47600	heat shock protein-related		-1.268
at1g44160	DNAJ chaperone		-1.934
at3g54510	ERD protein-related		-1.813
at4g21850	ATMSRB9		1.235
at5g39160	GLP2a		1.488
at5g39100	GLP6		1.933
at1g18970	GLP4		2.509
at1g72610	GLP1	-1.29	
at5g39120	germin-like protein, putative		2.889
at5g39180	germin-like protein, putative		2.552
at5g39130	germin-like protein, putative		1.364

3.3.5 Microarray validation and effects of *Beauveria bassiana* on *Arabidopsis thaliana* defence signalling pathways

A set of seven genes were selected to validate the microarray results using qPCR. The seven genes were related to different defence responses and identified as differentially expressed in the AtFRh2 transcriptome data. Their expression levels were measured by qPCR under the same condition and time point as used in the microarray analysis. Three biological replicates independent from the ones used in the microarray were used to compare the pattern of expression of the selected genes between the microarray and qPCR data.

All seven genes followed the same pattern of expression shown by the microarray data. Averaging the expression level of each gene over the three biological replicates and over the three reference genes showed that *AXR5, ACS4* and *ARR1* expression was downregulated whereas *GLIP1*, chitinase gene, *MYB122* and *WRKY63* expression was upregulated as suggested by the microarray data (Figure 3-10).

The relative expression calculation and the Ct values can be viewed in Tables G3 and G4-G6 Appendix G respectively.

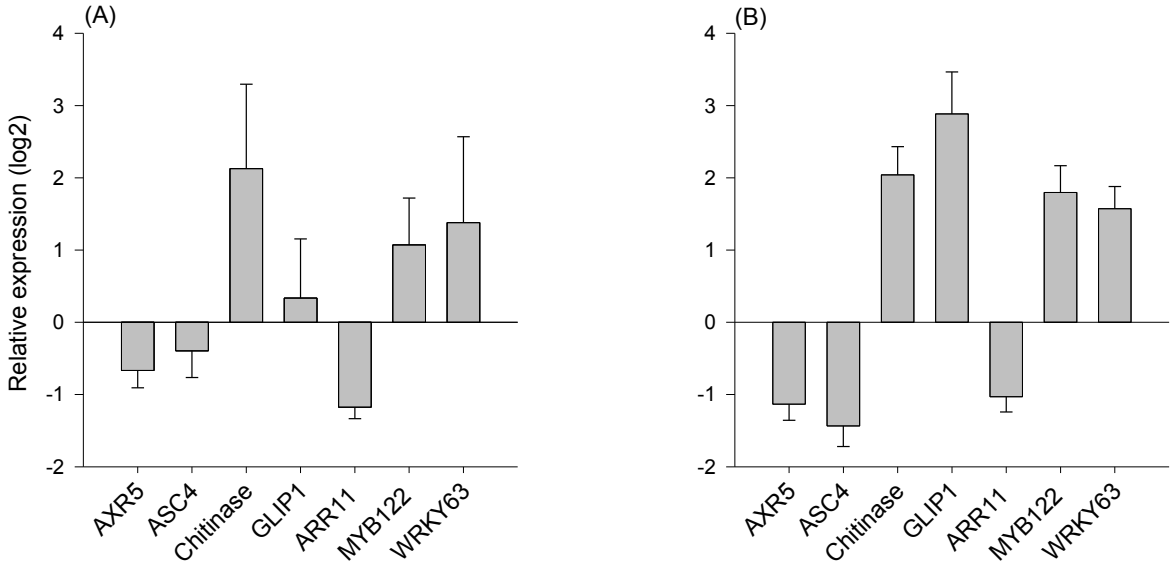


Figure 3-10 Validation of microarray analysis and comparison for relative expression of *AXR5, ACS4*, *chitinase, GLIP1, ARR1, MYB122* and *WRKY63* by quantitative polymerase chain reaction-qPCR (A) and by microarray (B).
qPCR data presented as log2 relative levels of gene expression calculated over three biological replicates and three reference genes using the $2^{-\Delta\Delta CT}$ method. Microarray data for expression level are calculated over four biological replicates and presented as log fold change >1 and <-1. Error bars present standard errors of the mean.

3.4 Conclusions: Interpretation of transcriptome analyses

Genome expression analysis of *Arabidopsis* provided evidence for the transcriptional reprogramming of plant defence pathways following *B. bassiana* colonisation.

This genome-wide analysis for *Arabidopsis* following colonisation by *B. bassiana* strains FRh2 and BG11 and its subsequent validation for FRh2 colonised plants with qPCR demonstrated that application of *B. bassiana* can enhance various defence pathways in the plant. In addition, the discrepancies in FRh2 and BG11 effects on *Arabidopsis* hormone pathways and resistance against abiotic stress showed that not all *B. bassiana* associations with plants behave in the same manner with regards to their effects on the plant host. These results showed that plant responses to *B. bassiana* colonisation resemble those of a symbiotic endophyte and aligned with many reports on *Arabidopsis* interaction with other PGPF such as *Trichoderma* spp. and *P. indica,* known roots endophytes.

Beauveria bassiana* strains BG11 and FRh2 trigger MTI defence in *Arabidopsis

Microbial-associated molecular pattern (MAMP)-triggered immunity (MTI), the first line of defence in plants, is initiated by the recognition of primary pathogen-derived elicitors such as chitin, flagellin, glycoproteins and lipopolysaccharides by plant transmembrane pattern recognition receptors (PRRS) (Yang et al., 1997). This recognition leads to the activation of what is called basal resistance or innate immune response. The transcriptomic data and subsequently the GO analysis showed that *B. bassiana* strains BG11 and FRh2 can trigger MTI in *Arabidopsis*. A significant upregulation of chitinase encoding genes and induction of the DEGs categories “response to innate immune response”, ”chitin metabolic/catabolic process”, “positive regulation of innate immune response”, “response to chitin” for both AtFRh2 and AtBG11 was observed. As might be expected, this suggests that *Beauveria* MAMPs can be chitin related. In their work on *Trichoderma* spp.-*Arabidopsis* interaction, Mathys et al. (2012) and Brotman et al. (2013) reported responses to chitin as one of the significantly induced biological processes. Hermosa et al. (2012) discussed that chitin can act as elicitors of defence responses in plants and chitinases can trigger defence responses by releasing the active polymers from the cell walls of invading fungi. Moreover, AtFRh2 and AtBG11 transcriptome data revealed the induction of a number of receptor-like kinase and proteinase genes including a LysM domain-containing protein gene. Recently, a lysine motif receptor-like kinase CERKI was identified in *Arabidopsis* as a chitin receptor for *Trichoderma* recognition (Iizasa et al., 2010; Petutschnig et al., 2010). In addition to CERK1, CERK4 was identified in *P. indica* recognition (Nongbri et al., 2012) and *cerk1* mutants were over-colonised by *P. indica* (Jacobs et al., 2011), indicating its involvement in restricting root colonisation by this plant growth promoting fungus.

It is known that endoplasmic reticulum quality control (ER-QC) for pattern recognition receptor accumulation is required for plant innate immunity (Li et al., 2009). AtFRh2 transcriptome data showed an induction of *BiP* genes involved in the first system of this QC as reflected in the GO analysis by the induction of "protein folding". Mathys et al. (2012) showed that *T. hamatum* T382 induced MAMP defence involved induction of protein folding. Additionally, the increased Ca^{2+} influx in the cell through the calcium-transporting ATPase and glutamate binding receptors as reflected in the GO analysis by the induction of ""calcium ion homeostasis" and "cellular calcium ion homeostasis" suggested the involvement of AtFRh2 in MTI.

Both AtFRh2 and AtBG11 data showed the induction of many NB-LRRs genes indicating that *B. bassiana* colonisation can affect plant defence through increasing effector-triggered immunity (ETI). MAMP recognition often activates mitogen-activated protein kinase cascades that mediate a wide range of responses that connect to other signalling molecules such as hormones. Interestingly, the data showed no significant effect on MAPK cascades related genes contrary to what was reported with some *Trichoderma* spp. (Shoresh et al., 2010; Mathys et al., 2012).

Beauveria bassiana* strains BG11 and FRh2 control ROS damage in *Arabidopsis

Oxidative burst is a hallmark of successful recognition of plant pathogens, characterised by the rapid generation of ROS and the accumulation of H_2O_2 (Lamb & Dixon, 1997). ROS accumulation is usually accompanied by host cell death and as a signalling molecule can induce a MAPK cascade eliciting a defence response (Hancock et al., 2002). The transcriptome data for AtBG11 and AtFRh2 indicated an induction of multiple ROS scavenger encoding genes such as peroxidase, glutathione-S-transferases and protective enzymes against oxidative stress and cell death. Comparable results were reported for the *T. hamatum* T382-*Arabidopsis* interaction (Mathys et al., 2012). Similarly, Salas-Marina et al. (2011) reported an induction in peroxidase expression in both root and leaf after treatment of *Arabidopsis* with *T. atroviride.* AtBG11 transcriptomic data showed an induction in the key regulator gene of the ascorbate-glutathione pathway, the mono-dehydro-ascorbate reductase *MHDAR* gene. A comparable result was reported for *T. hamatum* T382-*Arabidopsis* leaves interaction and in roots of both *Arabidopsis* and cucumber after inoculation with *T. asperelloides* T203 (Mathys et al., 2012; Brotman et al., 2013). Interestingly *MHDAR* was shown to be crucial for the interaction between *Arabidopsis* roots and *P. indica* (Vadassery et al., 2009). Waller et al. (2005) reported that *P. indica* infested barely had enhanced antioxidant capacity and an activated glutathione-ascorbate cycle. Additionally to the *MHDAR* gene, AtBG11 was characterised with the upregulation of *OXI1*; another enzyme linked to the interaction with endophytes. Mathys et al. (2012) reported the induction of *OXI1* by *T. hamatum* T382. Through a genetic screen of *Arabidopsis* which did not show a *P. indica*-

induced growth response Camehl et al. (2011) showed that the OXI1 pathway seems to influence plant growth promotion by the endophyte *P. indica*.

Beauveria bassiana strain BG11 induces salicylic and jasmonic-ethylene pathways in _Arabidopsis_

AtBG11 data suggested an activation of the SA-pathway as reflected in the GO analysis by the induction of "response to salicylic acid", "systemic acquired resistance (SAR)" and by the induction of SA marker genes such as *PR1, PR2, GRX48* and *WRKY6* (Maleck et al., 2000; Robatzek & Somssich, 2002; Ndamukong et al., 2007). The SAR pathway is usually associated with a hypersensitive response (HR) resulting in rapid cell death (Ryals et al., 1996). This was also reflected in the GO analysis showing the induction of "cell death" associated genes. Additionally, the GO analysis of BG11 colonised *Arabidopsis* showed induction of "response to jasmonic acid stimulus", "response to ethylene stimulus", "ethylene mediated signalling pathway" and "jasmonic acid mediated signalling pathway" associated genes. Furthermore, the induction of *PDF1.2, ORA 59, ERF1, ERF2* and *WRKY 40* confirmed the involvement of JA and ET pathways in the BG11-*Arabidopsis* interaction (Pre et al., 2008). This aligns with many reports of the capability of *Trichoderma* spp. to activate both SA and JA-ET pathways. Mathys et al. (2012) investigated *T. hamatum* T382-*Arabidopsis* leaf interaction after 48 hours of root inoculation. They reported an induction in the SA pathway whereas response to JA and ET were not affected. Their GO analysis indicated an induction of 'SAR' and 'regulation of SAR'. This was further highlighted by the induction of *WRKY6, PR1, PR2 and PR5* genes belonging to the SA pathway. The Salas-Marina et al. (2011) study showed an increased expression of SA-inducible *PR* genes (*PR1* and *PR2*) and *PDF1.2*, the marker gene for JA-ET-mediated signalling in *Arabidopsis* after treatment with *T. atroviride,* both locally in roots and systemically in leaves. Yoshioka et al. (2012) reported a significant increase in the expression of both SA-inducible genes *PR1, PR2* and *PR5* and JA-ET inducible genes, such as *PDF1.2a*, in *Arabidopsis* leaves after root treatment with *T. asperellum* SKT-1 Likewise, Brotman et al. (2013) reported a role for JA signalling, in the roots 24 hours after *T. asperelloides* inoculation, by showing an enhanced expression of *WKRY18* and *WRKY40*, which stimulate JA signalling via suppression of JAZ repressors.

Despite AtFRh2 showing induction in the expression of jasmonate-ethylene-regulated genes, FRh2 colonisation did not affect the expression of marker genes such as *PDF 1.2* contrary to BG11. This might be due to the downregulation of *ERF5, ERF6* and *ERF104* (Son et al., 2012; Meng et al., 2013; Wang et al., 2013). Similarly, AtFRh2 showed no effect on SA-pathway marker genes. The unaltered expression of SA and JA-ET markers does not exclude the involvement of both pathways in the FRh2-triggered defence and/or resistance. Although none of the JA-pathway marker genes were induced, Mathys et al. (2012) showed through mutant analyses the involvement of JA-pathway in *T. hamatum* T382-*Arabidopsis* interaction. Interestingly, (Van Wees et al., 1999) reported that a maximum level of

induced resistance against *P. syringae* pv. tomato DC3000 was achieved at five- to 100-fold lower concentrations of methyl jasmonate (MeJA) and the ethylene precursor 1-aminocyclopro- pane-1-carboxylate (ACC) than needed for the induction of the expression of the marker genes. Thus, resistance can be also elicited in the absence of detectable changes in the expression of SA and JA-ET marker genes.

Surprisingly, AtFRh2 showed a repression in ethylene pathway as reflected in the GO analysis for the downregulated process with "response to ethylene stimulus" and "ethylene mediated signalling pathway". In addition the downregulation of *ACS4*, the key regulatory enzyme of ethylene biosynthesis, might add to the repression of this pathway. It is well established that some plant growth-promoting bacteria and fungi (Viterbo et al., 2010) can alter ethylene production by the production 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCD). ACCD cleaves ACC, the immediate precursor of the plant hormone ethylene (Todorovic & Glick, 2008) protecting plants against a wide range of environmental stresses such as extremes of temperature, drought or high salt concentrations. This might explain the role of *B. bassiana* strain FRh2 in enhancing plant defence against abiotic stress as reflected in the GO analysis by the induction of "response to abiotic stimulus, to heat, to temperature stimulus, to hydrogen peroxide and to light stimulus" and by the induction of many genes related to heat and drought-salt stress. Analysis of integrated microbial genomes metagenomics database suggested that among domain Eukarya, the ACCD gene is prevalent in members of phylum Ascomycota (including *B. bassiana)* and Basidiomycota (Singh et al., 2015). Thus it remains to test the FRh2 strain for the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Additionally, the GA pathway was repressed in AtFRh2 as reflected in GO analysis for the downregulated process with "response to gibberellin stimulus" and "gibberellin mediated signalling pathway". Similar repression was reported in *P. indica* infested barley (Schafer et al., 2009). Jacobs et al. (2011) reported that *P. indica* recruits GA signalling to help establish proapoptotic root cell colonisation. A *quintuple-DELLA* mutant and the GA biosynthesis mutant showed higher and lower degrees of colonisation, respectively.

Further investigation, such as mutant analysis, is required to determine the involvement of the hormonal signalling pathway in *B. bassiana* induced defence and/or resistance.

Beauveria bassiana* strains BG11 and FRh2 induce camalexin pathway genes in *Arabidopsis

In *Arabidopsis* MTI relies additionally on the accumulation of antimicrobial camalexin, the most abundant phytoalexin in *Arabidopsis* (Glawischnig, 2007). Both *B. bassiana* strains FRh2 and BG11 induce camalexin biosynthesis pathway via the induction of *CYP71A12/13* and *CYP71B15* (*PAD3*) respectively. Similarly, Salas-Marina et al. (2011) reported an upregulation in *PAD3* expression in both root and leaves after treatment of *Arabidopsis* with *T. atroviride.* Contreras-Cornejo et al. (2011) found that *Arabidopsis* seedlings colonised by *T. virens* and *T. atroviride* accumulate camalexin in their leaves.

On contrast to camalexin, the AtFRh2 and AtBG11 transcriptomic data showed the absence of induction in the phenylalanine ammonia-lyase (PAL) coding gene. PAL catalyses the first step of the phenylpropanoid pathway (another secondary metabolite pathway which can lead to a number of secondary metabolite products). This is the same as Mathys et al. (2012) and Martinez-Medina et al. (2013) who reported they did not observe an increase in the expression of *PAL* in *Arabidopsis* leaves after colonisation with *T. hamatum* T382 and in tomato leaves after colonisation with *T. harzianum* T78 respectively. However, Brotman et al. (2013) found an increased expression of several genes involved in the phenylpropanoid pathway, such as *PAL1, PAL2* and *4CL*, in *Arabidopsis* roots after inoculation with *T. asperelloides* T203.

It remains to be tested if this induction in various defence pathways is actually accompanied with an increase in the production of defence molecules. Thus, *B. bassiana* colonisation effects on secondary metabolites are discussed in the following chapter. The focus was given to SA, JA and glucosinolates. In addition, the following chapter reports on assessment of their production levels in a tripartite interaction between *Arabidopsis* -*B. bassiana* and the herbivore *P. xylostella*.

Chapter 4

Beauveria bassiana colonisation effects on *Arabidopsis thaliana* secondary metabolism

4.1 Introduction

Arabidopsis thaliana developed an enhanced defence capacity when colonised by *B. bassiana* through the induction of different defence-related pathways. This defence was putatively more aligned to endophyte-induced defence against other organisms than a defence against a plant pathogen, suggesting that *B. bassiana* could enhance resistance to other threats to the plant.

Recently, it was shown that major changes in the expression of defence and biosynthesis of secondary metabolites genes were only visible in plants inoculated with ISR-inducing beneficial microorganisms and upon herbivore or pathogen challenge (Cordier et al., 1998; Pozo et al., 2002). Molitor et al. (2011) proved that plants colonised with *Piriformospora indica* were more resistance to the biotrophic leaf pathogen *Blumeria graminis* compared to those with non-*P. indica* plants. In Molitor et al. (2011) study *P. indica*-colonised plant showed higher expression level of 22 transcripts including those of pathogenesis- related genes and genes encoding heat-shock proteins only after *B. graminis* inoculation. Moreover, Mathys et al. (2012) showed that activation of genes involved in the biosynthesis of JA was only observed when ISR-inducing plants were challenged by the plant pathogen *B. cinerea.*

Thus, to further investigate the role of induced SA and JA signalling pathways in *B. bassiana* colonised *Arabidopsis*, the level of these signalling molecules were determined in *Arabidopsis* upon colonisation by *B. bassiana* . Since many studies showed that extensive changes in the expression of defence and biosynthesis of secondary metabolites genes were visible only when colonised plants where challenged, the level of SA and JA were also measured in *B. bassiana* inoculated plants challenged with the Brassicaeae specialist leaf-chewing caterpillar *P. xylostella*.

Similar to SA and JA, glucosinolates, the most intensively studied secondary metabolite of *Arabidopsis* (Sonderby et al., 2010), were also measured in colonised and *P. xylostella* challenged plants.

4.2 Materials and Methods

4.2.1 Quantification of salicylic acid (SA) and jasmonic acid (JA)

Salicylic acid and jasmonic acid levels were measured in inoculated and mock-inoculated *Arabidopsis thaliana* leaves with and without herbivore damage. The experiment was carried out in a randomised block design with six treatments: control plants (C), herbivore challenged plants (H), FRh2-inoculated plants (F), FRh2-inoculated and herbivore challenged plants (FH), BG11-inoculated plants (B), BG11-inoculated and herbivore challenged plants (BH). Each treatment consisted of six independent replicates (plants) resulting in a total of 36 replicates.

4.2.1.1 Plant inoculation

Five-week-old *Arabidopsis* roots were dipped in *B. bassiana* conidia suspension (final concentration of 1 x 10^8 conidia per ml) as described in 3.2.1.

4.2.1.2 Herbivore challenge

Fifteen days post inoculation, *B. bassiana* inoculated and mock-inoculated plants were challenged with third instar *P. xylostella*. Using a fine camel hair brush, a total of ten caterpillars were transferred onto fully expanded leaves of each plant and caterpillars were allowed to feed on the plants for 24 hours. Caterpillars were then carefully removed and *Arabidopsis* leaves were detached, immediately frozen and pulverized in liquid N_2 using mortar and pestle.

4.2.1.3 Salicylic acid (SA) and jasmonic acid (JA) measurement

Determination of SA and JA levels in *Arabidopsis* leaves was performed by vapour-phase extraction and subsequent gas chromatography-mass spectrometry (GC-MS) analysis as described by Schmelz et al. (2004).

Homogenised plant tissue (150 mg) was further pulverised using a FastPrep®-24 System (MP Biomedicals, USA) with the FastRNA®Pro Green Kit after addition of 300 µl of 70 ^oC preheated extraction buffer (water:1-propanole:HCl 1:2:0.005), 1 ml of methylene chloride and 10 µl/mg of each of the two internal standards: D6-salicylic acid (CDN Isotopes, Quebec, Canada) and dihydrojasmonic acid (TCI America, USA). Following extraction, the mixture was centrifuged at 13,000 g for 30 s for phase separation. The lower, organic phase was then treated with 2 M trimethylsilyldiazomethane in hexane (Sigma-Aldrich) for 5 min at room temperature to convert carboxylic acids into their corresponding methyl esters. After stopping the methylation reaction with 2-M acetic acid in hexane, the sample was subjected to a vapour-phase extraction procedure using a volatile collection trap packed with Super-Q absorbent (Analytical Research Systems, FL, USA). The

final evaporation temperature was set to 200 °C and the sample was eluted from the collection trap with methylene chloride.

The sample mixtures were separated using a Shimadzu GC-MS-QP2010 gas chromatograph-mass spectrometrer fitted with a Restek Rxi-1ms fused silica capillary column (30.0 m x 0.25 mm i.d. x 0.25 µm, Bellefonte). Three µl of each sample was injected in pulsed splitless mode at a temperature of 220 ^{o}C and with a pulse of 168 KPa for 40 s. Oven temperature was held at 50 ^{o}C for 3 min and then raised to 320 ^{o}C at 8 ^{o}C min^{-1} and held at this temperature for 8 min. Helium was used as carrier gas at a constant flux of 1.5 ml min^{-1}. All mass spectra were recorded in selected ion monitoring (SIM) mode to increase the detector's sensitivity (Table 4-1).

The recorded spectra were analysed using GCMS Postrun in LabSolutions, Version 2.5 (Shimadzu Corporation, Japan). The peaks of the quantifying ions for the two compounds of interest and for the two internal standards, respectively, were integrated, and JA as well as SA contents were calculated according the following formula:

$$leaf\ content\ (ng\ g^{-1}\ FW) = \frac{area_{m/z}\ (compound)\ X\ 200\ X\ correction\ factor}{area_{m/z}\ (internal\ standard)\ X\ FW}$$

Table 4-1 Characteristic ions of the compounds as detected in GC-MS.

Compound (Methyl ester)	Retention time (min)	Quantifying ion (m/z)	Confirming ion (m/z)	Correction factor
D6-salicylic acid	11.28	124	156	
Salicylic acid	11.30	120	92,152	1.1
Jasmonic acid	18.57	151	156,193,224	3.5
Dihydrojasmonic acid	18.69	153	156,195	

4.2.2 Glucosinolate analyses

Glucosinolate levels were measured in inoculated and mock-inoculated *Arabidopsis* leaves with and without herbivore damage. The experiment followed the SA and JA quantification experiment design with six treatments: control plants (C), herbivore challenged plants (H), FRh2-inoculated plants (F), FRh2-inoculated and herbivore challenged plants (FH), BG11-inoculated plants (B), BG11-inoculated and herbivore challenged plants (BH). Each treatment consisted of six independent replicates (plants) resulting in a total of 36 replicates.

4.2.2.1 Plant inoculation

Five-week-old *Arabidopsis* roots were dipped in *B. bassiana* conidia suspension (final concentration of 1 x 10^8 conidia per ml) as described in 3.2.1.

4.2.2.2 Herbivore challenge

Fifteen days post inoculation, *B. bassiana* inoculated and mock-inoculated plants were challenged with third instars *P. xylostella*. Using a fine camel hair brush, a total of ten caterpillars were transferred onto fully expanded leaves of each plant and caterpillars were allowed to feed on plants for 24 hours. Caterpillars were then carefully removed from *Arabidopsis* leaves.

4.2.2.3 Glucosinolates measurement

To obtain optimal glucosinolate induction, plants were harvested one day after herbivore feeding (Mewis et al., 2005). Leaves were frozen in liquid N_2 and kept at -80 ^oC. Frozen plant leaves were then freeze-dried for four days. Freeze dried plant samples were transferred into 2 ml microcentrifuge tubes containing 2.5 mm zirconia/silica beads (dnature, NZ) and were further pulverised using a bead mill (TissueLyser II, Qiagene) for 1 min. Ten mg of dry weight of each sample were sent to the lab of Prof. Caroline Müller (Bielefeld University, Germany) for glucosinolate measurement. Dried samples were extracted three times in 80% methanol, adding 2-propenyl glucosinolate (Phytoplan, Heidelberg, Germany) as internal standard at the first extraction. A sulfatase was used to convert glucosinolates to desulfoglucosinolates which were then analysed by high performance liquid chromatography (HPLC) coupled to a DAD detector (HPLC-1200 Series, Agilent Technologies, Inc., Santa Clara, CA, USA) as described by Abdalsamee and Muller (2012). Desulfoglucosinolate separation was performed on a reverse phase Supelcosil LC 18 column (3 µm, 150×3 mm, Supelco, Bellefonte, PA, USA) using a gradient of water to methanol, starting at 5% methanol for 6 min, and increasing from 5 to 95% methanol within 13 min with a hold at 95% for 2 min. Metabolites were identified by comparison of retention times and UV spectra to purified standards (Phytoplan, Heidelberg, Germany; Glucosinolates.com, Copenhagen, Denmark) or by

confirming the identities by ultra-HPLC coupled with a time of flight mass spectrometer (1290 Infinity UHPLC and 6210 TOF-MS Agilent, Technologies, Santa Clara, CA, USA). Desulfoglucosinolates were quantified using the integrated area at 229 nm, applying the response factors as in Brown et al. (2003).

4.2.3 Statistical analyses

Data from JA and glucosinolate measurements were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD and Fisher's least significant difference (LSD) post hoc tests. To meet assumptions of normality and homogeneity of variance, SA, 4-hydroxyindol-3-ylmethyl-GLS (4OHI3M) and 5-methylsulfinylpentyl-GLS (5MSOP) data were analysed using the nonparametric Kruskal-Wallis test followed by a Median test. Statistical analyses were performed using IBM ® SPSS statistics 22 and Statistica 13 software. Graphs were generated using SigmaPlot 13.0.

4.3 Results

4.3.1 Salicylic acid (SA) and jasmonic acid (JA) measurement

For the six distinct treatments, no significant differences ($P > 0.05$) were found in SA contents (Kruskal-Wallis Test, chi-square = 4.976, d.f. = 5, $P = 0.419$, Figure 4-1). JA levels at 24 hours post infestation showed a clear herbivore dependent-response in *Arabidopsis*. The highest levels of JA were observed for plants challenged with *P. xylostella* irrespective of *B. bassiana* colonisation (one-way ANOVA, d.f. = 5, $F = 3.044$, $P = 0.026$, Figure 4-1).

JA and SA levels for each treatment are shown in Table D4 Appendix D and the statistical analyses results in E4 Appendix E.

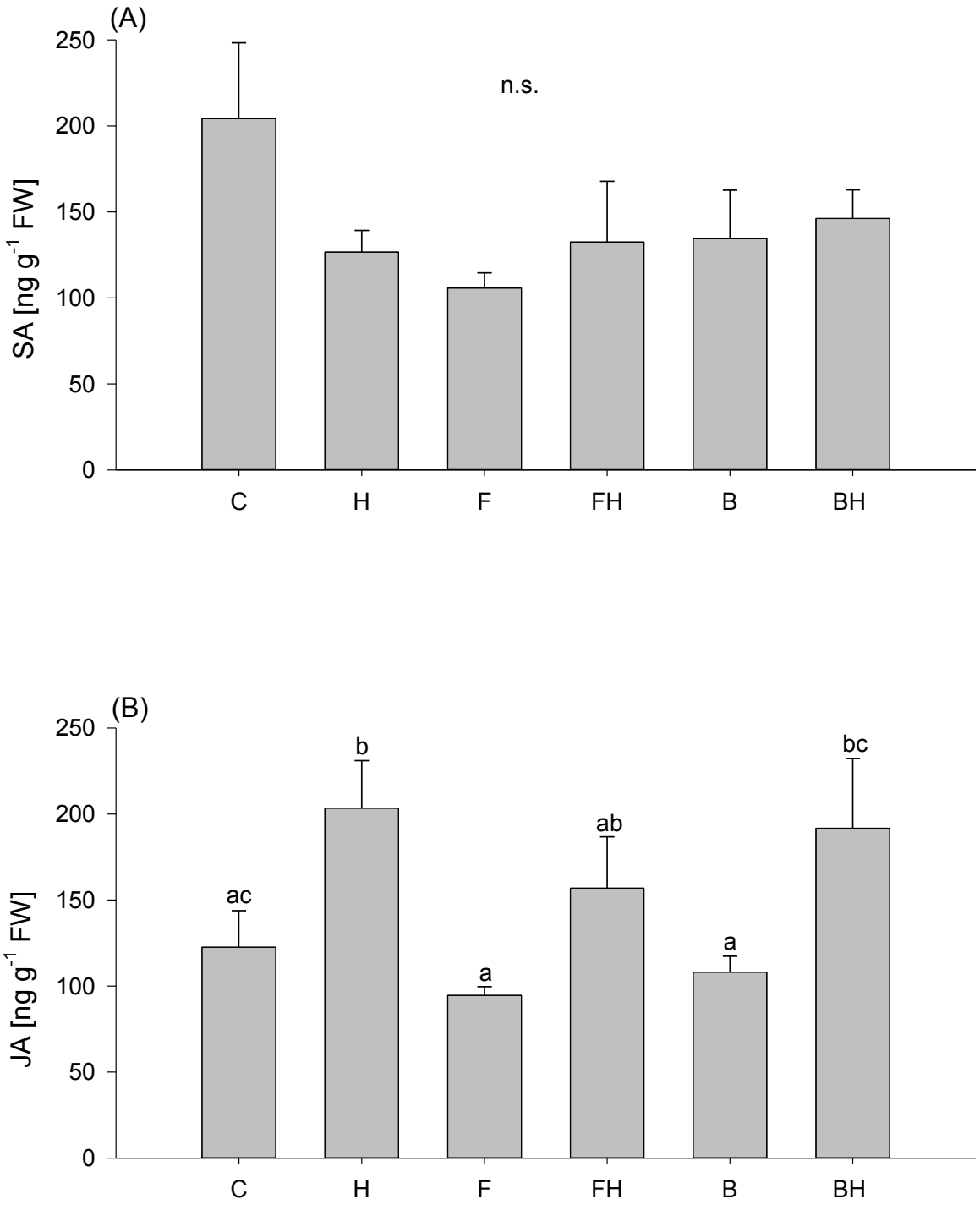


Figure 4-1 Levels of salicylic acid (A) and jasmonic acid (B) (means ±SE) measured in *Arabidopsis thaliana* leaves.
Plant treatments: C = control, H = *Plutella xylostella* caterpillars feeding for 24 hours, F = FRh2 colonised plants, FH = FRh2 colonised plants with caterpillars feeding for 24 hours, B = BG11 colonised plants, BH = BG11 colonised plants with caterpillars feeding for 24 hours
Error bars represent the standard error of the mean (N = 6), bars with different letters differ significantly, (salicylic acid: NPar tests, $P = 0.419$, jasmonic acid: one-way ANOVA, $P = 0.026$), n.s. = not significant.

4.3.2 Glucosinolate analyses

There were significant differences ($P \leq 0.05$) between the six treatments regarding indole and aliphatic glucosinolate levels (One-way ANOVA, IGs: d.f. = 5, $F = 3.162$, $P = 0.021$; AGs: d.f. = 5, $F = 4.360$, $P = 0.004$, Figure 4-2). FRh2 colonised plants showed lower levels of total aliphatic glucosinolates (AGs) compared to controls with a significantly low level of 4-methylsulfinylbutyl-GLS (4MSOB) (one-way ANOVA, d.f. = 5, $F = 3.81$, $P = 0.009$, Table 4-2). Despite the absence of a significant change in the total amount of AGs when compared to controls, BG11 colonised plants showed a significant increase in the level of 7-methylsulfinylheptyl-GLS (7MSOH), 4-methylthiobutyl-GLS (4MTB) and 8-methylsulfinyl-octyl-GLS (8MSOO) (one-way ANOVA, 7MSOH: d.f. = 5, $F = 4.942$, $P = 0.002$; 4MTB: d.f. = 5, $F = 4.876$, $P = 0.002$, 8MSOO: d.f. = 5, $F = 3.336$, $P = 0.016$, Table 4-2). BG11 colonised and herbivore damaged plants showed significantly lower levels of total IGs when compared to controls. Among the four measured IGs, indol-3-ylmethyl-GLS (I3M) showed the lowest significant level (one-way ANOVA, I3M: d.f. = 5, $F = 3.014$, $P = 0.036$, Table 4-2).

Quantities of individual indole and aliphatic glucosinolates are shown in Table D5 Appendix D and the statistical analyses results in E4 Appendix E.

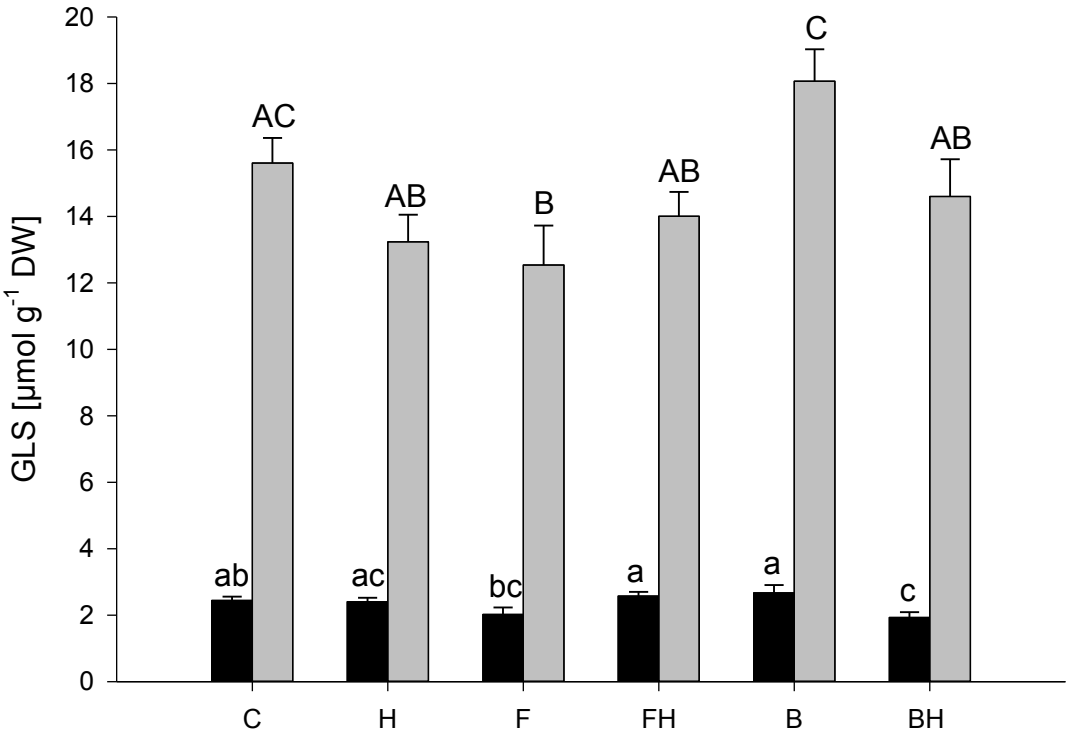


Figure 4-2 Indole (black bars) and aliphatic (grey bars) glucosinolate (GLS) levels (means ±SE) measured in *Arabidopsis thaliana* leaves.
Plant treatments: C = control, H = *Plutella xylostella* caterpillars feeding for 24 hours, F = FRh2 colonised plants, FH = FRh2 colonised plants with caterpillars feeding for 24 hours, B = BG11 colonised plants, BH = BG11 colonised plants with caterpillars feeding for 24 hours
Error bars represent the standard error of the mean (N = 6), bars with different letters differ significantly, (one-way ANOVA, $P \leq 0.05$).

Table 4-2 Mean ± SE glucosinolates (µmol g^{-1} DW) measured in *Arabidopsis thaliana* leaves.
Plant treatments: C = control, H = *Plutella xylostella* caterpillars feeding for 24 hours, F = FRh2 colonised plants, FH = FRh2 colonised plants with caterpillars feeding for 24 hours, B = BG11 colonised plants, BH = BG11 colonised plants with caterpillars feeding for 24 hours.
An asterisk indicates a statistically significant difference compared to untreated control plants using a one-way ANOVA followed by Tukey HSD and Fisher's least significant difference with significance at $P \leq 0.05$.

Glucosinolates[a]	C	H	F	FH	B	BH
Total AGs	15.60±0.75	13.23±0.81	12.54±1.18*	14±0.7	18.07±0.95	14.60±1.12
Total IGs	2.44±0.11	2.40± 0.12	2.01±0.21	2.5±0.12	2.67±0.23	1.92±0.16*
3MSOP	1.27 ± 0.05	1.03±0.08	0.99±0.12	1.16±0.09	1.47±0.12	1.18±0.10
4MSOB	12.32±0.56	10.11±0.68*	9.91±0.91*	10.84±0.58	13.79±0.76	11.51±0.93
5MSOP	1.07±0.03	0.95±0.10	0.90±0.10	0.92±0.08	1.17±0.06	0.92±0.06
6MSOH	0.08±0.02	0.08±0.02	0.06±0.01	0.07±0.01	0.09±0.01	0.10±0.02
7MSOH	0.19±0.03	0.21±0.02	0.13±0.02	0.20±0.03	0.30±0.02*	0.21±0.03
4MTB	0.15±0.04	0.21±0.08	0.11±0.02	0.14±0.03	0.38±0.08*	0.06±0.02
8MSOO	0.52±0.09	0.65±0.06	0.43±0.08	0.68±0.11	0.88±0.07*	0.62±0.08
4MOI3M	0.47±0.04	0.51±0.07	0.48±0.06	0.59±0.07	0.62±0.08	0.63±0.05
1MOI3M	0.29±0.08	0.31±0.07	0.15±0.03	0.40±0.11	0.31±0.09	0.22±0.03
4OHI3M	0.02±0.01	0.04±0.01	0.04±0.01	0.02±0.01	0.00±0.00	0.00±0.00
I3M	1.66±0.10	1.54±0.10	1.34±0.20	1.56±0.18	1.74±0.021	1.18±0.07*

[a]Abbreviations for individual glucosinolates (GLS) are: 3MSOP, 3-methylsulfinylpropyl-GLS; 4MSOB, 4-methylsulfinylbutyl-GLS; 5MSOP, 5-methylsulfinylpentyl-GLS; 6MSOH, 6-methylsulfinyl-heptyl-GLS; 7MSOH, 7-methylsulfinylheptyl-GLS; 4MTB, 4-methylthiobutyl-GLS; 8MSOO, 8-methylsulfinyl-octyl-GLS; 4MOI3M, 4-methoxyindol-3-ylmethyl-GLS; 1MOI3M, 1-methoxyindol-3-ylmethyl-GLS; 4OHI3M, 4-hydroxyindol-3-ylmethyl-GLS; I3M, indol-3-ylmethyl-GLS.

4.4 Discussion

Beauveria bassiana colonisation did not result in higher levels of JA and SA in *Arabidopsis* leaves. JA levels were only increased in *P. xylostella* damaged plants regardless of the presence or absence of *B. bassiana,* indicating an herbivore dependent-response in *Arabidopsis*. Contrary to pathogen attack, SA levels wouldn't be expected to be altered by herbivores with a chewing feeding mode as they are known to induce mainly JA-dependent defence responses (Chapter 1). The unaltered level of the herbivore-induced phytohormone suggested that *B. bassiana* strains FRh2 and BG11 presence did not prime the plant for JA-mediated defences which possibly explain the non-protective role of *B. bassiana* against *P. xylostella* (Chapter 2).

Pieterse et al. (2000) reported that after treatment of *Arabidopsis* roots with the ISR-inducing *P. fluorescens* WCS417r bacteria, neither JA content, nor the level of ethylene were altered. This indicates that induction in JA and ET dependent plant responses can be triggered without an increase in the levels of these hormones (Van der Ent et al., 2009). Schweizer et al. (1997) showed that rice infection with the fungal pathogen *Magnaporthe grisea* activated jasmonate inducible genes without an increase in the level of endogenous JA. Similarly, *B. bassiana* can induce a SA and JA-ET dependent response in *Arabidopsis* without altering the production of these hormones. Moreover, other physiological process involved the induction of hormonal signalling pathways rather than the actual production of the hormone in question. Tsai et al. (1996) showed that an increase in ethylene sensitivity and not ethylene production is the event triggering JA enhanced senescence in detached rice leaves. In addition, Pieterse et al. (2000) demonstrated the infiltration of leaves with ISR inducing rhizobacteria elicited JA- and ET-dependent resistance against *P. syringae* pv. tomato DC3000, but this was not accompanied by a local increase in the production of these hormones indicating that resistance can be accompanied by no increased levels of these hormones . Moreover, they were able to show that transgenic S-12 *Arabidopsis* that were affected in the production of JA in response to wounding expressed a significantly induced resistance against *P. syringae* pv. tomato DC3000 after root treatment with *P. fluorescens* WCS417r. Pathogen- induced resistance is characterised by the accumulation of SA and by the activation of a sepecific set of pathogenesis-related (*PR*) genes (Chapter 1). However, Shoresh et al. (2005) showed that the levels of SA did not vary between *T. asperellum* T203 inoculated and control plants even post challenge with the leaf pathogen *P. syringae* pv. *lachrymans* and SA levels increased only in *P. syringae* pv. *lachrymans*-infected cucumbers leaves. They further showed that challenge of *Trichoderma*-preinoculated plants with *P. syringae* pv. *lachrymans* resulted in higher systemic expression of the pathogenesis-related genes encoding for chitinase 1, β-1,3-glucanase, and peroxidase indicating that resistance can be achieved without the actual increase in SA levels

Thus, it is not surprising that levels of SA and JA were not altered by *B. bassiana* colonisation although transcription data showed induction in many SA and JA pathway related genes (Chapter 3). Future work will need to assess the levels of JA and SA in *B. bassiana*-colonised *Arabidopsis* that are challenged with the plant pathogen *S. sclerotiorum* and the phloem feeder *M. persicae* in order to elucidate any priming effects due to endophyte colonisation (Chapter 2).

Similarly, *B. bassiana* colonisation did not result in major changes in glucosinolates profile. Lower content of total aliphatic glucosinolates were only recorded in FRh2 colonised *Arabidopsis* but this did not seem to favour resistance against the crucifer-feeding specialist *P. xylostella* as reflected in the result of the feeding bioassay (Chapter 2). Maag et al. (2014) showed that foliar glucosinolate levels did not differ between *T. atroviride* LU132 inoculated oilseed rape (*Brassica napus*) roots and non-inoculated plants even post *P. xylostella* feeding challenge. Muller et al. (2010) reported that decreased levels of aliphatic and/or indole glucosinolates content do not cause major changes in the larval performance of. *P.xylostella*. In addition, higher levels of aliphatic and indole glucosinolates respectively conferred resistance to the generalists *S. exigua* and *S. littoralis* but not against the specialist *P. xylostella* (Stotz et al., 2000; Kroymann et al., 2003). Metabolically engineered glucosinolate profiles using *CYP79* genes in *Arabidopsis* did not appear to have any unequivocal effect on *P. xylostella* (Sarosh et al., 2010). Mewis et al. (2005) reported that methylsulfinylalkyl glucosinolate levels were negatively correlated with *S. exigua* weight gain thus it will be interesting to further investigate the effects of the increased level of each of 7MSOH, 4MTB, 8MSOO in BG11 colonised plant on generalist herbivores.

Chapter 5

General Discussion

In the recent decade numerous studies suggested the potential use of endophytic *B. bassiana* as a biocontrol agent against herbivores and plant pathogens. The published literature revealed a possible protective role of *Beauveria* against herbivores and pathogens in some cases (Chapter 1). However the mechanisms behind such effects are largely unknown. This thesis examined the interactions between *A. thaliana*, the entomopathogenic fungus *B. bassiana*, and three plant antagonists.

The protective role of *B. bassiana* strains FRh2 and BG11 against the plant pathogen *S. sclerotiorum* was demonstrated in this study. However this was not the case for the insects *P. xylostella* and *M. persicae*. FRh2 and BG11 as endophytes had no detrimental effects against either species (Chapter 2). Moreover, this study is the first to describe an enhanced defence state in *Arabidopsis* colonised by *B. bassiana*. The extensive transcriptomic analyses (Chapter 3) showed that *B. bassiana* strains FRh2 and BG11 can evoke a MTI defence in *Arabidopsis* leaves 15 days post inoculation. This MTI resulted in the induction of many genes related to SA and JA signalling pathways, to the camalexin pathway and to ROS damage control in *A. thaliana*. The induction of SA and JA signalling related genes by *B. bassiana* colonisation did not result in higher levels of SA and JA metabolites This is in agreement with previous studies showing that ISR inducing beneficial *P. fluorescens* WCS417r can induce or potentiate genes related to the JA and ET signalling pathways without increasing the levels of these phytohormones in colonised *Arabidopsis* (Pieterse et al., 2000). To investigate further the role of JA and ET signalling pathways in *P. fluorescens* WCS417r-ISR, Van der Ent et al. (2009) showed that despite the unaltered levels of JA and ET in WCS417r colonised plants, many genes related to JA and ET signalling pathways were induced and that genes coding for the AP2/ERF family of transcription factors implicated in the regulation of JA- and ET-dependent defences (Lorenzo et al., 2003) were overrepresented. Moreover, induction of the phenylalanine ammonia-lyase (PAL) coding gene, known to be involved in SA related defences (Sticher et al., 1997; Kim & Hwang, 2014), did not result in higher levels of SA in *Trichoderma*-inoculated cucumber plants (Shoresh et al., 2005) . Equally, no priming of these phytohormones was found when infected plants were challenged with the leaf chewing caterpillar *P. xylostella* (Chapter 4). Moreover, glucosinolate profile did not show major changes following *B. bassiana* strains FRh2 and BG11 colonisation (Chapter 4).

Even though both *B. bassiana* strains FRh2 and BG11 induced plant defence genes and had similar ecological effects on *S. sclerotiorum*, *P. xylostella* and *M. persicae* (both reduced *S. sclerotiorum* disease symptoms while having no detrimental effects on *P. xylostella* and *M. persicae*) the plant gene expression data seemed to vary with *B. bassiana* strains. The number of differentially expressed genes was greatest for FRh2 colonised plants and, as reflected in the GO analysis, only the expression data for FRh2 colonised plants showed an increase in the induction of abiotic stress related genes and a suppression in genes related to ethylene, gibberellin and auxin stimulus (Chapter 3). Furthermore, FRh2 and BG11 colonisation affected different genes within the same defence pathway. For example, FRh2 and BG11 colonisation affected the camalexin biosynthesis pathway via the induction of *CYP71A12/13* and *CYP71B15 (PAD3)* respectively (Chapter 3). Glucosinolate profiles varied too, depending on the *B. bassiana* strain. For example, only FRh2 colonised *Arabidopsis* had decreased total aliphatic glucosinolate levels.

The reasons for the discrepancy in the genetic responses to these *B. bassiana* strains are a matter of speculation. Differences in the biology of the two strains could explain this observation. Similar findings have been reported from other systems. *Arabidopsis* colonised by the non-pathogenic root coloniser *P. fluorescens* strains GM30 and Pf-5, for instance, showed strain-dependent and strain-independent host plant responses (Weston et al., 2012) . Also, *P. fluorescens* strains WCS417r and FPT9601-T5 induced plant defence responses and protected *Arabidopsis* against *Pseudomonas syringae* pv. tomato, however both strains had different effects on the *Arabidopsis* transcriptome and only five genes were found to respond similarly (Verhagen et al., 2004; Wang et al., 2005b). The strain-dependent responses described here could be attributed to the possibility that FRh2 and BG11 produced different effector or elicitor molecules that can manipulate plant responses differently by inducing specific genes. These microbial molecules are specifically recognised by the plant and can either elicit plant immune responses and/or suppress the host plant's defensive system to facilitate further infection or colonisation (Maffei et al., 2012).

Contrary to plant-pathogen interactions, effector biology is poorly understood in plant-beneficial microbe relationships (Zamioudis & Pieterse, 2012) and only recently studies have shown that beneficial microbes can produce such molecules. For example, mutualistic root symbionts such as *P. indica* and *Laccaria bicolor* can produce effector-like molecules such as small secreted proteins (SSP) (Martin et al., 2008; Plett et al., 2011; Zuccaro et al., 2011). Moreover, Kloppholz et al. (2011) showed that the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* produces a protein, SP7, that interacts with the pathogenesis-related transcription factor ERF19 in the plant nucleus. Many endophytic *Trichoderma* spp., which are capable of priming the plant's immune system, can produce such molecules but their roles remain to be fully elucidated (Hermosa et al., 2012; Schmoll et al., 2016). Strain-specific differences were found in the case of non-pathogenic *Xanthomonas arboricola*

strains CFBP7651 and CFBP7634, which were characterised with and without *hrp/hrc* genes, coding for the type three secretion system (T3SS), which is known to play a basic role in pathogenicity.

Whether differential effects of FRh2 and BG11 on plant responses correlated with the host from which the strains were isolated FRh2 is an insect-derived strain while BG11 was isolated from a plant), remains unknown. When testing the association between insect-host and *B. bassiana*, many studies showed that in general this should be viewed as a coincidental co-occurrence of the pathogen with a certain insect (St Leger et al., 1992; Bidochka et al., 2002; Wang et al., 2005a) . Moreover, there was no clear evidence that *B. bassiana* virulence is host-dependent. Studies showed that virulence of *B. bassiana* strains was not stronger toward the insect host species from which they were isolated (Todorova et al., 2002; Talaei et al., 2006) Thus, it is more likely that plant responses to FRh2 and BG11 do not relate to the fact that both strains derived from different organisms. However, further investigation is required to assess if there is any correlation between plant responses and *B. bassiana* hosts.

In the light of these findings, how can *B. bassiana* induction of plant defence be explained? And why did *B. bassiana* induce resistance only against *S. sclerotiorum* but not against insects?

Arabidopsis colonisation by *B. bassiana* BG11 and FRh2 resulted in the induction of many defence related genes such as ROS scavenger, camalexin and SA-JA signalling pathway related genes. The induction of such plant defence responses could explain the resistance against *S. sclerotiorum* infection. Previously it has been shown that plant defence against *S. sclerotiorum* depends on a complex network of hormonal and signalling defence responses. Stotz et al. (2011) demonstrated that mutant lines deficient in camalexin biosynthesis were hyper-susceptible to *S. sclerotiorum*. Guo and Stotz (2007) clearly showed that SA and JA-ET signalling pathways are involved in defence against *S. sclerotiorum*. In addition, the induction of *WRKY75* in both BG11 and FRh2 colonised plants and the reduction in GA pathway in FRh2 colonised plants might have contributed to this resistance. *WRKY75* overexpression lines conferred enhanced resistance to *S. sclerotiorum* (Chen et al., 2013). Navarro et al. (2008) demonstrated that *Arabidopsis* mutants blocked in GA signalling showed enhanced resistance against the necrotrophic fungus *A. brassicicola*. Moreover, the induction of camalexin and SA-JA signalling related genes could indicate a possible activation of priming mechanism in the plant. However, further investigation is required to confirm priming as a mechanism behind *B. bassiana* induced resistance against *S. sclerotiorum*. Transcriptional analysis of the tripartite interaction between *B. bassiana*, *Arabidopsis* and *S. sclerotiorum* interaction and combining such analysis with metabolic profiles of SA, JA and camalexin will help in gaining a comprehensive picture of the protective role of *B. bassiana* against this plant necrotic pathogen.

Although *B. bassiana* BG11 and FRh2 enhanced plant resistance against *S. sclerotiorum*, both *B. bassiana* strains had no detrimental effects against *P. xylostella* and *M. persicae*. In *Arabidopsis* plants, resistance against *M. persicae* is known to be mediated through the JA pathway (Ellis et al., 2002). Despite the induction of many JA-signalling related genes, *B. bassiana* colonisation neither reduced *M. persicae* infestation in *Arabidopsis* nor resulted in higher levels of JA. Therefore, it is likely that *B. bassiana* did not induce plant defences involved in resistance against *M. persicae*. Beside JA-related defences, studies identified additional key players in modulating plant defence against the phloem sap-feeding aphid *M. persicae*. Kim et al. (2008a) reported that *M. persicae* population size was smaller on *Arabidopsis* mutant plants, which accumulated elevated levels of indole glucosinolates, thus suggesting that indole glucosinolates are detrimental to *M. persicae*. As reported in Chapter 4, both *B. bassiana* strains FRh2 and BG11 used herein did not increase the level of indole glucosinolates. Pegadaraju et al. (2005) demonstrated that *M. persicae* population size on the camalexin-deficient *pad3* mutant was comparable to that on the wild type plant indicating that PAD3 and camalexin are not essential for controlling *M. persicae* infestation on *Arabidopsis*. Recently, genetic studies reported PAD4 (Phytoalexin Deficient 4) and not PAD3 as a converging point in modulating defence against *M. persicae* in *Arabidopsis*. However, PAD4 involvement in defence against *M. persicae* was not related to its role in SA and camalexin metabolism (Pegadaraju et al., 2007; Louis et al., 2012; Louis & Shah, 2014).

P. xylostella larvae feeding drastically reprogrammed the *Arabidopsis* transcriptome (Ehlting et al., 2008) showing major induction in the secondary defence metabolism and signalling. However, this brassica specialist acquired adaptations to circumvent any negative effect associated with plant defence. Thus, enhancing plant defence for resistance against this destructive specialist is a challenge. *B. bassiana* colonisation neither enhanced resistance (Chapter 2) nor primed *P. xylostella* damaged plant for JA defence (Chapter 4) In addition, glucosinolate profiles did not show major changes following *B. bassiana* colonisation (Chapter 4).

Despite the non-protective role of BG11 and FRh2 strains against the third instar *P. xylostella* and *M. persicae,* the capacity of *B. bassiana* to induce and/or prime for resistance in *Arabidopsis* against other insects cannot be excluded. Thus, testing a broader range of herbivores and assessing different response variables such as pupation, oviposition, and different developmental stages are required to evaluate the protective role of *B. bassiana* against insects. It is probable that the effectiveness of *B. bassiana* in protecting plants from insects depends on the sensitivity of the insect to the induced plant defence responses (Van Oosten et al., 2008). Therefore, the protective role of *B. bassiana* against insects is likely to be specific and should be tested individually for each type of insect (Pozo & Azcon-Aguilar, 2007; Pineda et al., 2013).

The transcriptome data of FRh2 and BG11 colonised *Arabidopsis* could be used as a starting point to further assess *B. bassiana* effects on other insects. BG11 colonised *Arabidopsis* showed induction in the genes *PR1*, *PR2*, *WRKY38*, *WRKY6, WRKY26, WRKY40*, and *WRKY75*, which were involved in defence against the cabbage aphid *Brevicoryne brassicae* (Kusnierczyk et al., 2008). Genes involved in camalexin biosynthesis (*PAD3/CYP71B15* and *CYP71A13*) that were upregulated in BG11 and FRh2-colonised *Arabidopsis*, were also strongly induced in cabbage aphid-infested *Arabidopsis* (Kusnierczyk et al., 2011), highlighting the possibility of *B. bassiana* to induce or prime for camalexin against cabbage aphid. Moreover, the transcriptome data suggested the possibility that BG11 colonisation can enhance resistance against other leaf chewing insects. *WRKY40* and *ANAC055* induced in BG11 colonised plants are known to play a role in the resistance against the generalist *Spodoptera littoralis* (Schweizer et al., 2013). In addition, BG11 colonised *Arabidopsis* showed induction in calmodulin-like protein coding genes, known to be involved in plant defence following treatment with oral secretions of *S. littoralis* (Guo et al., 2013).Therefore it would be worthwhile to test BG11 and FRh2 against *B. brassicae* and *S. littoralis*

This study provided evidence of enhanced resistance in *Arabidopsis* colonised by *B. bassiana*, suggesting induction of plant defences as a possible mechanism that contributed to disease reduction. However, other mechanisms could be involved in the resistance against *S. sclerotiorum* too. Competition, antibiosis and mycoparasitism are modes of direct interaction by which *B. bassiana* strains FRh2 and BG11 could have exerted an inhibitory effect on the pathogen.

Competition for space was suggested as a mechanism of enhanced resistance against the plant pathogen *Rhizoctonia solani* in *B. bassiana* colonised cotton and tomato. The degree of disease correlated with the population density of *B. bassiana* conidia on seeds (Ownley et al., 2008). Jaber and Salem (2014) reported on the potential of endophytic *B. bassiana* to confer protection against the plant virus Zucchini yellow mosaic virus (ZYMV) in cucurbits. They attributed the reduction in ZYMV symptoms to the interference of *B. bassiana* colonisation with the systemic movement of ZYMV from cell to cell.

Although antibiosis of *B. bassiana* against plant pathogens has been reported in many studies (Renwick et al., 1991; Griffin et al., 2005; Clark et al., 2006; Ownley et al., 2008), the antimicrobial compounds were not identified. Beauvericin is a well-studied secondary metabolite in *Beauveria* (Wang & Xu, 2012; Scharf et al., 2014). Xu et al. (2008) showed that beauvericin, from the endophytic fungus, *Fusarium redolens,* has inhibitory effects on three plant pathogens: *Pseudomonas lachrymans*, *Agrobacterium tumefaciens*, and *Xanthomonas vesicatoria*. Thus, it will be interesting to test whether beauvericin from endophytic *Beauveria* can affect plant pathogens.

Volatile organic compounds (VOC) released from *B. bassiana* cultures were also characterised but not evaluated against plant pathogens (Crespo et al., 2008; Hussain et al., 2010). Interestingly, and as discussed in Chapter 2, it is unknown whether these fungal metabolites are produced within the plant and at concentrations that can influence insects or pathogens. Thus, more focused research needs to assess the metabolic profiles of *B. bassiana* in *planta* taking into consideration the plant role in altering such profiles.

Recently, Luo et al. (2015) showed that *B. bassiana* adapted to different environments by metabolic regulation. The study contributed to the differential metabolic responses of *B. bassiana* in pupae extracts and root exudates to the lack of insect components and/or to the presence of components in the root exudates without characterising the nature of these components.

Many studies reported mycoparasitism as a primary direct mechanism employed by some fungi against plant pathogens. Mycoparasitism is a term introduced by Butler (1954) to describe the relationship when one fungus parasitizes another. The mycoparasitic fungus then obtains nutrients from its live or dead host (Jeffries, 1995). Askary et al. (1997) showed that resistance of *Lecanicillium lecanii*-inoculated cucumber leaves against the powdery mildew pathogen *Sphaerotheca fuliginea* is associated with parasitism activity on leaf cell surface. In their study to evaluate potential biological control agents against *Sclerotinia sclerotiorum*, Abdullah et al. (2008) showed *in vitro* that *T. harzianum* inhibited the growth and the production of mycelia and sclerotia through mycoparasitism rather than antibiosis. Scanning electron microscopy (SEM) showed hyphae of *T. harzianum* coiling and producing an ‘‘appressoria-like’’ structure around *S. sclerotiorum* hyphae.

Card et al. (2009) showed that *Trichoderma atroviride* LU132 inhibits *Botrytis cinerea* on strawberry leaves through competition for glucose, through the production of volatile and non-volatile compounds and with an undefined level of inhibition through direct parasitism. In the Card et al. (2009) study, light micrograph and SEM studies on dual culture showed that *T. atroviride* LU132 coiled around hyphae of *B. cinerea* and within four days of inoculation *B. cinerea* collapse and die.

So far, mycoparasitism activity was suggested as a mode of direct action for *B. bassiana* but not tested (Ownley et al., 2010). Recently and in liquid culture, Paredes et al. (2016) and Jhoel Montoya et al. (2016) reported on the induction of *B. bassiana* chitinase and β-1,3-glucanase coding genes in the presence of plant pathogens. In parallel, many recent studies confirmed the importance of cell wall-degrading enzymes (CWDEs) such as chitinase and β-1,3-glucanase in *Trichoderma* mycoparasitism against *B. cinerea* (Sanz et al., 2005; Yang et al., 2009; Seidl et al., 2009; Shoresh et al., 2010; Kubicek et al., 2011; Mukherjee et al., 2013).

From the above it is clear that it cannot be entirely excluded that more than one mechanism contributed to the observed reduction in disease symptoms caused by the plant pathogen *S. sclerotiorum* when *Arabidopsis* was pre-treated with *B. bassiana* stains FRh2 and BG11. Therefore, more research is required to evaluate competition, the potential production of metabolites by *B. bassiana* and their effects on this plant pathogen and to elucidate the likelihood of mycoparasitism.

In summary, this study showed the ability of the entomopathogenic fungus *B. bassiana* FRh2 and BG11 as an endophyte to protect against the necrotrophic plant pathogen *S. sclerotiorum* but not against the insects pest *P. xylostella* and *M. persicae*. Results also showed that *B. bassiana* strains FRh2 and BG11 evoked MAMP-triggered defence responses in *Arabidopsis* leaves suggesting induction of plant defences as a possible mechanism that contributed to disease reduction. Furthermore, no priming was found for JA-related defences and for glucosinolates in *B. bassiana* colonised and *P. xylostella* challenged plants. Future work should focus on camalexin-induction by *B. bassiana*, and possibly priming for SA- and/or JA-mediated defences as a mechanism for enhanced resistance against *S. sclerotiorum*. Research should also expand its focus on testing a wider range of insects with different life histories and, in particular, degree of specialisation. In addition, investigating *B. bassiana* direct effects and not only plant-mediated changes is essential to fully elucidate the mechanisms that govern *B. bassiana* protective role against pathogens and insects. The presented thesis is one of the first studies reporting plant responses to endophytic entomopathogens and will contribute to a better understanding of the mechanisms that confer resistance against plant antagonists.

Appendix A

Media preparation

A 1 Murashige-Skoog Basal Medium (MS) Sigma- Aldrich

- 0.43% basal salts MS
- 1% sucrose
- 0.8% Agar
- Adjust pH at 7
- Autoclave: 121 ^{o}C at 15 psi for 15 min.

A 2 Potato Dextrose Agar (PDA)-Merck

- Dissolve 39 g in 1 L of distilled water
- Autoclave: 121 ^{o}C at 15 psi for 15 min.

A 3 *Beauveria* semi selective medium (BSM)

- Dissolve 9.25 g of PDA in 1 L of with 11 g agar
- Autoclave: 121 ^{o}C at 15 psi for 15 min.
- Add aseptically each:

  50 mg/L Tetracycline chloride = 3.33 ml of the stock solution

  350 mg/L Streptomycin sulphate = 3.5 ml of the stock solution

125 mg/L Cyclohexamide

A 4 Antibiotics preperation

Tetracycline chloride 1.5 % stock solution

- Dissolve 1.5 g of Tetracycline chloride- Sigma- Aldrich in 100 ml of methanol
- Store at -20 ^{o}C

Streptomycin sulphate 10% stock solution

- Dissolve 10 g of Streptomycin sulphate - Sigma- Aldrich in 150 ml of filter sterile distilled water
- Store at -20 ^{o}C

Cyclohexamide 1.56% prepared fresh

- Dissolve 0.125 g of Cyclohexamide - Sigma- Aldrich in 4ml of methanol.
- Once dissolved, add 4 ml of filter sterile distilled water.

Appendix B

Molecular detection of *Beauveria bassiana*

Table B 1 Primer sequences used for *Beauveria bassiana* identification and detection

Name		Sequence (5'–3')	Reference
Co-Acetyl	CoA_1100dR	ATG CCC TCA CCA GAA TCC G	This study
	CoA_1433F	GGG ATT AGC AGG TGT CGC A	
EF1-α gene	EF1685 R	ATG TCA CGG ACG GCC AAA	This study
	EF349 F	TGG CCA CCA GCA CTC ACT AC	
SCAR markers			
$SCA14_{445}$	OPA14 F_{445}	TCT GTG CTG GCC CTT ATC G	(Castrillo et al., 2003)
	OPA14 R_{445}	TCT GTG CTG GGT ACT GAC GTG	
$SCA15_{441}$	OPA15 F_{441}	TTC CGA ACC CGG TTA AGA GAC	
	OPA15 R_{441}	TTC CGA ACC CAT CAT CCT GC	
$SCB9_{677}$	OPB9 F_{677}	TGG GGG ACT CGC AAA CAG	
	OPB9 R_{677}	TGG GGG ACT CAC TCC ACG	
Modified ITS F		GAACCTACCTATYGTTGCTTC	(Griffin, 2007)
Modified ITS R		ATYCGAGGTCAACGTTCAG	

Table B 2 PCR components for *Arabidopsis thaliana* background check for *Beauveria bassiana*

Components	Volume (µl)
Taq Buffer (10x)+ MgCl (2mM)	2.5
dNTPs (2.5 mM)	2
Primer-F (10µM)	1
Primer-R (10µM)	1
BSA	0.5
Taq polymerase (5U/µl)	0.25
gDNA template	2
ddH2O	15.75
Total	25

Table B 3 PCR programme for *Arabidopsis thaliana* background check for *Beauveria bassiana*

Step	Temperature (°C)	Time	
Denaturation	95	5’	
Denaturation	95	45”	40 cycles
Annealing	55	45”	
Extension	72	2’	
Extension	72	7’	

Table B 4 *Beauveria bassiana* strains used in this study

Isolate code	Species	Origin	Isolated from	Associated plant
FRh2	*B. bassiana*	Riverheads - New Zealand	*Hylastes ater* (Coleoptera: Curculionidae) cadaver pine bark beetle (Reay et al.,2010)	*Pinus radiata*
BG 11	*B. bassiana*	Botanical gardens –Christchurch - New Zealand	*Bellis perennis* (Asteraceae) Annabel Clouston (Bio-Protection Research Centre, Lincoln University)	*Bellis perennis* (Asteraceae)

Table B 5 PCR components used for detection of *Beauveria bassiana* in *Arabidopsis thaliana*

Components	Volume (µl)
Tag Buffer (10x)+ MgCl (2mM)	2.5
dNTPs (2.5 mM)	2
Primer-F(10µM)	1
Primer-R (10µM)	1
BSA	0.5
Taq polymerase (5U/µl)	0.25
gDNA template	2
ddH2O	15.75
Total	25

Table B 6 PCR programme used for *Beauveria bassiana* colonisation detection in *Arabidopsis thaliana*

Step	Temperature (°C)	Time	
Denaturation	95	5’	
Denaturation	95	45”	40 cycles
Annealing	58	45”	
Extension	72	2’	
Extension	72	7’	

Appendix C

Conidia suspensions preparation

Suspensions of conidia from FRh2 and BG11 strains at a time were prepared in 0.05% Tween 80 at a concentration of 10^8 conidia per ml

- Add 6 ml of 0.05% Tween 80 to a strain culture plate. Mix Tween thoroughly with hockey stick and (filter through Mira cloth for conidia only) into a universal/falcon tube.

- Add another 4 ml of 0.05% Tween 80 to the same plate and repeat to make up a 10 ml conidia suspension from each plate (approximately).

- Make six 1/10 series dilutions in 0.05% Tween 80 from the suspension (500µl of suspension to 4.5 ml Tween), 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}.

- Use the 10^{-3} for haemocytometer conidia counts in order to estimate concentration of conidia per ml (Calculate by counting spores per medium square where 16 small squares make up a medium square, for 5 medium squares (repeat), then average the medium squares and multiply by 250,000).

- Use the $10^{-5}$and 10^{-6} to add 100 µl of each to a PDA plate for a spore viability check.

- Using the haemocytometer counts, calculate the volume of suspension required to achieve a concentration of 1 x 10^8 conidia per ml in 10 ml following the equation $C_1xV_1=C_2xV_2$.

Appendix D

Effects of *Beauveria bassiana* colonisation on herbivorous insects, a plant pathogen and levels of jasmonic acid, salicylic acid and glucosinolate in *Beauveria bassiana* colonised plants

Table D 1 Third instar *Plutella xylostella* caterpillar body mass after 48, 72 and 96 hours feeding on mock-inoculated (C) and *Beauveria bassiana* (F=FRh2, B=BG11) inoculated *Arabidopsis thaliana*.

See Supplement material 1

Table D 2 *Myzus persicae* population on mock-inoculated (C) and *Beauveria bassiana* (F=FRh2, B=BG11) inoculated *Arabidopsis thaliana*.

See supplement material 1

Table D 3 *Sclerotinia sclerotiorum* lesion area in control (C) and *Beauveria bassiana* (F=FRh2 and B= BG11) inoculated *Arabidopsis thaliana.*

See supplement material 1

Table D 4 Levels of salicylic acid (A) and jasmonic acid (B) (means ±SE) measured in *Arabidopsis thaliana* leaves. Plant treatments: C = control, H = *Plutella xylostella* caterpillars feeding for 24 hours, F = FRh2 colonised plants, FH = FRh2 colonised plants with caterpillars feeding for 24 hours, B = BG11 colonised plants, BH = BG11 colonised plants with caterpillars feeding for 24 hours

See supplement material 1

Table D 5 Levels of indole and aliphatic glucosinolates measured in *Arabidopsis thaliana* leaves. Plant treatments: C = control, H = *Plutella xylostella* caterpillars feeding for 24 hours, F = FRh2 colonised plants, FH = FRh2 colonised plants with caterpillars feeding for 24 hours, B = BG11 colonised plants, BH = BG11 colonised plants with caterpillars feeding for 24 hours

See supplement material 1

Appendix E

Statistical analyses

Table E 1 Statistical analysis for *Plutella xylostella* caterpillar body mass after 48, 72 and 96 hours feeding on mock-inoculated (C) and *Beauveria bassiana* (F=FRh2, B=BG11) inoculated *Arabidopsis thaliana*

See supplement material 1

Table E 2 Statistical analysis for *Myzus persicae* population on mock-inoculated (C) and *Beauveria bassiana* (F=FRh2, B=BG11) inoculated *Arabidopsis thaliana*.

See supplement material 1

Table E 3 Statistical analysis for *Sclerotinia sclerotiorum* lesion area in control (C) and *Beauveria bassiana* (F=FRh2 and B= BG11) inoculated *Arabidopsis thaliana.*

See supplement material 1

Table E 4 Statistical analysis for *Beauveria bassiana* colonisation effects on levels of salicylic acid, jasmonic acid, indole and aliphatic glucosinolates (means ±SE) measured in *Arabidopsis thaliana* leaves. Plant treatments: C = control, H = *Plutella xylostella* caterpillars feeding for 24 hours, F = FRh2 colonised plants, FH = FRh2 colonised plants with caterpillars feeding for 24 hours, B = BG11 colonised plants, BH = BG11 colonised plants with caterpillars feeding for 24 hours

See supplement material 1

Table E 5 Statistical analysis for Residence times and first choice of *Trissolcus basalis* females to the Y-olfactometer linked to *Nezara viridula* challenged *Beauveria bassiana*-free plants (C) and *Nezara viridula* challenged *Beauveria bassiana*-colonised plants

See supplement material 1

Appendix F

Analysis of microarray, gene ontology, enrichment analysis and MapMan analysis

Figure F 1 Boxplots from AtCO_vs_AtFRh2 data and AtCO_vs_AtBG11 data before and after normalisation

See supplement material 3

Figure F 2 Heat map of all DEGs for AtCO_vs_AtFRh2.

See supplement material 3

Figure F 3 Heat map of the top 100 DEGs for AtCO_vs_AtFRh2.

See supplement material 3

Figure F 4 Heat map of the top 50 DEGs for AtCO_vs_AtFRh2

See supplement material 3

Figure F 5 Heat map of all DEGs for AtCO_vs_AtBG11

See supplement material 3

Figure F 6 Heat map of the top 100 for AtCO_vs_AtBG11

See supplement material 3

Figure F 7 Heat map of the top 50 for AtCO_vs_AtBG11

See supplement material 3

Table F 1 Differentially expressed genes (DEGs) for AtCO_vs_AtFRh2

See supplement material 2

Table F 2 Differentially expressed genes (DEGs) AtCO_vs_AtBG11

See supplement material 2

Table F 3 Gene ontology and enrichment analysis for AtFRh2 DEGs involved in BP

See supplement material 2

Table F 4 Gene ontology and enrichment analysis clustering for AtFRh2 DEGs involved in BP

See supplement material 2

Table F 5 Gene ontology and enrichment analysis for AtFRh2 upregulated DEGs involved in BP

See supplement material 2

Table F 6 Gene ontology and enrichment analysis clustering for AtFRh2 upregulated DEGs involved in BP

See supplement material 2

Table F 7 Gene ontology and enrichment analysis for AtFRh2 upregulated DEGs involved in Cellular component (CC)

See supplement material 2

Table F 8 Gene ontology and enrichment analysis for AtFRh2 upregulated DEGs involved in Molecular function (MF)

See supplement material 2

Table F 9 Gene ontology and enrichment analysis for AtFRh2 upregulated DEGs involved in protein domains

See supplement material 2

Table F 10 Gene ontology and enrichment analysis for AtFRh2 downregulated DEGs involved in BP

See supplement material 2

Table F 11 Gene ontology and enrichment analysis clustering for AtFRh2 downregulated DEGs involved in BP

See supplement material 2

Table F 12 Gene ontology and enrichment analysis for AtFRh2 downregulated DEGs involved in Cellular component (CC).

See supplement material 2

Table F 13 Gene ontology and enrichment analysis for AtFRh2 downregulated DEGs involved in molecular function (MF)

See supplement material 2

Table F 14 Gene ontology and enrichment analysis for AtFRh2 downregulated DEGs involved in protein domains

See supplement material 2

Table F 15 Gene ontology and enrichment analysis for AtBG11 DEGs involved in BP

See supplement material 2

Table F 16 Gene ontology and enrichment analysis clustering for AtBG11 DEGs involved in BP.

See supplement material 2

Table F 17 Gene ontology and enrichment analysis for AtBG11 upregulated DEGs involved in BP.

See supplement material 2

Table F 18 Gene ontology and enrichment analysis clustering for AtBG11 upregulated DEGs involved in BP.

See supplement material 2

Table F 19 Gene ontology and enrichment analysis for AtBG11 upregulated DEGs involved in Cellular component (CC)

See supplement material 2

Table F 20 Gene ontology and enrichment analysis for AtBG11 upregulated DEGs involved in Molecular function (MF)

See supplement material 2

Table F 21 Gene ontology and enrichment analysis for AtBG11 upregulated DEGs involved in protein domains

See supplement material 2

Table F 22 Gene ontology and enrichment analysis for AtBG11 downregulated DEGs involved in BP.

See supplement material 2

Table F 23 Gene ontology and enrichment analysis clustering for AtBG11 downregulated DEGs involved in BP.

See supplement material 2

Table F 24 Gene ontology and enrichment analysis for AtBG11 downregulated DEGs involved in Cellular component (CC)

See supplement material 2

Table F 25 Gene ontology and enrichment analysis for AtBG11 downregulated DEGs involved in molecular function (MF)

See supplement material 2

Table F 26 Gene ontology and enrichment analysis for AtBG11 downregulated DEGs involved in protein domains.

See supplement material 2

Table F 27 FDR correction for AtFRH2 DEGs

See supplement material 2

Table F 28 FDR correction for AtBG11 DEGs

See supplement material 2

Table F 29 AtFRh2 DEGs as assigned by Mapman

See supplement material 2

Table F 30 AtBG11 DEGs as assigned by Mapman

See supplement material 2

Appendix G

Quantitative real time PCR

Table G 1 Primer sequences used in qRT-PCR

See supplement material 2

Table G 2 qRT-PCR reaction components

Components	Volume (µl)
Tag Buffer (10x)	1.6
$MgCl_2$ (25mM)	2.56
dNTPs (10mM)	1
Primer-F(10µM)	0.8
Primer-R (10µM)	0.8
ROX (50X)	0.32
SYBR Green (1:30000)	1
Taq polymerase (5U/µl)	0.25
cDNA template (1:10)	1
ddH2O	6.67
Total	16

Table G 3 Relative gene expression calculations for the target genes over three reference genes in three independent biological replicates

See supplement material 2

Table G 4 Real time Ct values for the analysed target genes over the three reference genes in biological replicate I (BRI)

See supplement material 2

Table G 5 Real time Ct values for the analysed target genes over the three reference genes in BRII

See supplement material 2

Table G 6 Real time Ct values for the analysed target genes over the three reference genes in BRIII

See supplement material 2

Appendix H

Olfactory response of the egg parasitoid *Trissolcus basalis to cabbage plants treated with Beauveria bassiana* (strain ATCC 74040 - NATURALIS®) and host eggs

This work was carried out in the lab of Prof Stefano Colazza, DEMETRA Department, University of Palermo, Italy as part of the Better Understanding of Bugs for Improved Environment (BUGSIE) project. The aim of this assay was to explore whether the egg parasitoid *Trissolcus basalis* (Hym., Encyrtidae) changes its olfactory preference for plant volatiles induced by eggs of its host *Nezara viridula* (Hem., Pentatomidae) when plants are treated with *B. bassiana* (Colazza et al., 2004). Parasitoid and fungus are biological control agents and negative or positive interactions between both could have implications for simultaneous application in crop protection.

H.1 Materials and Methods

Plant material

Four to six leaf stage *Brassica oleracea* var Capitata were used in this assay and supplied by Palermo University nursery.

Conidia suspention

Conidia suspensions at a concentration of 2.3 x 10^7 conidia per ml were prepared from NATURALIS® - BIOGARD® (active ingredient *B. bassiana* strain ATCC 74040) according to the manufacturer's instructions.

Herbivore treatment

Four to six leaf stage *Brassica oleracea* var Capitata plants were challenged with mated *N. viridula* females in a pre-oviposition state (7–10 days after mating), defined as those whose abdomens appeared enlarged and slightly bloated. Three adults were transferred using a fine brush onto cabbage leaves per plant. Plants then were covered with meshed bags to allow close contact between plant leaves and bugs. Adults were allowed to feed and oviposit at least one egg cluster per plant for 54 hours.

Females *T. basalis* were isolated almost 24 hours before the assays and provided with a drop of honey–water solution for survival.

Plant inoculation

After 54 hours of *N. viridula* challenge, the adults were carefully removed and only plants harbouring at least a single *N. viridula* egg cluster were sprayed with *B. bassiana* conidia suspension (concentration of 2.3 x 10^7 conidia per ml) using lab trigger bottles. Control plants were mock-inoculated in the same manner with 0.05% Tween 80 in sterile water. After three days, control and inoculated plants were used in the olfactometer assay

Olfactometer assays

The Y-tube olfactometer used was similar to that described by (Colazza et al., 1999). Control and inoculated plants were caged in glassed jars and assigned to the right and the left arm of the Y tube respectively. A single female *T. basalis* was placed near the orifice of the Y-tube and tracked by a video camera. X bug software, developed by Prof Stefano Colazza's team, was used to process the tracks. Each wasp was allowed to choose one of the arms of the olfactometer for a period of 10 min. After that the tracking is stopped and tracks were analysed according to the software manual.

The software calculates the duration that a wasp spends in each arm or segmentation of the Y-olfactometer. The residence time in each arm was calculated as the sum of the duration in the corresponding segmentation. A choice was considered to have been made when a wasp remained in the designated arm for 20 s.

The experiment consisted in total of 14 plants divided into seven sets. Each set consisted of one control and one inoculated plant. Each set of plants was tested with 5 to 10 female wasps resulting in observations of 46 individual female *T. basalis.*

Residence time and first choice data were analysed using t-test and χ2 test respectively.

H.2 Results

B. bassiana colonisation did not affect *T. basalis* first choice towards volatiles emitted by plants treated with *N. viridula* alone or together with *B. bassiana* ($\chi 2 = 2.631579$, d.f. = 1, $P = 0.104758$ Figure H1). Also, there was no significant difference in the residential time spent by the female wasps in the Y-olfactometer arms ($t=0.105545$, $P=0.916413$, n =46, Figure H 1).

Residence times and first choice of *T. basalis* females to the Y-olfactometer linked to *N. viridula* challenged *B. bassiana*-free plants (C) and *N. viridula* challenged *B. bassiana*-colonised plants and the statistical analyses can be viewed in Table H1 and Table E5 respectively.

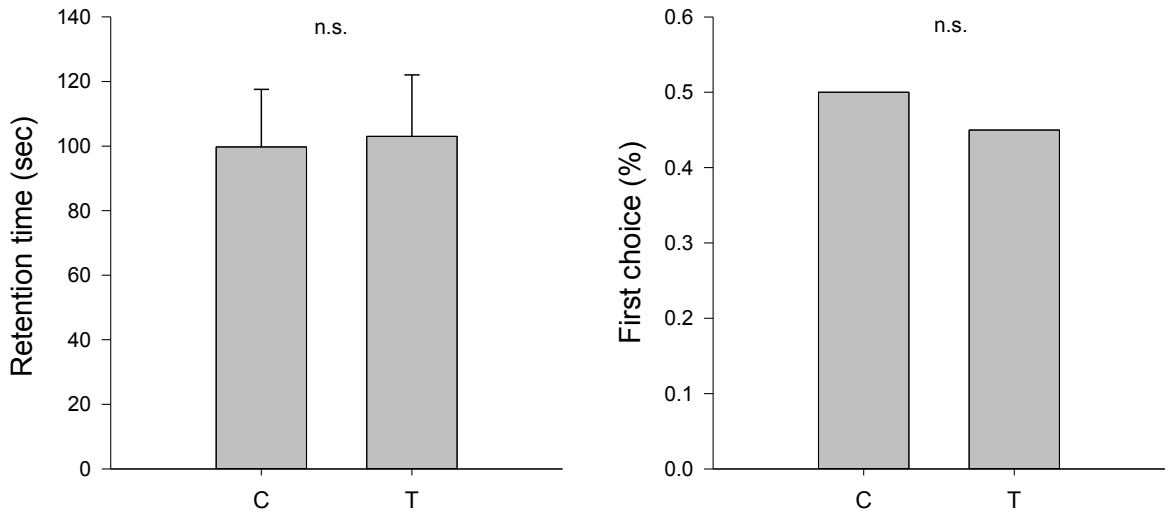


Figure H 1 Residence times (means ±SE) of *Trissolcus basalis* females (right) and percentage of *Trissolcus basalis* females first choices (left) in the Y-olfactometer arms linked to *Nezara viridula* challenged *Beauveria bassiana*-free plants (C) and *Nezara viridula* challenged *Beauveria bassiana*-colonised plants (T). No significant differences between treatments (N =46 individuals of females *T. basalis*, *t*-test, χ2 test, $P < 0.05$). n.s. = not significant.

H.3 Discussion

Many parasitoids, including *T. basalis*, are attracted to volatile chemicals produced by plants infested with their host (Nafisi et al., 2007; McKinnon, 2011). This preliminary experiment suggests that *B. bassiana* treatment of cabbage did not alter the attractive plant volatile blend induced by *N. viridula* feeding and oviposition. The parasitoids showed no preference, regardless whether the fungus was present or not. Whether this makes both biocontrol agents compatible or incompatible depends on the susceptibility of *T. basalis* when in contact with *B. bassiana* and requires further investigation.

References

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