Signaling in Arabidopsis roots in response to beneficial rhizobacteria

Christos Zamioudis

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Signaaltransductie in Arabidopsis wortels
in reactie op goedaardige rhizobacteriën

(met een samenvatting in het Nederlands)

Proefschrift

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to my wife Evi,

my parents Ditsios and Eugenia,

my sister Anastasia

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CHAPTER 1

General introduction

Modulation of host immunity by beneficial microbes

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In nature, plants abundantly form beneficial associations with soil-borne microbes that are important for plant survival and as such affect plant biodiversity and ecosystem functioning. Classical examples of symbiotic microbes are mycorrhizal fungi that aid in the uptake of water and minerals, and *Rhizobium* bacteria that fix atmospheric nitrogen for the plant. Several other types of beneficial soil-borne microbes, such as plant growth-promoting rhizobacteria and fungi with biological control activity, can stimulate plant growth by directly suppressing deleterious soil-borne pathogens, or by priming above-ground plant parts for enhanced defense against foliar pathogens or insect herbivores. The establishment of beneficial associations requires mutual recognition and substantial coordination of plant and microbial responses. A growing body of evidence suggests that beneficial microbes are initially recognized as potential invaders after which an immune response is triggered, whereas at later stages of the interaction mutualists are able to short-circuit plant defense signaling to enable successful colonization of host roots. In this Chapter we review our current understanding of how symbiotic and non-symbiotic beneficial soil microbes modulate the plant immune system and discuss the role of local and systemic defense responses in establishing the delicate balance between the two partners.

SYMBIOTIC AND NON-SYMBIOTIC BENEFICIAL MICROBES IN THE RHIZOSPHERE

In nature, plants are exploited by a plethora of microbial pathogens and insect herbivores that can cause severe diseases and pests. Beneficial relationships between plants and microorganisms are frequent in nature as well. They often occur in the rhizosphere and improve plant growth or help the plant to overcome biotic or abiotic stress. Each gram of soil contains billions of microbes. However, the microbial community on plant roots is very different from that in bulk soil, suggesting that plants are able to shape their microbiome (Bais et al., 2004; Badri and Vivanco, 2009; Bisseling et al., 2009; Mendes et al., 2011). Well-known examples of beneficial microbes are mycorrhizal fungi that can form a symbiosis with approximately 80% of all terrestrial plant species (Van der Heijden et al., 1998; Harrison, 2005). They acquire nutrients from the soil and deliver these to plant roots in return for photosynthates. *Rhizobium* bacteria form an intimate symbiotic relationship with leguminous plants, which leads to mutual recognition and development of symbiotic structures in which the *Rhizobium* bacteria fix atmospheric nitrogen for the plant (Spaink, 2000). Beneficial associations also include non-symbiotic plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF) of diverse genera, which can stimulate plant growth through degradation of soil pollutants, the production of phytostimulators, or by suppressing plant diseases or pests (Harman et al., 2004; Kloepper et al., 2004; Pozo and Azcon-Aguilar, 2007; De Vleesschauwer and Höfte, 2009; Lugtenberg and Kamilova, 2009; Van der Ent et al., 2009a; Pineda et al., 2010; Shoresh et al., 2010). The latter is often achieved through the elicitation of an induced systemic resistance (ISR) that is effective against a broad spectrum of foliar pathogens and even insect herbivores (Van Loon et al., 1998; Van Oosten et al., 2008; Van Wees et al., 2008; Pineda et al., 2010). The establishment of beneficial associations requires mutual recognition and substantial coordination of plant and microbial responses. Rhizobial and mycorrhizal symbioses share a common symbiosis signaling (Sym) pathway which is activated in plant cells upon perception of the rhizobial Nod and mycorrhizal Myc factors, respectively (Oldroyd and Downie, 2008; Oldroyd et al., 2009; Bonfante and Genre, 2010; Maillet et al., 2011). Interestingly, it appears that certain signaling components of the Sym pathway may also be activated by non-symbiotic beneficial microbes, such as PGPR, suggesting that plant signaling pathways triggered by different beneficial microbes are partly converged (Sanchez et al., 2005).

IMMUNE SIGNALING IN THE RHIZOSPHERE

Both symbiotic and non-symbiotic beneficial microbes are initially recognized as alien organisms. Hence, active interference with the plant immune system is fundamental for the establishment of intimate mutualistic relationships. Immune signaling in plants is initiated upon receptor-mediated perception of non-self molecules that are often conserved among different classes of microbes, both pathogenic and beneficial. These molecules are called microbe-associated molecular patterns (MAMPs) and MAMP-induced defense responses mounted in host plants are collectively referred to as MAMP-triggered immunity (MTI) (Jones and Dangl, 2006; Boller and Felix, 2009). Despite the fact that innate immune signaling in leaves has been extensively studied over the past years, very little is known about MTI in roots where the majority of the plant-beneficial microbes reside. Only recently, Millet et al. (2010) demonstrated that Arabidopsis roots respond to different MAMPs in a tissue-specific manner and that MAMP-triggered immune signaling in the roots is very similar to that observed in the leaves. To establish a mutualistic interaction with the plant, beneficial microbes need to cope with host immune responses that are triggered locally in the roots upon MAMP perception. In this review we focus on the strategies that symbiotic and non-symbiotic beneficial microbes evolved to reduce stimulation of host immune responses and actively suppress MTI.

MODULATION OF HOST IMMUNITY IN THE *RHIZOBIUM*-LEGUME SYMBIOSIS

Rhizobia have evolved to reduce stimulation of the host's immune system. In order to evade detection by the host immune system, successful microbes evolved ways to minimize recognition of their MAMPs. Flagellin, the major structural protein of flagella, is one of the best-studied bacterial proteins that is recognized as a MAMP by the plant immune system (Boller and Felix, 2009). In Arabidopsis, flagellin is perceived by the FLAGELLIN-SENSING 2 (FLS2) receptor, after which an intracellular signaling cascade is initiated leading to the activation of a defense program against the invading bacteria. The immunogenic properties of flagellin reside in the highly conserved N-terminus of the molecule. Flg22, a synthetic 22-amino acid peptide that corresponds to the conserved N-terminus of flagellin, is a potent elicitor of defense responses in Arabidopsis and other plant species (Felix et al., 1999). Recently, Lopez-Gomez et al. (2011) demonstrated that flg22-triggered defense responses in the roots of *Lotus japonicus* negatively influence the nodulation by inhibiting rhizobial infections and delaying the nodule organogenesis. However, flagellins from the legume symbiont *Sinorhizobium meliloti* are exceptionally divergent in the otherwise conserved flagellin epitope and neither the crude flagellin extracts nor the corresponding flg22 synthetic peptide are able to elicit defense responses (Felix et al., 1999). Likewise, the roots *of L. japonicus*, although well able to respond to flg22, do not respond to purified flagellin from *Mesorhizobium loti*, suggesting that LjFLS2 is unable to sense flagellin molecules from the symbiotic partner (Lopez-Gomez et al., 2011). Taken together, it is tempting to speculate that immune selective pressure exerted by the putative FLS2 orthologs of the leguminous hosts, forced the emergence of escape mutations within the active flagellin epitope to provide the microsymbionts with an evolutionary advantage of reducing stimulation of the host's immune system.

Rhizobia initially elicit a MTI response which is subsequently suppressed. Large scale gene expression profiling studies of early nodulation stages in the model legumes *L. japonicus* and *Medicago truncatula* revealed a significant induction of defense- and stress-related genes, indicating that the leguminous hosts initially recognize their symbiotic partners as a potential threat. However, the same cluster of genes was found to be down-regulated at later stages of root nodule formation, indicating that the microsymbionts have evolved to actively suppress host defense responses (El Yahyaoui et al., 2004; Kouchi et al., 2004; Lohar et al., 2006; Moreau et al., 2011). Maunoury et al.

(2010) recently reported two waves of transcriptional reprogramming in *M. truncatula* involving repression of defense-related genes followed by the activation of a nodule-specific transcriptome. By using a collection of plant and bacterial mutants, the authors demonstrated that this transcriptome switch is dependent upon a molecular dialogue between both partners. In Figure 1 we provide a summarizing model for the modulation of host immunity in the *Rhizobium*-legume symbiosis.

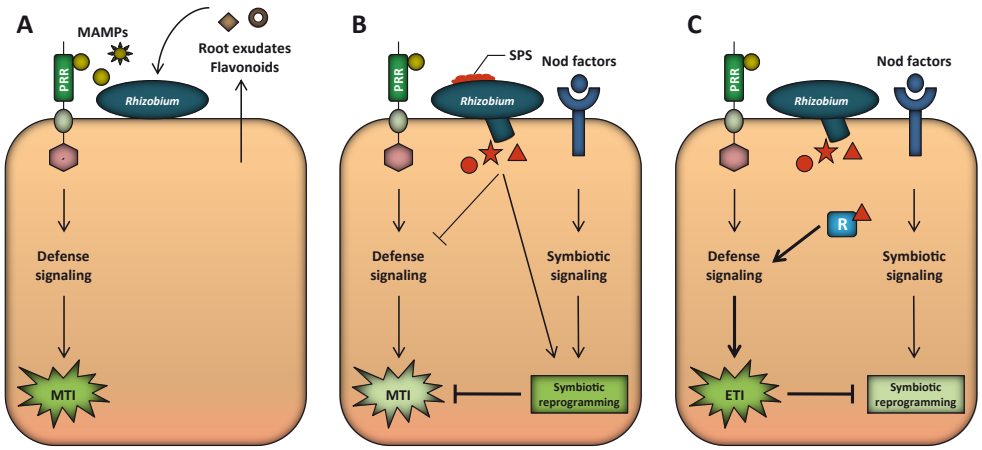

Figure 1. Model for the modulation of host immunity in the Rhizobium-legume symbiosis. (A) Root exudates recruit *Rhizobium* bacteria and secreted flavonoids prime the microsymbionts for the interaction. Host plants initially recognize rhizobia as potential invaders; pattern-recognition receptors (PRRs) in the host perceive microbe-associated molecular patterns (MAMPs, yellow-colored shapes) and a signaling cascade is initiated that results in MAMP-triggered immunity (MTI). **(B)** Surface polysaccharides (SPS) function early during the interaction, most likely as extracellular effectors to facilitate immune evasion. At later stages, the establishment of the symbiotic program in the plant cells, which is activated upon perception of the rhizobial Nod factors, counteracts the MTI with mechanisms yet to be defined. Rhizobial effectors that are secreted through the type III secretion system (brown-colored shapes) may assist in the suppression of the MTI response or act as symbiotic determinants. **(C)** In the case that a host resistance protein (R) recognizes a cognate rhizobial effector, effector-triggered immunity (ETI) is activated that in turn terminates the interaction as incompatible.

Rhizobial extracellular polysaccharides suppress host defense responses. Several rhizobial MAMPs have been identified that play a role in immune suppression in leguminous hosts, including common structural components such as lipopolysaccharides (LPS) and exopolysaccharides (EPS) (Fig. 1B). LPS are structural components of the outer membrane of gram-negative animal- and plant-associated bacteria and potent activators of host immune responses (Zeidler et al., 2004). Purified LPS of *S. meliloti* bacteria were shown to provoke typical immune responses in cell cultures of the non-host tobacco. However, cell cultures of the host plant *Medicago sativa* only weakly responded to *S. meliloti* LPS (Albus et al., 2001). Surprisingly, simultaneous application of *S. meliloti* LPS and a yeast elicitor to alfalfa cells resulted in the suppression of the elicitor-induced oxidative burst, indicating that LPS may act to suppress host defenses (Albus et al., 2001; Scheidle et al., 2005). Importantly, the ability of *S. meliloti* LPS to suppress elicitor-triggered defense responses is not only restricted to early events, such as the oxidative burst, it is also related to a global down-regulation of the elicitor-induced transcriptional reprogramming (Tellstrom et al., 2007). The mechanism underlying the immune-suppressive effects of rhizobial LPS is yet to be identified. However, the fact that *S. meliloti* LPS suppresses host defenses in alfalfa hosts, while it typically triggers immune responses in non-host plants, suggests that legumes evolved a sophisticated LPS perception system able to differentially respond to pathogens and symbionts.

EPS are high-molecular weight sugar polymers secreted by gram-negative and gram-positive bacteria with well documented roles in bacterial physiology and pathogenicity. Acidic EPS are essential for rhizobia to establish symbiosis with host plants. Early studies have shown that failed infections by EPS-deficient rhizobial mutants are associated with the induction of defense responses, such as callose deposition and production of antimicrobial compounds, indicating that EPS are involved in suppressing plant defense responses in the *Rhizobium* symbiosis (Niehaus et al., 1993). Microarray analysis further revealed that *M. truncatula* roots inoculated with the *S. meliloti* EPS-deficient mutant *exoY* more strongly induced the expression of defense-related genes than roots inoculated with the wild type strain, indicating that EPS are required for efficient down-regulation of host defense responses during early stages of infection (Jones et al., 2008). Aslam et al. (2008) demonstrated that the virulence function of EPS is based on its ability to chelate calcium anions, thereby blocking calcium influx into the cytosol, which is an essential step for the activation of a plethora of MAMP-trigged responses. Hence, calcium chelation may be an important mechanism underlying the immune-suppressive effects of rhizobial EPS.

Rhizobia suppress SA-dependent defense responses by utilizing the Nod signaling pathway. Salicylic acid (SA) is an important regulator of defense signaling against biotrophic and hemibiotrophic pathogens (Vlot et al., 2009). SA signaling is predominantly transduced via the NPR1 protein, which upon its nuclear translocation acts as transcriptional co-activator of SA-responsive genes (Dong, 2004) and functions as a master regulator in the plant immune signaling network (Leon-Reyes et al., 2009; Pieterse et al., 2009). Rhizobia are mutualistic organisms and therefore it is likely that they are sensitive to SA-regulated defense responses. Indeed, several studies report negative effects of SA signaling on the rate and intensity of rhizobial infection and nodulation (Martinez-Abarca et al., 1998; Van Spronsen et al., 2003; Stacey et al., 2006). Interestingly, Peleg-Grossman et al. (2009) demonstrated that overexpression of *NPR1* in *M. truncatula* suppressed root hair deformation in response to *S. meliloti*, whereas RNAi-mediated reduction in the levels of NPR1 resulted in accelerated root hair curling. This is the first evidence that SA affects symbiosis through NPR1 and highlights the latter as a negative regulator in *Rhizobium*-legume symbiosis.

On the other hand, rhizobia have evolved mechanisms to efficiently control the SA levels of host plants and establish successful infections. Martinez-Abarca et al. (1998) showed that alfalfa plants responded to incompatible *Rhizobium* strains by increasing the endogenous SA content, whereas in response to compatible strains the SA level remained unchanged. Importantly, this same report also demonstrated that a *Rhizobium* mutant unable to synthesize Nod factors was also unable to reduce the endogenous SA levels, indicating that Nod-mediated signaling is required to suppress SA-triggered responses. Likewise, the expression of the β-glucanase *MtBGLU1* gene, which is homologous to the pathogenesis-related *PR2* gene of tobacco, was found to be down-regulated in the roots of *M. truncatula* after inoculation with *S. meliloti,* but not after inoculation with mutant that is unable to synthesize Nod factors (Mitra and Long, 2004). Nod factors were found to be not only essential but also sufficient to down-regulate *MtBGLU1* expression. Moreover, the non-nodulating *dmi1* mutant of *M. truncatula* was shown to be defective in *MtBGLU1* suppression, indicating that Nod-dependent *MtBGLU1* suppression is part of the symbiotic signaling pathway (Mitra and Long, 2004). Collectively, in addition to the essential role of Nod factors as signaling molecules in the nodulation process, evidence is accumulating pointing to a critical role of Nod signaling in overriding host defense responses triggered during rhizobial invasion (Fig. 1B). The molecular mechanisms by which Nod factors interfere with the host immune system are currently unknown. Nod factor perception at the epidermis leads to localized increases in levels of cytokinin and auxin in cortical cells (Oldroyd and Downie, 2008). Thus, one possibility is that Nod factors locally affect the strength of the SA signaling pathway through hormonal-cross talk mechanisms, as demonstrated in diverse plant-pathogen interactions (Pieterse et al., 2009; Verhage et al., 2010).

Type III secreted rhizobial effectors as host-range specificity determinants. Plant and animal pathogenic bacteria possess a specific secretion machinery, called the Type III Secretion System (TTSS), to directly inject virulence effectors into the host cell (McCann and Guttman, 2008). Many effectors are able to suppress MAMP-triggered immunity or manipulate the host's metabolic program to the pathogen's advantage (Boller and Felix, 2009). Functional TTSSs have been identified in several rhizobial species. In TTSS-harboring rhizobia, the *type three secretion* (*tts*) genes cluster together and are homologous to the *hypersensitive response conserved* (*hrc*) genes of pathogenic bacteria (Marie et al., 2001). Rhizobial proteins secreted through the TTSS are known as Nops (for Nodulation outer proteins) (Marie et al., 2001). Nops can be further grouped into proteins that are structurally associated with secretion appendages, and effector proteins that are secreted into the host cell where they are postulated to interfere with biological functions of the host. An important issue of whether rhizobial effectors are directly targeted into the host cell has been recently resolved. Translocation of rhizobial Nop proteins into the cytoplasm of plant cell has been experimentally confirmed for NopP of *Sinorhizobium fredii* and NopE1 and NopE2 of *Bradyrhizobium japonicum* (Schechter et al., 2010; Wenzel et al., 2010). NopL of the *Rhizobium* sp. strain NGR234 is the best characterized effector to possess defense-suppressive functions. NopL acts as virulence factor when ectopically expressed in tobacco plants by down-regulating virus-induced PR protein accumulation (Bartsev et al., 2004). Several Nop effectors show homology to effectors from plant and animal pathogens (Deakin and Broughton, 2009), supporting the idea that the targets of bacterial effectors inside the host cells are conserved (Buttner and Bonas, 2003).

In contrast to bacterial pathogens that depend on a functional TTSS for pathogenesis, rhizobial effectors are not essential for the symbiosis because *Rhizobium* strains lacking a TTSS are still able to establish successful infections, form nodules, and fix nitrogen. Rhizobial type III effectors are likely to assist in the suppression of the MTI response, a role that is most likely superimposed on the dominant defense-suppressive functions of surface polysaccharides and Nod factors. Certain effectors may also act as symbiotic determinants as demonstrated for the Y4IO effector of *Rhizobium* sp. strain NGR234 (Yang et al., 2010a) (Fig. 1B). Despite their elusive role in symbiosis, rhizobial type III effectors have long been considered to function as host range specificity determinants. Only recently, this level of specificity was found to be controlled by host TIR-NBS-LRR resistance (R) proteins that recognize cognate rhizobial effector(s) resulting in effector triggered immunity (ETI) (Fig. 1C) (Yang et al., 2010a). The study of Yang et al. (2010a) highlights a common recognition mechanism underlying symbiotic and pathogenic interactions and further suggests that rhizobia have evolved to suppress ETI in order to establish successful infections. It is therefore anticipated that in the cocktail of effectors that a certain *Rhizobium* injects into the host cytoplasm, certain effectors may possess avirulence activities while others have evolved to suppress the ETI response.

MODULATION OF HOST IMMUNITY IN MYCORRHIZAL ASSOCIATIONS

Symbiotic mycorrhizal fungi reduce stimulation of the host's immune system. Besides through MAMPs, microbes can also be detected via damage-associated molecular patterns (DAMPs), which are endogenous plant-derived molecules that arise from damage or enzymatic degradation of cell walls, such as caused by invading alien organisms. Interestingly, the genome of the ectomycorrhizal fungus (EMF) *Laccaria bicolor* lacks several gene families that encode for enzymes involved in the degradation of plant cell walls that could otherwise elicit immune responses (Martin et al., 2008). Likewise, the EMF *Tuber melanosporum* (black truffle) carries a relatively small number of carbohydrate cleaving enzymes (Martin et al., 2010). This particular genomic feature is also apparent in the genomes of the fungal pathogen *Ustilago maydis* (Kamper et al., 2006) and the oomycete *Hyaloperonospora arabidopsidis* (Baxter et al., 2010) and mirrors an evolutionary adaptation of symbiotic fungi and certain plant pathogens to a symbiotic and biotrophic lifestyle, respectively.

Mycorrhizal fungi initially elicit a MTI response which is subsequently suppressed. Early studies regarding the interaction between plants and arbuscular mycorrhizal fungi (AMF) revealed that expression of defense- and stress-related genes is prominent during early stages of the interaction and subsequently declines as the symbiosis develops (Kapulnik et al., 1996). Most research based on large-scale transcriptional profiling in various symbiotic plant-mycorrhiza interactions revealed that defense-related gene expression in host plants follows similar expression patterns (Liu et al., 2003; Heller et al., 2008). Thus, the transcriptional response activated in roots upon mycorrhization may be similar to the two-wave transcriptional reprogramming reported for the *Rhizobium*-legume symbiosis. These data suggest that hosts initially treat symbiotic fungi as potential invaders and activate a defense program (Fig. 2A), whereas in later stages of the interaction this defense program is countered by the mycorrhizal symbionts (Fig. 2B and 2C).

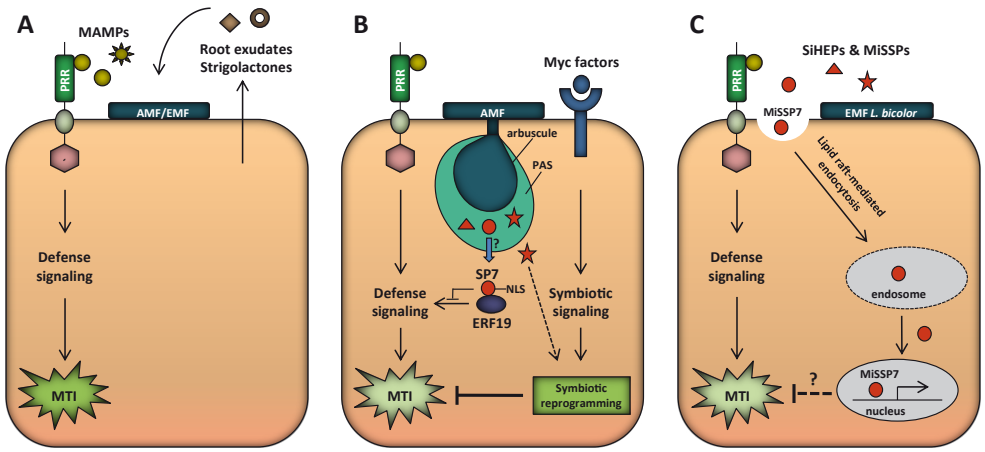

Figure 2. Model for the modulation of host immunity in the ecto- and endomycorrhizal symbioses. (A) Root exudates recruit symbiotic mycorrhizal fungi and prime them for the interaction. Host plants initially recognize ectomycorrhizal (EMF) and arbuscular mycorrhizal fungi (AMF) as potential invaders; pattern-recognition receptors (PRRs) in the host perceive microbe-associated molecular patterns (MAMPs, yellow-colored shapes) and a signaling cascade is initiated that results in MAMP-triggered immunity (MTI). **(B)** The EMF *Laccaria bicolor* secretes proteins similar to the haustoria-expressed proteins of pathogenic basidiomycetes (SiHEPs) and several cysteine-rich mycorrhiza-induced small secreted proteins (MiSSPs) that may function as extra- or intracellular effectors to suppress host immune responses. The MiSSP7 effector is perceived by the plant cell through lipid-raft mediated endocytosis and translocated into the nucleus where it promotes auxin-related gene expression. It remains to be demonstrated whether upregulation of auxin signaling has a negative impact on plant defenses as demonstrated for pathogenic interactions. **(C)** In the AM symbiosis the establishment of the symbiotic program in plant cells, which is activated upon perception of the mycorrhizal Myc factors, counteracts MTI with mechanisms yet to be defined. Molecules (brown-colored shapes) that are secreted in the apoplastic or periarbuscular space (PAS) may act either as apoplastic or cytoplasmic effectors to suppress the MTI response or promote the symbiotic program. The AMF *Glomus intraradices* secretes the SP7 effector which is translocated into the plant cytosol; a nuclear localization signal (NLS) targets SP7 to the nucleus where it interacts with the defense-related transcription factor ERF19 to block the ERF19-mediated transcriptional program.

Insights into the genome of mycorrhizal fungi: secretion of effector-like molecules. Sequencing of the genomes of the EMF *L. bicolor* and *T. melanosporum* provided the first important evidence that symbiotic fungi may use similar strategies as pathogenic fungi to evade host immunity. In *L. bicolor*, whole-genome sequence analysis combined with genome-scale expression profiling revealed candidate molecules that may act as effectors in modulating plant innate immunity as demonstrated for effectors of several pathogenic fungi and oomycetes (Martin et al., 2008). Twelve predicted proteins of the symbiotic fungus share significant similarity with haustoria-expressed secreted proteins that are involved in the pathogenesis of pathogenic basidiomycetes

(Martin et al., 2008). In addition, the genome of *L. bicolor* encodes a number of small secreted proteins (SSPs), many of which are induced during the symbiotic interaction. Several cysteine-rich mycorrhiza-induced SSPs (MiSSPs) show significant similarity to cysteine-rich apoplastic effectors of pathogenic fungi (Martin et al., 2008), suggesting that they may function as extracellular or intracellular effector-type molecules to suppress defense signaling pathways. Recently, Plett et al. (2011) demonstrated that the effector MiSSP7 of *L. bicolor* is perceived by the plant cell through lipid-raft mediated endocytosis and is translocated into the nucleus where it promotes auxin-related gene expression (Fig. 2B). Whether upregulation of auxin signaling has a negative impact on plant defenses as demonstrated for pathogenic interactions (Kazan and Manners, 2009), remains to be demonstrated. It should be noted that in the *T. melanosporum* ectomycorrhizas, MiSSPs are not induced (Martin et al., 2010), indicating that in different symbiotic systems different molecules may operate as effectors.

In contrast to the EMF, the genomes of the AMF are not assembled yet, and thus predictions for putative effectors in the AM symbioses remain elusive. AMF on the one hand, and certain biotrophic fungal and oomycete pathogens on the other hand, employ similar invasion strategies to infect their hosts (Paszkowski, 2006). The transcriptional responses mounted in host plants in response to biotrophic pathogens and AMF significantly overlap, pointing to the existence of conserved molecules that execute similar functions (Paszkowski, 2006). It is, therefore, anticipated that certain molecules secreted by the AMF in the apoplastic or periarbuscular space during the interaction with the host, act either as apoplastic or cytoplasmic effectors in order to short-circuit the plant defense program. The SP7 effector of the AMF *Glomus intraradices* is the first endomycorrhizal effector described so far to possess defense-suppressive activity (Kloppholz et al., 2011). *SP7* expression is induced upon contact with the host roots and the secreted protein is translocated into the plant cytosol. A nuclear localization signal (NLS) subsequently targets SP7 to the nucleus where it interacts with the defense-related ET-responsive transcription factor ERF19 to block the ERF19-mediated transcriptional program (Fig. 2C). The identification of the SP7 protein as a mycorrhizal effector that interferes with the ethylene (ET) signaling pathway is in line with recent data that highlight the importance of this hormone in MTI (Clay et al., 2009; Boutrot et al., 2010; Mersmann et al., 2010; Millet et al., 2010).

Mycorrhizal fungi suppress SA-mediated defense responses by utilizing the Myc signaling pathway. Similar to rhizobium symbiosis, SA signaling has been shown to negatively affect root mycorrhization by AMF (Blilou et al., 1999; Medina et al., 2003). On the other hand, strict regulation of the SA content is a prerequisite for efficient colonization. Recently, López-Ráez et al. (2010) demonstrated that the SA content, and accordingly the expression of the SA-responsive gene *PR1a,* was exclusively elevated in tomato roots colonized by the AMF *Glomus mossae* but not by *Glomus intraradices*, a fungus known to colonize tomato plants at higher rates. Likewise, SA only transiently accumulated during early stages of the interaction between pea and the AMF *G. mosseae* (Blilou et al., 1999). In the non-mycorrhizal P2 mutant of pea (*Pisum sativum*) the initial increase in the SA levels persisted, indicating that, like rhizobia, AMF modulate SA-mediated responses by utilizing the Myc signaling pathway. Interestingly, in the *M. truncatula-Gigaspora margarita* interaction, the activity of the Ca^{+2}/calmodulin kinase DMI3 is required to repress the expression of early-induced defense-related genes (Siciliano et al., 2007), suggesting that perception of Myc factors also contributes to the repression of early MTI responses (Fig. 2C).

MODULATION OF HOST IMMUNITY IN NON-SYMBIOTIC BENEFICIAL INTERACTIONS

PGPR may use phase variation to avoid stimulation of the host's immune system. Like rhizobia and mycorrhizal fungi, non-symbiotic beneficial microbes, such as PGPR that often grow endophytically

inside the roots, should also minimize stimulation of their host's immune system. Phenotypic variation or phase variation is an adaptive process by which bacteria can reversibly switch between colonies with different morphology (Davidson and Surette, 2008). At the molecular level, phase variation is controlled by diverse genetic mechanisms including site-specific DNA rearrangements and epigenetic modifications (Hallet, 2001; Wisniewski-Dye and Vial, 2008). Either of these mechanisms generates bacterial subpopulations within a clonal population that differentially express surface molecules (e.g. flagella, LPS) or express surface molecules with altered structure (Van der Woude and Baumler, 2004). Phase variation provides bacteria with a significant advantage of adaptation to different environments and has been extensively documented in several studies as a mechanism that animal pathogens employ to escape immune detection (Kingsley and Baumler, 2000). Phenotypic variation is also common among rhizosphere Pseudomonads and has been reported as a conserved strategy that bacteria have evolved in order to increase their overall fitness in the rhizosphere (Van den Broek et al., 2005). Rhizosphere *Pseudomonas* bacteria may use antigen variation to reduce their antigenic potential and therefore minimize stimulation of the host's immune system. For instance, the PGPR *Pseudomonas brassicacearum* shows two distinct morphological variants designated as Phase I and Phase II (Achouak et al., 2004). Phase I cells are found on the basal parts of the root and produce significantly lower amounts of flagellin compared to Phase II cells, which are predominantly found on secondary roots and root tips (Achouak et al., 2004). It is possible that once colonization of new root niches is achieved, *P. brassicacearum* shifts into phase I cells in order to mask flagellin recognition by the host. Interestingly, *Pseudomonas aeruginosa* was recently found to excrete an alkaline protease (AprA) that degrades flagellin monomers that serve as ligands for the immune receptors FLS2 in plants and TLR5 in mammals, thereby evading host immune activation in both plants and mammals (Bardoel et al., 2011). In *P. brassicacearum, AprA* was demonstrated to be expressed in phase I cells (Achouak et al., 2004), supporting the hypothesis that phase variation plays a role in immune evasion.

Non-symbiotic microbes initially elicit a MTI response. Immune responses to elicitor molecules derived from PGPR are best characterized for selected ISR-inducing strains of fluorescent Pseudomonads (Bakker et al., 2007; Van Wees et al., 2008). Cell wall preparations of various ISR-inducing rhizobacteria all triggered typical immune responses in tobacco suspension cells, including a burst of reactive oxygen species, extracellular medium alkalization, rapid elevation of cytoplasmic Ca^{+2}, and defense related gene expression (Van Loon et al., 2008). Furthermore, heat-killed *Pseudomonas fluorescens* WCS417 bacteria were shown to activate the expression of MAMP-responsive reporters and trigger callose depositions in Arabidopsis roots (Millet et al., 2010). Recently, Jacobs et al. (2011) demonstrated that the PGPF *Piriformospora indica* is also recognized by the root immune system through its MAMPs. Thus, both PGPR and PGPF possess a pallet of MAMPs able to elicit MTI in the roots of host plants (Fig. 3A). On the other hand, evidence is accumulating that non-symbiotic beneficial microbes actively suppress the defense responses that are initially mounted in roots upon MAMP perception (Fig. 3B and 3C).

Non-symbiotic microbes utilize effector molecules to suppress MTI: Recently, Millet et al. (2010) provided evidence that the PGPR *P. fluorescens* WCS417 is able to suppress flagellin-triggered MTI responses in Arabidopsis roots via apoplastic secretion of one or more low molecular weight molecule(s) (Fig. 3B). Bacterial determinants that have been demonstrated to be essential for rhizobial infections, such as LPS and EPS, may have similar functions in suppressing MTI during early stages of interaction between PGPR and host plants. Indeed, a *P. fluorescens* WCS417 mutant lacking the *O*-antigenic side chain of its outer membrane LPS was demonstrated to colonize tomato roots to a lesser extent than the wild type strain (Duijff et al., 1997), suggesting a role for wild-type LPS in immune suppression.

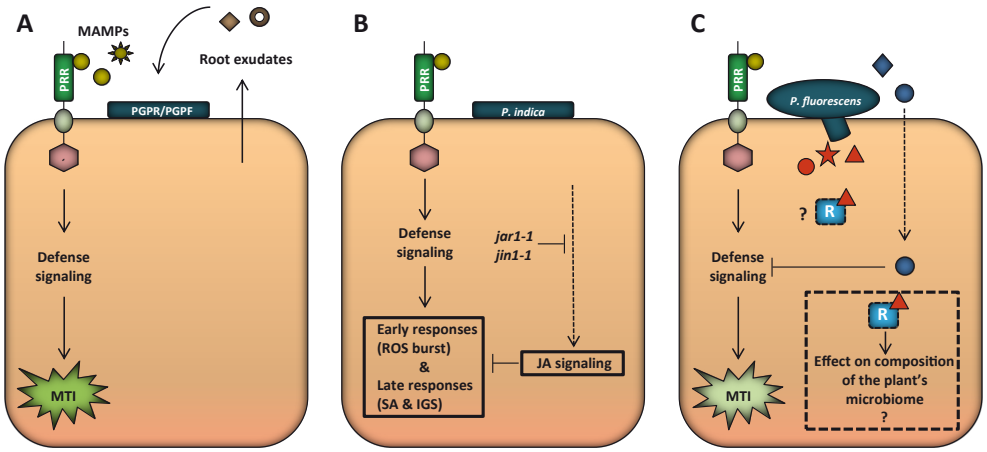

Figure 3. Model for the modulation of host immunity during interactions with non-symbiotic PGPR and PGPF. **(A)** Root exudates recruit plant growth promoting rhizobacteria (PGPR) and fungi (PGPF) and prime them for interaction. Host plants initially recognize PGPR and PGPF as potential invaders; pattern-recognition receptors (PRRs) in the host perceive microbe-associated molecular patterns (MAMPs, yellow-colored shapes) and a signaling cascade is initiated, resulting in MAMP-triggered immunity (MTI). **(B)** The PGPR *Pseudomonas fluorescens* WCS417 suppresses the MTI response via apoplastic secretion of one or more so far unidentified effector molecules (grey-colored shapes). Whether the secreted molecule(s) act as apoplastic or cytoplasmic effectors, and the mechanisms by which they interfere with the host immune system remain currently unknown. Effector molecules (brown-colored shapes) that are secreted via the type III secretion apparatus of *P. fluorescens* and other PGPR are likely to assist, but seem not to be essential for the MTI suppression. In analogy to root nodule symbiosis, certain type III effectors may be recognized by host resistance proteins (R), which in turn may impact the composition of the microbial community in the rhizosphere. **(C)** The PGPF *Piriformospora indica* recruits the JA signaling pathway to suppress both early (reactive oxygen species (ROS) production) and late (SA-mediated responses and indole glucosinolate (IGS) production) defense responses. This is mediated via the JA signaling components JAR1 and MYC2 (JIN1) because the JA-related mutants *jar1-1* and *jin1-1* of Arabidopsis are unable to suppress MTI.

Presuming the importance of ET in MTI (Clay et al., 2009; Millet et al., 2010), another plausible scenario is that *P. fluorescens*-secreted molecules target ET-dependent defense processes. A wide range of PGPR have been shown to secrete the enzyme ACC deaminase, which degrades the ET precursor ACC resulting in reduced ET production in the plant (Glick et al., 2007). A significant reduction in the expression of genes encoding ET-related transcription factors has been reported for Arabidopsis roots colonized by PGPR *P. fluorescens* WCS417 and FPT9601-T5 (Verhagen et al., 2004; Wang et al., 2005), which supports the hypothesis that PGPR modulate host immune responses by interfering with the ET signaling pathway.

In the *P. indica*-Arabidopsis interaction, the beneficial fungus recruits the jasmonic acid (JA) signaling pathway to suppress both early- and late-activated defense responses (Fig. 3C). Also suppression of the flg22-mediated MTI in the roots by the plant pathogen *Pseudomonas syringae* was demonstrated to depend on a functional JA signaling pathway (Millet et al., 2010). In both cases, suppression of MTI was mediated via the JA signaling components JAR1 and MYC2, suggesting that activation of the JA pathway may be a common strategy to affect host immunity in the roots.

Hormone-like compounds produced by PGPR may contribute to the suppression of the SA signaling pathway. Induction of SA-mediated responses have been demonstrated to reduce bacterial abundance in the plant rhizosphere (Kniskern et al., 2007; Doornbos et al., 2011). Also colonization of Arabidopsis roots by the PGPF *P. indica* is affected by the SA signaling (Jacobs et al., 2011). Many PGPR and PGPF are able to produce substantial amounts of phytohormone-like compounds, such as auxins and gibberellins (Sirrenberg et al., 2007; Lugtenberg and Kamilova,

2009). Several phytohormones have been demonstrated to negatively cross-communicate with the SA signaling pathway and affect the outcome of the immune response (Pieterse et al., 2009; Verhage et al., 2010). Hence, it is tempting to speculate that non-symbiotic microbes may produce phytohormones in order to attenuate the relative strength of the SA signaling via hormonal cross-talk mechanisms.

Type III secreted PGPR effectors: host-specificity determinants? The existence of a classical TTSS is not only restricted to pathogenic bacteria and rhizobia, but also occurs in other nonpathogenic, root-associated bacteria. TTSS has been first described for the PGPR *P. fluorescens* SBW25 (Rainey, 1999), but other fluorescent Pseudomonads are also equipped with a TTSS (Preston et al., 2001; Mazurier et al., 2004; Rezzonico et al., 2004; Mavrodi et al., 2011). In *P. fluorescens* SBW25, genes encoding for TTSS reside in a 20-kb *tts* cluster (named *rsc/rsp*) and several ORFs in the cluster display a significant degree of similarity to the *hrc/hrp* genes of pathogenic bacteria (Preston et al., 2001). *P. fluorescens* SBW25 is believed to secrete at least one effector and this is the RopE of the AvrE family, a widely conserved family of effectors. There is also evidence for the existence of additional effectors, since *ropE* mutants are still able to trigger HR in *Nicotiana clevelandii*. *P. fluorescens* Q8r1-96 was recently reported to secrete the type III effectors RopAA of the HopAA1-1 family of effectors, RopM of the HopM1 family, and the unique RopB effector (Mavrodi et al., 2011). Interestingly, all three type III effectors were found to be capable of suppressing typical innate immune responses when ectopically expressed in *N. benthamiana* (Mavrodi et al., 2011). Several mutations in components of the TTSS of *P. fluorescens* SBW25 have been shown to affect the competitive colonization capacity of the bacterium in the rhizosphere of sugar beets (Jackson et al., 2005). However, it is worth noticing that all of these mutants displayed altered *in vitro* growth compared to the wild type strain, suggesting a more general role of the TTSS in the physiology of *P. fluorescens* SBW25 (Jackson et al., 2005). On the other hand, *P. fluorescens* Q8r1-96 mutants lacking functional TTSS are not altered in their rhizosphere competence (Mavrodi et al., 2011). Overall, the significance of type III secretion in PGPR remains far from clear. Effectors delivered via the TTSS of PGPR may either assist in the suppression of the MTI response or may manipulate certain host's metabolic processes for their own benefit. Further research is needed to address whether resistance proteins in the host are evolved to recognize PGPR effectors, and thereby may be instrumental in shaping the plant's microbiome in the rhizosphere (Fig. 3B). Earlier work already demonstrated that plant immune responses triggered by PGPR depends on the host/PGPR combination (Van Wees et al., 1997; Van Loon et al., 1998; Ton et al., 1999; Ton et al., 2002b), suggesting a gene-for-gene-type relationship between plants and mutualistic rhizobacteria.

LOCAL AND SYSTEMIC DEFENSE RESPONSES CONTRIBUTE TO BALANCING THE COSTS OF MUTUALISM

Despite their net fitness benefit, mutualistic interactions often also come with fitness costs. Therefore, host plants have evolved strategies to control the extent to which a mutualistic association develops. For instance, in the *Rhizobium*-legume symbiosis, the host is able to partly control the level of infection through selective allocation of resources (Kiers et al., 2003). However, evidence is emerging for an important role of induced plant defense responses in the balancing of mutualistic associations.

Local JA and ET signaling in the control of beneficial interactions. Plant oxylipins comprise an important class of oxidized fatty acids commonly involved in pathogenic interactions, either as antimicrobial agents or as signaling molecules regulating plant defense responses (Wasternack, 2007). The oxylipin JA and its derivatives (collectively called jasmonates) play an important role in

the regulation of plant-beneficial microbe interactions (Van der Ent et al., 2009a). In the mycorrhizal symbiosis, JAs were shown to accumulate (Hause et al., 2002; Isayenkov et al., 2005; Stumpe et al., 2005; López-Ráez et al., 2010), hence a role for JAs in restricting mycorrhizal colonization via the onset of a defense program was accordingly proposed (Hause et al., 2007; Gutjahr and Paszkowski, 2009; López-Ráez et al., 2010). Similarly, the PGPF *P. indica* was shown to upregulate JA biosynthetic genes in the roots (Schafer et al., 2009), suggesting that activation of the JA signaling pathway may be a widely-used strategy that plants employ to control colonization by beneficial endophytic fungi.

ET also appears to have a crucial role in maintaining a delicate balance between host plants and microsymbionts. In *Rhizobium* symbiosis the number of rhizobial infections within the nodulation zone is under regulation and only a small number of infection threads persist and colonize nodules. The ET-insensitive mutant *sickle* of *Medicago* is defective in this level of regulation and is hyperinfected by its microsymbiont *S. meliloti* (Penmetsa and Cook, 1997). Since arrested infections often display characteristics of a hypersensitive response (Vasse et al., 1993), it is possible that ET regulates the number of successful infections by signaling certain defense responses within the nodulation zone. Supportive of this hypothesis is that *MtSkl1* mediates defense responses against a subset of *Medicago* pathogens (Varma Penmetsa et al., 2008). ET is also important in regulating the progress of root colonization by beneficial endophytic fungi. Camehl et al. (2010) recently demonstrated that overexpression of the ET-responsive transcription factor ERF1 in Arabidopsis resulted in reduced root colonization by the PGPF *P. indica* due to the potentiated activation of defense responses. Conversely, *P. indica* more effectively colonized roots of the ET-related mutants *etr1*, *ein2* and *ein3/eil1* (Camehl et al., 2010). Importantly, in both cases the benefits for the host were diminished, indicating that ET homeostasis within the plant is crucial to control the extent of fungal colonization by regulating the expression of defense-related genes.

Systemic signaling in the control of beneficial interactions. In addition to these local responses, plants that establish a symbiosis with rhizobia can also control the number of infections and nodules through a systemic mechanism called autoregulation of nodulation (AON) (Oka-Kira and Kawaguchi, 2006). AON is a two-step process and is mediated by long-distance signaling which involves both root- and shoot-derived signal(s) (Fig. 4A). The root-derived signal is generated in response to primary rhizobial infections locally in the roots and is then translocated to the shoot. Upon perception, a second signal is generated and then translocated back to the root to activate a defense program that restricts further nodulation. A specific group of hypernodulating mutants defective in the shoot-regulated AON has been identified (Oka-Kira and Kawaguchi, 2006). These mutants are all defective in homologs of *CLAVATA1* (*CLV1*), a LRR-RLK involved in the regulation of shoot and floral meristem size of Arabidopsis. Recent work in *M. truncatula* and *L. japonicus* provided important evidence that Clavata3/endosperm surrounding region (CLE) peptides are candidate molecules for the root derived signal (Okamoto et al., 2009; Mortier et al., 2010).

The AON phenomenon has striking mechanistic similarities to induced systemic resistance (ISR) and systemic acquired resistance (SAR), as in all these cases a microbial infection in local tissues leads to resistance against secondary infections in distant plant parts (Durrant and Dong, 2004; Van Wees et al., 2008; Vlot et al., 2009). A possible connection between AON and defense signaling is supported by the fact that several autoregulation mutants are hypersusceptible to nematode and pathogen infection (Lohar and Bird, 2003; Tazawa et al., 2007). In line with this, Kinkema and Gresshoff (2008) reported that in soybean, a subset of defense-related genes is regulated via the AON receptor GmNARK, albeit in a nodulation-independent manner. Interestingly, the CLAVATA3 peptide of Arabidopsis was recently reported to be capable of triggering immune signaling via the flagellin receptor FLS2 (Lee et al., 2011), raising the possibility that CLE peptides may also be perceived in systemic tissues independent of the AON receptor kinase to trigger an immune response. Elicitation of induced resistance in the above-ground tissues was shown to impact

beneficial plant-microbe interactions in the rhizosphere (Faessel et al., 2010; De Roman et al., 2011), which strengthens the notion that systemic defense signaling may be an intrinsic part of the AON phenomenon and functions in balancing the costs and benefits of the mutualistic relationship.

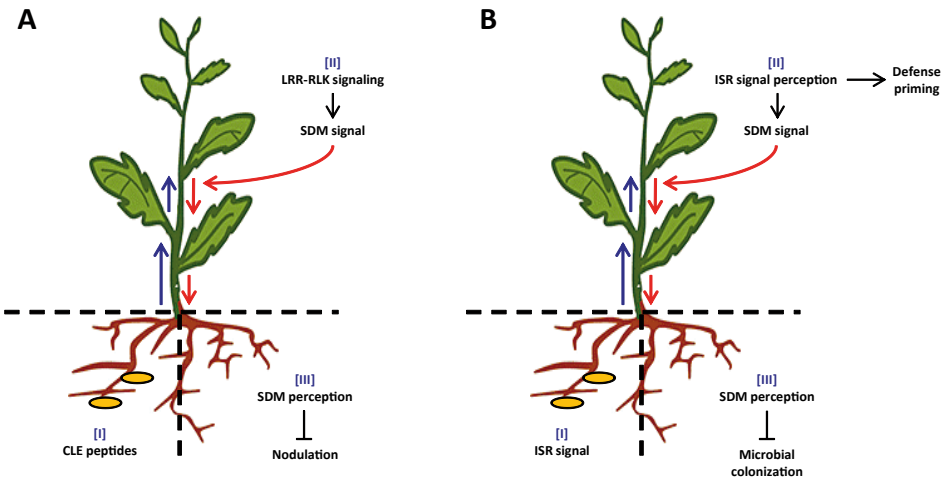

Figure 4. Parallels between autoregulation of nodulation (AON) and induced systemic resistance (ISR). (A) Proposed model for AON in the root nodule symbiosis in a split-root system (adapted from Oka-Kira and Kawaguchi, 2006; Staehelin et al., 2011). Clavata3/endosperm surrounding region (CLE) peptides are produced upon primary rhizobial infections and subsequently transported to the shoot, most likely through the xylem (stage I). Perception of CLE peptides in the shoot by the corresponding leucine-rich repeat receptor-like kinase (LRR-RLK) initiates a signaling cascade that results in the production of a phloem-mobile shoot-derived molecule (SDM) (stage II). SDM perception in the roots restricts nodulation (stage III). **(B)** Proposed model for ISR in a split-root system. Root colonization by selected strains of plant growth promoting rhizobacteria (PGPR) results in the production of the ISR signal which travels via the xylem to the above ground tissues. Signal perception in the shoot may result in the production of a phloem-mobile shoot-derived molecule (SDM) and priming for enhanced defense, which is effective against a broad spectrum of pathogens (stage II). SDM perception in the root results in enhanced resistance against root pathogens, and possibly further restricts colonization by the beneficials (stage III). Blue and red arrows indicate movement via the xylem and phloem, respectively.

INDUCED SYSTEMIC RESISTANCE: THE RESULTANT OF AN AUTOREGULATION SIGNALING PATHWAY?

Split-root experiments with AON mutants of soybean revealed that autoregulation in the rhizobial and the mycorrhizal symbiosis is controlled in a similar manner (Meixner et al., 2005; Staehelin et al., 2011). Also in the interaction with PGPR there is evidence that plants utilize systemic autoregulation mechanisms to control the beneficial interaction. For instance, in split-root experiments it was shown that colonization of one half of the roots by beneficial PGPR leads to enhanced defenses in the other part of the root system, which should have been mediated via a systemic signal (Leeman et al., 1996; Siddiqui and Shaukat, 2002). Often these systemically activated defenses are also expressed in above-ground plant tissues, giving rise to an induced systemic resistance that is typically effective against a broad spectrum of plant pathogens and even insect herbivores (Van Loon et al., 1998; Van Wees et al., 2008; Pineda et al., 2010). In contrast to pathogen-induced SAR, which is dependent upon SA signaling and is associated with enhanced expression of a large set of pathogenesis-related genes (Durrant and Dong, 2004; Vlot et al., 2009), PGPR- and PGPF-triggered ISR is often SA-independent and not associated with major changes in defense-related gene expression (Van Wees et al., 2008; Van der Ent et al., 2009a). Instead, a relatively mild systemic immune reaction is triggered that is associated with priming for enhanced defense (Pozo et al.,

2008; Van der Ent et al., 2008; Van der Ent et al., 2009b). PGPR-primed plants do not express costly defenses but display an accelerated, often JA/ET-dependent defense response upon pathogen or insect attack (Van Hulten et al., 2006; Pozo et al., 2008; Stein et al., 2008). Because priming only gives rise to activation of defense upon recognition of a potential intruder, it is an ideal mechanism to control the delicate interaction with invading beneficial microbes. It is, therefore, tempting to speculate that in analogy to rhizobia and mycorrhizal symbiosis, beneficial associations with non-symbiotic microbes are controlled by an autoregulation strategy of which the resultant is the ISR phenomenon that provides systemic protection in roots and shoots against a broad spectrum of pathogens (Fig. 4B).

CONCLUDING REMARKS

The boundaries between mutualism and pathogenesis are fluid. A nice example of this was recently demonstated for the pathogenic bacterium *Ralstonia solanacearum*. Inactivation of the master virulence regulator *HrpG* of this pathogen, which controls the expression of TTSS components and associated effectors, with concomitant transfer of the symbiotic plasmid of the microsymbiont *Cupriavidus taiwanensis*, was sufficient to shift the pathogen's behavior from pathogenic towards mutualistic (Marchetti et al., 2010). Moreover, the recent findings that mycorrhizal fungi employ effector-like molecules to manipulate the host's immune response (Kloppholz et al., 2011; Plett et al., 2011), and that host resistance genes control specificity in the *Rhizobium* symbiosis (Yang et al., 2010a) indicate that mutualistic and pathogenic interactions involve strikingly similar principles.

To date, research on plant responses to pathogenic and beneficial microbes has been largely separated. Due to the numerous commonalities in mechanisms and principles of both types of plant-microbe interactions, scientists from both fields start to join forces to foster an integrated view on the molecular mechanisms that evolved in pathogenic and mutualistic plant-microbe interactions. Effector biology, which currently is a major topic in plant-pathogen interactions research, is becoming an active area in the field of beneficial plant-microbe interactions as well, and is anticipated to significantly improve our understanding of how mutualistic associations develop. With this review we aimed to provide an overview of the mechanisms involved in the modulation of host immunity by symbiotic and non-symbiotic beneficial microbes. We tried to identify commonalities between plant responses to different symbiotic beneficials and non-symbiotic PGPR and generate an integrated view on how modulation of local and systemic defense responses aid in establishing the delicate balance between the two partners. Ultimately, research on the interplay between plants and beneficial microbes will provide detailed insight into how plants are able to shape the microbiome in their rhizosphere in order to maximize the profitable functions of their "second genome".

THESIS OUTLINE

Root colonization by selected strains of beneficial soil-resident bacteria and fungi is known to improve plant growth and trigger a systemic immune response that is effective against a broad range of pathogens, known as induced systemic resistance (ISR). Significant progress has been made during the past years regarding the signaling pathways involved in rhizobacteria-mediated ISR in systemic tissues. However, the molecular mechanisms underpinning beneficial interactions between plants and ISR-inducing bacteria in roots, received little attention. In this thesis we aimed to explore signaling mechanisms that are activated in local tissues in response to ISR-inducing bacteria.

In Chapter 2, we investigate the molecular mechanisms underlying the effects of soil-borne *Pseudomonas* spp. strains on root system architecture. We demonstrate that *Pseudomonas fluorescens* WCS417-secreted molecules exert a negative impact on primary root growth of Arabidopsis, whereas they positively influence lateral root and root hair formation. By employing confocal microscopy and genetic analysis, we highlight auxin signaling as the central hormonal pathway to mediate these effects, and we discuss the potential impact of such modification on root morphology for both plants and bacteria.

Previously, *P. fluorescens* WCS417- and *Trichoderma*-mediated ISR was found to depend on the root-specific MYB72 transcription factor, which is further known to be specifically induced under iron limited conditions. Based on this, in Chapter 3, we investigate possible connections between ISR and iron-deficiency inducible mechanisms. We demonstrate that rhizobacteria capable of triggering ISR in Arabidopsis, hijack the iron-sensing system in roots to trigger iron-deficiency inducible mechanisms (known as Strategy I) and *MYB72* expression. By assessing the disease resistance phenotype of mutants with overactive Strategy I locally in roots, a link between rhizobacteria-mediated ISR and iron-deficiency inducible mechanisms was established.

In Chapter 4, we employed microarray analysis in order to identify WCS417-responsive genes that are regulated in a MYB72-depedent manner, in an effort to identify downstream components that are essential for the ISR establishment. Our experiments led to the identification of five WCS417-responsive genes that are regulated in a MYB72-dependent manner, several of which are also regulated under iron starvation. One of the identified genes encodes the β-glucosidase BGLU42, for which we demonstrate that it is an indispensable component for the establishment of ISR. Data presented in this Chapter, further points to a central role of root trichoblasts in the initiation of rhizobacteria-mediated ISR.

In Chapter 5, we initiated an effort to identify MYB72-interacting partners. Our analysis revealed the closely-related MYB10 transcription factor as MYB72-interacting partner. In this Chapter, we also show that MYB72 and MYB10 have redundant roles in regulating the expression of genes involved in the shikimate pathway and the phenylpropanoid pathway, whereas their overexpression results in the suppression of a large set of defense-related genes in the Arabidopsis root. A role of MYB72 and MYB10 as targets of rhizobacterial effectors will be discussed.

In Chapter 6, results presented in this thesis are discussed under the prism of the current status of knowledge on beneficial interactions between plants and soil-borne microbes.

CHAPTER 2

Dissection of root developmental program initiated by plant growth-promoting bacteria

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ABSTRACT

Root colonization by beneficial soil-resident bacteria and fungi is known to trigger a systemic immune response that is effective against a broad range of pathogens, called induced systemic resistance (ISR). In this study, we demonstrate that selected ISR-inducing *Pseudomonas* spp. strains are further capable of improving plant growth and confering notorious alterations in the root system architecture, independent of their ability to trigger ISR in Arabidopsis. By employing an *in vitro* co-cultivation system and focusing in the model strain *Pseudomonas fluorescens* WCS417, we demonstrate that WCS417 releases diffusible factors that exert negative effects on primary root growth whereas possitively influence lateral root and root hair formation. Detailed analysis on root meristems by employing tissue- and cell-type specific markers revealed that WCS417-secreted molecules do not disrupt the overall meristem organization, however, they do possitively affect cell divisions and negatively influence cell expansion. WCS417-mediated alteration in root morpholgy is accompanied by an augmented auxin response as evident by confocal microscopy of the corresponding auxin-response lines. Mutations in genes involved in auxin signaling as well as chemical inhibition of polar auxin transport severely affected the ability of WCS417 to exert its effects on root developmental programs, pointing to a crital role of auxin in WCS417-mediated developmental root plasticity. Jasmonic acid and ethylene signaling mutants and mutants *myb72* and *npr1,* known to be affected in their ability to mount ISR in response to WCS417, respond similary to wild type WCS417, indicating that ISR and alterations in root system architecture stimulated by WCS417 are mediated by different signaling components.

INTRODUCTION

The rhizosphere is the narrow zone of soil immediately surrounding plant roots. It is an energy-rich habitat that supports high populations of soil microbes. Non-symbiotic beneficial rhizosphere bacteria, collectively referred to as plant growth-promoting rhizobacteria (PGPR), positively affect plant performance by improving growth and conferring local protection against root pathogens (Lugtenberg and Kamilova, 2009). Root colonization by selected strains of PGPR is known to result in a systemic immune response, called induced systemic resistance (ISR), that is effective against a broad spectrum of foliar pathogens and even insects (Van Wees et al., 2008; Pineda et al., 2010). The ISR signaling pathway is controlled by the plant hormones jasmonic acid (JA) and ethylene (ET) and depends on the transcriptional (co-)activators NPR1, MYB72, and MYC2 (Pieterse et al., 1998; Pozo et al., 2008; Van der Ent et al., 2008)

Significant progress has been made over the past years regarding the plant-growth promoting effects mediated by PGPR. So far, several mechanisms for rhizobacteria-mediated plant growth-promotion have been proposed and various bacterial determinants have been suggested to function as plant-growth stimulants (Lugtenberg and Kamilova, 2009). Amongst them, the volatile substances acetoin and 2,3-butanediol emitted from *Bacillus* spp. have been demonstrated to be potent stimulators of plant growth (Ryu et al., 2003). Other PGPR are known to improve plant nutrition by increasing phosphate solubility or upregulating NO_3^- uptake mechanisms (Mantelin and Touraine, 2004; Browne et al., 2009). Importantly, root colonization by PGPR and plant-growth promoting fungi (PGPF) is commonly accompanied with alterations in root morphology (Mantelin and Touraine, 2004; Lopez-Bucio et al., 2007; Felten et al., 2009; Splivallo et al., 2009).

The root system architecture is affected by three main processes: (i) indeterminate growth of the main root, a process orchestrated by the root meristem, (ii) lateral root formation, and (iii) root hair formation. Post-embryonic root development is controlled by cell divisions in the meristematic

zone, cell expansion in the elongation zone, and functional differentiation into specialized cell types in the differentiation zone of the root (Bennett and Scheres, 2010). The population of mitotic cells in the root meristem originates from single stem cells whose identity is controlled by an organizing center called quiescent center (QC) (Weigel and Jurgens, 2002; Ohlstein et al., 2004; Li and Xie, 2005). Auxin gradients as those established by the PIN-FORMED (PIN) auxin efflux facilitator network and a genetic program regulated by WUSCHEL RELATED HOMEOBOX 5 (WOX5), SCARECROW (SCR), SHORT-ROOT (SHR), and PLETHORA (PLT) transcription factor proteins are crucial for stem cell maintenance and function (Di Laurenzio et al., 1996; Helariutta et al., 2000; Aida et al., 2004; Blilou et al., 2005; Sarkar et al., 2007).

Lateral roots (LR) and root hairs (RH) constitute important traits of the root architecture that facilitate plant anchorage and increase the root's exploratory capacity for water and minerals. LR originate from xylem pole pericycle cells that are primed in the basal meristem to become LR founder cells (Casimiro et al., 2003). LR initiation occurs in more distal parts of the root where an initial anticlinal and assymetrical division of a pair of adjacent founder cells is followed by a series of cell divisions to form higher order LR primodia (LRP). At the later stages, the LR emerges from the parental root by concurrent expansions of cells within the LRP and cell wall modifications in the surrounding tissues (Casimiro et al., 2003; Benková and Bielach, 2010). Local auxin accumulation and signaling has a critical role during LR formation by regulating developmental processes from founder cell specification to LR emergence (De Smet et al., 2007; Dubrovsky et al., 2008; Fukaki and Tasaka, 2009).

Root hairs originate from a subset of epidermal cells, referred to as H cells, positioned in the cleft between two cortical cells. Epidermal cells that are located over a single cortical cell do not develop into RH and are generally referred to as N cells (Dolan et al., 1994; Galway et al., 1994). This particular epidermal patterning is determined by positional cues controlled by the leucine-rich repeat receptor-like kinase SCRAMBLED (SCM) and the zing finger protein JACKDAW (JKD) (Kwak et al., 2005; Hassan et al., 2010). Several signaling components that determine the H and N fate specification in the root epidermis have been identified (Schiefelbein, 2003; Ueda et al., 2005; Ishida et al., 2008; Grebe, 2012). N fate is controlled by a transcriptional complex formed by WEREWOLF (WER)-GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), and TRANSPARENT TESTA GLABRA (TTG1) which promote the expression of GLABRA (GL2) in N cells to specify hairless cell differentiation (Galway et al., 1994; Rerie et al., 1994; Masucci et al., 1996; Lee and Schiefelbein, 1999; Walker et al., 1999; Bernhardt et al., 2003). The MYB-like transcription factor proteins CAPRICE (CPC), TRIPTYCHON (TRY) and ENHANCER OF CPC (ETC), on the other hand, act redundantly in H cells to promote the hair fate (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004). In H cells, CPC competes with WER for binding to the GL3-EGL3-TTG1 complex; consequently, the expression of *GL2* is reduced and the H fate is adopted by those cells (Bernhardt et al., 2003; Bernhardt et al., 2005).

Many PGPR have been shown to produce and secrete substantial amounts of plant growth-regulators, such as auxins, cytokinins and gibberellins that could potentially influence the root system architecture (Lugtenberg and Kamilova, 2009). However, little is known about the biological activity of these bacterially-produced molecules or the molecular mechanisms underpinning the morphological root alteration in response to rhizobacteria-derived signals. Recent data that report on hormonal crosstalk between auxin and JA/ET during primary root elongation (Ortega-Martinez et al., 2007; Chen et al., 2011) and LR development (Ivanchenko et al., 2008; Sun et al., 2009) raises the question about a plausible connection between ISR and rhizobacteria-induced alterations in the root architecture. In this study, we dissected the root developmental program that is initiated by the ISR-inducing PGPR *Pseudomonas fluorescens* WCS417 in *Arabidopsis thaliana*. We demonstrate that the significant morphological alterations in the root system architecture is caused by the promotion of LR formation and the increase in RH density and length. By employing genetic and

pharmacological approaches, our study highlights auxin transport and signaling as key regulators that drive root phenotypic plasticity in response to ISR-inducing bacteria. We further provide evidence that ISR, plant growth promotion, and alterations in root morphology are governed by distinct signaling pathways.

RESULTS

Selected strains of ISR-inducing rhizobacteria promote growth in Arabidopsis and influence root system architecture

Three well-characterized PGPR, *P. fluorescens* WCS417, *P. fluorescens* WCS374, and *Pseudomonas putida* WCS358 (hereafter called WCS417, WCS358 and WCS374) that have been previously shown to differentially trigger ISR in Arabidopsis and other plant species, were tested for their ability to promote plant growth. Both WCS417 and WCS358 are capable of triggering ISR in Arabidopsis. WCS374 does not commonly stimulate systemic resistance in this plant but does trigger ISR in radish (Leeman et al., 1995b; Van Wees et al., 1997). The plant growth-promoting effects of each strain were investigated on Arabidopsis Col-0 seedlings growing vertically on 1 × Murashige and Skoog (MS) agar-solidified medium supplemented with 0.5% sucrose. To test the possibility that diffusible compounds could affect plant growth, bacterial suspensions of each strain were applied at a 5-cm distance from the root tip of 4-day old seedlings. All bacterial strains tested were capable of stimulating plant biomass production, although to a different extend (Fig. 1A-D).

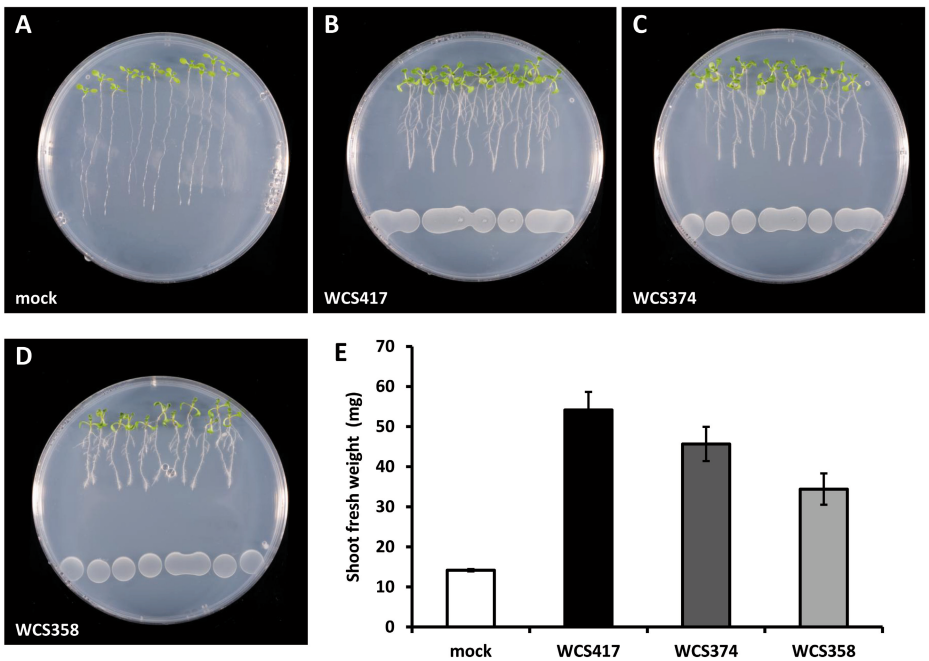


Figure 1. Effects of molecules released by selected strains of ISR-inducing bacteria on plant growth and root system architecture of Arabidopsis Col-0 seedlings. (A, B, C and D) Representative pictures of seedlings growing in control plates and plates containing *P. fluorescens* WCS417, *P. putida* WCS358 and *P. fluorescens* WCS374 bacteria. Surface sterilized seeds were sown on 1 × MS agar-solidified medium supplemented with 0.5 % sucrose. At 4 d post germination, 240 µl of bacterial suspension was spotted on the opposite side of the plate at a 5-cm distance from the root tip. Pictures were taken 8 d post co-cultivation. **(E)** Shoot biomass production measured after 8 d of co-cultivation with the indicated bacterial strains. Data represents the mean weight of 3 groups of seedlings each consisting of 10 excised shoots. Bars represent standard errors.

More specifically, 8 d after co-cultivation we measured an increase of approximately 3.9-fold and 3.2-fold in the shoot fresh weight of seedlings growing in the presence of WCS417 or WCS374, respectively, whereas WCS358 stimulated shoot biomass by 2.4-fold (Fig. 1E). Thus, under the *in vitro* conditions employed in this study, selected strains of rhizosphere bacteria have the potential to promote growth in Arabidopsis independent of their ability to trigger ISR in this plant.

Besides stimulating shoot fresh weight, all bacterial strains tested also induced a number of developmental alterations as evident by the reduction of primary root length and the increased number of LR in seedling exposed to rhizobacteria-derived compounds. To more closely analyze the molecular mechanisms underpinning these effects on root development, we focused our analysis on developmental alterations stimulated by WCS417, a PGPR that has been extensively used as a model bacterial strain for studying ISR in Arabidopsis. To specifically look at the effects of WCS417-released molecules we employed the co-cultivation system described above in which plants and bacteria grow separately at the opposite sides of circular MS-containing Petri dishes.

WCS417-induced reduction in primary root elongation is caused by reduced cell expansion in the elongation zone

After 8 d of co-cultivation, the primary root length of roots exposed to WCS417 was reduced by approximately 40% compared to mock-treated roots, suggesting a suppressive effect of WCS417 on primary root elongation (Fig. 2A). We reasoned that the short-root phenotype of WCS417-treated roots may be derived from defects in the organization of the root meristem. To this end, we used the QC-localized marker *pWOX5::GFP* (Sarkar et al., 2007), as well as the endodermis/QC localized marker *pSCR::YFP*$_{ER}$ (Sabatini et al., 1999) in order to detect any apparent differences in the meristem organization of WCS417-treated roots. Expression patterns of those marker genes were similar to that of mock-treated seedlings, suggesting that WCS417 does not have profound effects on the organization of the stem cell niche (Fig. 2B, 2C). Likewise, confocal microscopy of the QC-localized markers *pQC46::YFP*$_{ER}$ (Sabatini et al., 1999) and *pQC25::CFP*$_{ER}$ (Sabatini et al., 1999) further confirmed the neutral effects of WCS417 in QC identity (Supplemental Fig. S1).

In order to test whether reduced meristem size accounts for WCS417-induced primary root growth inhibition, we used the cell cycle marker *pCYCB1;1::GUS* (Colón-Carmona et al., 1999), which allows the visualization of cells at the G2/M phase of the cell cycle and reports for cell division rates in the root meristem. We observed enhanced expression of the reporter in the meristematic zone of WCS417-treated roots indicating that WCS417 promotes rather than inhibits cell division (Fig. 2D). Accordingly, the number of meristematic cells (as defined by the number of isodiametric epidermal cells in the meristematic zone) was found to be significantly higher in WCS417- compared to mock-treated roots (Fig. 2E). To further test the possibility that reduction of root length in WCS417-treated roots is due to defects in cell elongation, the length of root epidermal cells in the elongation and differentiaton zone was assessed after 8 d of co-cultivation with WCS417. This number was found to be reduced by approximately 40% in the WCS417-treated roots when compared to mock-treated roots (Fig. 2F). Collectively, our data indicate that reduced root elongation in response to WCS417 is due to defects in cell elongation rather than in the organization and function of root meristem.

WCS417 promotes LR formation by influencing early and late stages of LR development

One of the most prominent WCS417-mediated morphological alteration in the root system architecture is the stimulation of LR formation (Fig. 3A). In order to follow the kinetics of LR production in response to WCS417, LR formation was assessed over a 10-d time period. We measured a 2-fold increase in the number of emerged LR on WCS417-treated roots after 4 d of co-cultivation, whereas WCS417-treated roots formed approximately 3-fold more LR compared to mock-treated roots after 10 d (Fig. 3B). To further investigate the stage of LRP that is affected by

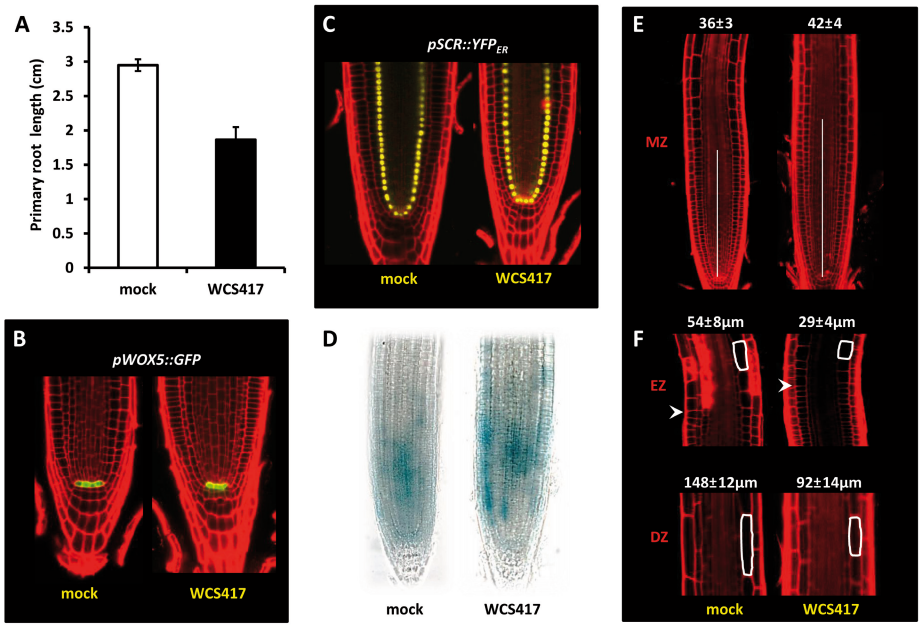


Figure 2. Effects of WCS417 on primary root development of Arabidopsis Col-0 seedlings, after 8 d of co-cultivation using the experimental set-up described in Fig. 1. (A) Primary root length of seedlings growing in $MgSO_4$ (mock) and WCS417-treated plates (*n*=20). **(B, C)** Representative confocal images showing the expression pattern of the QC-localized marker *pWOX5::GFP* and the endodermis/QC-localized marker *pSCR::YFP$_{ER}$* under control and WCS417-induced conditions. (**D**) *pCYCB1;1::GUS* expression in roots growing on plates treated with either $MgSO_4$ (mock) or WCS417 bacteria. **(E)** Meristem size of mock- and WCS417-treated roots. Values represent the average number of cortical cells in the meristem zone (MZ) (*n*=15). **(F)** Cortical cell elongation in the elongation zone (EZ) and differentiaton zone (DZ) of roots growing in the presence or absence of WCS417. Arrows indicate the boundary between MZ and EZ. Values represent the average length of 45 cells in each developmental zone (*n*=15).

WCS417, the developmental stage of each LRP of WCS417-treated and control roots was classified according to (Malamy and Benfey, 1997). We used GUS expression patterns of the *pCYCB1;1::GUS* reporter in order to precisely localize LRP across the primary root and accurately define each LRP stage. After 8 d of co-cultivation, the average number of total LPR in roots exposed to WCS417 was 1.5-fold more than in control roots (Fig. 3C). Interestingly, in clear contrast to mock treated-roots in which the developmental stages of LRP were almost uniformly distributed over the seven classes, in WCS417-treated roots a clear overrepresentation of early (I to III) and late (VI) LRP stages could be observed (Fig. 3D). Collectively, these data indicate that WCS417 promotes LR formation by stimulating both LR initiation and LR outgrowth.

WCS417 promotes RH development

In addition to positive effects on LR formation, WCS417 has a strong impact on RH development as evidenced by the increased RH density and length in WCS417-treated roots (Fig. 4A). In particular, at day 8 after co-cultivation, we measured a 3-fold increase in the RH number and a 2-fold increase in the average RH length of WCS417-exposed roots (Fig. 4B). A close inspection of RH topology in WCS417-treated roots revealed accelerated RH formation in cells located in H positions, but also ectopic RH formation in adjacent epidermal cell files (Fig. 4C). This latter correlates with the expression of the RH specific marker *CPC* as revealed by the expression patterns of the corresponding p*CPC::GUS* reporter line (Wada et al., 1997) (Fig. 4D). We then tested whether WCS417 is able to

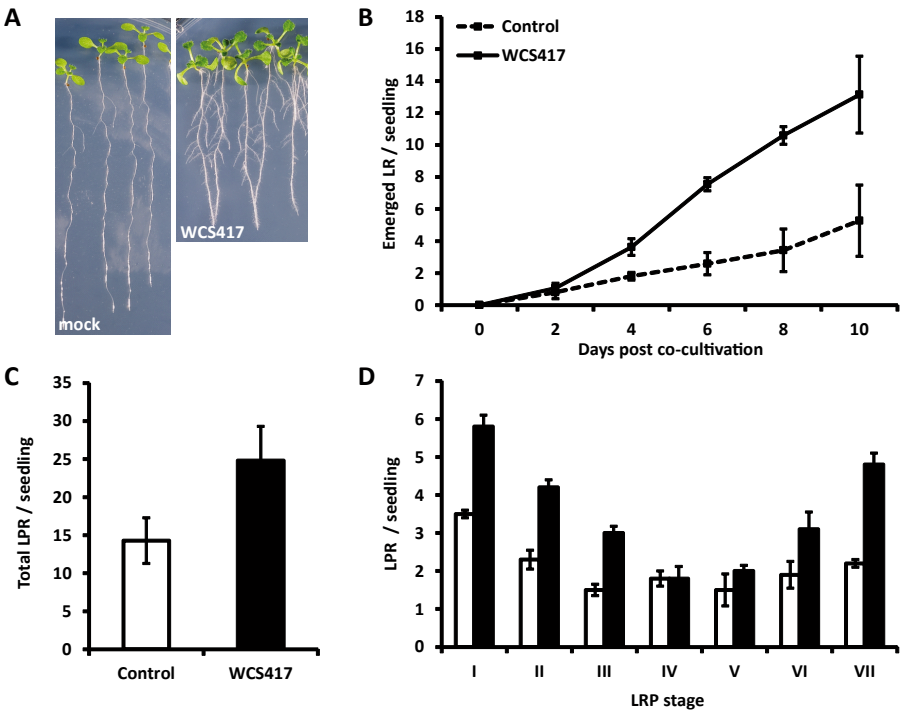


Figure 3. Effects of WCS417 on LR formation in Col-0 seedlings by employing the experimental set-up described in Fig 1. (A) Representative pictures of seedlings growing in control and WCS417-containing plates after 8 d of co-cultivation. **(B)** Time course of LR formation in response to WCS417. Time point 0 corresponds to the time the bacteria were spotted on plates. **(C)** LRP density marked by the *pCYCB1;1::GUS* activity after 8 d of co-cultivation. **(D**) Distribution of LRP in seven developmental classes (Malamy and Benfey, 1997). For each experiment, values represents the mean of at least 20 seedlings. Bars represent SD.

rescue the RH-defective phenotype of the *cpc1* mutant lacking the central transcription factor that promotes differentiation in hair-forming cells. In this mutant background, WCS417 was unable to promote RH formation neither in H nor in ectopic positions, indicating that WCS417-induced RH formation depends on a canonical RH fate specification pattern (Fig. 4E).

To further test whether the ectopic formation of RH in WCS417 treatment is due to cell fate re-specification from the N to the C fate, or is due to increased number of cells allocated in H position, cross sections of mock- and WCS417-treated roots were examined. WCS417 increased the number of cortical cells and accordingly the number of epidermal cells located in H positions (Table 1), suggesting that induction of RH is conditionally mediated via a cell-autonomous pathway. Collectively, these data indicate that WCS417 promotes RH initiation in cells located in H files and further increases the number of RH via the allocation of more epidermal cells in H positions.

Table 1. Numbers (average ± SD) of cortical and H-positioned epidermal cells per cross-section of roots growing under standard (mock) and WCS417-induced conditions. Transverse root sections were done after 8 d of co-cultivation by employing the experimental set-up described in Fig 1. At least 10 cross-sections per condition were examined.

	mock	WCS417
Number of cortical cells per cross-section	8.20 ± 0.3	9.5 ± 0.5
Number of cells in H possition per cross-section	8.00 ± 0.5	9.8 ± 0.7

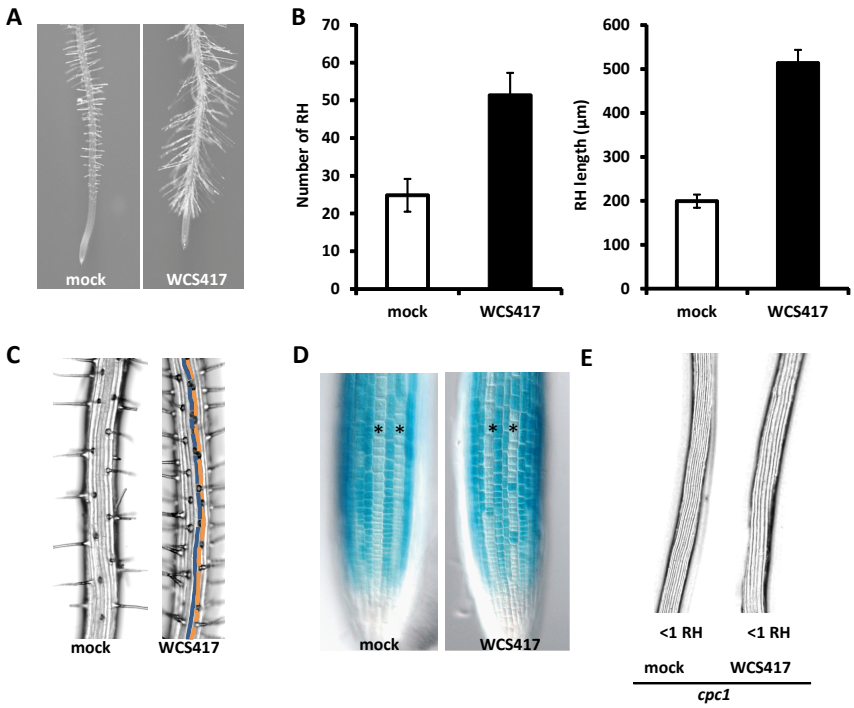


Figure 4. Effects of WCS417 on RH formation after 8 d of co-cultivation using the experimental set-up described in Fig 1. (A) Representative images of Col-0 root tips showing RH formation in $MgSO_4$- (mock) and WCS417-treated roots. **(B)** RH density expressed as the average RH number in the root segment located 1.0 cm above the root tip (*n*=20) and average RH length (80 RH, *n*=20 roots) of Col-0 seedlings growing on control and WCS417-containing plates. **(C)** Binocular views of RH distribution in mock and WCS417-treated Col-0 roots. Note that RH formation occurs in adjacent epidermal cell files in WCS417-treated roots (false-colored blue and orange lines). **(D)** p*CPC::GUS* expression patterns in the root tip of mock- and WCS417-treated seedlings. Asterisks indicate N epidermal files. **(E)** Functional *CPC1* is required for WCS417-induced RH formation. Binocular views of segments located 1.0 cm above the root tip of the *cpc1* mutant under control and WCS417-induced conditions.

WCS417 enhances auxin-regulated gene expression

The effects of WCS417 on the primary root length and the abundance of LR and RH are similar to that described for exogenous auxin-induced developmental alterations. In order to investigate whether WCS417-treated roots exhibit an enhanced auxin response, transgenic plants expressing the synthetic auxin reporter *DR5::vYFP* (nuclear) (Laskowski et al., 2008) and the auxin-regulated *pAUX1::AUX1-YFP* reporter (Swarup et al., 2001) were subjected to confocal imaging. In mock-treated *DR5::vYFP* expressing seedlings, a strong auxin response was detected towards the root tip in the root meristem of primary root tips and at the distal end of the vasculature. Similar spatial expression patterns were also observed in the roots of seedlings growing in the presence of WCS417. However, the YFP fluorescence towards and upwards the root meristem was remarkably enhanced (Fig. 5A). Likewise, AUX1-YFP more strongly accumulated at the plasma membrane of meristematic and lateral root cap cells of WCS417-treated roots, pointing to positive effects of WCS417 on auxin-regulated gene expression (Fig. 5B). Imaging of both reporter lines over time under a binocular fluorescent microscope further revealed increased density of auxin response maxima along the primary root of WCS417-treated seedlings (Fig. 5C and 5D).

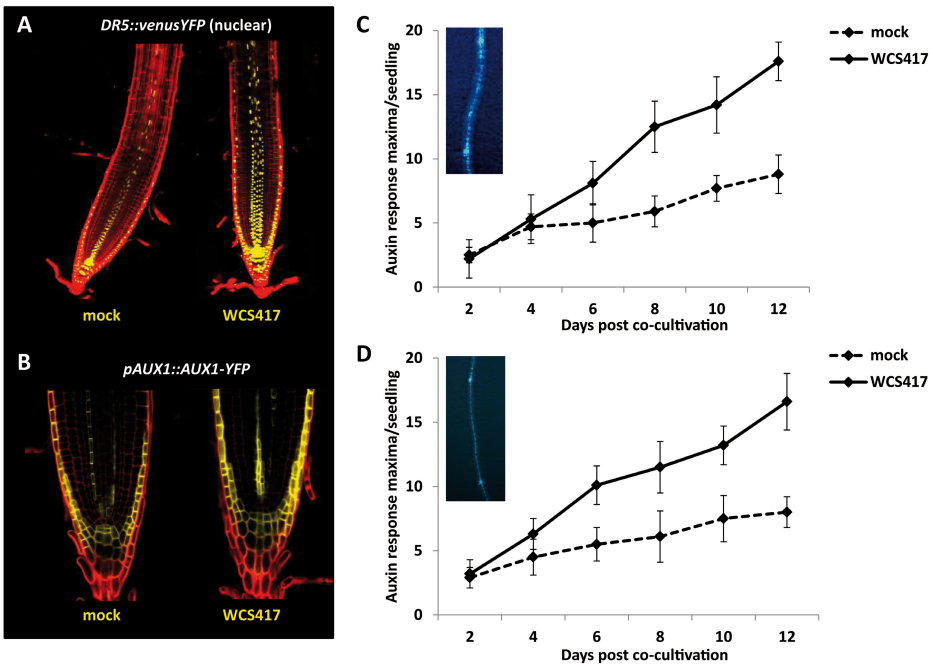


Figure 5. Effects of WCS417 on auxin-regulated gene expression. (**A, B)** Representative confocal images of *DR5::venusYFP (nuclear)* and *pAUX1::AUX1-YFP* expression at 8 d post co-cultivation with WCS417. **(C, D)** Time course analysis on the density of auxin response maxima across the primary root, marked by the *DR5::venusYFP (nuclear)* and *pAUX1::AUX1-YFP* activity. Data represents the mean of fluorescence spots in the primary root of at least 20 seedlings. Bars represent SD.

WCS417-triggered LR formation is affected in auxin perception and signaling mutants

In order to address the role of auxin signaling in WCS417-induced LR formation, we assessed LR development in the auxin perception triple mutant *tir1afb2afb3,* and in the auxin signaling mutants *axr1-12* and *axr2-1*. Stimulation of LR formation by WCS417 was also analyzed in the single *arf7-1* and *arf19-1* mutants and in the *arf7arf19* double mutant, carrying mutant alleles of the auxin response factors ARF7 and AFR19 that are essential for LR development (Okushima et al., 2007) (Fig. 6A). The *tir1afb2afb3* mutant, which did not develop LR under control conditions, was insensitive to WCS417-stimulated LR formation. Likewise, the *axr1-12* mutant that formed a significantly reduced number of LR under non-induced conditions, responded to WCS417 by producing approximately 40% the LR number of Col-0 under the same conditions. By contrast, no differences could be detected in LR formation in the *axr2-1* mutant, both under basal and WCS417-induced conditions when compared to the wild type background. Regarding the role of ARF7 and ARF19 transcription factors, LR formation was severely compromised in *arf7-1*, moderately affected in *arf19-1*, and completely abolished in the *arf7arf19* double mutant, further suggesting that WCS417-triggered LR formation operates via a canonical auxin response pathway.

In order to address the role of auxin transport in WCS417-induced LR formation, the LR phenotype of the auxin influx mutant *aux1-7* was assessed (Fig. 6B). Although this mutant formed 50% less LR compared to Col-0 under control conditions, WCS417-treated roots developed wild-type numbers of LR, reaching approximately 18 LR / seedling after 8 d of co-cultivation. To further address the role of polar auxin transport (PAT) in WCS417-mediated LR formation, LR number was quantified in the presence of the polar auxin transport inhibitor 1-N-Naphthylphthalamic acid

(NPA). At 1 µM NPA the ability of WCS417 to stimulate LR formation was severely affected, whereas in the presence of 5 µM NPA, LR formation in response to WCS417 was completely abolished. It can thus be concluded that a functional auxin efflux machinery is required for WCS417-induced LR formation. Previously, it was reported that stimulation of LR in Arabidopsis by the ectomycorrhizal fungus *Laccaria bicolor* requires PIN2-mediated auxin transport (Felten et al., 2009). To investigate the role of PIN proteins, the LR phenotype of the triple *pin2pin3pin7* mutant was analyzed in order to test whether basipetal auxin transport is involved in WCS417-mediated LR formation. No differences could be detected in the LR production under basal and WCS417-induced conditions when compared to wild type (data not shown), suggesting that *L. bicolor* and WCS417 utilize different components of the efflux machinery to confer alterations in the root system architecture.

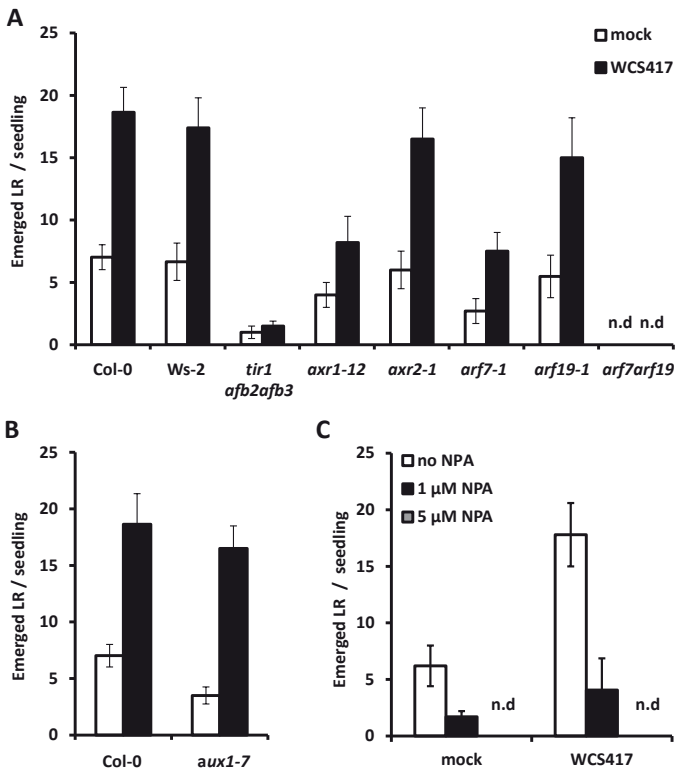


Figure 6. Influence of auxin signaling and transport on WCS417-mediated LR formation. (A) LR formation under mock and WCS417-induced conditions in wild type roots (Col-0 and Ws-2) and roots of auxin perception (*tir1afb2afb3*) and signaling mutants (*axr1-12*, *axr2-1*, *arf7-1*, *arf19-1*, *arf7arf19*), after 8 d of co-cultivation using the experimental set-up described in Fig 1. **(B)** Assessment of LR formation in the auxin influx mutant *aux1-7* after 8 d of co-cultivation. **(C)** Effects of the polar auxin transport inhibitor NPA on WCS417-induced LR formation. The same experimental setup was employed including NPA at the indicated concentrations. Values represent the mean of at least 20 seedlings ±SD.

WCS417 promotes RH development through a mechanism that involves auxin signaling

Auxin and ET have been demonstrated to play key roles in regulating processes related to RH initiation and outgrowth (Rahman et al., 2002). We set out to investigate the role of auxin signaling in WCS417-mediated RH formation by evaluating the RH phenotypes of the auxin perception triple mutant *tir1afb2afb3*, the auxin signaling mutants *axr1-12* and *axr2-1,* and the auxin influx mutant *aux1-7*. Under non-induced conditions, *aux1-7* and *axr1-12* produced significantly fewer RH, whereas RH formation was severely affected in the *axr2-1* and *tir1afb2afb3* mutants. In response to WCS417, the absolute increase in the RH number of the *axr1-12* and *aux1-7* mutants did not differ significantly from that of Col-0, whereas both the *tir1afb2afb3* and *axr2-1* mutant failed to initiate new RH, suggesting that WCS417-triggered RH initiation requires a functional auxin perception machinery and AXR2-mediated signaling (Fig. 7A).

In order to address the role of ET signaling, we assessed RH development in the ET signaling mutant *ein2-1*. Althought no clear difference could be detected in the RH number of *ein2-1* when compared

to wild-type roots under basal or induced conditions (Fig. 7A), RH elongation was significantly compromised in both conditions (Fig. 7B). RH formation in response to WCS417 was further assessed in the *rhd6* mutant that is defective in RH initiation and is known to be rescued by exogenous application of either synthetic auxins or the ethylene precursor ACC (Masucci and Schiefelbein, 1994). After 8 d of co-cultivation with WCS417, the *rhd6* mutant responded similar to wild type roots, indicating that WCS417 is capable of rescuing the RH phenotype stemming from this mutation (Fig. 7C).

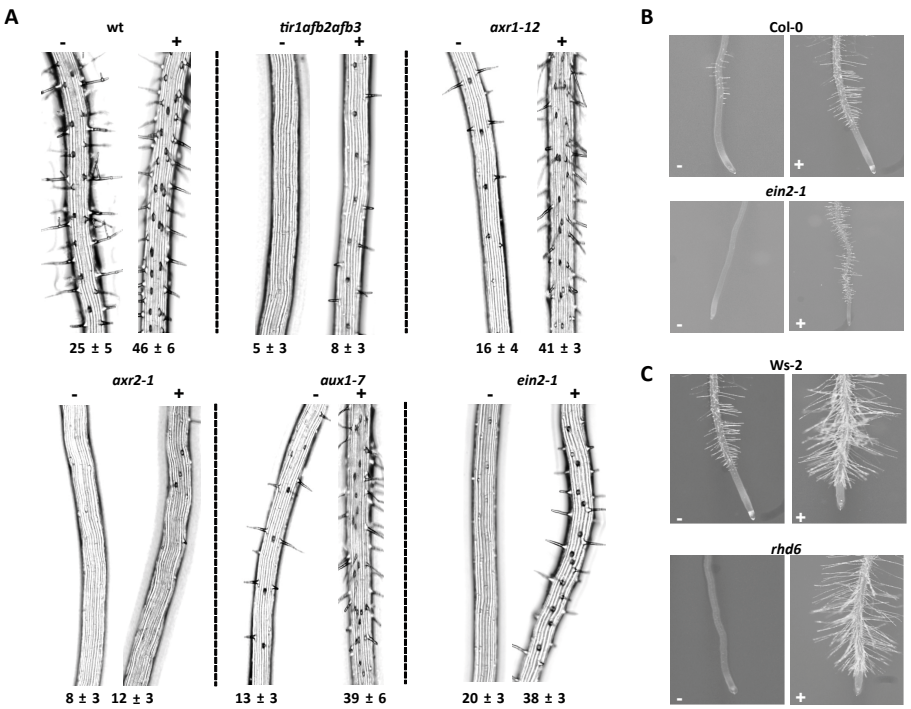


Figure 7. Influence of auxin and ET signaling on WCS417-induced RH formation. (A) Binocular views of segments located 1 cm above the root tip of seedlings of wild type, various auxin mutants, and the ET-signaling mutant *ein2-1* growing under control (-) and WCS417-induced (+) conditions. Data were obtained after 8 d of co-cultivation by using the experimental set-up described in Fig. 1. Values represent the average number of RH of at least 15, 1-cm root segments ±SD. Note the absence of ectopic RH formation in the auxin perception triple mutant *tir1afb2afb3* and signaling *axr2-1*. **(B)** RH formation in Col-0 and *ein2-1* in absence and presence of WCS417. **(C)** RH formation in Col-0 and the RH defective phenotype mutant *rhd6*.

ISR-defective mutants are not affected in WCS417-induced developmental plasticity and plant-growth promotion

The crucial role of ET and JA signaling in systemic immune responses that are triggered by beneficial microbes, including WCS417, is well- documented (Pieterse et al., 1998; Van Loon et al., 1998; Van der Ent et al., 2009a). In addition, both ET and JA have been shown to regulate aspects of root development, as both hormones play a role in the inhibition of primary root elongation and differentially influence LR formation through crosstalk with auxin transport and signaling. In particular, ethylene, which has a negative impact on LR formation (Ivanchenko et al., 2008; Negi et al., 2008), was shown to inhibit primary root growth by triggering local auxin biosynthesis in the root tip via WEI2/ASA1 (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007), a rate-limiting enzyme in the biosynthetic pathway of IAA. On the other hand, jasmonates have

been suggested to positively regulate LR formation through WEI2/ASA1 (Sun et al., 2009), and to inhibit primary root elongation in a MYC2-depedent manner (Chen et al., 2011). Considering the crucial role of the ET and JA signaling pathways in WCS417-mediated ISR, we were prompted to

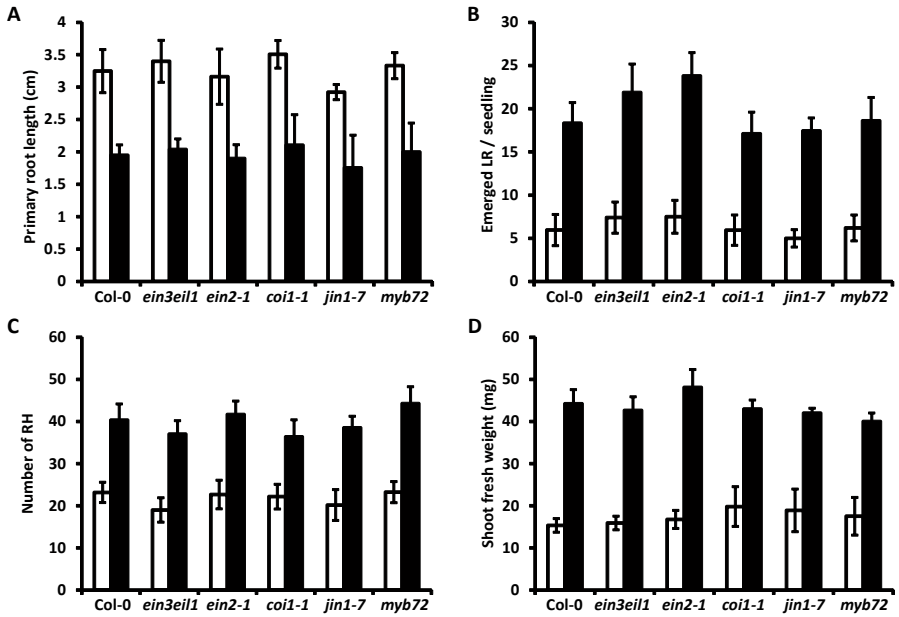


Figure 8. WCS417-induced developmental plasticity and plant-growth promotion in ISR-impaired mutants after 8 d of co-cultivation. Wild-type Col-0, the ET signaling mutants *ein3eil1* and *ein2-1*, the JA-related mutants *coi1-1* and *jin1-7(myc2)* and the *myb72-1* mutant were tested in the absence (white bars) and presence (black bars) of WCS417. **(A)** Primary root elongation. **(B)** LR density. **(C)** RH density in the 1-cm segment located 1 cm above the root tip. **(D)** Shoot biomass production.

investigate whether WCS417 exerts its effects on auxin signaling in the roots by recruiting either of these signaling pathways. To this end, we assessed primary root elongation and LR formation in the ET signaling mutants *ein3ein1* and *ein2-1*, in the JA-response mutants *coi1-1* and *jin1-7 (myc2)*, and in the *myb72-1* mutant, which are all defective in their ability to mount ISR in response to WCS417. In all these mutants, we measured a significant reduction in the primary root length after 8 d of co-cultivation, which was comparable to that of Col-0 (Fig. 8A). Furthermore, all mutants responded to WCS417 similarly to wild type by increasing the average LR number by 3-fold after 8 d of co-cultivation (Fig. 8B). Likewise neither WCS417-triggerd RH formation nor WCS417-mediated shoot growth promotion was affected in the ISR mutants tested (Fig. 8C, 8D). Therefore, it can be concluded that auxin-associated developmental alteration stimulated by WCS417 are mediated in a ET- and JA-independent manner and that WCS417 rather directly influences auxin signaling. These results also indicate that induction of ISR and plant growth promotion are two unlinked traits.

DISCUSSION

Over the past years significant progress has been made in our understanding of how broad-spectrum systemic immunity develops in response to colonization of plant roots by PGPR (Van Wees et al., 2008; Zamioudis and Pieterse, 2012). However, little is known about the molecular details involved in the plant growth-promoting effects of these beneficial rhizosphere bacteria. Although, mutant analyses previously identified auxin as an important signaling molecule in the

growth-promoting effect of PGPR and PGPF (Idris et al., 2007), a detailed analysis of the remarkable changes in the plant root system architecture has to our knowledge never been described. In this study, we dissected the significant alterations in root morphology that are triggered by PGPR, and describe in detail the specific role of auxin signaling and transport in different stages of the PGPR-induced root developmental program.

WCS417 enhances auxin responses in Arabidopsis roots

Auxin production is widespread among PGPR strains (Lugtenberg and Kamilova, 2009). It is, therefore, very likely that WCS417 produces and secretes auxin-like compounds that augment auxin signaling in the roots of Arabidopsis, resulting in the stimulation of auxin-mediated developmental programs. This is clearly supported by the fact that the RH initiation defect of the *rhd6* mutant could be rescued by co-cultivation with WCS417, consistent with previous studies reporting that exogenous application of auxin is capable of rescuing the *rhd6* phenotype (Masucci and Schiefelbein, 1994). Other bacterial determinants may also contribute to the modifications observed in root morphology. It has recently been demonstrated that diketopiperazines (DKPs), which are quorum sensing signals of *Pseudomonas aeruginosa,* function as structural auxin analogues capable of inducing auxin-dependent alterations in the root system architecture of Arabidopsis (Ortiz-Castro et al., 2011). Hence, a variety bacterially derived molecules can enhance auxin responses in the roots of host plants.

Effects of WCS417 bacteria on the root meristem and primary root elongation

Auxin is an essential plant hormone in the maintenance of stem-cell identity and function (Benjamins and Scheres, 2008b). Various experimental manipulations that alter the auxin gradient in the root tip of Arabidopsis, such as chemical inhibition of polar auxin transport by NPA and exogenous application of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), have profound effects on processes related to cell fate specification and tissue polarity (Sabatini et al., 1999). Our study demonstrates that WCS417 enhances the auxin response in the root tip of Arabidopsis, but this does not influence the overall pattern of auxin distribution or the organization of the root meristem as revealed by confocal microscopy studies of cell- and tissue-specific type developmental markers. Differences in the transport mechanisms of different auxin structural analogs may explain these effects. For instance, in contrast to 2,4-D that is not redistributed by export carriers and predominantly induces *DR5::GUS* activity in the epidermal layers (Sabatini et al., 1999), our data indicate that auxin-like molecules secreted by WCS417 are transported via polar auxin transport. It is, therefore, unlikely that they generate an auxin maximum in the outermost root cell layer that is sufficient to define cell patterning. However, we did observe enhanced expression of the *pCYCB1;1::GUS* reporter in the meristematic zone of WCS417-treated roots, consistent with the role of auxin in promoting cell divisions (Campanoni and Nick, 2005). Importantly, it appears that enhanced local auxin signaling in the elongation zone of WCS417-roots results in reduced cell elongation and primary root growth. We therefore conclude that auxin levels mounted in roots upon co-cultivation with WCS417 are optimal to promote cell divisions, yet inhibitory for cell elongation. As a result, co-cultivation of Arabidopsis roots with WCS417 reduces primary root elongation.

Effects of WCS417 on lateral root formation: role of auxin signaling and efflux

Roots possess a high degree of phenotypic plasticity that facilitates adaptation to unfavorable soil environments. For instance, enhanced production of LR commonly mediate the plant's adaptation to nutrient deficiencies, such as phosphate starvation (López-Bucio et al., 2003; Perez-Torres et al., 2008). Changes in the root system architecture in response to microbial signals are also common. Not surprisingly, microbial-driven morphological alterations are commonly mediated by

mechanisms involving auxin transport and signaling. For instance, rhizobia and parasitic nematodes form nodules and specialized feeding structures, respectively, in roots of host plants by targeting elements of the auxin transport machinery (Grunewald et al., 2009). Beneficial soil fungi also recruit auxin signaling to promote LR initiation and emergence (Felten et al., 2009; Splivallo et al., 2009). In this study, we show that WCS417 utilizes a canonical auxin signaling pathway to promote LR formation. Interestingly, LR formation induced by WCS417 did not require the auxin influx carrier AUX1, a member of the amino acid permease family of proton-driven transporters that functions in the uptake of the auxin molecules indole-3-acetic acid (IAA) and 2,4-D, but not the lipophilic auxin 1-naphthaleneacetic acid (1-NAA) (Marchant et al., 1999). Therefore, it is tempting to speculate that auxin-like substances produced by WCS417 adopt a diffusion-based mode of entry into plant cells similarly to 1-NAA. However, treatment with the polar auxin transport inhibitor NPA severely affected the ability of WCS417 to promote LR formation, suggesting that WCS417-produced auxins are substrates for auxin carriers. Our data indicate that PIN2, PIN3 and PIN7 carriers, known to mediate basipetal auxin transport (Blilou et al., 2005), are not involved in this process. Hence, other member of the PIN or MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (MDR/PGP) family of auxin efflux carriers are likely to mediate auxin export towards the innermost cells of the root.

Effects of WCS417 on RH formation

In Arabidopsis, epidermal cell fate is determined by positional cues, whereas initiation and elongation are under the control of auxin and ET signaling (Schiefelbein, 2003). RH development is sensitive to external cues such as nutrient deficiencies and suboptimal availability of either phosphate, iron or manganese (López-Bucio et al., 2003). Here, we demonstrate that WCS417 increases RH density, and that it does so by affecting processes related both to cell fate specification and RH initiation. Ectopic RH formation in WCS417-treated roots depends on CPC, the central transcription factor that promotes differentiation in hair-forming cells, but is not induced via a cell autonomous pathway. Instead, it is rather based on an increase in the number of cortical cells that in turn generate additional epidermal files located in H positions. This effect appears to be auxin-dependent and correlates with the observation that WCS417 augments cell divisions in the root meristem. Our study further demonstrates that WCS417 bacteria utilize the intrinsic genetic program that involves AXR2-mediated signaling to promote RH initiation downstream of a pre-established H cell fate. In addition, we found that RH elongation in WCS417-exposed roots was significantly compromised in the ET signaling mutant *ein2*, suggesting that auxin acts upstream of ET during WCS417-induced RH outgrowth.

Distinct molecular mechanisms govern ISR, alterations in root architecture, and plant growth promotion

Under the experimental conditions employed in this study, the ISR-inducing rhizobacterial strains WCS417 and WCS358, and the ISR-noninducing strain WCS374 were able to confer severe alterations in root morphology and to promote shoot growth. In addition, Arabidopsis mutants unable to mount ISR responded equally well to WCS417 as wild-type plants with respect to plant growth promotion and changes in the root morhology. It can thus be concluded that the induced root developmental program and enhanced shoot growth are PGPR-mediated processes that function independently of the rhizobacterial ability to systemically stimulate the plant immune system. The latter suggest that although both ET and JA share the potential to influence root development by affecting processes related to auxin signaling and transport (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007; Chen et al., 2011), WCS417 does not recruit either of these signaling pathways to exerts its effects on the root architecture.

Biological significance of auxin signaling and root architecture in beneficial interactions

What could be the biological significance of the observed PGPR-mediated alterations in root morphology? The composition of microbial communities in the rhizosphere significantly differs from that in bulk soil and is highly depended on low and high-molecular weight compounds secreted from the roots, collectively referred to as root exudates (Bais et al., 2006; Mendes et al., 2011; Berendsen et al., 2012). Plants excrete up to 20% of all phytosynthetically fixed carbon via their root system. Hence, root exudation represents a significant carbon cost for the plant. However, important biological processes that are mediated by root exudates, such as communication with symbiotic microorganisms via secretion of semiochemicals and alterations in the chemical and physical properties of the soil via secretion of organic and inorganic substances, suffice to justify such an energy investment (Bais et al., 2004; Bais et al., 2006). Although the chemical composition of these compounds considerably differs among plant species, energy-rich compounds such as sugars and organic acids encompass a significant proportion. Importanlty, most of the root exudation is considered to occur in the elongation zone of newly formed roots (Bais et al., 2006). It is therefore reasonable to speculated that enhanced LR formation in response to WCS417 and other beneficial microbes may be a conserved mechanism that soil microbes employ for their own benefit in order to enhance root exudation, and thus increase the energy flow from the roots of host plants.

Furthermore, a role of RH in plant-microbe interactions is emerging. Internal colonization of RH by certain types of soil-borne beneficial bacteria other than rhizobia may be a more general phenomenon as recent studies demonstrate (Paungfoo-Lonhienne et al., 2010; Prieto et al., 2011). Induction of RH formation may facilitate entry of certain strains of rhizosphere bacteria into internal root tissues. In any case, considering that LR and RH formation are typical responses to nutrient-limited conditions (López-Bucio et al., 2003), it is expected that under natural growth conditions, rhizobacteria-induced alterations in the root morphology would greatly facilitate plant nutrition, thus conferring significant ecological benefits to host plants.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana accessions Col-0 and Ws-2 were used as wild type plant genotypes. The following mutants were used in this study: *cpc-1* (Lee and Schiefelbein, 2002), *tir1afb2afb3* (Dharmasiri et al., 2005), *axr2-1* (Timpte et al., 1994), *axr1-12* (Leyser et al., 1993), *aux1-7* (Pickett et al., 1990), *pin2pin3pin7* (Blilou et al., 2005), *arf7-1*, *arf19-1* and *arf7arf19* (Okushima et al., 2007), *rhd6* (Masucci and Schiefelbein, 1994), *ein3eil1* (Chao et al., 1997; Binder et al., 2007), *ein2-1* (Guzmán and Ecker, 1990), *jin1-7* (Lorenzo et al., 2004), *coi1-1* (Feys et al., 1994) and *myb72-1* (Van der Ent et al., 2008). Seeds were surface sterilized and sown on 1 × Murashige and Skoog (MS) agar-solidified medium supplemented with 0.5% sucrose at a density of 8 - 10 per plate. After 2 d of stratification at 4°C, the Petri dishes were transferred and positioned vertically in a growth chamber under a long-day photoperiod (16 h of light, light intensity 100 µmol m^{-2} s^{-1}) at 22°C. For inhibition of polar auxin transport, the PAT inhibitor 1-N-naphthylphthalamic acid (NPA) was added at the final concentrations of 1 or 5 µM.

Cultivation of rhizobacteria and induction treatments

Pseudomonas fluorescens WCS417r, *P. fluorescens* WCS374r, and *Pseudomonas putida* WCS358r were cultured on King's medium B (KB) agar plates supplemented with 50 µg mL^{-1} rifampicin at 28°C. After 24 h growth, cells were collected in 10 mM MgSO4, washed twice by centrifugation for 5 min at 5.000 × g, and finally resuspended in 10 mM MgSO4. The bacterial titer was adjusted to the OD_{600} of 0.002 (10^6 cfu mL^{-1}) and 240 µl of bacterial suspension was spotted at a 5-cm distance from the root tip of 4-d old seedlings using a multichannel pipet.

GUS histohemical staining

Histochemical detection of GUS in the *pCPC::GUS*, *pGL2::GUS* and *pCYCB1;1::GUS* reporter lines was performed in a GUS staining solution (50 mM sodium phosphate [pH 7], 10 mM EDTA, 0.5 mM K4[Fe(CN)6], 0.5 mM K3[Fe(CN)6], 0.5 mM X-Gluc, and 0.01 % Silwet L-77) at 37^{o}C for defined periods. Stained roots were cleared in a mixture of chloral hydrate/glycerol/water (8:1:2) and observed with Nomarski optics.

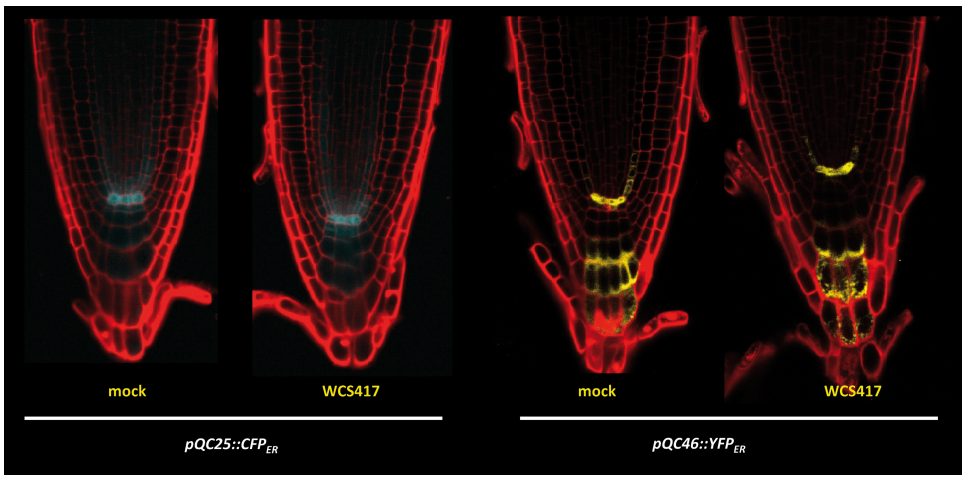
Fluorescence microscopy

Local auxin-response maxima across the primary root of *DR5::vYFP*$_{NLS}$ and *pAUX1::AUX1-YFP* lines were observed under a Leica MZ16F fluorescence stereoscope. Confocal laser-scanning microscopy in the *DR5::vYFP*$_{NLS}$, *pAUX1::AUX1-YFP pWOX5::GFP*, *pSCR::YFP*$_{ER}$, *QC46::YFP*$_{ER}$ and *QC25::CFP*$_{ER}$ transgenic lines was performed using a Leica SP2 inverted microscope. As counterstain, roots were stained in 10 μg mL^{-1} propidium iodide (PI) solution for 2 min. Chromophores were excited using the 488 nm Argon laser and fluorescence was detected at 500-550 nm (GFP), 550-615 nm (YFP), 465-500 nm (CFP) and 570-620 nm (PI).

Phenotypic and data analysis

For shoot fresh weight measurements, seedlings were sectioned at the root-shoot junction and the weight of 3 groups of 10 excised shoots was immediately measured on an analytical balance. For root length measurements, digital images of Petri dishes of Arabidopsis seedlings were captured using a gel documentation system and the primary root length of at least 20 seedlings was calculated with the ImageJ software (http://rsb.info.nih.gov/nih-image/). The number of emerged LR (>0.5 mm) of at least 20 seedlings was counted using a dissecting microscope. The LRP developmental stages were classified according to Malamy and Benfey (1997) as follows: Stage I: the LRI stage in which the first anticlinal divisions in the pericycle occur. In the longitudinal axis, approximately 8 to 10 short pericycle cells are formed. Stage II: the formed LRP is divided into two layers (inner and outer layer) by a periclinal division. Stage III: the outer layer cells undergo periclinal divisions to create a three-layered LRP. Stage IV: the inner layer cells undergo periclinal divisions to form a LRP with four-cell layers. Stage V: the LRP is halfway through the parental cortex. Stage VI: the LRP has passed through the parent cortex layer and has penetrated the epidermis. Stage VII: the LRP appears to be just about to emerge from the primary root. For RH measurements, digital images were obtained from the primary root segment located 1.0 cm above the root tip under a microscope at a magnification of 40 times. Root hair density and length was then quantified with ImageJ. For determination of RM size and cell length measurements, digital confocal images of PI-stained roots were analyzed with ImageJ software. RM size was assessed as the number of cortical cells between the QC and the first cell that was twice the length of the immediately preceding cell. Cell length measurements were performed on single cortical cells located in the EZ and DZ. For measurements of cortical and H-positioned epidermal cells, plastic root sectioning was done as previously described (Hassan et al., 2010).

SUPPLEMENTAL DATA



Supplemental Figure S1. Representative confocal images showing the expression pattern of the indicated QC-localized markers under mock- and WCS417-induced conditions, after 8 d of co-cultivation using the experimental set-up described in Fig. 1.

CHAPTER 3

Pseudomonas fluorescens WCS417 hijacks iron-limitation induced signaling to trigger systemic resistance in Arabidopsis

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ABSTRACT

Root colonization by selected strains of non-pathogenic rhizobacteria can trigger an induced systemic resistance (ISR) that is effective against a broad spectrum of pathogens and even insects. In Arabidopsis, ISR triggered by the plant-growth promoting rhizobacterium *Pseudomonas fluorescens* WCS417 requires the root-specific transcription factor MYB72 that locally operates in the generation or translocation of a thus-far unidentified systemic signal. *MYB72* gene expression is strongly activated upon colonization of the roots by WCS417, but it is also known to be specifically induced under iron limited conditions. Here, we demonstrate that rhizobacteria capable of triggering ISR in Arabidopsis upregulate the iron-deficiency response Strategy I, independently of the iron availability status. Similar to the expression of *MYB72,* the iron-limitation marker genes *FRO2* and *IRT1*, and ferric reductase activity were strongly upregulated in roots colonized by ISR-inducing stains WCS417 and *Pseudomonas putida* WCS358, but not by the ISR-non-inducing strain *Pseudomonas fluoresecens* WCS374. We further demonstrate that rhizobacteria-induced *MYB72* expression depends on FIT1, the central transcription factor of Strategy I that orchestrates plant responses to iron limitation. A search for bacterial determinants involved in *MYB72* induction revealed that volatile organic compounds (VOCs) emitted by WCS417 are potent elicitors of the iron deficiency response in roots. WCS417-derived VOCs activated *MYB72, FRO2,* and *IRT1* expression in the ethylene mutants *ein2* and *ein3eil1* and the auxin mutants *tir1afbafb3* and *aux1* in comparable levels to wild type roots, indicating that the volatile blend of WCS417 functions downstream of the ethylene and auxin signaling pathways, both reported to be important for regulating transcriptional responses to iron limitation. In agreement with its critical signaling role in mounting the Strategy I response in roots, nitric oxide (NO) seemed to accumulate to higher levels in VOC-treated roots, pointing to a role for NO in WCS417-mediated activation of the iron-deficiency response in the roots. Arabidopsis mutants *opt3-2* and *frd3-1* that constitutively express Strategy I in roots, display enhanced resistance against the necrotrophic pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*. Collectively, these data indicate that induction of the iron-sensing signaling pathway in roots plays an important role in the onset of rhizobacteria-mediated broad-spectrum systemic resistance in foliar tissues.

INTRODUCTION

Plants interact with a plethora of microorganisms in the dynamic environment of the soil rhizosphere (Mendes et al., 2011). Beneficial interactions between plants and microbes are complex and largely affect processes related to plant nutrition and resistance towards various types of biotic and abiotic stresses (Marx, 2004; Lugtenberg and Kamilova, 2009). An important class of soil-borne beneficial microbes represents those living in close association with plant roots either as epiphytes or endophytes. These organisms are collectively called plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF). PGPR and PGPF, along with symbiotic mycorrhizal fungi, are well-documented to exert positive effects on plant growth and can confer systemic protection against foliar pathogens and even insects (Pozo and Azcon-Aguilar, 2007; Van Wees et al., 2008; Pineda et al., 2010). This form of systemic resistance is called induced systemic resistance (ISR) and widely occurs in monocotyledonous and dicotyledonous plant species (Van Loon et al., 1998; Van Loon and Bakker, 2005).

The molecular mechanisms underpinning rhizobacteria-mediated ISR are well-studied in the model plant *Arabidopsis thaliana* (Pieterse et al., 2002; Van der Ent et al., 2009a). In Arabidopsis, ISR triggered upon root colonization by the PGPR *Pseudomonas fluorescens* WCS417 depends on

an intact jasmonic acid (JA) and ethylene (ET) signaling pathway (Pieterse et al., 1996; Pieterse et al., 1998) and further requires the transcriptional regulators MYC2 and NPR1 (Pieterse et al., 1998; Pozo et al., 2008). In contrast to plant defenses activated by pathogens or insects, the establishment of ISR is not correlated with substantial reprogramming of the host's transcriptome (Verhagen et al., 2004). Instead, upon pathogen attack, immunized plants display a boosted immune reaction resulting in enhanced resistance to the attacker encountered (Van Wees et al., 1999; Verhagen et al., 2004; Pozo et al., 2008). This phenomenon is called priming and shares striking similarities with the potentiation of cellular defense responses in primed monocytes and macrophages in mammals (Conrath et al., 2002; Conrath et al., 2006; Jung et al., 2009). In roots, initiation of ISR is regulated by the root-specific transcription factor MYB72 (Van der Ent et al., 2008), a member of the R2R3 family of MYB transcription factors, and components of the ET signaling pathway that locally act in the generation or translocation of a thus-far unidentified systemic signal (Knoester et al., 1999). Importantly, MYB72 is also required for ISR triggered upon root colonization by the beneficial fungus *Trichoderma asperellum* strain T34, suggesting that this transcription factor is a node of convergence in signaling pathways induced by diverse types of beneficial soil-borne microbes (Segarra et al., 2009).

Besides its role in ISR, *MYB72* has been reported to be specifically induced in roots under iron limitation and growth conditions that distort iron uptake, such as high zinc concentrations (De Mortel et al., 2008; Buckhout et al., 2009). Primary root growth of Arabidopsis *myb72* mutants is significantly more affected under iron-starved conditions than that of wild type roots, illustrating the importance of MYB72 in the adaptive response of Arabidopsis to iron limitation (De Mortel et al., 2008). Iron is an essential element for plant and animal life and despite the fact that it is one of the most abundant elements on Earth, iron availability is limited for plants, especially in calcareous soils because it is only slightly soluble. On the other hand, plants have evolved sophisticated mechanisms to cope with iron-limited conditions (Connolly and Guerinot, 2002). The responses activated upon iron limitation differ between monocotyledonous and dicotyledonous plant species. Monocotyledonous plants, such as barley, maize and rice, secrete phytosiderophores, molecules that chelate iron from the soil environment. The chelator-iron complex is imported to root tissues by plasma membrane-localized transporters and subsequently distributed to internal tissues (Walker and Connolly, 2008; Morrissey and Guerinot, 2009; Kobayashi and Nishizawa, 2012). In Arabidopsis and other dicotyledonous plants, iron limitation induces a set of coordinated responses, collectively referred to as Strategy I that aims to foster iron mobilization and uptake under iron-limited conditions (Fig. 1A). Strategy I includes three main steps that take place in the epidermal cells of the root: (i) rhizosphere acidification via proton extrusion mediated by members of plasma membrane-localized H^+-ATPases (AHA) to enhance solubility of ferric iron in the soil, (ii) reduction of ferric to ferrous iron via the plasma membrane ferric reductase FRO2, and (iii) transport of ferrous ion from the soil environment within the plant tissues via the high-affinity iron transporter IRT1 (Walker and Connolly, 2008; Morrissey and Guerinot, 2009; Kobayashi and Nishizawa, 2012).

Whole-genome transcriptional responses to iron deficiency at the whole-organ and tissue-specific level have been reported (Colangelo and Guerinot, 2004; Dinneny et al., 2008; Buckhout et al., 2009). The central transcription factor that orchestrates transcriptional reprogramming under iron starvation in Arabidopsis is the basic helix-loop-helix (bHLH) transcription factor FIT1 (bHLH29) (Colangelo and Guerinot, 2004; Jakoby et al., 2004). FIT1 is known to regulate the ferric reductase FRO2 at the transcriptional level and the iron transporter IRT1 both at the transcriptional and the protein level (Colangelo and Guerinot, 2004). Recently, FIT1 was demonstrated to also induce the expression of AHA2, the major H^+-ATPase involved in proton extrusion (Ivanov et al., 2012). Despite its requirement for the induction of the iron uptake genes, overexpression of *FIT1* does not lead to constitutive expression of the iron deficiency markers *FRO2* and *IRT1* (Colangelo and Guerinot,

2004; Jakoby et al., 2004). In fact, only upon heterodimerization with at least one of the transcription factors bHLH38 and bHLH39 (Yuan et al., 2008), and most likely the closely-related transcription factors bHLH100 and bHLH101 (Wang et al., 2007b), FIT1 induces constitutive expression of *FRO2* and *IRT1*. FIT1 is also known to regulate its own transcription (Colangelo and Guerinot, 2004) and recent studies demonstrate an additional level of regulation at the protein level that ensures synchronized transcriptional responses of iron uptake genes upon iron starvation (Lingam et al., 2011; Meiser et al., 2011; Sivitz et al., 2011). Importantly, *MYB72*, which is rapidly induced upon iron starvation (Buckhout et al., 2009), has been identified amongst the iron deficiency-regulated genes induced in a FIT1-depedent manner (Colangelo and Guerinot, 2004).In this study, we aimed to investigate the relationship between rhizobacteria-mediated ISR and the iron-deficiency response. We demonstrate that rhizobacteria capable of triggering ISR in Arabidopsis activate the iron deficiency response in the roots, irrespective of the iron availability status. By using Arabidopsis genotypes that constitutively express iron deficiency responses in roots, we demonstrate that activation of Strategy I locally in roots is associated with enhanced disease resistance in systemic tissues. Our study further reveals a prominent role of airborne signals emitted from ISR inducing *Pseudomonas* in the induction of *MYB72* expression and the iron deficiency response in the roots of Arabidopsis. Collectively, our results suggest that ISR-inducing *Pseudomonas* bacteria hijack the iron-deficiency response to activate systemic immunity in foliar plant parts.

RESULTS

ISR-inducing rhizobacteria upregulate the iron deficiency response in Arabidopsis

The root-specific transcription factor MYB72 is essential for the establishment of rhizobacteria-mediated ISR in Arabidopsis (Van der Ent et al., 2008). Considering that *MYB72* expression is also induced in the roots upon iron limitation (Colangelo and Guerinot, 2004; Dinneny et al., 2008; Buckhout et al., 2009), we set out to investigate whether selected strains of rhizosphere bacteria are capable of activating the iron-deficiency response in Arabidopsis. To this end, three well-characterized rhizobacterial strains, *Pseudomonas fluorescens* WCS417, *Pseudomonas putida* WCS358 and *Pseudomonas fluorescens* WCS374 (hereafter called WCS417, WCS358 and WCS374), which have been previously shown to trigger ISR in Arabidopsis (WCS417 and WCS358) or not (WCS374) (Van Wees et al., 1997), were tested for their ability to induce the expression of the well-documented iron deficiency marker genes *FRO2* and *IRT1*. Ten-day-old seedlings growing vertically in non-iron-limiting 1 × Hoagland agar-solidified medium containing 20 μM Fe(III)EDTA were treated with the above-mentioned bacterial strains and root samples were collected 2 days post treatment (dpt). Gene expression analysis revealed that both *FRO2* and *IRT1* were specifically induced in response to ISR-inducing bacteria WCS417 and WCS358, but not by the ISR-non-inducing strain WCS374 (Fig. 1B). Likewise, ferric reductase activity in WCS417- and WCS358-treated roots was enhanced, whereas in $MgSO_4$- and WCS374-treated roots it remained at basal levels (Fig. 1C and 1D). These results indicate that ISR-inducing rhizobacteria activate the iron-deficiency response in roots of Arabidopsis.

WCS417-induced *MYB72* expression depends on FIT1 and is similarly regulated as *FRO2* and *IRT1*

In order to determine whether the same signaling components that operate during the iron-deficiency response are also involved in rhizobacteria-mediated activation of Strategy I, we assessed the impact of root colonization by WCS417 on the expression of genes encoding major transcriptional regulators of Strategy I. To this end, we assessed the expression of the transcription factor genes *FIT1 (bHLH29)*, *bHLH38*, and *bHLH39* 2 days after root colonization by WCS417. We found that root colonization significantly elevates the expression of *bHLH38* and *bHLH39* and marginally induces *FIT1* transcription (Fig. 2A). Because FIT1 was previously reported

to regulate the expression of *MYB72* upon iron limitation (Colangelo and Guerinot, 2004), we investigated whether FIT1 is essential for WCS417-induced expression of *MYB72*. In the loss-of-function mutant *fit1-1,* WCS417-mediated *MYB72* induction was severely compromised (Fig. 2B), indicating that FIT1 is an important regulator of rhizobacteria-induced expression of *MYB72*.

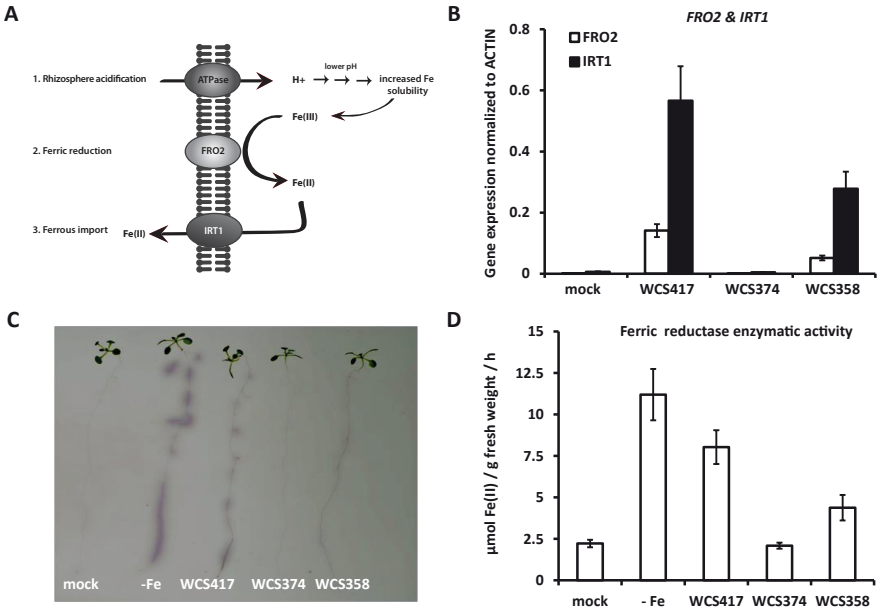


Figure 1. ISR-inducing rhizobacteria upregulate iron deficiency mechanisms in Arabidopsis. **(A**) Schematic representation of Strategy I response. Upon iron-limited conditions, roots acidify the soil environment via proton extrusion to solubilize Fe(III) which is then reduced to Fe(II) by the action of ferric chelate reductase FRO2; Fe(II) is then imported into epidermal cells via the IRT1 transporter. **(B)** Quantitative RT-PCR analysis of *FRO2* and *IRT1* transcript levels in the roots of Col-0 seedlings treated with $MgSO_4$ or the indicated bacterial strains. Seedlings were treated when 10-day-old and samples were collected 2 dpt. **(C)** Visualization of ferric reductase activity. **(D)** Quantitative ferric reductase activity in mock- and rhizobacteria-treated roots 2 dpt. Iron deprived plants were used as positive control. Data in all graphs represent the mean of three replicates (± SD).

FIT1 is known to heterodimerize with either bHLH38 or bHLH39 to induce the transcription of *FRO2* and *IRT1* in the epidermal root layer (Yuan et al., 2008). In order to determine whether the same heterodimers are involved in the induction of *MYB72*, we assessed *MYB72* expression in transgenic lines overexpressing FIT1 (*oxFIT1*), bHLH38 (*oxbHLH38*), bHLH39 (*oxbHLH39*) or both FIT1 and bHLH38 (oxFIT1/bHLH38). Figure 2C shows that overexpression of either *FIT1*, *bHLH38* or *bHLH39* alone does not affect basal levels of *MYB72* transcription. However, simultaneous overexpression of *FIT1* and *bHLH38* results in constitutive expression of *MYB72* at high levels. Similar results were obtained for *FRO2* and *IRT1* (Fig. 2C), indicating that expression of *MYB72* is similarly regulated as the iron uptake genes *FRO2* and *IRT1*.

Induction of Strategy I by WCS417 is not caused by iron starvation

Siderophores are high-affinity iron chelating compounds produced and secreted by a plethora of beneficial and pathogenic microbes to facilitate uptake of iron from the environment. Fluorescent *Pseudomonas* spp. produce large amounts of siderophores and these molecules have been reported to be capable of triggering ISR in Arabidopsis and other plant species (Meziane et al., 2005; Ran et al., 2005; Bakker et al., 2007; Van Loon et al., 2008). We reasoned that Fe-

chelating siderophores of ISR-inducing rhizobacteria may be the causal agents of the induction of the iron deficiency response in colonized roots. Therefore, we tested whether siderophores are responsible for WCS417-triggered expression of *MYB72* by using two siderophore-defective *Tn5* transposon-insertion mutants of WCS417 (WCS417-S680 and WCS417-M634) (Duijff et al., 1993). Figure 3A shows that both siderophore-deficient mutants activated *MYB72* expression to the same extent as wild type WCS417, suggesting that iron chelation by bacterially-secreted siderophores is not essential for *MYB72* activation in the roots of ISR-expressing seedlings. This is further supported by the fact that the strain WCS374, which produces large amounts of siderophores (Leeman et al., 1995a) but does not induce ISR in Arabidopsis (Van Wees et al., 1997), was incapable of activating *MYB72* and the Strategy I response in Arabidopsis roots (Fig. 1B).

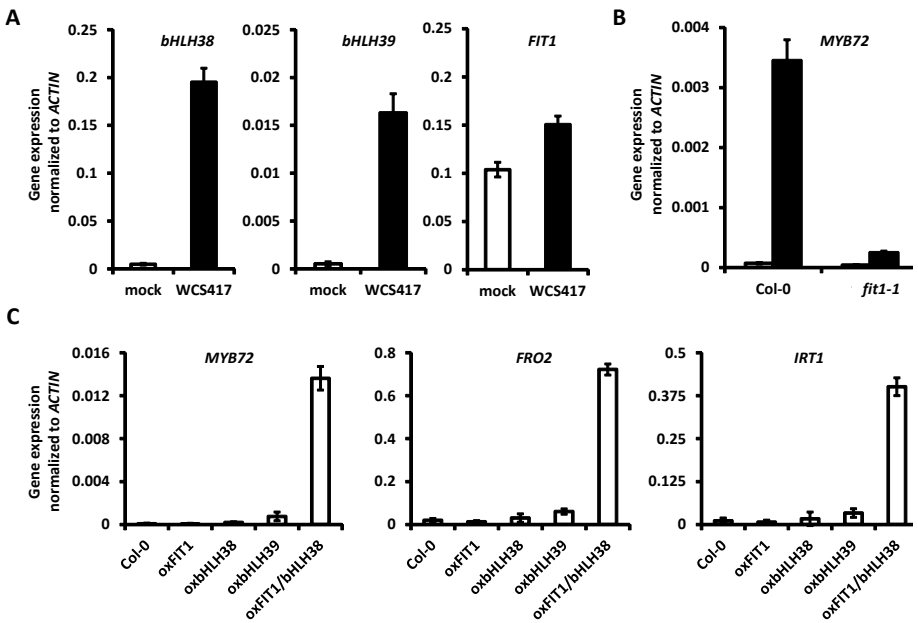


Figure 2. Role of FIT1 in WCS417-mediated induction of *MYB72* in the roots of Arabidopsis. (A) Quantitative RT-PCR analysis of *bHLH38, bHLH39* and *FIT1* transcript levels in mock- and WCS417-treated roots of Col-0. **(B)** *MYB72* expression levels in mock- and WCS417-treated roots of Col-0 and mutant *fit1-1*. Seedling were treated when 10-day-old and samples were collected 2 dpt. **(C)** *MYB72*, *FRO* and *IRT1* transcript abundance in the roots of Col-0 and transgenic lines overexpressing *FIT1* (oxFIT1), *bHLH38* (oxbHLH38), *bHLH39* (oxbHLH39), and *FIT1* and *bHLH38* (oxFIT1/bHLH38). Data in all graphs represent the mean of three replicates (± SD).

Organic volatiles emitted by ISR-inducing rhizobacteria activate *MYB72* expression

In addition to siderophores, other microbe-derived immune-elicitors (Van Wees et al., 1997; Meziane et al., 2005; Van Loon et al., 2008) and volatile organic compounds (VOCs) (Nass et al., 2005) have been reported to trigger systemic ressitance in Arabidopsis. In order to test whether the well-characterized defense elicitors flagellin and chitosan are capable of activating *MYB72* expression, hydroponically grown seedlings were treated with either 250 nM flagellin (flg22) or 0.001% chitosan after which *MYB72* expression was monitored over time. While *CYP71A12,* a gene previously shown to be induced in roots upon treatment with flg22 and chitosan (Millet et al., 2010), was upregulated, we found that both flg22 and chitosan down-regulate the expression of *MYB72* (Fig. 3B). Likewise, the expression of the iron deficiency marker gene *FRO2* was down-regulated in response to flg22

and chitosan (Fig. 3B). Hence, the onset of a defense program in the roots of Arabidopsis, such as triggered by the MAMPs flg22 and chitosan, does not account for the activation of *MYB72* expression and the iron-deficiency response in roots colonized by ISR-inducing rhizobacteria.

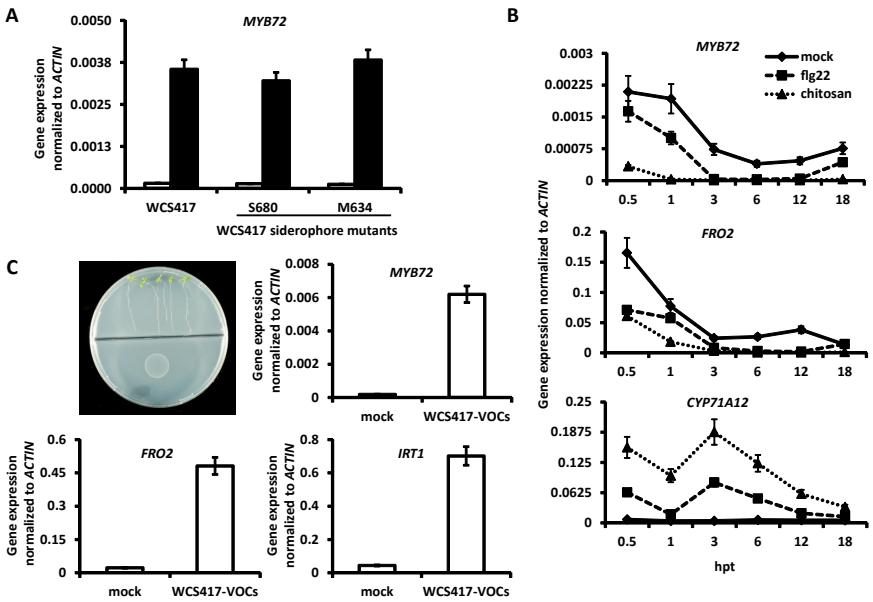


Figure 3. Bacterial determinants involved in WCS417-mediated induction of *MYB72* in roots of Arabidopsis. (A) Quantitative RT-PCR analysis of *MYB72* transcript levels in roots of Col-0 seedlings treated with $MgSO_4$, wild-type WCS417, or the siderophore-impaired mutants WCS417-S680 or WCS417-M634. Seedling were treated when 10-day-old and samples were collected 2 dpt. **(B)** Quantitative RT-PCR analysis of *MYB72*, *FRO2* and *CYP71A12* (positive control) transcript levels in the roots of 2-week-old Col-0 seedling that were either mock-treated or treated with 250 nM flg22 or 0.001% chitosan. Samples were collected for gene expression analysis at the indicated time points after treatment. **(C)** An overview of the experimental set-up for VOC studies. Quantitative RT-PCR analysis of *FRO2*, *IRT1* and *MYB72* transcripts in the roots of Col-0 seedlings upon exposure to WCS417-derived VOCs. Eight-day-old growing seedlings were exposed to VOCs in a split-plate assay and samples were collected for gene expression analysis 2 d post treatment. Data in all graphs represent the mean of three replicates (± SD).

In order to test whether bacterial VOCs are involved in WCS417-triggered expression of *MYB72*, 8-day-old seedlings were exposed to VOCs produced by WCS417 while growing on 1 × Murashige and Skoog (MS) agar-solidified medium supplemented with 0.5 % sucrose. After 2 days of WCS417-VOC exposure, root samples were collected for gene expression analysis. Figure 3C demonstrates that WCS417-derived bacterial VOCs strongly activate the expression of *MYB72* and the Strategy I markers *FRO2* and *IRT1.* These results further demonstrate that activation of the iron deficiency response in the roots of Arabidopsis is not caused by iron starvation. An initial analysis of VOCs produced by WCS417 and that of the ISR-inducing microbes WCS358 and *T. asperellum* strain T34 is provided in Supplemental Figure S1.

WCS417-mediated activation of Strategy I is independent of ethylene and auxin signaling

The plant hormones ET and auxin have been implicated in the regulation of iron acquisition and homeostasis (Romera et al., 2011). Exogenous application of ET stimulates the expression of several genes involved in iron acquisition and homeostasis in Arabidopsis, including *MYB72* (Garcia et al., 2010). Likewise, the auxin analog naphthyleneacetic acid (NAA) has been shown to enhance the

expression of the iron uptake genes *FRO2* and *IRT1* (Chen et al., 2010). Furthermore, the expression of Strategy I marker genes is impaired in the auxin transport mutants *aux1-7* (Chen et al., 2010) and the ET signaling double mutant *ein3eil1* (Lingam et al., 2011). Based on these priors, we reasoned that WCS417-VOCs may activate the iron deficiency response by affecting auxin or ET signaling. To test this, we assessed *FRO2*, *IRT1* and *MYB72* expression in the auxin signaling-deficient triple mutant *tir1afb2afb3*, the auxin transport mutant *aux1-7*, and the ET signaling mutants *ein2-1* and *ein3eil1*. Figure 4A shows that WCS417-VOC-induced *MYB72, FRO2,* and *IRT1* transcription was not impaired in either of the ET and auxin signaling mutants. Also the polar auxin transport inhibitor NPA, previously demonstrated to severely compromise the expression of *FRO2* and *IRT1* upon iron limitation (Chen et al., 2010), did not affect WCS417-VOC-mediated induction of *MYB72, FRO2,* and *IRT1* (data not shown). Together these data indicate that activation of the iron-deficiency response by WCS417-VOCs is not mediated via modulation of auxin or ET signaling.

In addition to ET and auxin, the gaseous molecule nitric oxide (NO) was shown to have a critical signaling role in the physiological responses mounted in roots of Stategy I-expressing plants in response to iron limitation (Graziano and Lamattina, 2007; Jin et al., 2009; Chen et al., 2010; Garcia et al., 2010; Meiser et al., 2011). To test whether WCS417-VOCs influence NO levels locally in roots, NO was determined by 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FMDA) fluorescence. As illustrated in Figure 4B, NO staining was more intense in roots exposed to bacterial VOCs compared to mock treatment, pointing to a potential role of NO signaling in VOC-mediated induction of *MYB72*.

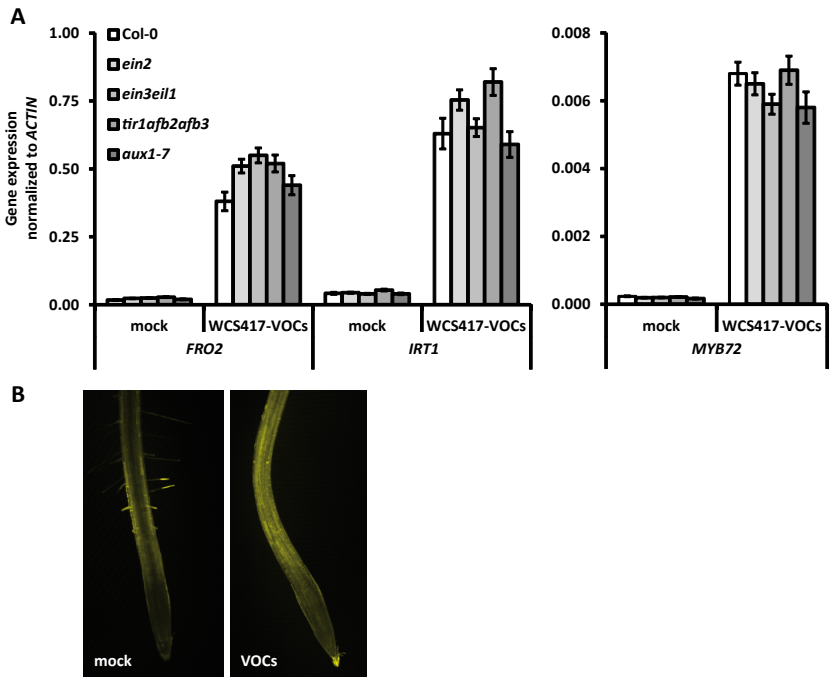


Figure 4. WCS417-VOCs induce the expression of Strategy I markers independent of the auxin and ET signaling pathways. (A) Quantitative PCR analysis of *FRO2*, *IRT1* and **(B)** *MYB72* transcripts in the roots of Col-0 and roots of the ET- and auxin-related mutants *ein2*, *ein3eil1*, *tir1afbafb3*, *aux1*. Eight-day-old seedlings were exposed to VOCs in a split-plate assay and samples were collected for gene expression analysis 2 dpt. Data represent the mean (± SD) of three replicates. **(C)** DAF-FMDA fluorescence (yellow color) in roots of control seedlings and seedlings exposed to WCS417-VOCs. Five-day-old seedlings were exposed to WCS417-VOCs in a split-plate assay and roots were subjected to confocal microscopy 2 d post treatment.

Constitutive Strategy I expressing mutants *opt3-2* and *frd3-1* exhibit enhanced resistance against necrotrophic pathogens

Despite its essential role in the initiation of ISR, overexpression of *MYB72* does not lead to enhanced disease resistance, suggesting that other components are co-required (Van der Ent et al., 2008). Considering that *MYB72* is induced in the roots of ISR-expressing plants as part of an orchestrated induction of the iron deficiency response, we assessed whether constitutive activation of Strategy I locally in roots would result in enhanced disease resistance in systemic tissues. To this end, the Arabidopsis mutants *opt3-2* (Stacey et al., 2008) and *frd3-1* (Rogers and Guerinot, 2002), which constitutively express the Strategy I response in the roots, were tested for resistance against the necrotrophic pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*. Quantitative RT-PCR analysis revealed that both mutants constitutively express *MYB72* under basal conditions (Fig. 5C, F). Figure 5 shows that both *opt3-2* and *frd3-2* display a significantly higher level of resistance against both necrotrophic fungi compared to the wild-type genetic backgrounds Col-0 and Col-5, respectively. These results indicate that activation of Strategy I locally in roots is associated with enhanced disease resistance in systemic tissues.

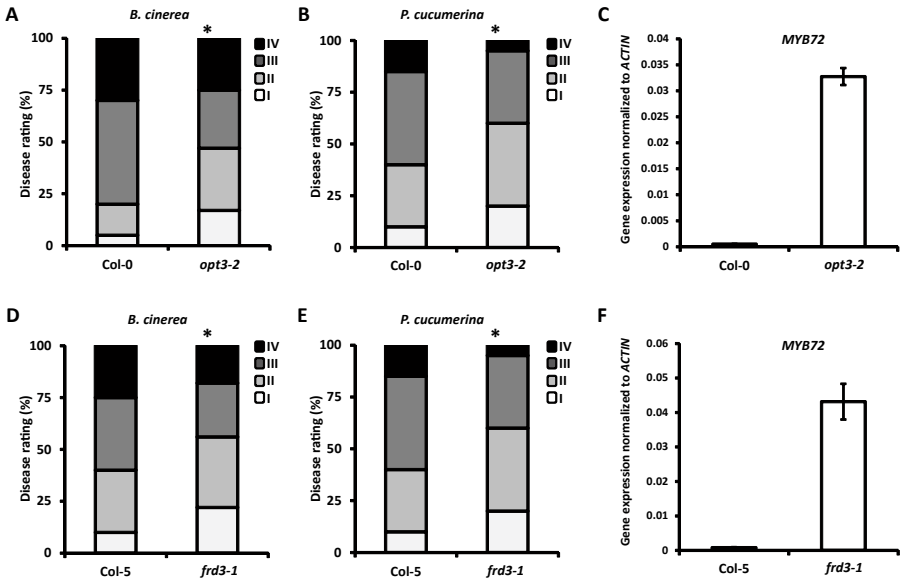


Figure 5. Constitutive Strategy I expressing mutants *opt3-2* and *frd3-1* display enhanced resistance against necrotrophic pathogens. Quantification of disease in **(A, D)** *B. cinerea* infected and **(B, E)** *P. cucumerina* infected plants. Disease severity was determined 3 d after inoculation for *B. cinerea* and 5 d after inoculation for *P. cucumerina*. Disease ratings are expressed as the percentage of leaves in disease-severity classes: I arrested lesion; II spreading lesion; III spreading lesion surrounded by a chlorotic halo; spreading lesion with extensive chlorosis and tissue maceration. Asterisks indicate statistically significantly different distributions of the disease severity classes (Chi-square, α=0.05). **(C, F)** Quantitative RT-PCR of *MYB72* transcripts in roots of 12-day-old seedlings of mutants *opt3-2* and *frd3-1*.

DISCUSSION

MYB72 has been identified as an iron deficiency-regulated gene that is strongly activated in response to iron deprivation. In this study, we demonstrate that upregulation of *MYB72* by ISR-inducing rhizobacteria is part of an orchestrated stimulation of the iron deficiency response, even when plants are not deprived of iron. Firstly, the central regulator of Strategy I, FIT1 (Colangelo and

Guerinot, 2004; Jakoby et al., 2004), appeared to be essential for rhizobacteria-mediated induction of *MYB72*. Secondly, we showed that *MYB72* transcription upon root bacterization is co-regulated with the iron deficiency markers *FRO2* and *IRT1,* and requires a combination of the basic helix-loop-helix transcription factors FIT1 and bHLH38 (or bHLH39). A working model for the regulation of *MYB72* in Arabidopsis roots in response to colonization by WCS417 is presented in Figure 6.

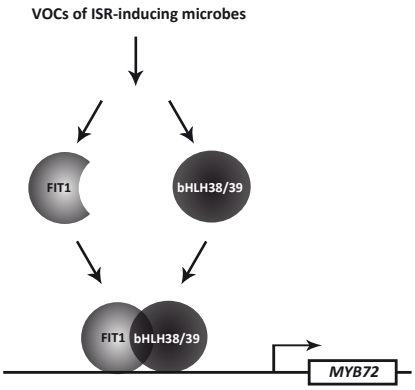


Figure 6. Model for *MYB72* regulation in *Arabidopsis* roots in response to VOCs of ISR-inducing rhizobacteria.WCS417-induced *MYB72* transcription requires FIT1. VOCs of WCS417 induce the expression of the transcription factor genes *bHLH38* and *bHLH39.* FIT1 heterodimerizes with either bHLH38 or bHLH39 to induce *MYB72* expression.

Despite the fact that microbe-derived immune-elicitors of ISR-inducing bacteria, such as flagellin and lipopolysaccharides (Van Wees et al., 1997; Meziane et al., 2005; Bakker et al., 2007; Van Loon et al., 2008), were previously shown to induce systemic resistance when locally applied to roots, our results demonstrate that the onset of a defense program in the roots of Arabidopsis negatively influences *MYB72* expression. However it should be emphasized that, although bacterial and fungal MAMPs have long been considered as molecular determinants for the elicitation of ISR in Arabidopsis and other plant species, there is no evidence demonstrating that this form of systemic resistance is manifested similarly to rhizobacteria-stimulated ISR (De Vleesschauwer and Höfte, 2009). Instead, the identification of *MYB72* as essential component of WCS417- and *Trichoderma*-mediated ISR in Arabidopsis (Van der Ent et al., 2008; Segarra et al., 2009), together with experimental data from this study placing *MYB72* induction in the frame of an orchestrated induction of the iron deficiency response, support the concept that WCS417-mediated ISR is based on the activation of iron-limitation-related signaling pathways.

Several lines of evidence indicate that activation of Strategy I in bacterized roots is not based on iron deficiency. Firstly, bacterial siderophores that have been previously reported as resistance-inducing determinants (Meziane et al., 2005; Ran et al., 2005; De Vleesschauwer et al., 2008; Van Loon et al., 2008), do not appear to have a role in the induction of *MYB72*, despite their high potential to chelate iron from the soil environment. Secondly, we identified VOCs emitted by WCS417 to potently activate *MYB72* and the associated iron-deficiency response. In other words, induction of the iron-deficiency response upon exposure to bacterial VOCs does not represent a condition of iron starvation in plant tissues, but rather reflects an active manipulation of host's iron homeostasis. Such manipulation of iron-limitation inducible mechanisms locally in roots by VOCs of the soil bacterium *Bacillus subtilis* GB03 has been demonstrated to ultimately result in increased iron assimilation and improvement of host's photosynthetic capacity (Zhang et al., 2009). Interestingly, it was recently shown that the PGPR *Pseudomonas sp.* G62 triggers an artificial sugar-starvation-like transcriptional response resulting in increased sugar contents in bacterized roots (Schwachtje et al., 2011). Hence, hijacking deficiency responses locally in roots upon colonization by PGPR may be an important aspect of plant responses to PGPR.

The molecular mechanism by which bacterial VOCs trigger the iron deficiency response, and thus induce *MYB72* expression for ISR initiation, remains elusive. Previously, it was demonstrated

that VOCs of *B. subtilis* GB03 are capable of causing rhizosphere acidification, an effect ascribed to the presence of organic acids in the volatile blend of GB03 (Zhang et al., 2009). Despite the potential impact that such modifications may have on plant signaling processes related to iron assimilation, rhizosphere acidification or other modifications in the growth substrate do not appear to be essential for *MYB72* induction. On the other hand, several lines of evidence point to the existence of iron-sensing mechanisms in the roots that monitor the iron status in the root vicinity and/or the iron distribution within plant tissues, and signal transcriptional responses when appropriate to ensure iron supply in plant tissues (Schmidt, 2003; Vert et al., 2003). Thus, a direct interference with components of these iron-sensing systems is likely to be a mechanism of action for microbial VOCs. The plant hormones ET and auxin that are emerging as intrinsic components of iron-sensing networks by either transducing or amplifying iron deficiency signals (Romera et al., 2011), do not appear to be essential for VOC-mediated induction of *MYB72*. Therefore, it is reasonable to speculate that VOCs influence cellular signaling downstream of those hormonal pathways. NO has been proposed to act downstream of auxin and upstream of FIT1 towards Strategy I activation upon iron starvation (Chen et al., 2010). In our experiments we observed that NO accumulates in roots exposed to WCS417-VOCs, pointing to a possible role of this signal molecule in VOC-mediated activation of Strategy I. However, whether NO accumulation is the cause or consequence of Strategy I activation, remains to be demonstrated.

A link between ISR and iron-deficiency inducible mechanisms is highlighted by the fact that the *opt3-2* and *frd3-1* mutants of Arabidopsis that constitutively express *MYB72* and iron-limitation responses locally in roots (Rogers and Guerinot, 2002; Stacey et al., 2008), possess enhanced resistance against necrotrophic pathogens. It is further supported by previous findings highlighting the ability of rhizobacterial VOCs to trigger systemic resistance in Arabidopsis (Nass et al., 2005). How activation of the iron deficiency-response in roots leads to the establishment of a systemic immune response and the nature of the components that function downstream of MYB72 in facilitating broad-spectrum systemic protection in foliar tissues is a major challenge. Future research should reveal the chemical identity of the microbial VOCs that trigger the iron-deficiency response and the molecular mechanism by which this leads to ISR. Understanding the chemical and biological properties of these molecules will be highly instrumental in developing novel combinatorial strategies for disease resistance and impoved iron nutrition.

MATERIALS AND METHODS

Plant material and growth conditions

The following *Arabidopsis thaliana* genotypes were used in this study: wild type Col-0, Col-5, Ws-2 and the mutants *fit1-1* (Colangelo and Guerinot, 2004), *tir1afb2afb3* (Dharmasiri et al., 2005), *aux1-7* (Pickett et al., 1990) *ein2-1* (Guzmán and Ecker, 1990), *ein3eil1* (Chao et al., 1997; Binder et al., 2007), *opt3-2* (Stacey et al., 2008) and *frd3-1* (Rogers and Guerinot, 2002). The following overexpressor lines were used: *oxFIT1*, *oxbHLH38*, *oxbHLH39* and *oxFIT1/bHLH38* (Yuan et al., 2008). Seeds were surface sterilized and sown on agar-solidified Hoagland growth medium containing the following ingredients: KNO_3 (3 mM), $MgSO_4$ (0.5 mM), $CaCl_2$ (1.5 mM), K_2SO_4 (1.5 mM), NaH_2PO_4 (1.5 mM), H_3BO_3 (25 µM), $MnSO_4$ (1 µM), $ZnSO_4$ (0.5 µM), $(NH_4)_6Mo_7O_{24}$ (0.05 µM) $CuSO_4$ (0.3 µM), Fe(III) EDTA (20 µM) (Hoagland and Arnon, 1938). The pH of the medium was adjusted to 6.0 with KOH. Seeds were sown at a density of 18 - 20 per plate. After 2 d of stratification at 4°C, the Petri dishes were transferred and positioned vertically in a growth chamber under a long-day photoperiod (16 h of light per day, light intensity 100 µmol m^{-2} s^{-1}) at 22°C. For experiments involving bacterial VOCs, two-compartment circular plates with a center partition were used; bacterial treatments were done by applying the cell suspensions into the plant-free compartment containing 1 × Murashige and Skoog (MS) agar-solidified medium supplemented with 0.5% sucrose.

Cultivation of beneficial microbes and induction treatments

Pseudomonas fluorescens WCS417, *Pseudomonas putida* WCS358, *P. fluorescens* WCS374 (Van Wees et al., 1997), and the WCS417 siderophore mutants S680 and M634 (Duijff et al., 1993) were cultured on King's medium B (KB) agar plates (King et al., 1954) supplemented with 50 µg mL^{-1} rifampicin at 28°C. After 24 h of growth, cells were collected in 10 mM $MgSO_4$, washed twice by centrifugation for 5 min at 5.000 × g, and finally resuspended in 10 mM $MgSO_4$. The bacterial titer was then adjusted to the OD_{600} of 0.002 (10^6 cfu mL^{-1}). Ten µl of bacterial suspension was then applied on each root of 10-day-old seedlings, right below the hypocotyl. For the VOC experiments, 150 µl of bacterial suspension was used. *Trichoderma asperellum* strain T34 was grown on 10 g l^{-1} malt agar plates for five days at 22°C (Segarra et al., 2009). Conidia were collected in distilled water and resuspended in 10 mM $MgSO_4$.

For studies involving treatments with defense elicitors, flagellin (flg22) and chitosan were applied to hydroponically grown seedlings at a final concentration of 250 nM and 0.001 % (v/v), respectively. Root samples were collected at defined time points post application by cutting the seedlings at the root-shoot junction and flash freezing in liquid nitrogen.

Root ferric reductase activity

Root ferric reductase activity was visualized by transferring mock- and rhizobacteria-treated seedling on agar-solidified Hoagland medium containing 0.5 mM $CaSO_4$, 0.5 mM FerroZine, and 0.5 mM Fe(III)EDTA for 20 min (Schmidt et al., 2000). Quantification of ferric reductase was performed by transferring root tissues into liquid medium containing 0.1 mM Fe(III)EDTA and 0.3 mM FerroZine (Yi and Guerinot, 1996). After 20 min incubation the absorbance of the Fe(II)-FerroZine complex was recorded at the 562 nm, as described. Reduction rates were calculated using an extinction coefficient of 28.6 mM^{-1} cm^{-1}. Roots were briefly rinsed in 10 mM $MgSO_4$ solutions before starting of the assays.

Detection of endogenous NO

Seven-day-old seedlings were incubated in a solution containing 5 µM of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FMDA) in buffer (10 mM Tris-HCl, pH 7.4) for 1 h at 25 °C in the dark. Seedlings then were washed three times for 15 min with fresh buffer (10 mM Tris-HCl, pH 7.4). Finally, fluorescence emitted by DAF-FMDA was detected under a Leica confocal microscope by excitation at 495 nm and emission at 515 nm (Fernández-Marcos et al., 2011).

Pathogen bioassays

Botrytis cinerea strain B0510 and *Plectosphaerella cucumerina* were cultivated on half-strength Potato Dextrose Agar (PDA) plates for 10 d at 22°C. *B. cinerea* spores were collected and resuspended in half-strength potato dextrose broth to a final density of 5 x 10^5 spores ml^{-1}. *P. cucumerina* spores were collected and suspended in 10 mM $MgSO_4$ to a final density of 10^6 spores ml^{-1}. Five-week old plants were infected by applying 5-µl drops per leaf. Symptoms were scored 4 days after inoculation with *B. cinerea* and 8 days after inoculation with *P. cucumerina*. Disease ratings were expressed as the percentage of leaves in disease-severity classes: I arrested lesion; II spreading lesion; III spreading lesion surrounded by a chlorotic halo; spreading lesion with extrensive chlorosis and tissue maceration.

Q-PCR analysis

Total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions and treated with Ambion® TURBO™ DNase. Subsequently, cDNA was synthesized using SuperScript-III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Cycle tresholds

were determined in duplicate per transcript in three biological replicas per sample using the ABI PRISM 7700 sequence detection system (Applied Biosystems) and SYBR Green I as reporter dye. The data was normalized using *Actin7*. The following gene-specific primers were used in this study:

MYB72_F	ACGAGATCAAAAACGTGTGGAAC
MYB72_R	TCATGATCTGCTTTTGTGCTTTG
FIT1_F	ACCTCTTCGACGAATTGCCTGACT
FIT1_R	TTCATCTTCTTCACCACCGGCTCT
bHLH38_F	GACGGTACCACAGACTTATGAAGT
bHLH38_R	TAAGCTCTTTGAAACCGTTTCAGGA
bHLH39_F	GACTTATGGAGCTGTTACAGCGGT
bHLH39_R	CTTCAAGCTTCGAGAAACCGTCGCA
IRT1_F	ACCCGTGCGTCAACAAAGCTAAAG
IRT1_R	TCCCGGAGGCGAAACACTTAATGA
FRO2_ F	TGTGGCTCTTCTTCTCTGGTGCTT
FRO2 _R	TGCCACAAAGATTCGTCATGTGCG

SUPPLEMENTAL DATA

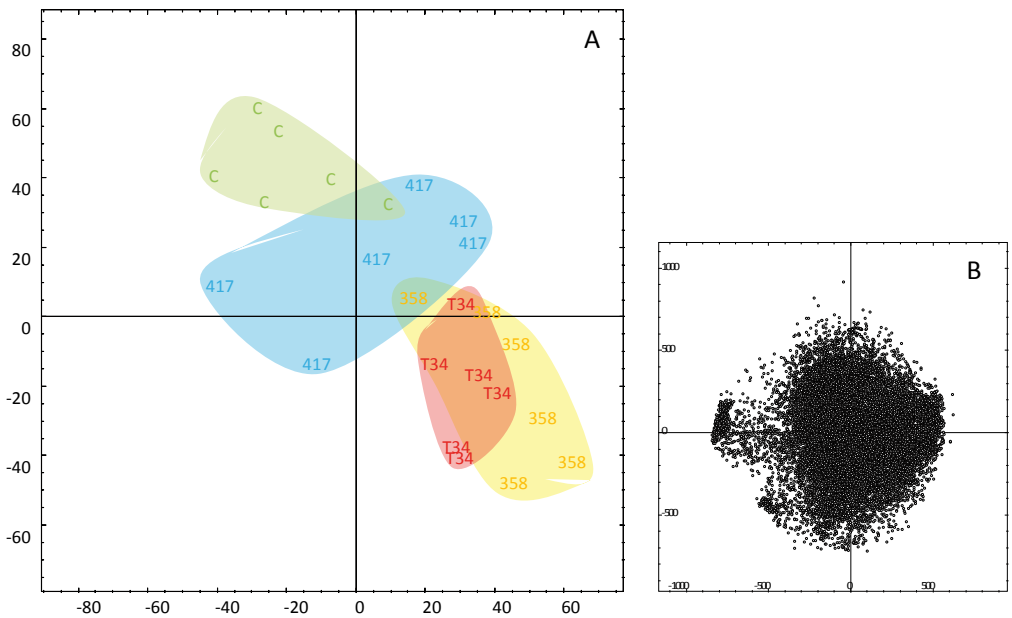
VOLATILE PROFILING

The role of rhizobacterial VOCs in the induction of *MYB72* prompted us to employ an initial GC-MS analysis to profile the VOCs of WCS417, WCS358, and the beneficial fungus *T. asperellum* T34, all microbes shown to trigger systemic resistance in Arabidopsis in a MYB72-dependent manner (Segarra et al., 2009).

WCS417, WCS358 and T34 strains were grown on 1 × MS agar-solidified medium supplemented with 0.5% sucrose for 48 h in 50-mL glass chambers under sterile conditions at 22°C. For the mock treatment an equal volume of sterile 10 mM $MgSO_4$ solution was added on the plates. The glass chambers contained a PFTE septum through which a needle could be punged. Inlet air was filtered by passing through stainless steel cartridges (Markes, Lantrisant, UK) filled with 200 mg Tenax TA (20/35 mesh, Grace-Alltech, Deerfield, USA). Bacterial volatiles were collected by pulling 100 ml min^{-1} of air through a similar cartridge filled with 200 mg Tenax TA for 30 min. Samples were obtained in six replicas. Headspace samples were analyzed with a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, USA) quadrupole mass spectrometer. Before desorption of the trapped volatiles, the Tenax cartridges were dry-purged with nitrogen at 30 ml min^{-1} for 20 min at ambient temperature to remove water. Volatiles were desorbed from the cartridges using thermal desorption at 250°C for 3 min (Model Ultra Markes Llantrisant, UK) with a Helium flow of 30 ml min^{-1}. Analytes were focused at 0°C on an electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK). Volatiles were transferred in a split less mode to the analytical column (Rtx-5ms, 30 m, 0.25 mm i.d., 1.0 µm film thickness, Restek, Bellefonte, USA) by rapid heating of the cold trap to 250°C. The GC was held at an initial temperature of 40°C for 3.5 min followed by a linear thermal gradient of 10°C min^{-1} to 280°C and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45-400 m/z with a scan rate of 3 scans s^{-1}. Bacterial volatiles were putatively annotated by comparison of their retention index and mass spectrum with those known from the NIST library database and an in-house mass spectral database for GC-TOF-MS (PRI-PPH, WUR, The Netherlands). GC-MS acquired headspace data were processed by MetAlign software (Lommen, 2009) for noise detection and alignment of the data points. Quantification was based on comparison of the area of m/z fragments under the GC curve. For comparisons of the same compound under different treatments, response factors for individual compounds were assumed to be equal. Data were Log10 transformed, normalized and scaled symmetrically by the standard deviation of corresponding sample using GeneMath software.

To obtain more insight in those volatile compounds that may be responsible for the differences in the volatile signature, multivariate exploratory analysis was performed on the headspace compositions using Principal Component Analysis (PCA). Our analysis revealed consistent differences in the composition of the VOC signature emitted by the *Pseudomonas* spp. strains and the *Trichoderma* strain compared with VOCs produced by the control MS plates (Fig. S1). The first two principal components of the PCA explained 47% of the total variance found in the data set. The loading plot of the first two principal components is shown in Figure S1B and can be used to identify which compounds contribute most to the separation of the bacterial blends in Figure S1A. However, as hundreds of different volatile compounds can be detected in a headspace sample, quantitative alignment of the different metabolites results in a dataset that comprises almost 27.000 mass fragments (Fig. S1B). Therefore, a two-tailed T-test was performed to assign those molecular fragments that significantly differ between each bacterial or fungal strain and the mock treatment ($p < 0.05$). This resulted in 536 molecular fragments for WCS417, 824 fragments for WCS358 and 1111 fragments for T34 that differed significantly with those obtained from the

control plates. Mass spectroscopic analysis of these fragments and compounds, and comparison with the 147.000 compounds comprising the NIST library (http://peptide.nist.gov) and our in-house database and their retention index, allowed the identification of only a few compounds, such as dodecanal, 1-undecene, 2,4-dimethyl hexane, 1,10-undecadiene, decanal and isovalaldehyde. A dedicated search for volatile terpenoids or phenylpropanoids did not result in the annotation of any compound, and these classes of compounds are apparently not present in the head space of these bacteria.



Supplemental Figure S1. GC-MS profiling of VOCs produced by ISR-inducing microbes.

CHAPTER 4

Transcript profiling of Arabidopsis roots identifies the MYB72-dependent β-glucosidase gene *BGLU42* as a key component of rhizobacteria-induced systemic resistance

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ABSTRACT

Root colonization by selected strains of non-pathogenic rhizobacteria triggers an induced systemic resistance (ISR) in diverse plant species that is effective against a broad spectrum of pathogens and even insects. The root-specific transcription factor MYB72 of *Arabidopsis thaliana* is required for ISR. Here, we report that under basal conditions *MYB72* is predominantly expressed in the xylem parenchyma cells of the vascular bundle. However, in response to colonization of the roots by ISR-inducing *Pseudomonas fluorescens* WCS417 bacteria, *MYB72* transcripts specifically accumulate in the root trichoblasts and cortical cells. By whole-genome transcript profiling of control- and WCS417-treated roots of wild-type Col-0, mutant *myb72,* and *MYB72*-complemented *35S::FLAG-MYB72*(*myb72*) plants, we identified the β-glucosidase *BGLU42*, the cytochrome P450 monooxygenase *CYP71B5*, the oligopeptide transporter *NRT1*.8 and the *AT5G55620* gene, as WCS417-responsive genes that are regulated in a MYB72-dependent manner. The T-DNA knockout mutant *bglu42* appeared to be abolished in its ability to mount WCS417-ISR against the pathogen *Pseudomonas syringae* pv. *tomato* DC3000, indicating that BGLU42 is an essential component of ISR downstream of MYB72. In addition, overexpression of *BGLU42*, in the absence of rhizobacteria, conferred a significant level of protection against the pathogens *Botrytis cinerea*, *Pseudomonas syringae* pv. *tomato* and *Hyaloperonospora arabidopsidis*.

INTRODUCTION

Plants have a multicomponent defense system to protect themselves against pathogens (Spoel and Dong, 2012). Constitutively expressed physical barriers such as the plant cell wall and the waxy cuticle, as well as chemical obstacles, constitute the first line of defense by hindering pathogen entry into internal plant tissues (Osbourn, 1996; Juge, 2006; Skamnioti and Gurr, 2007). Plants further rely for their protection against pathogenic microorganisms on specialized surveillance mechanisms that appear to be evolutionary conserved amongst the eukaryotes (Nürnberger and Brunner, 2002; Nürnberger et al., 2004). These defense systems have evolved to recognize and perceive non-self molecules that are conserved amongst different classes of pathogens and are collectively referred to as general elicitors or microbe-associated molecular patterns (MAMPs) (Boller and Felix, 2009). MAMPs are perceived by the corresponding pattern recognition receptors (PRRs) after which signaling cascades are initiated, ultimately leading to transcriptional reprogramming towards defense-related metabolic and cellular responses (Pedley and Martin, 2005; Zipfel, 2008; Zipfel, 2009). Plant hormones appear to have central roles in orchestrating such defense networks. In particular, a considerable body of research identified the stress-related hormones salicylic acid (SA), ethylene (ET) and jasmonates (JAs) as the primary hormonal signals in the regulation of the plant's immune response (Bari and Jones, 2009; Pieterse et al., 2009; Pieterse et al., 2012). Although the above mentioned mechanisms provide an efficient protection against the vast majority of harmful microorganisms, certain pathogens can eventually infect host plants and cause disease resulting in significant agricultural and economic losses. It is well established that the capacity of a pathogen to establish compatible interactions with host plants is based on its ability to overcome static barriers and further suppress inducible defense responses by utilizing effector molecules (He et al., 2007; Stassen and Van den Ackerveken, 2011; Thomma et al., 2011). Interestingly, it appears that not only pathogenic microorganisms but also beneficial microbes have evolved to manipulate plant innate immunity in order to establish successful associations with host plants (Zamioudis and Pieterse, 2012).

A primary infection caused by a pathogenic microorganism in local tissues is known to generate

a systemic defense response that aims to protect the whole plant against future pathogenic attack. This phenomenon is called systemic acquired resistance (SAR) and phenotypically resembles the immunological memory in organisms of the animal kingdom (Sticher et al., 1997; Durrant and Dong, 2004). In addition to SAR, a different form of systemic resistance, called induced systemic resistance (ISR), is commonly activated in the aboveground plant parts upon root colonization by selected strains of beneficial root-colonizing bacteria (Van Loon et al., 1998; Van Wees et al., 2008; De Vleesschauwer and Höfte, 2009), but also by mycorrhizal and free-living fungi (Harman et al., 2004; Pozo and Azcon-Aguilar, 2007). Rhizobacteria-mediated ISR has been demonstrated in many plant species, including bean, carnation, cucumber, radish, tobacco and tomato (Van Loon et al., 1998; Van Loon and Bakker, 2005), suggesting that the activation of a systemic immune response upon root colonization by beneficial bacteria is a common plant response. Most of the knowledge related to the molecular mechanisms underpinning the ISR phenomenon stems from studies on the model plant *Arabidopsis thaliana* (Arabidopsis) and its interaction with the soil-borne bacterium *Pseudomonas fluorescens* WCS417. WCS417-mediated ISR is effective against a broad variety of pathogens and even herbivorous insects (Pieterse et al., 1996; Van Wees et al., 1997; Ton et al., 2002a; Van Oosten et al., 2008; Pineda et al., 2010). It depends on functional JA and ET signaling pathways and further requires the transcriptional regulators NPR1 and MYC2 (Pieterse et al., 1998; Pozo et al., 2008).

Large scale gene expression analysis revealed that the establishment of ISR in foliar tissues is not associated with detectable changes in gene expression (Verhagen et al., 2004). Instead, ISR-expressing plants are potentiated for accelerated JA/ET-regulated gene expression (Verhagen et al., 2004; Pozo et al., 2008) and the accumulation of callose-rich papillae at the sites of pathogen entry (Van der Ent et al., 2009b), responses that become apparent only upon microbial attack. This phenomenon is known as priming and is analogous to the interferon-γ-induced conditioning of human monocytes and macrophages towards enhanced responsiveness to pathogen-derived lipopolysaccharides (Conrath et al., 2006). Ultimately, ISR and other priming phenomena triggered by various agents such as the non-protein amino acid β-amino-butyric acid (BABA) (Ton et al., 2005), provide a cost-effective mechanism of protection against pathogens (Van Hulten et al., 2006).

In contrast to leaves, roots respond to colonization of the roots by WCS417 via the onset of a transcriptional program that involves significant alterations in the expression of 97 genes (Verhagen et al., 2004). Amongst them, the root-specific MYB72 transcription factor (TF) of the R2R3-type MYB family emerged as an important component for the onset of ISR. *MYB72* is locally induced upon WCS417-colonization and T-DNA *myb72* mutants *myb72-1* and *myb72-2* are abolished in their ability to generate systemic resistance against a broad range of pathogens (Van der Ent et al., 2008). Despite its essential role in ISR, constitutive *MYB72* expression does not lead to increased levels of resistance in the absence of rhizobacteria, suggesting that either MYB72 undergoes post-translational activation or other interacting partners are co-required (Van der Ent et al., 2008). Interestingly, not only beneficial rhizobacteria, but also the beneficial fungus *Trichoderma asperellum* T34 utilizes MYB72-mediated signaling for ISR elicitation, pointing to the existence of a conserved mechanism that operates locally in roots during ISR triggered by different types of beneficial microbes (Segarra et al., 2009). Here, we report on the tissue-specific expression pattern of *MYB72* and the whole-genome transcriptome changes that are regulated by MYB72 in the roots of Arabidopsis. We identified the MYB72-regulated β-glucosidase gene *BGLU42* as an important novel component of the ISR signaling pathway.

RESULTS

Tissue specific expression pattern and subcellular localization of MYB72

In order to gain a better understanding of MYB72 function during ISR initiation locally in roots,

we first examined its spatial expression pattern under basal and WCS417-induced conditions. To this end, we generated reporter lines expressing the GFP-GUS fusion protein under control of the 1.7-kb *MYB72* promoter region. Confocal imaging of the reporter line revealed that under uninduced conditions, *MYB72* is predominantly expressed in the vascular tissue (Fig. 1A). This finding is in line with cell-type specific gene expression data obtained from the AREX database that reports on maximum *MYB72* expression in the xylem parenchyma cells (XPCs) within the stele (Supplemental Fig. S1). Upon WCS417 application, we observed massive accumulation of the GFP fluorophore in the epidermal cells and to a lesser extend in the cortical cells of the bacterized roots (Fig. 1B). Interestingly, *MYB72* expression in the epidermal tissues is restricted to trichoblasts, cells that are located over the cleft between two cortical cells and are destined to make root hairs. We further asked for the subcellular localization of MYB72 by inspecting the subcellular localization of the YFP (Yellow Fluorescent Protein)-MYB72 chimeric protein in transgenic lines stably expressing the corresponding construct under the CaMV 35S promoter. Consistent with its function as TF, we found MYB72 to localize in the nucleus of plant cells (Fig. 1C).

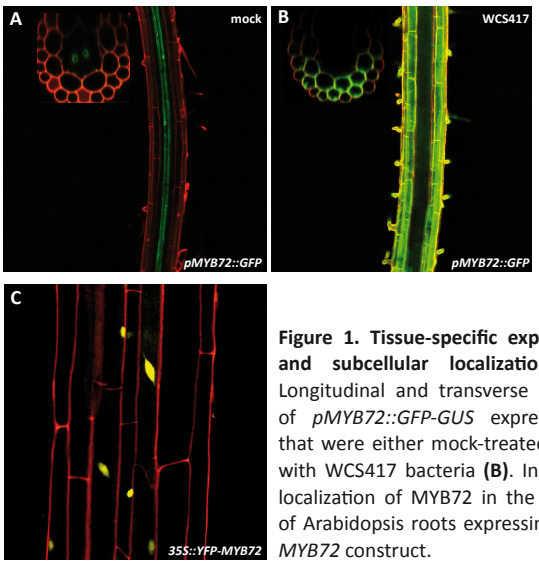


Figure 1. Tissue-specific expression pattern and subcellular localization of MYB72. Longitudinal and transverse optical sections of *pMYB72::GFP-GUS* expressing seedlings that were either mock-treated **(A)** or treated with WCS417 bacteria **(B)**. In **(C)**, subcellular localization of MYB72 in the epidermal cells of Arabidopsis roots expressing the *35S::YFP-MYB72* construct.

Genome-wide transcriptional responses to root colonization by WCS417

To identify WCS417-responsive genes that are regulated in a MYB72-dependent manner, we generated global gene expression profiles in the roots of wild type and *myb72-2* seedlings growing vertically on 1 × Hoagland agar-solidified medium. We also included in our analysis a *35S::Flag-MYB72* overexpressor line that was generated in the *myb72-2* genetic background (hereafter referred to as oxMYB72). Root material was harvested in three biological replicas, at 2 days post treatment (dpt) when *MYB72* transcript levels reach their maximum level in WCS417-treated wild-type Col-0 plants (data not shown). RNA was isolated for each replica and treatment, and the corresponding cRNAs were hybridized to Affymetrix ATH1 GeneChips for whole-genome transcript profiling analyses. By setting as criteria for differentially expressed genes a statistical significance p value of < 0.05, and an additional cut-off value of more than 2-fold regulation, we identified 301 up-regulated and 209 down-regulated genes in wild type Col-0 roots upon WCS417 colonization. Amongst the 301 up-regulated WCS417-responsive genes, three major groups can be distinguished (Fig. 2): (i) a set of 37 genes that are upregulated in the oxMYB72 line under uninduced conditions (green and purple cluster); (ii) a large cluster of 120 genes that are induced in roots of both wild-type and mutant seedling but show compromised expression in the roots

of oxMYB72 (blue cluster); and (iii) a set of 5 MYB72-dependent genes that show compromised WCS417-induced expression in *myb72-2* compared to Col-0, but are recovered in oxMYB72 (red cluster). The 209 genes that were significantly down-regulated in Col-0 by WCS417 were also down-regulated in *myb72-2* and oxMYB72 (data not shown) and are further disregarded in this study.

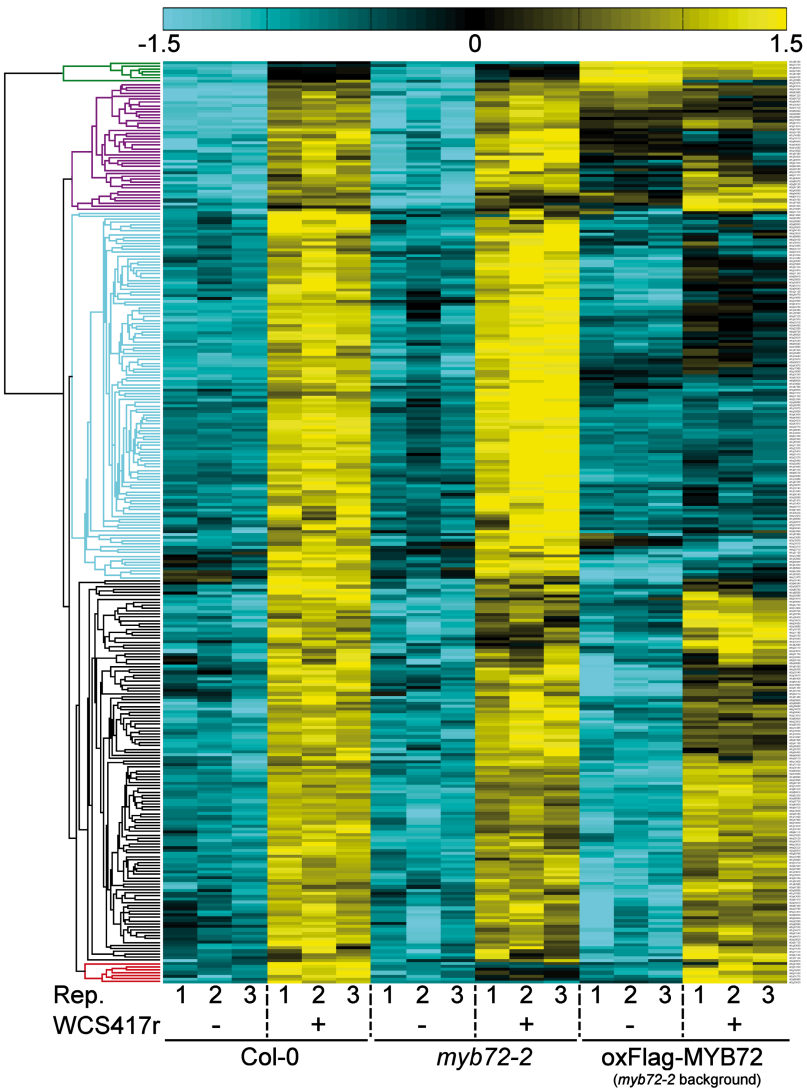
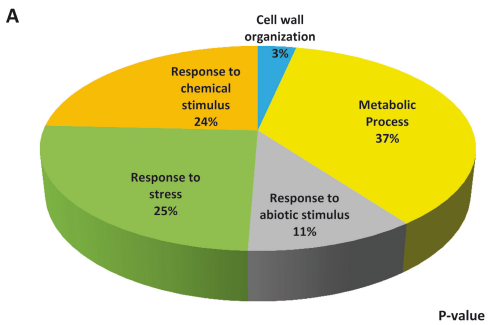


Figure 2. Summary of microarray analysis performed on control- and WCS417-treated roots of Col-0, *myb72-2* and oxMYB72. Heat-map of differentially expressed genes (more than 2-fold upregulation, $p < 0.05$) in the roots of wild type Col-0 seedlings and the corresponding gene expression signatures in the roots of *myb72-2* and oxMYB72. The green and purple clusters represents WCS417-responsive genes that show constitutive upregulation in oxMYB72 under non-induced conditions. The blue cluster represents genes that are significantly induced by WCS417 in Col-0 but are repressed in WCS417-treated oxMYB72. The red cluster represents WCS417-responsive genes, whose WCS417-induced expression is compromised in *myb72-2* and restored in oxMYB72 (oxFlag-MYB72 in *myb72-2* background).

To gain insight into the specific functions of the identified gene clusters, we classified the 301 WCS417-upregulated genes into biological categories using the AmiGO Term Enrichment software. As depicted in Figure 3, the dominant categories represent those related to biotic and abiotic stress. Metabolic signatures commonly involved in plant defense responses, such as biosynthetic processes of indole-glucosinolate and phenylpropanoid compounds, are also overrepresented. Considering the emerging relationship between ISR and iron deficiency response (Chapter 3), we further asked for the proportion of WCS417-upregulated genes that are also responsive to iron limitation. To this end, we compared transcriptional profiles obtained from our microarray data with those obtained from Dinneny et al. (2008). We found that almost 1/3 of the WCS417-induced genes are also induced in response to iron deprivation, further pointing to a connection between plant responses to ISR-inducing rhizobacteria and iron homeostasis. Collectively, the transcriptional signature of Col-0 roots upon root colonization by WCS417 possess predominantly, characteristics of defense- and iron deficiency-associated transcriptional reprogramming.



	P-value
GO:0071555 cell wall organization	2.89E-03
GO:0042435 indole-containing compound biosynthetic process	6.63E-09
GO:0009699 phenylpropanoid biosynthetic process	3.27E-04
GO:0000162 tryptophan biosynthetic process	1.63E-03
GO:0080167 response to karrikin	5.97E-05
GO:0050832 defense response to fungus	9.57E-11
GO:0009611 response to wounding	1.44E-04
GO:0009814 defense response, incompatible interaction	1.01E-03
GO:0042742 defense response to bacterium	1.39E-03
GO:0071456 cellular response to hypoxia	1.81E-21
GO:0071281 cellular response to iron ion	9.62E-09
GO:0071732 cellular response to nitric oxide	3.93E-07
GO:0006979 response to oxidative stress	7.48E-07
GO:0071369 cellular response to ethylene stimulus	7.84E-06

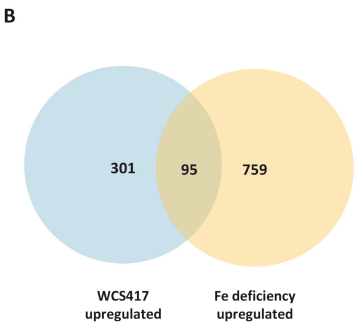


Figure 3. GO term analysis of WCS417-induced genes in Col-0. (A) Annotation of the 301 WCS417-induced genes depicted in Fig. 2 into biological GO terms using the AmiGO Term Enrichment software. Each caterogy is shown in a different color in the pie chart with a corresponding color-coded list below, specifying the overrepresented cellular responses (*p* value < 0.05). **(B)** Venn-diagram showing numbers of unique and overlapping genes between WCS417- and iron deficiency-induced genes (Dinneny et al., 2008).

In order to gain insight into the role of MYB72 in the regulation of WCS417-ISR we focused on the small set of genes that is specifically upregulated by WCS417 in a MYB72-dependent manner (red cluster in Fig. 2). The large group of genes that is suppressed by *MYB72* overexpression (blue cluster), will be further discussed in Chapter 5.

MYB72 regulates the expression of iron-deficiency inducible genes in response to WCS417

To identify WCS417-responsive genes that are regulated in a MYB72-dependent manner, we selected for those genes that were differentially expressed between wild-type and *myb72-2* upon colonization of the roots by WCS417 (p value < 0.05). The resultant list of candidate genes is provided in Supplemental Table 1. To further focus on a more robust gene-set, we asked for genes that are additionally induced or repressed by 2-fold in wild-type roots upon WCS417 application, and further show a 2-fold de-regulation in the *myb72-2* mutant under the same conditions (p < 0.05). Our analysis resulted in a total of 5 genes that fulfilled these selection criteria (Fig. 4), which correspond to the red cluster in Figure 2. Overexpression of *MYB72* in the mutant *myb72-2* background (oxFLag-

MYB72), restored the WCS417-responsiveness of these genes (Fig. 4). Moreover, by employing quantitative PCR analysis we confirmed these 5 genes as MYB72-targets (Supplemental Fig. S2).

A

Locus	Annotation
AT5G55620	unknown protein
*AT5G36890	BGLU42 (BETA GLUCOSIDASE 42) beta-glucosidase/ catalytic/ cation binding / hydrolase, hydrolyzing O- glycsyl compounds
*AT3G53280	CYP71B5 (CYTOCHROME P450 71B5) electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
*AT4G21680	NRT1.8 proton-dependent oligopeptide transport (POT) family protein
AT3G56980	BHLH039 DNA binding / transcription factor

* FIT1-regulated genes upon iron deficiency

B

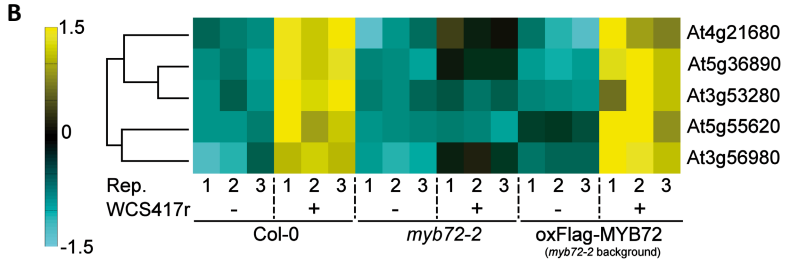


Figure 4. WCS417-induced gene expression regulated in a MYB72-dependent manner. **(A)** List of genes that are strongly regulated by MYB72 upon root colonization by WCS417 bacteria, as revealed by microarray analysis of Arabidopsis roots. Asterisks indicate genes previously identified to be regulated in a FIT1-dependent manner upon iron limitation (Colangelo and Guerinot, 2004). **(B)** A heat-map of gene expression that is regulated by MYB72 upon root colonization by WCS417 as revealed by microarray analysis. Genes that are induced more than 2-fold in wild type Col-0 roots and show more that 2-fold deregulation in the *myb72-2* mutant are depicted (*p* value < 0.05).

The MYB72-dependent, WCS417-responsive genes *BGLU42*, *CYP71B5* and *NTR1.8* genes have been previously identified as iron deficiency-induced genes regulated in a FIT1-dependent manner (Colangelo and Guerinot, 2004). FIT1 is a central transcription factor that orchestrates physiological responses to iron starvation (Colangelo and Guerinot, 2004; Jakoby et al., 2004), previously reported to be essential for WCS417-mediated induction of *MYB72* (Chapter 3). *AT5G5560*, although not FIT1-regulated, was reported as an early-induced, iron deficiency-responsive gene (Buckhout et al., 2009). The basic helix-loop-helix transcription factor bHLH39 and the closely related bHLH38 have been shown to heterodimerize with FIT1 (Yuan et al., 2008) for the induction of the iron uptake genes *FRO2* (Yi and Guerinot, 1996) and *IRT1* (Eide et al., 1996) upon iron limitation. A probe for *bHLH38* is not included in the ATH1 gene array. Quantitative PCR analysis of *bHLH38* upon root colonization by WCS417 revealed that MYB72 also partially regulates *bHLH38* expression (Supplemental Fig. S2). However, we could not detect significant differences in the expression of the iron deficiency markers *FRO2* and *IRT1* upon root bacterization in the *myb72-2* mutant, suggesting previously described redundancy amongst members of the bHLH family of transcription factors (Wang et al., 2007b) in the regulation of these iron uptake genes (Supplemental Fig. S2).

Amongst the MYB72-target genes, only the expression of *AT5G55620* is completely abolished in the *myb72* mutant background. The expression of *BGLU42* and *CYP71B5*, and to lesser extend of *NRT1.8* and *bHLH39*, although compromised, are still induced in the *myb72-2* roots, suggesting that other factors co-operatively with MYB72 regulate the expression of those genes.

Overexpression of *BGLU42* results in constitutive disease resistance

Consistent with previous findings that overexpression of *MYB72* does not lead to constitutive disease resistance (Van der Ent et al., 2008), none of genes that are mis-regulated in the *myb72-2* mutant show constitutive expression in the oxMYB72 line. However, overexpression of *MYB72* in the *myb72-2* mutant background, restored the expression of MYB72 target-genes in response to root colonization by WCS417 (Fig. 4 and Supplemental Fig. S2). In Chapter 3, we demonstrated that the *opt3-2* (Stacey et al., 2008) and *frd3-1* (Rogers and Guerinot, 2002) mutants of Arabidopsis, which constitutively express iron deficiency responses and *MYB72* locally in roots, display a significant level of resistance against the necrotrophic fungi *Botrytis cinerea* and *Plectospaerella cucumerina*. By employing quantitative PCR analysis we found that all MYB72-target genes are also constitutively upregulated in the *opt3-2* and *frd3-1* mutants (Fig. 5). Therefore, we hypothesized that upregulation of at least one of the

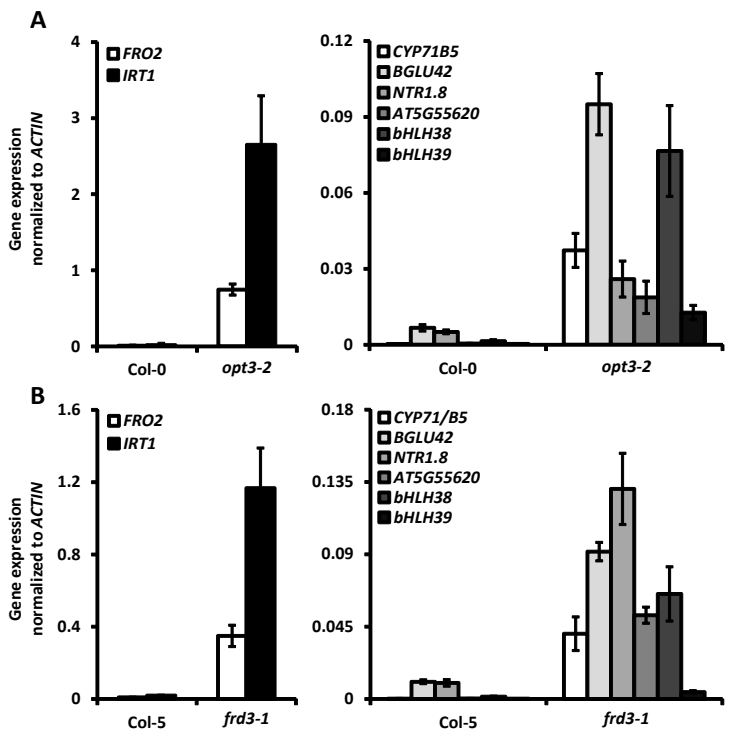


Figure 5. MYB72-target genes are constitutively expressed in *opt3-2* and *frd3-1*. Quantitative PCR analysis of the MYB72 target genes *CYP71B5, BGLU42, NTR1.8, AT5G55620, bHLH38,* and *bHLH39,* and the iron-deficiency response marker genes *FRO2* and *IRT1* in the roots of 12-day-old seedlings of the constitutive iron deficiency response expressing mutants *opt3-2* and *frd3-1* and their respective wild types Col-0 and Col-5. Gene expression analysis was perfomed with non-bacterized roots. Error bars represent the SE of three biological repetitions.

downstream MYB72-targets may be the limiting step for the initiation of ISR locally in roots. To this end we obtained transgenic lines overexpressing *NRT1.8*, *bHLH38* and *bHLH39* and we further generated transgenic lines overexpressing *AT5G55620*, *BGLU42* and *CYP71B5*. Independent T3 transformants were then tested for enhanced resistance against *B. cinerea* infection. Results in Figure 6A show that, amongst the transgenic lines tested, only plants overexpressing *BGLU42* (*o*xBGLU42) displayed a significant level of resistance. We further assessed whether ectopic overexpression of *BGLU42* also confers enhanced disease resistance against the biotrophic pathogens *P. syringae* pv. *tomato* DC3000 and *Hyaloperonospora arabidopsidis*. Again, a significant level of resistance against

both pathogens was observed in all independent oxBGLU42 lines tested (Fig 6B, C). These results collectively suggest a role of the MYB72 target gene *BGLU42* in broad spectrum disease resistance.

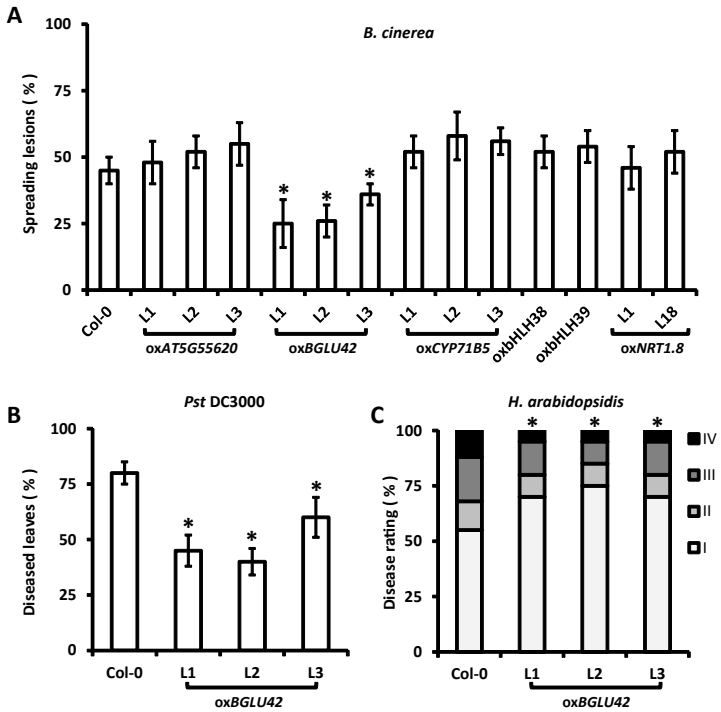


Figure 6. Level of disease resistance in transgenic *Arabidopsis* lines overexpressing the MYB72 target genes *AT5G55620, BGLU42, CYP71B5, bHLH38, bHLH39, and NRT1.8.* Levels of disease severity in Col-0 and independent transgenic lines overexpressing *AT5G55560*, *BGLU42*, *CYP71B5*, *bHLH38*, *bHLH39* or *NRT1*.8 upon inoculation with **(A)** *B. cinera,* **(B)** *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), and **(C)** *H. arabidopsidis*. For *B. cinereas* infections, disease symptoms were determined 5 d after inoculation. Disease ratings were expressed as percentage of leaves showing spreading lesions. Asterisks indicate statistically significant differences compared to Col-0 (Student's *t* test, $p < 0.05$). For *P. syringae* pv. *tomato* DC3000 infections, disease symptoms were determined 4 d after inoculation. Disease ratings were expressed as the percentage of leaves showing necrotic or water-soaked lesions surrounded by chlorosis. Asterisks indicate statistically significant differences compared to Col-0 (Student's *t* test, $p < 0.05$). For *H. arabidopsidis* infections, disease severity was determined 10 d after inoculation. Disease ratings are expressed as the percentage of leaves in disease-severity classes: I, no sporulation; II, trailing necrosis; III, <50% of the leaf area covered with sporangia; IV, >50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse. Asterisks indicate statistically significant different distributions of the disease severity classes compared to Col-0 (χ^2-test, $\alpha = 0.05$).

Knockout mutant *bglu42* is defective in WCS417-mediated ISR

To examine whether *BGLU42* is required for WCS417-mediated ISR, we obtained an homozygous knockout line from the SALK collection (Alonso et al., 2003) carrying a T-DNA insert in the 12th exon in sense orientation (Fig. 7A). We then tested the ability of *bglu42* to express WCS417-ISR against *P. syringae* pv. *tomato* DC3000. Col-0, *myb72-2* and *bglu42* plants were grown in the presence or absence of WCS417 bacteria and disease severity was assessed four days after pathogen inoculation. Colonization of Col-0 roots by WCS417 resulted in a moderate but significant reduction in the development of disease symptoms caused by *P. syringae* pv. *tomato* DC3000 (Fig. 7B). By contrast, mutant *myb72-2* did not develop ISR, confirming previous findings (Van der Ent et al., 2008) (Fig. 7B). Similarly to *myb72-2*, *bglu42* plants growing in the presence of WCS417, did not mount enhanced resistance

against *P. syringae* pv. *tomato* DC3000 (Fig. 7B). These data collectively indicates that downstream of MYB72, BGLU42 is an essential component for the onset of WCS417-mediated ISR in Arabidopsis.

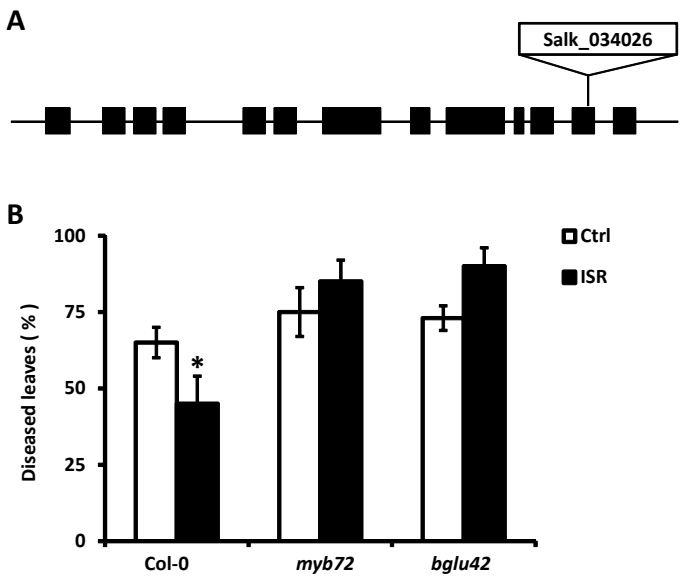


Figure 7. WCS417-ISR against *P. syringae* pv. *tomato* DC3000 is blocked in *blgu42*. **(A)** Structure of *BGLU42* gene and potition of the T-DNA insertion. Exons are indicated as black boxes. **(B)** Levels of disease severity in control- and WCS417-treated Col-0, *myb72-2* and *bglu42* plants upon inoculation with *P. syringae* pv. *tomato* DC3000. ISR was induced by growing plants for three weeks in soil containing WCS417 bacteria. Five-week-old plants were challenge-inoculated with a *P. syringae* pv. *tomato* DC3000 bacterial suspension. The percentage of diseased leaves was assessed four days after inoculation. Asterists indicate statistically significant differences compared to non-induced plants (Student's *t*-test, $p<0.05$).

BGLU42 is predominantly expressed in root trichoblasts

In order to get additional insights into the expression pattern of the MYB72 target gene *BGLU42*, we generated transgenic lines expressing a GFP-GUS fusion under the approximate 2-kb promoter region of the *BGLU42* gene and examined its expression patterns locally in roots under basal and WCS417-induced conditions. We observed that in the absence of WCS417 bacteria, *BGLU42* is expressed at low levels, predominantly in the epidermal cells of the mature root (Fig. 8A). Upon root colonization by WCS417, strong *BGLU42* expression was detected in root trichoblasts, and to a lesser extend in cortical cells (Fig. 8B). This expression pattern is consistent with that of the WCS417-induced expression pattern of *MYB72* in the epidermal and cortical root tissues (Fig. 1B). These data suggests that transcriptional and metabolic responses upon root colonization that occur in the root trichoblasts may have critical roles for the initiation of ISR.

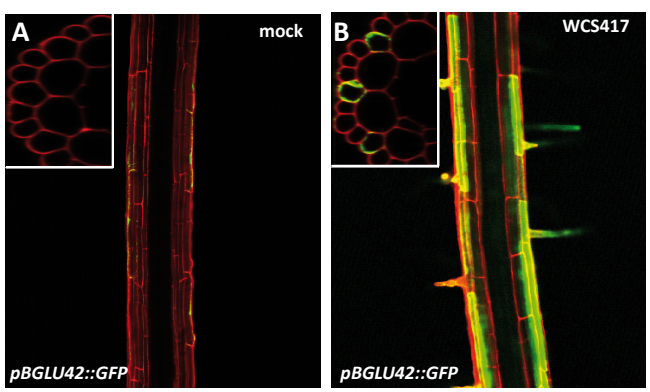


Figure 8. Tissue-specific expression pattern of *BGLU42*. Longitudinal and transverse optical sections of roots of *pBGLU42::GFP-GUS* expressing Col-0 seedlings that were either mock-treated **(A)** or treated with WCS417 bacteria **(B)**.

DISCUSSION

Defense and iron deficiency-related responses dominate the Arabidopsis root transcriptome in response to WCS417.

By employing whole-genome microarray analysis we have demonstrated that root colonization by WCS417 activates a transcriptional program in the roots of Arabidopsis that is predominantly characterized by the induction of defense-associated and iron deficiency-responsive genes. A significant portion of genes that show induced expression by WCS417 are annotated as MAMP-responsive, indicating that Arabidopsis recognizes WCS417 as a potential invader and consequently activates a defense program similar to that induced against pathogenic microbes. Recently, Millet and associates reported on the ability of WCS417 to suppress the flg22-mediated activation of MAMP-responsive promoters and callose deposition in the elongation zone of the Arabidopsis roots (Millet et al., 2010). Since suppression of MAMP-triggered immunity occurs at early stages of the interaction, it is possible that our data reflects a delayed, mild activation of MAMP-responsive genes. Alternatively, WCS417 may be able to suppress only a subset of immune responses while allowing others to develop. Importantly, our microarray analysis revealed that in addition to defense-related transcriptional responses, and consistent with a putative involvement of MYB72 in iron deficiency responses, bacterized roots undergo transcriptional reprogramming that significantly overlaps with transcriptional responses to iron limitation. This is clearly evident by the fact that almost one third of the WCS417-induced genes locally in roots have been previously reported as iron deficiency-responsive genes (Dinneny et al., 2008).

MYB72 target genes encode components of the iron deficiency response

By comparing transcriptional responses mounted in wild type roots with those mounted in the *myb72*-2 mutant upon colonization of the roots by WCS417, we were able to identify a cluster of 5 genes that is regulated in a MYB72-dependent manner. While impaired in *myb72-2,* WCS417-induced expression of these genes was restored in the complemented mutant overexpressing MYB72 in the *myb72-2* background. In particular, we found MYB72 to regulate the expression of *AT5G55620*, significantly affect the expression of *CYP71B5* and *BGLU42* and partially regulate the expression *NRT1.8*. We further identified the *bHLH38* and *bHLH39* transcription factor genes to be partially regulated by MYB72. Considering that regulation of *MYB72* in response to WCS417 is similarly regulated as the iron deficiency markers *FRO2* and *IRT1* by the FIT1/bHLH38 or FIT1/bHLH39 heterodimer (Chapter 2), it is reasonable to speculate that MYB72 participates in a feedback mechanism to regulate its own expression. Importantly, amongst the MYB72 targets, the *BGLU42*, *CYP71B5* and *NTR1.8* genes have also been identified to be regulated in a FIT1-dependent manner upon iron limitation (Colangelo and Guerinot, 2004), suggesting that these genes are not primary FIT1 targets, but rather FIT1 indirectly regulates their expression via MYB72. Collectively, the identification of iron-deficiency responsive genes as MYB72-targets indicates that rhizobacteria-mediated ISR utilizes components of the iron deficiency response for its establishment (see also Chapter 3).

A role of MYB72 in regulating plastid-related processes

The gene *AT5G5560* encodes an unknown protein. Its transcript abundance changes rapidly in response to iron deficiency. Despite its elusive role in iron homeostasis, bioinformatics analysis recently predicted that the AT5G55620 protein has a central role in metabolic processes that occur in plastids (Yang et al., 2010b), but how these plastid-related processes connect to iron deficiency responses remains elusive. *CYP71B5* encodes a putative enzyme of the cytochrome P450 monooxygenase superfamily, one of the largest protein families in higher plants. Members

of this family are involved in a plethora of biological processes, such as hormone synthesis and breakdown, as well as primary and secondary metabolic processes (Bak et al., 2011). CYP71B5 shows high homology with the CYP71B15, which is involved in the biosynthesis of camalexin, an indole-derived phytoalexin with antifungal activity (Nafisi et al., 2007). Together with CYP82C4 and CYP82C3, CYP71B5 constitute the only three P450 monooxygenases that are induced under iron deficient conditions through a FIT1-dependent pathway (Colangelo and Guerinot, 2004). In contrast to *CYP82C4*, *CYP71B5* does not to show any association with genes involved in metal uptake or transport (Murgia et al., 2011). Instead, its expression pattern significantly correlated with that of several genes coding for plastidial proteins, suggesting that it may be involved in similar metabolic processes as *AT5G55620*.

An emerging role of MYB72 in responses to cadmium tolerance

Our data also demonstrate that WCS417 stimulates *NRT1.8* expression in a MYB72-dependent manner. NRT1.8 is a member of the nitrate transporter family 1 (NRT1) of Arabidopsis (Dechorgnat et al., 2011). In addition to their role in nitrate transportation, nitrate transporters appear to have critical roles in signaling processes, including plant defense responses. For instance, the NRT1.1 (Remans et al., 2006b) and NRT2.1 (Remans et al., 2006a) transporters of Arabidopsis are involved in nitrate-sensing systems that enable the plant to detect and exploit nitrate-rich soil niches, whereas mutations in the *NRT2.1* gene appears to influence metabolomic and transcriptomic responses to *P. syringae* pv. *tomato*. NRT1.8 was recently identified as a proton-dependent low-affinity transporter specialized to unload nitrate from xylem vessels to xylem parenchyma cells (Li et al., 2010). Interestingly, its transcript abundance dramatically increases upon exposure to high concentration of cadmium (Cd), indicating that nitrate distribution mediated by NRT1.8 has an important role in Cd tolerance (Li et al., 2010). Considering that high concentration of Cd may trigger iron deficiency-related transcriptional responses in Arabidopsis (Van de Mortel et al., 2008), it is likely that the effect of Cd on the expression of *NRT1.8* stems from an iron deficiency response mounted locally in roots. This in turn implies an interconnection between iron homeostasis and nitrate distribution and further points to a potential role of MYB72 in this process as this transcription factor partially regulates *NRT1.8* expression.

BGLU42 has a prominent role in the establishment of ISR

Importantly, our study revealed that downstream of MYB72, BGLU42 functions as an essential component for the establishment of ISR. *BGLU42* encodes for a β-glucosidase of the glycoside hydrolase (GH) family 1 (Xu et al., 2004). Glycoside hydrolases hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. In plants, β-glucosidases play important roles in diverse aspects of plant physiology. Their role in chemical defense against insects is well documented. For instance, many plant defense compounds, collectively referred to as phytoanticipins, are often stored in a non-active glucosylated form and converted to bio-active molecules by the corresponding glucosidases (Morant et al., 2008). Well-studied phytoanticipins include the benzoxazinoid glucosides of non-Brassicaceous plants (Frey et al., 2009) and glucosinolates metabolites (Sønderby et al., 2010) of the order Brassicales. The latter can be hydrolyzed by endogenous β-thioglucoside glucohydrolases, called myrosinases, that cleave off the glucose group, liberating effector molecules with biocidal activity. Importantly, both glucosinolates and benzoxazinoid glucosides appear to have additional important roles as signaling molecules in MAMP-triggered immunity and defense responses against pathogenic microbes (Bednarek et al., 2009; Clay et al., 2009; Ahmad et al., 2011). Glucosidases are also involved in phytohormone activation by releasing hormonal molecules from their glucosides. For instance, the Arabidopsis BG1 (BGLU18) and BG2 (BGLU33) glucosidase homologs, have been shown to hydrolyze Glc-conjugated ABA (ABA-GE) towards ABA production during osmotic stresss (Lee et al.,

2006; Xu et al., 2012). Not surprisingly, β-glucosidases have additional important functions in cell wall metabolism either by releasing monolignols from their glucosides to promote lignification of secondary cell walls (Escamilla-Treviño et al., 2006), or degrading oligosaccharides during cell-wall turnover (Leah et al., 1995). A survey in the Arabidopsis transcriptome using the microarray database and analysis toolbox Genevestigator (https://www.genevestigator.com) reveals that *BGLU42* is specifically and strongly upregulated upon iron limited conditions and during seed germination, whereas its expression is severely compromised upon treatment with ABA. It is therefore tempting to speculate a role of BGLU42 in cell wall metabolic processes needed for cell expansion during germination. In this case, BGLU42 may direcly act on cell walls or may hydrolyse oligosaccharides released by cell wall β-glucans. The plant cell wall can play an active role in defense as a source of signals for the establishment of the systemic wound response (Narváez-Vásquez et al., 2005). Therefore BGLU42, by regulating cell wall-related processes might function in systemic defense.

MATERIALS AND METHODS

Plant material and growth conditions

For *in vitro* growth conditions, seeds were surface sterilized and sown on 1 × Hoagland agar-solidified medium at a density of 18 - 20 per plate. After 2 d of stratification at 4°C, the Petri dishes were transferred and positioned vertically in a growth chamber under a long day photoperiod (16 h of light, light intensity 100 µmol m^{-2} s^{-1}) at 22°C. For experiments performed in soil, seeds were sown in quartz sand and transferred when two-week-old in 60-mL pots containing sand/potting soil mixture (autoclaved twice for 20 min with 24-h interval). The following genotypes were used: wild types Col-0 and Col-5, and mutants *myb72-2* (Van der Ent et al., 2008), *opt3-2* (Stacey et al., 2008), and *frd3-1* (Rogers and Guerinot, 2002). Knock-out mutant *bglu42* (SALK-034026) carrying a T-DNA insertions in the 12th gene-exon was obtained from the SALK collection (Alonso et al., 2003) and validated by PCR analysis on genomic DNA using a primer located in the T-DNA left border (LBA1) and *BGLU42* gene-specific primers (data not shown). The following transgenic lines, overexpressing the indicated genes, were used: *35S::Flag-MYB72* (oxMYB72) in the *myb72-2* background, and *35S::YFP-MYB72*, *35S::YFP-BGLU42*, *35S::YFP-AT5G55620*, *35S::YFP-CYP71B5*, *35S::bHLH38-GUS* (Yuan et al., 2008), *35S::bHLH39-GUS* (Yuan et al., 2008), and *35S::NRT1.8* (L1 and L18) (Li et al., 2010) in the Col-0 background. The following reporter lines were used: *pMYB72::GFP-GUS* and *pBGLU42::GFP-GUS,* both in the Col-0 background.

Cloning procedures and generation of transgenic lines

The approximate 2-kb genomic region upstream the start codon of *MYB72* and *BGLU42* was amplified from genomic Col-0 DNA, captured into the pDONR221-pGEMT-Easy vector using the BP reaction and recombined into the destination pBGWFS7.0 vector using the LR reaction (Invitrogen). The cDNAs of *MYB72*, *BGLU42*, *AT5G55620*, and *CYP71B5* were amplified from wild-type Col-0 cDNA and captured into the pDONR221-pGEMT-Easy vector. The corresponding cDNAs were then recombined either into the pEarleyGate202 (35S-FLAG tag-Gateway-OCS-3'), or pEarleyGate104 (35S-YFP-Gateway-OCS-3') plasmids (Earley et al., 2006). For generation of transgenic lines, recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90. *A. tumefaciens*-mediated plant transformation was performed using the floral dip method (Clough and Bent, 1998) in Col-0 plants except the 35S::FLAG-MYB72 construct which was transformed in the *myb72-2* genetic background.

Induction treatments

Pseudomonas fluorescens WCS417 was cultured on King's medium B (KB) agar plates supplemented with 50 µg mL^{-1} rifampicin at 28°C. After 24 h growth, cells were collected in 10 mM $MgSO_4$, washed

twice by centrifugation for 5 min at 5.000 × *g*, and finally resuspended in 10 mM $MgSO_4$. For in vitro assays, the bacterial titer was adjusted to the OD_{600} of 0.002 (10^6 cfu mL^{-1}); 10 µl of bacterial suspension was then applied on each root of 10-day-old seedlings, right below the hypocotyl. Induction of ISR with WCS417 was performed by mixing ISR-inducing rhizobacteria through the soil as described (Pieterse et al., 1996).

Pathogen cultivation an bioassays

Pseudomonas syringae pv. *tomato* DC3000 was cultured on King's medium B (KB) agar plates supplemented with 50 µg mL^{-1} rifampicin at 28°C. After 24 h growth, cells were collected in 10 mM $MgSO_4$, washed twice by centrifugation for 5 min at 5.000 × *g*, and finally resuspended in 10 mM $MgSO_4$. Plants were pathogen inoculated when 5-week-old by spraying leaves until run-off with a solution of 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 containing 10^8 cfu mL^{-1} *P. syringae* pv. *tomato* DC3000 bacteria. Four days after inoculation, disease severity was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. The disease index was calculated by determining the proportion of leaves with disease symptoms per plant (*n* = 24) as described (Van Wees et al., 1997). *Botrytis cinerea* strain B0510 was cultivated on half-strength PDA plates for 10 d at 22°C. *B. cinerea* spores were collected and resuspended in half-strength potato dextrose broth to a final density of 5 $x10^5$ spores ml^{-1}. Five-week old plants were infected by applying 5 µl drops per leaf. Symptoms were scored the 4th day after infection with *B. cinerea*. Disease ratings were expressed as the percentage of leaves showing spreading lesions as described (Van der Ent et al., 2008). For *Hyaloperonospora arabidopsidis* bioassays, three-week-old Arabidopsis seedlings were misted with a *H. arabidopsidis* spore suspension containing 5 × 10^4 sporangiospores mL^{-1}. Disease symptoms were scored at 10 d after inoculation. Disease ratings were expressed as severity of disease symptoms and pathogen sporulation on each leaf: I, no sporulation; II, trailing necrosis; III, <50% of the leaf area covered by sporangia; IV, >50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse as described (Van der Ent et al., 2008).

Microarray and data analysis

RNA samples for microarray analysis were collected 2 d after the induction with WCS417. RNA purity and integrity were confirmed by using a RNA 6000 Nano Assay (Agilent, http://www.home.agilent.com) and gel electrophoresis. cRNA labeling, hybridization, washing and scanning of Affymetrix ATH1 GeneChips® (Affymetrix, http://affymetrix.com) was performed according to Affymetrix OneCycle Lab protocols. Data were analyzed statistically using the R language environment for statistical computing (http://www.r-project.org) version 2.11.1 and Bioconductor release 2.6 (Gentleman et al., 2004). Data were normalized using the Robust Multichip Average (RMA) expression measure in the Affy package (Gautier et al., 2004). Differentially expressed genes were identified using the LIMMA package (Smyth, 2004). The obtained *p*-values were corrected for multiple testing errors using the BH procedure (Benjamini and Hochberg, 1995), yielding q-values. Lists of q-values were transferred to Microsoft Excel™ and sorted. The probe set sequences were aligned to the TAIR9 gene model database of transcripts (www.arabidopsis.org). For gene annotations into biological categories, the AmiGO Term Enrichment software was used (Carbon et al., 2009)

Fluorescence microscopy

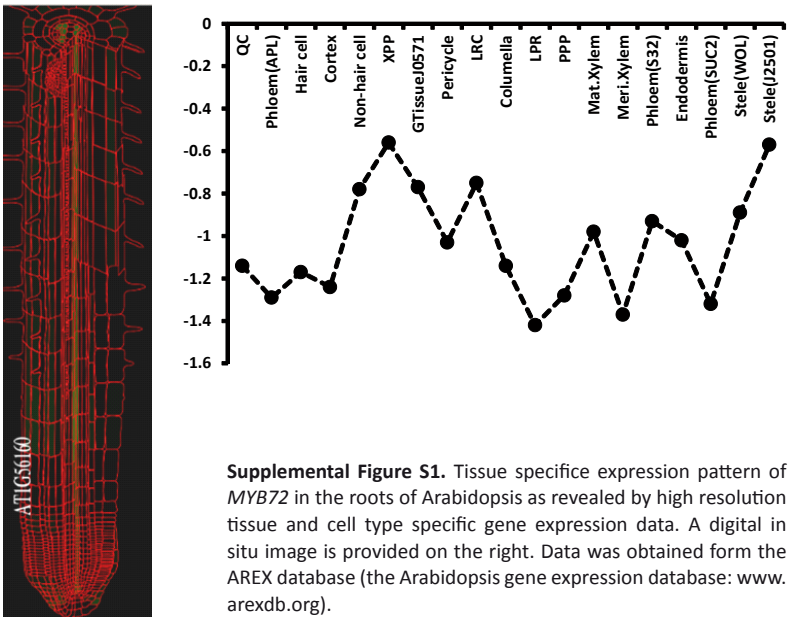
Confocal laser-scanning microscopy on the *pMYB72::GFP-GUS* and *pBGLU42::GFP-GUS* reporter lines was performed in a Leica SP2 inverted microscope. As counterstain, roots were stained in 10 µg mL^{-1} propidium iodide (PI) solution for 2 min. Chromophores were excited using the 488 nm Argon laser and fluorescence was detected at 500-550 nm (for GFP), 550-615 nm (for YFP) and 570-620nm (for PI).

Q-PCR analysis

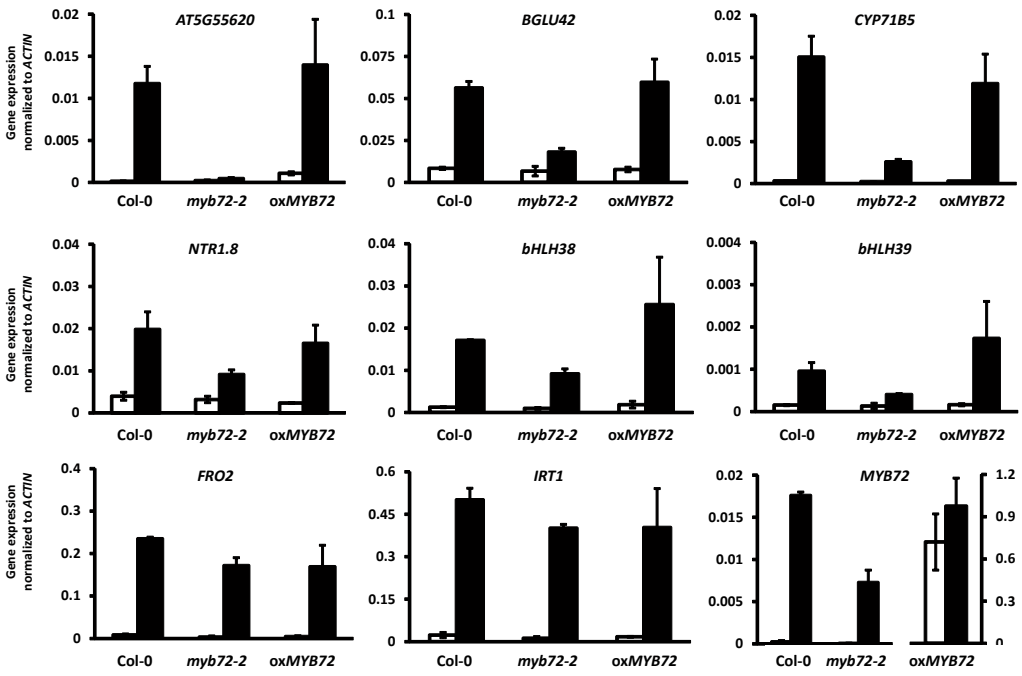
Total RNA was extracted using the RNeasy kit and treated with the Ambion® TURBO™ DNase. cDNA was synthesized with the SuperScript-III reverse transcriptase (Invitrogen). Cycle tresholds were determined in duplicate per transcript in three biological replicas per sample using the ABI PRISM 7700 sequence detection system (Applied Biosystems) and SYBR Green I as reporter dye. The data was normalized using *Actin7*. Q-PCR and data analysis was performed as described (Van der Ent et al., 2009b). The following gene-specific primers were used in this study:

MYB72_F	ACGAGATCAAAAACGTGTGGAAC
MYB72_R	TCATGATCTGCTTTTGTGCTTTG
NRT1.8_F	GGCTTCAGATTCTTGGATAG
NRT1.8_R	AACCACAGAGTAGAGGATGG
CYP71B5_F	GACGAGAGCCAATTGTTGGT
CYP71B5_R	GAGGTTGGTCTCCGATTCAA
BGLU42_F	ATGGCCTGGGAACTGAAGTC
BGLU42_R	ATTTGTCCAACCTCCGATTG
AT5G55620_F	TCCCAAGTGGATTCCAAGTC
AT5G55620_R	AGCTGATCAGGCCAGAGAAA
bHLH38_F	GACGGTACCACAGACTTATGAAGT
bHLH38_R	TAAGCTCTTTGAAACCGTTTCAGGA
bHLH39_F	GACTTATGGAGCTGTTACAGCGGT
bHLH39_R	CTTCAAGCTTCGAGAAACCGTCGCA
IRT1_F	ACCCGTGCGTCAACAAAGCTAAAG
IRT1_R	TCCCGGAGGCGAAACACTTAATGA
FRO2_ F	TGTGGCTCTTCTTCTCTGGTGCTT
FRO2 _R	TGCCACAAAGATTCGTCATGTGCG

SUPPLEMENTAL DATA



Supplemental Figure S1. Tissue specifice expression pattern of *MYB72* in the roots of Arabidopsis as revealed by high resolution tissue and cell type specific gene expression data. A digital in situ image is provided on the right. Data was obtained form the AREX database (the Arabidopsis gene expression database: www.arexdb.org).



Supplemental Figure S2. Validation of microarray analysis. Quantitative PCR analysis of *AT5G55620*, *BLU42*, *CYP71B5*, *NRT1.8*, *bHLH38*, *bHLH38, FRO2*, *IRT1* and *MYB72* transcript levels in mock- (white) and WCS417-treated (black) roots. Roots of the indicated genotypes were treated with either $MgSO_4$ or WCS417 bacteria and samples were collected 2 dpt. Error bars represent the SE of three biological repetitions.

Supplemental Table S1. WCS417-responsive genes that are de-regulated in the *myb72-2* mutant background ($p < 0.05$) as revealed by microarray analysis of Arabidopsis roots.

Gene Annotation	Fold Change [WCS417 vs mock] Col-0	*myb72-2*	35S::MYB72
UP-regulated			
AT5G26270	6.41	5.59	8.32
CYP71B5	4.55	1.11	3.40
BGLU42	4.51	1.58	5.09
BHLH039	3.52	1.73	3.54
NTR1.8	3.47	1.86	3.47
AT5G55620	3.47	1.00	3.04
protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2.90	1.80	0.97
PHT3 (PHOSPHATE TRANSPORTER 3)	2.06	1.25	1.38
pEARLI 1; lipid binding	1.73	1.21	2.33
unknown protein	1.56	1.21	1.58
EDA36 (EMBRYO SAC DEVELOPMENT ARREST 37)	1.44	1.65	1.04
caffeoyl-CoA 3-O-methyltransferase, putative	1.33	1.23	0.85
PDR9 (PLEIOTROPIC DRUG RESISTANCE 9)	1.23	0.76	1.30
short-chain dehydrogenase/reductase (SDR) family protein	1.23	1.14	1.43
PR-1-LIKE (PATHOGENESIS-RELATED PROTEIN-1-LIKE)	1.18	0.79	2.25
TPLATE; binding	1.11	1.14	1.20
mitochondrial processing peptidase beta subunit, putative	1.07	1.09	1.09
OTU-like cysteine protease family protein	1.05	1.02	0.97
DOWN-regulated			
acid phosphatase class B family protein	0.95	0.88	1.52
QQS (QUA-QUINE STARCH)	0.83	1.11	0.84
MD-2-related lipid recognition domain-containing protein	0.78	0.66	0.70
unknown protein	0.65	0.45	0.73
Bet v I allergen family protein	0.56	0.41	0.97
SULTR4;2; sulfate transmembrane transporter	0.54	0.39	0.70
PSAO (photosystem I subunit O)	0.39	0.31	0.53

CHAPTER 5

Combinatorial and overlapping functions of MYB72 and MYB10 transcription factors in the Arabidopsis root

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ABSTRACT

Selected strains of root-colonizing bacteria and fungi are known to trigger a systemic immune response in diverse plant species, called induced systemic resistance (ISR). In the model plant Arabidopsis *thaliana*, ISR triggered by the plant-growth promoting microbes *Pseudomonas fluorescens* WCS417 and *Trichoderma asperellum* strain T34 depends on the root-specific transcription factor MYB72. Previously, microarray analysis identified the WCS417-responsive genes *BGLU42*, *CYP71B5*, *NRT1.8*, *AT5G55620* and *bHLH39* as MYB72-target genes. Consistent with the fact that overexpression of MYB72 does not lead to constitutive disease resistance, none of the MYB72-target genes was found to be constitutively upregulated in roots of plants overexpressing *MYB72*. This suggests that MYB72 may function synergistically with other cellular components for the establishment of ISR. Here, we demonstrate that MYB72 interacts with the closely-related MYB10 transcription factor, and co-operatively trigger the expression of the *AT5G55620* gene. In addition we demonstrate that MYB72 and MYB10 may function redundantly in regulating the expression of genes involved in the shikimate pathway, the general phenylpropanoid pathway, and the lignin biosynthetic pathway. We further demonstrate that overexpression of MYB72 or MYB10 suppresses the expression of a large group of defense-related genes upon root colonization by WCS417 bacteria, suggesting that these transcription factors may be targets of bacterial effectors that function during the interaction in order to suppress innate immune responses and enable bacteria to establish long-term associations with host roots.

INTRODUCTION

Plants have a sophisticated multicomponent system to protect themselves against pathogens. In general, both constitutively expressed barriers and induced responses contribute to the host's overall defense. Induced defense responses rely on the recognition of microbe-associated molecular patterns (MAMPs), which are general elicitor molecules that are conserved amongst different classes of pathogenic microbes (Boller and Felix, 2009; Nürnberger and Kemmerling, 2009). Upon receptor-mediated perception of MAMPs (Zipfel, 2008), a signaling network is recruited that is fine-tuned by small-molecule hormones (Pieterse et al., 2012), and rapidly activates the appropriate defense responses. These plant defense responses are commonly manifested as extracellular medium alkalization, reactive oxygen species (ROS) production, cell wall reinforcement via callose deposition, and production of antimicrobial compounds (collectively referred to as phytoalexins) (Hammond-Kosack and Jones, 1996; Zipfel, 2009). On the other hand, successful pathogens have evolved to suppress plant innate immunity by means of delaying or reducing the intensity of defense-related gene expression and cellular immune responses. To date, it is well established that the mechanisms by which pathogenic bacteria, fungi and oomycetes establish pathogenicity is based on the production and secretion of effector molecules that either as apoplastic or cytoplasmic determinants interfere with the host's defense machinery (He et al., 2007; Stassen and Van den Ackerveken, 2011; Thomma et al., 2011; Bozkurt et al., 2012).

Roots are also equipped with an immune system to cope with microbial infections in the soil environments (Millet et al., 2010). Like foliar pathogens, root pathogenic bacteria and fungi employ effector molecules to manipulate host immunity (Mukaihara and Tamura, 2009; Takken and Rep, 2010; Klosterman et al., 2011). Evidence is emerging that beneficial soil-borne microbes have evolved decoy strategies to short-circuit immune responses triggered locally in roots upon initial recognition, which paves the way for prolonged mutualistic associations (Zamioudis and Pieterse, 2012). Recent work in the field of mycorrhizal biology revealed that effectors secreted during

interaction with host roots are essential for symbiosis (Kloppholz et al., 2011; Plett et al., 2011). In addition, the beneficial fungus *Piriformospora indica* has been shown to suppress host immune responses by utilizing the jasmonic acid signaling pathway, pointing to the existence of effector molecules that possess immune-suppressive functions (Jacobs et al., 2011). Similarly, the beneficial rhizobacterium *Pseudomonas fluorescens* WCS417 suppresses the activation of MAMP-responsive promoters, as well as callose deposition locally in roots of Arabidopsis *thaliana* (Arabidopsis), in a manner that does not require the type III secretion machinery that is commonly utilized by pathogenic bacteria to deliver effectors in the cytoplasm of host cells (Millet et al., 2010).

While suppressing local immune responses in the roots, interactions with soil-resident beneficial bacteria and fungi often results in the onset of a systemic immune response in the aerial parts of host plants, called induced systemic resistance (ISR) (Van Loon et al., 1998; Van Wees et al., 2008; De Vleesschauwer and Höfte, 2009). Induction of ISR in Arabidopsis by the root-colonizing bacterium *P. fluorescens* WCS417 is well-studied. WCS417-mediated ISR is effective against a broad range of pathogens and is based on a potentiated activation of host defenses upon pathogenic attack (Van Wees et al., 1999; Verhagen et al., 2004; Pozo et al., 2008). This phenomenon is known as priming (Conrath et al., 2006), it depends on intact jasmonic acid and ethylene signaling pathways, and further requires the transcriptional co-activator NPR1 (Pieterse et al., 1998). In roots, initiation of WCS417-ISR is regulated by the root-specific transcription factor (TF) MYB72 (Van der Ent et al., 2008), a member of the R2R3 family of MYB TFs, and components of the ethylene signaling pathway (Knoester et al., 1999) that locally act in the generation or translocation of a thus-far-unidentified systemic signal.

In Chapter 4 we identified WCS417-responsive genes that depend on MYB72 for their induction and we further identified BGLU42 as an essential component of WCS417-mediated ISR. Consistent with previous findings that *MYB72* overexpression does not result in constitutive disease resistance, gene expression analysis did not reveal constitutive upregulation of MYB72-target genes (including *BGLU42*) in the 35S::*MYB72* overexpressing line (Chapter 4). This points to synergistic interactions between MYB72 and other components in the induction of those genes. Based on this, we initiated an effort to identify MYB72-interacting partners. Here, we report on the identification of the MYB10 TF as MYB72-interacting partner. We further demonstrate that MYB72, redundantly with MYB10, promotes the expression of genes involved in the shikimate and the general phenylpropanoid pathway, while it attenuates the expression of defense-related genes upon root colonization by WCS417.

RESULTS

Search for MYB72-interacting partners

Combinatorial interactions among TFs are critical to induce and specify gene expression in developmental- and stress-related transcriptional programs. Members of the R2R3 family of MYB TFs are commonly involved in such interactions and often found as part of large transcriptional complexes (Yanhui et al., 2006). In order to identify MYB72-interacting partners, we first tested putative interactions between MYB72 and TFs involved in the ethylene signaling pathway. Ethylene signaling is required for the initiation of ISR locally in roots (Knoester et al., 1999). Previously, yeast two-hybrid experiments revealed that MYB72 physically interacts with EIL3 (SLIM1) (Van der Ent et al., 2008), a member of the EIN3 family of TFs that play important roles in ethylene signal transduction (Stepanova and Ecker, 2000; Tieman et al., 2001; Guo and Ecker, 2004). In order to test whether MYB72 and EIL3 interact *in vivo* as well, we cloned the *MYB72* and *EIL3* cDNAs in frame with the N- and C-terminal fragment of the YFP protein, respectively, and the corresponding constructs were co-transfected to Arabidopsis mesophyll protoplasts. Subsequently, we employed Bimolecular Fluorescence Complementation (BiFC) to visualize protein-protein

interactions *in vivo*. A summary of the interactions tested in this study is provided Figure 1A. Consistent with the reported interaction between SHRSCARECROW (SCR) and SHOORT-ROOT (SHR) (Cui et al., 2007; Welch et al., 2007), fluorescence accumulated in nuclei of co-transfected-cells (Fig. 1B); however, we could not observe reconstitution of the YFP fluorophore in the nucleus of cells co-transfected with MYB72 and EIL3, indicating that MYB72 and EIL3 did not interact *in vivo* (Fig. 1B). We could also not obtain evidence for interactions between MYB72 and two other members of the EIN3 family of TFs: EIN3 and EIL1 (data not shown). Hence, we conclude that the previously identified interaction between MYB72 and EIL3 is not functional *in vivo.*

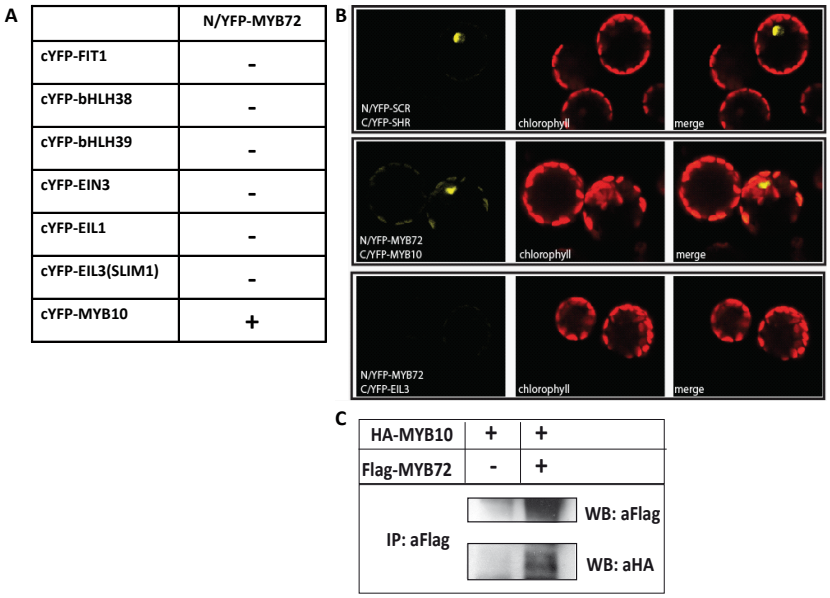


Figure 1. (A) Summary of the interactions tested between MYB72 and TFs involved in the ethylene signaling pathway (EIN3, EIL1, EIL3) and the iron deficiency response (FIT1, bHLH38, bHLH39, MYB10) by employing BiFC assays in Arabidopsis mesophyll protoplasts. Negative interactions are indicated with (–) and positive ones with (+). **(B)** MYB72 interacts with MYB10. BiFC assay illustrating interaction between the TFs MYB72 and MYB10 (middle panel). Protoplasts were co-transfected with the indicated constructs and YFP fluorescence was detected 18 h post transfection by confocal laser-scanning microscopy. The interaction between the root-specific TFs SCARECROW (SCR) and SHOORTROOT (SHR) served as positive control (top panel). MYB72 and EIL3 do not interact in the BiFC assay (bottom panel). **(C)** Co-IP analysis of MYB72 and MYB10 in Arabidopsis mesophyll protoplasts. Co-IP was performed with protoplasts co-expressing FLAG-MYB72 and HA-MYB10. The Co-IP assay was carried out with an anti-FLAG antibody (IP: α-FLAG), and proteins were analyzed by Western blot using an anti-HA antibody (WB: α-HA).

Secondly, we tested putative *in vivo* interactions between MYB72 and the basic helix-loop-helix (bHLH) leucine zipper TFs bHLH29 (FIT1), bHLH38 or bHLH39. These three bHLH TFs are central transcriptional regulators of the iron deficiency response (Colangelo and Guerinot, 2004; Yuan et al., 2008), and previously shown to regulate the expression of *MYB72* in response to the ISR-inducing rhizobacteria (Chapter 3). Again, in contrast to the positive interaction between SCR and SHR, we were not able to demonstrate interaction of MYB72 with either of these bHLH TF proteins (data not shown). We thus conclude that it is unlikely that MYB72 interacts with these bHLH TFs *in vivo.*

MYB TFs have been reported to form heterodimers with each other, as in the case of CCA1 and LHY, two single MYB domain (MYBR1) proteins that synergistically function as transcriptional

regulators of the Arabidopsis circadian clock (Lu et al., 2009). *MYB10* encodes the closest homologue of MYB72 (Yanhui et al., 2006; Van der Ent et al., 2008). Like *MYB72, MYB10* was originally identified as an iron-deficiency induced gene regulated by the bHLH TF FIT1 (Colangelo and Guerinot, 2004), and was also identified as a WCS417-induced gene (microarray data, Chapter 4). Confocal imaging of *pMYB10::GFP-GUS* roots showed that, like *MYB72, MYB10* is expressed in the vascular tissue of uninduced roots, whereas it is strongly induced in root trichoblasts upon colonization of the roots by WCS417 (Fig. 2). We therefore reasoned that it is likely that MYB10 possesses functions similar to MYB72 or has synergistic effects on MYB72, as reported for the CCA1 and LHY1 MYB TFs (Lu et al., 2009). We first tested the localization pattern of MYB10 in Arabidopsis mesophyll protoplasts. As expected for its role as a putative TF, MYB10 localizes in the nucleus of plant cells (data not shown). Subsequently, the interaction between MYB72 and MYB10 was assessed by BiFC. As shown in Figure 1B, YFP fluorescence was detected in the nuclei of co-transfected protoplasts, indicating

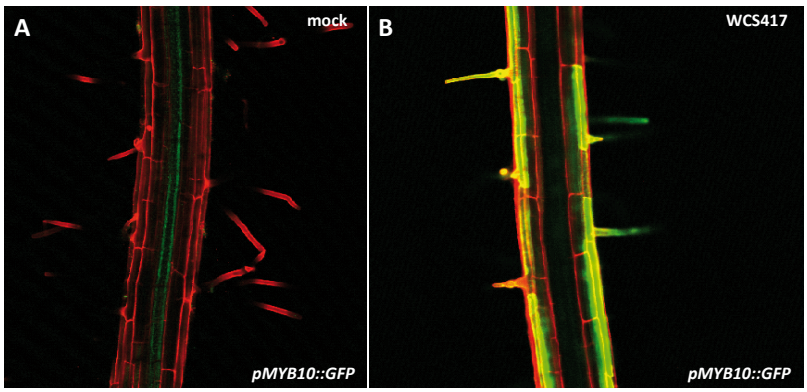


Figure 2. Tissue-specific expression of *MYB10* in Arabidopsis roots. Longitudinal sections of roots of *pMYB10::GFP-GUS* expressing seedlings that were either mock-treated (**A**) or treated with WCS417 bacteria (**B**).

that MYB72 and MYB10 physically interact *in vivo*. To further verify the interaction between MYB72 and MYB10, we performed co-immunoprecipitation assays in Arabidopsis protoplasts. To this end, *35S::FLAG-MYB72* and *35S::HA-MYB10* constructs were co-transfected into Arabidopsis protoplasts. We subsequently pulled-down MYB72-containing protein complexes in which we could detect MYB10 protein (Fig. 1C, lane 2). Importantly, MYB10 was specifically precipitated with MYB72 and the positive signal could not be attributed to background binding (Fig. 1C, lane 1). Together, these data indicate MYB10 as MYB72-interacting partner.

Previously, *BGLU42*, *CYP71B5*, *NRT1.8*, *AT5G55620* and *bHLH39* were identified as WCS417-induced genes, regulated in the roots upon WCS417 bacterization in a MYB72-dependent manner (Chapter 4). However, overexpression of *MYB72* in the corresponding *oxMYB72* line did not result in the constitutive upregulation of these MYB72-targets (Chapter 4). In order to assess the impact of the MYB72-MYB10 interaction on the expression of these genes, we generated transgenic plants overexpressing *MYB10* (*oxMYB10*) and transgenic lines overexpressing both *MYB72* and *MYB10* (*oxMYB72/oxMYB10*), and examined the expression of the MYB72-target genes in these genetic backgrounds. Gene expression analysis revealed that amongst the MYB72 target genes, specifically the expression of *AT5G55620* was upregulated in the double *oxMYB72/oxMYB10* overexpressor (Fig. 3).

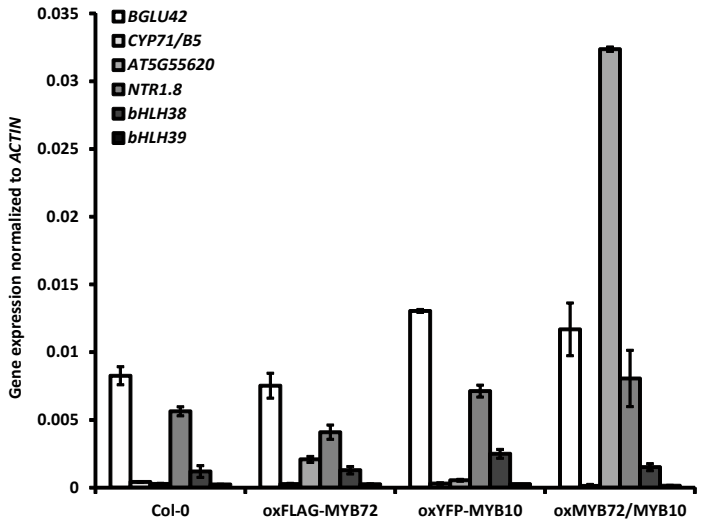


Figure 3. Expression of MYB72-target genes in *MYB72* and *MYB10* single and double overexpressors. Quantitative RT-PCR analysis of the indicated MYB72-target genes in roots of wild type Col-0 and the indicated overexpressors.

MYB72 and MYB10 redundantly activate genes involved in the shikimate, the general phenylpropanoid and the lignin biosynthetic pathway

Previously, we employed microarray experiments to identify downstream MYB72-targets with potential roles in rhizobacteria-mediated ISR, by comparing WCS417-induced transcriptional responses in wild type and *myb72-2* roots. In this analysis we also included oxMYB72 plants, in which we could identify two WCS417-responsive gene-sets that were differentially expressed compared to wild type (Chapter 4). In particular we identified a cluster of 37 WCS417-responsive genes that were also upregulated in roots of oxMYB72 plants under uninduced conditions compared to Col-0 (Chapter 4; Figure 2, green and purple color clusters) and a large cluster of 120 WCS417-responsive genes that were induced in roots of both wild-type and mutant seedlings, but showed compromised expression in the roots of oxMYB72 plants (Chapter 4; Figure 2, blue color cluster). This suggests that MYB72 regulates a more broad transcriptional program in roots of plants expressing ISR, and further suggests that other factors redundantly function with MYB72 in regulating the expression of those two gene clusters.

To identify genes differentially expressed under basal conditions in the oxMYB72 line, we analyzed gene expression data of the mock-treated plants from our microarray experiments (Chapter 4). By setting as selection criteria more than 2-fold regulation and p value < 0.05 we identified 195 genes that showed constitutive expression, and 63 genes that were repressed in the roots of mock-treated oxMYB72 in comparison to the roots of mock-treated Col-0 (data not shown). Amongst the genes constitutively upregulated in the roots of oxMYB72 under basal conditions many genes encode proteins involved in the shikimate pathway, the general phenylpropanoid pathway, and the lignin biosynthesis pathway (Fig. 4). Several genes encode for proteins involved in the biosynthesis of chorismate, the immediate precursor of the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp). In particular, we found genes encoding biosynthetic enzymes that are specifically involved in the conversion steps of chorismate towards Phe, but not Tyr or Trp, to show constitutive upregulation in roots of seedlings overexpressing *MYB72* (Fig. 4). Moreover, genes that encode enzymes catalyzing essential steps of the phenylpropanoid metabolism, i.e. phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-Coumarate:CoA ligase (4CL), that feed metabolic precursors to all subsequent branches of the phenylpropanoid pathway, were also upregulated (Fig. 4). This suggests that MYB72 specifically orchestrates transcriptional programs related to the phenylpropanoid pathway. Importantly, downstream of the coumaroyl-

coenzyme A (the end product of the general phenylpropanoid pathway), MYB72 appears to activate the expression of genes involved in lignin biosynthesis (CoA O-methyltransferase (CCOMT), 4-coumarate:CoA ligase (4CL), cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD)) (Fig. 4), pointing to a potential role of MYB72 in the regulation of cell-wall reinforcement.

To test whether MYB10 is similarly capable of inducing the expression of genes in the shikimate pathway, the general phenylpropanoid pathway and the lignin biosynthetic pathway, we employed quantitative RT-PCR analysis to assess steady-state transcript levels in the oxMYB10 line. Indeed, we found that *MYB10* overexpression activates the transcription of these genes to comparable levels, as does overexpression of *MYB72* (Fig. 4). These results collectively suggest that MYB72 and MYB10 have redundant roles in the induction of lignin biosynthesis genes in the roots of Arabidopsis.

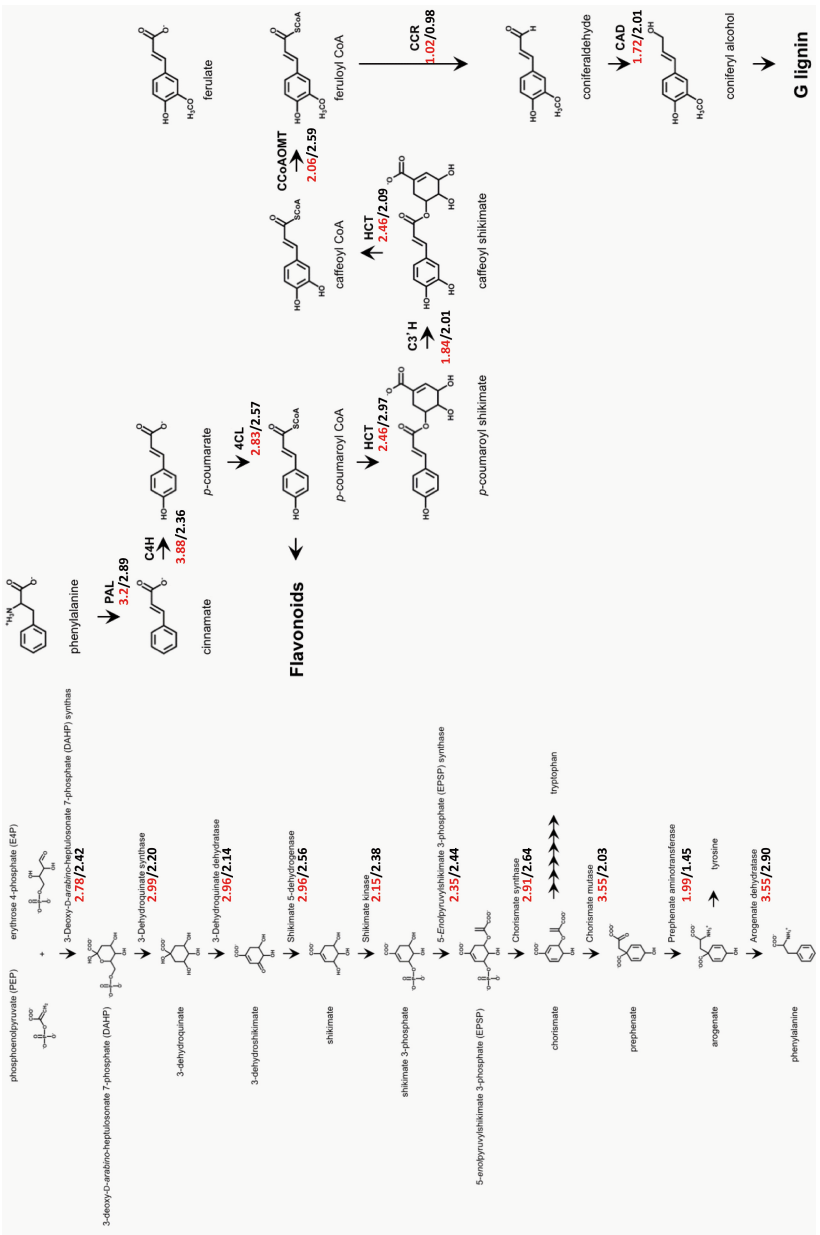
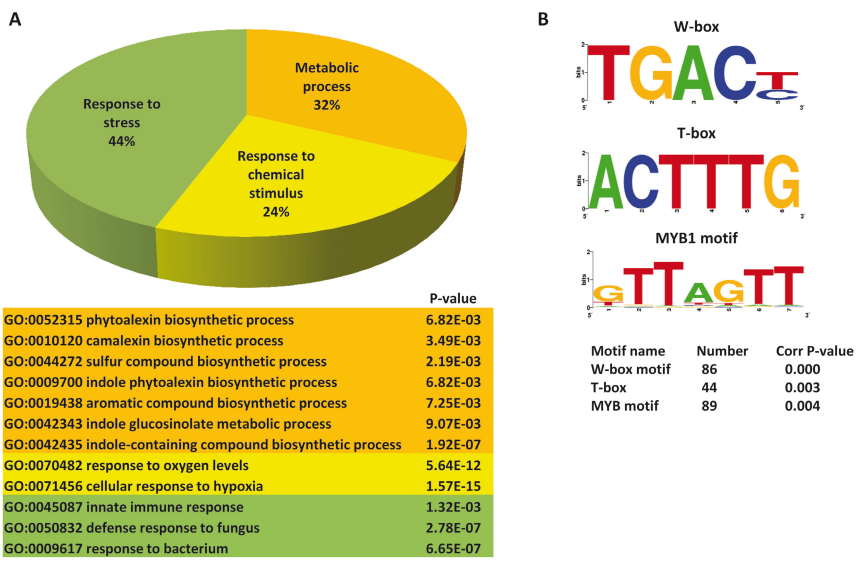


Figure 4. Coordinated transcriptional induction of genes encoding key enzymes of the shikimate, the phenylpropanoid and the lignin biosynthetic pathway by ectopic overexpression of *MYB72* or *MYB10*. Schematic representation of the shikimate pathway (left), the general phenylpropanoid pathway (middle) and the lignin biosynthetic pathway (right). Depicted are the major intermediates and enzymes. Numbers below the key enzymes indicate the average fold change induction (LOG_2 values) in gene expression compared to wild-type Col-0, in the roots of seedlings overexpressing *MYB72* (red) or *MYB10* (black). Expression levels were determined by quantitative RT-PCR.

Overexpression of *MYB72* and *MYB10* suppresses the expression of defense-related genes in roots

As mentioned above, the microarray analysis identified a second cluster of 120 genes (Chapter 4, Figure 2, blue cluster) that are induced in roots of both wild-type and *myb72* mutant seedlings upon root colonization by WCS417, but display compromised expression in the oxMYB72 roots. Classification of these 120 MYB72-suppressed genes into biological categories using the AmiGO Term Enrichment software (Carbon et al., 2009), revealed overrepresentation of genes encoding components of plant defense and innate immunity, especially processes related to indole glucosinolate metabolism (Fig. 5A) (Halkier and Gershenzon, 2006; Muller, 2009; Sønderby et al., 2010). In particular, overexpression of *MYB72* appears to suppress the expression of genes encoding defense-associated transcription factors, such as MYB15 (Ding et al., 2009), which is involved in the regulation of abscisic acid biosynthesis and signaling, MYB51 and its close homologue MYB122 (Gigolashvili et al., 2007), which are involved in glucosinolate metabolism, and the AP2/ERF-type TF ORA59 that positively regulates JA-mediated defense responses (Pré et al., 2008). Other genes encode disease resistance proteins, enzymes involved in camalexin biosynthesis, such as the PAD3 (Schuhegger et al., 2006), CYP71A12 (Nafisi et al., 2007; Millet et al., 2010) and CYP71A13 (Nafisi et al., 2007), and enzymes with antimicrobial activity, including hydrolases, cysteine proteases,



	P-value
GO:0052315 phytoalexin biosynthetic process	6.82E-03
GO:0010120 camalexin biosynthetic process	3.49E-03
GO:0044272 sulfur compound biosynthetic process	2.19E-03
GO:0009700 indole phytoalexin biosynthetic process	6.82E-03
GO:0019438 aromatic compound biosynthetic process	7.25E-03
GO:0042343 indole glucosinolate metabolic process	9.07E-03
GO:0042435 indole-containing compound biosynthetic process	1.92E-07
GO:0070482 response to oxygen levels	5.64E-12
GO:0071456 cellular response to hypoxia	1.57E-15
GO:0045087 innate immune response	1.32E-03
GO:0050832 defense response to fungus	2.78E-07
GO:0009617 response to bacterium	6.65E-07

Motif name	Number	Corr P-value
W-box motif	86	0.000
T-box	44	0.003
MYB motif	89	0.004

Figure 5. Overexpression of *MYB72* compromises the induction of defense-related genes in the roots of Arabidopsis. (A) Annotation of 120 WCS417-induced genes that showed compomised expression in oxMYB72 into biological GO term categories using the AmiGO Term Enrichment software. Each caterogy is shown in a different color in the pie chart with a corresponding color-coded list below, specifying the overrepresented cellular responses (p value < 0.05). **(B)** Overrepresentation of TF-binding motifs in the 1-kb promoter region of genes used in A.

proteases inhibitors and peroxidases. Bioinformatics analysis of the 1-kb promoter region of the MYB72-suppressed genes revealed overrepresentation of T-box (ACTTTG) and WRKY-binding (TGACY) motifs, the latter commonly found in the promoters of defense-associated genes (Fig. 5B). Also the MYB1-binding motif (GTTAGT) was found to be overrepresented, suggesting that MYB72 may directly bind to the promoter elements of defense genes to attenuate their expression (Fig. 5B).

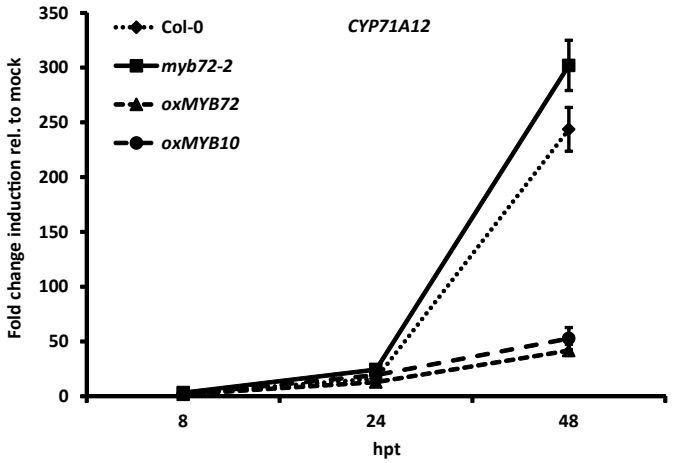


Figure 6. Overexpression of *MYB72* or *MYB10* results in the suppression of defense-related *CYP71A12* gene expression. Quantitative RT-PCR analysis of *CYP71A12* transcript levels in roots of Col-0, *myb72-2*, oxMYB72 and oxMYB10 seedlings, in response to treatment with WCS417 bacteria. Samples were collected at the indicated h post treatment (hpt). Data represents the mean of fold change induction of three biological replicas

In order to test whether MYB10 acts redundantly with MYB72 to negatively regulate the expression of the MYB72-suppressed defense-related genes, we monitored the expression of the MAMP-related marker *CYP71A12* (Millet et al., 2010) in the roots of Col-0, *myb72-2,* oxMYB72, and oxMYB10 plants upon colonization by WCS417. No differences could be detected in the amplitude by which *CYP71A12* was expressed in the roots of Col-0 and *myb72-2*, whereas in the roots of

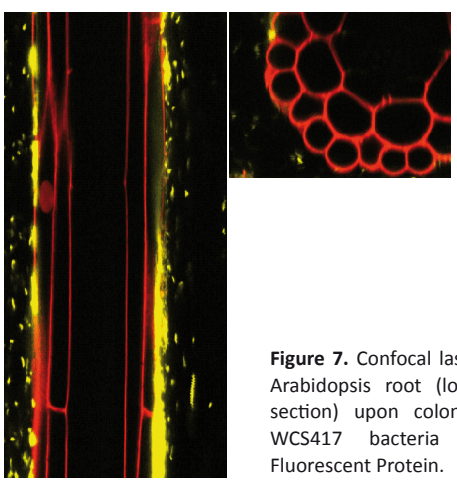


Figure 7. Confocal laser scanning microscopy of Arabidopsis root (longitudinal and transverse section) upon colonization by *P. fluorescens* WCS417 bacteria expressing the Yellow Fluorescent Protein.

oxMYB72 and oxMYB10 the expression of the *CYP71A12* markers was significantly compromised upon WCS417 application (Fig. 6). These data collectively suggest that MYB72 and MYB10 exert redundant functions upon root colonization by WCS417 to mitigate plant defense responses mounted locally in the roots. In line with these data, we found WCS417 bacteria to mainly colonize niches located on both sides of root trichoblasts, where MYB72 and MYB10 are predominantly expressed (Fig. 7).

DISCUSSION

In this study we identified MYB10 as an important MYB72-interacting partner. To our knowledge, this is the first study that reports on functional interactions between members of the R2R3 family of MYB TFs. We were further able to demonstrate that the interaction between MYB72 and MYB10 is sufficient for the upregulation of the *AT5G55620* gene, previously identified to be induced in a MYB72-dependent manner in WCS417-bacterized roots. However, the role of *AT5G55620* in rhizobacteria-mediated ISR remains elusive. Bioinformatics analysis recently revealed that the AT5G55620 protein may have central roles in metabolic processes occuring in plastids upon iron-limited conditions (Yang et al., 2010b). In addition to this synergistic interaction, we were further able to assign redundant functions of MYB72 and MYB10 in the upregulation of genes involved in the shikimate pathway and the general phenylpropanoid pathway, as well as in the downregulation of defense-related genes locally in roots upon colonization by WCS417.

Phenylpropanoids encompass an important class of secondary metabolites, wide-spread throughout the plant kingdom, with essential roles in a plethora of biological processes, from structural support to biotic and abiotic stress tolerance (Dixon and Paiva, 1995). They derive from the amino acid Phe, the end product of the shikimate pathway, which ultimately links the carbohydrate metabolism to the biosynthesis of aromatic compounds (Vogt, 2010; Fraser and Chapple, 2011). In Arabidopsis and other plant species, several members of the R2R3-MYB family have been found to regulate the expression of genes encoding biosynthetic enzymes of the shikimate and the general phenylpropanoid pathway (Vogt, 2010). Moreover, downstream of these pathways, different MYB TFs have been shown to differentially (co-)regulate the expression of genes involved in different metabolic sub-pathways, suggesting a role for MYB TFs in regulating specificity towards metabolic endproducts. For instance, the closely related MYB TFs MYB46 and MYB83 (McCarthy et al., 2009; Zhong and Ye, 2012), as well as the phylogenetically more distinct MYB TFs MYB58 and MYB63 (Zhou et al., 2009) have been shown to regulate secondary cell wall formation downstream of the NAC domain TF SND1 (Zhong et al., 2006; Mitsuda et al., 2007; Zhong et al., 2008) by activating the expression of lignin biosynthetic genes. In addition, the synthesis of anthocyanins in Arabidopsis is controlled by the closely related MYB transcription factors PAP1/MYB75, PAP2/MYB90, MYB113, and MYB114 that stimulate the transcription of both general phenylpropanoid- and anthocyanin-specific genes (Schwinn et al., 2006). Our study demonstrates that MYB72 and MYB10 redundantly activate genes involved in the shikimate pathway and the general phenylpropanoid pathway, which in turn drive the metabolic flow towards lignin biosynthesis.

Previously we demonstrated an intrinsic relationship between root colonization by WCS417 and the activation of the iron deficiency response. This latter response may results in enhanced iron uptake, which may lead to toxicity of certain transition metals. Lignin deposition is increased by a variety of stresses, including toxic concentrations of zinc and copper and unfavorable metals such as cadmium, aluminum, and nickel (Mao et al., 2004; van de Mortel et al., 2006; Kováčik and Klejdus, 2008; Xue et al., 2008; Moura et al., 2010). Recent studies demonstrates that phenylpropanoid metabolism and lignin biosynthesis is induced in iron-deprived roots (Lan et al., 2011). Lignification alters cell wall properties and thus it may serve to restrict the long-distance transport of toxic metals. This is in line with an increased lignification of the endodermis in response to zinc excess in *Thlaspi caerulescens* (van de Mortel et al., 2006). The expression patterns of *MYB72* and *MYB10* further indicate that lignification upon iron deficiency is likely to occur in the root trichoblasts and root hairs, cells that are directly involved in nutrient absorption from the soil environment. The role of the phenylpropanoid pathway in the onset of WCS417-mediated ISR remains elusive and future research is needed to address the impact of its local activation in systemic root to shoot directed immune responses.

Our study further revealed a role of MYB72 and MYB10 in the suppression of defense responses mounted locally in roots upon WCS417 bacterization, especially those associated with

indole glucosinolate metabolic processes. Indole glucosinolates comprise an important class of secondary metabolites with central roles in plant defense against insect herbivores and microbial attack. They are hydrolyzed by endogenous β-thioglucoside glucohydrolases that cleave off the glucose group, thereby liberating effector molecules that may either possess biocidal activity or have additional signaling roles in MAMP-triggered immunity (Bednarek et al., 2009; Clay et al., 2009; Hopkins et al., 2009; Muller, 2009). Specifically in the case of elicitor-triggered immunity, indole glucosinolate metabolism is channeled by the action the P450 enzyme CYP81F2 and the atypical myrosinase PEN2 towards the production of end-metabolic products that are transported to the apoplastic space (Lipka et al., 2005; Bednarek et al., 2009). Together with gene expression data demonstrating that MYB72 has a negative impact on the expression of genes involved in the biosynthesis of camalexin (Glawischnig, 2007), our data points to a prominent role of MYB72 and MYB10 in the transcriptional repression of genes involved in the indole glucosinolate metabolism. Importantly, their suppressive effects on gene expression are not limited to genes associated with the indole glucosinolate metabolism, but are further extended to other classes of defense-related genes. In Chapter 3, we demonstrated that bacterial volatiles are able to trigger iron deficiency regulated transcriptional responses. Data presented in this Chapter suggests that bacterial volatiles, via *MYB72* and *MYB10* induction, may function as effector molecules that contribute to the suppression of MAMP-triggered immunity locally in roots, to facilitate root colonization by ISR inducing bacteria. WCS417 bacteria are not defective in colonizing roots of the *myb72* mutant (Van der Ent et al., 2008), suggesting that MYB10 may compensate in defense suppression. Therefore, a double mutant lacking both transcription factors should be studied to assess their contribution in root colonization by beneficial ISR-inducing bacteria.

MATERIALS AND METHODS

Plant material and induction treatments

Seeds of Col-0, *35S::FLAG-MYB72* (*myb72-2* background) and *35S::YFP-MYB10* (Col-0 background) plants were surface sterilized and sown on 1 × Hoagland agar-solidified medium at the density of 18 - 20 per plate. After 2 d of stratification at 4°C, the Petri dishes were transferred and positioned vertically in a growth chamber under a long day photoperiod (16 h of light, light intensity 100 µmol m^{-2} s^{-1}) at 22°C. *Pseudomonas fluorescens* WCS417 was cultured on King's medium B (KB) agar plates (King et al., 1954) supplemented with 50 µg mL^{-1} rifampicin at 28°C. After 24 h growth, cells were collected in 10 mM $MgSO_4$, washed twice by centrifugation for 5 min at 5.000 × *g*, and finally resuspended in 10 mM $MgSO_4$. The bacterial titer was then adjusted to the OD_{600} of 0.002 (10^6 cfu mL-1); 10 µl of bacterial suspension was then applied on each root of 10 d old seedlings, right below the hypocotyl.

BiFC and Co-IP experiments

The coding regions of *MYB72*, *MYB10*, *FIT1(bHLH29)*, *bHLH38*, *bHLH39*, *SLIM1*(*EIL3*), *EIN3* and *EIL1* were amplified from wild-type Col-0 cDNA. The resulting PCR products flanked by attB recombination sites, were captured into the pDONR221-pGEMT-Easy vector using the BP reaction (Invitrogen) according to the manufacturer's instructions, and recombined into the plasmids pARC233 or pARC234 (Welch et al., 2007) to generate N-terminal YFP/N and YFP/C fusions. The resulting plasmids were pairwise co-transfected into Arabidopsis Col-0 mesophyll protoplasts. Protoplast isolation and transfections procedures were previously described (Yoo et al., 2007). The interacting root-specific SCARECROW (SCR) and SHOORTROOT (SHR) of the GRAS family TFs were used as positive control (Cui et al., 2007; Welch et al., 2007). For Co-IP experiments, the *MYB72* coding sequencing was recombined into the pEarleyGate202 vector (Earley et al., 2006) to generate a *35S::FLAG-MYB72* construct and the coding sequence of *MYB10* was recombined

into the pEarleyGate201 vector (Earley et al., 2006) to generate a *35S::HA-MYB10* construct. The resulting plasmids were co-transfected into protoplasts and Co-IP was performed as previously described (Zhu et al., 2010).

Generation of transgenic lines

The 2-kb genomic region upstream of the start codon of *MYB10* was amplified from genomic Col-0 DNA, captured into the pDONR221-pGEMT-Easy vector using the BP reaction and recombined into the destination vector pBGWFS7.0 using the LR reaction according to the manufacturer's instructions (Invitrogen). The coding sequence of *MYB10* was amplified from wild-type Col-0 cDNA and captured into the pDONR221-pGEMT-Easy vector. The corresponding cDNAs were then recombined into the pEarleyGate104 (35S-YFP-Gateway-OCS-3') plasmid (Earley et al., 2006). For generation of transgenic lines, recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90. *A. tumefaciens*-mediated plant transformation was performed in Col-0 plants, as described (Clough and Bent, 1998).

Fluorescence microscopy

Confocal laser-scanning microscopy on reporter lines and Arabidopsis mesophyll protoplasts (subcellular localization and BiFC assays) was performed using a Leica SP2 inverted microscope. As counterstain, roots were stained in 10 µg mL^{-1} propidium iodide (PI) solution for 2 min. Chromophores were excited using the 488 nm Argon laser and fluorescence was detected at 500-550 nm (for GFP), 550-615 nm (for YFP) and 570-620 nm (for PI). Arabidopsis mesophyll protoplasts were isolated and transfected as previously reported. Protoplasts were examined under a confocal microscope 18 h post transfection.

Microarray and Q-RT-PCR analysis

icroarrays experiments and analysis are described in Chapter 4. For quantitative PCR analysis, total RNA was extracted using the Qiagen's RNeasy kit and treated with the Ambion® TURBO™ DNase. cDNA was synthesized with the SuperScript-III reverse transcriptase (Invitrogen). Cycle tresholds were determined in duplicate per transcript in three biological replicas per sample using the ABI PRISM 7700 sequence detection system (Applied Biosystems) and SYBR Green I as reporter dye. The data were normalized using *Actin7*.

Chapter 6

Summarizing Discussion

Partly adapted from:

Pieterse, C.M.J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S.C.M.
Hormonal modulation of plant immunity.
Annual Review of Cell and Developmental Biology.
in press (2012)

Beneficial rhizobacteria drive phenotypic plasticity via auxin signaling

Plants are sessile organisms and as such cannot move away from hostile environmental conditions. For this reason, mechanisms of adaptation are well-developed in the plant kingdom. Roots are important for plant life since they feed photosynthetic tissues with water and nutrients, while providing mechanical support to the plant body. One of the best characterized examples of phenotypic plasticity are physiological and morphological responses of roots to nutrient deficiencies. Often, nutrients become limiting factors for plant growth and productivity. In turn, plants respond to nutrient-limited conditions by initiating developmental programs aimed to increase the root's exploratory capacity (López-Bucio et al., 2003).

It is known for years that analogous modifications in root morphology can be triggered by plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF), effects that were linked to the ability of these microbes to promote growth of host plants (Lugtenberg and Kamilova, 2009). However, only recently the molecular mechanisms underpinning those phenomena started to be dissected at the genetic and molecular level (Lopez-Bucio et al., 2007; Felten et al., 2009; Splivallo et al., 2009). In Chapter 2 of this thesis, we aimed to investigate the molecular mechanism by which PGPR modify root morphology and its relationship with the capacity of selected PGPR to trigger broad-spectrum induced systemic resistance (ISR) in foliar tissues of Arabidopsis *thaliana* (Arabidopsis). We demonstrated that, independent of their ability to trigger ISR in Arabidopsis, selected strains of *Pseudomonas* spp. rhizosphere bacteria are capable of conferring notorious alteration in root system architecture by affecting genetic programs related to primary root, lateral root and root hair development. Our results further demonstrated that, although rhizobacteria-mediated ISR depends on functional ET and JA signaling pathways (Pieterse et al., 1998; Ton et al., 2002c), induced alterations in the root system architecture are established independent of these pathways. Not surprisingly, auxin, with central roles in embryonic and post-embryonic root development (Benjamins and Scheres, 2008a), appeared to be the central molecule to orchestrate adaptive morphological response of roots in response to rhizobacteria-released signals.

The role of auxin in plant-microbe interactions has been well-documented (Kazan and Manners, 2009; Wang and Fu, 2011). For instance, global down-regulation of auxin responses by salicylic acid (SA) is an important aspect of the defense response of Arabidopsis against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Navarro et al., 2006; Wang et al., 2007a). Conversely, auxin can promote susceptibility to this pathogen, since treatment with the auxin analogs 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-naphthalacetic acid (NAA) enhanced the disease symptoms of *P. syringae* pv. *tomato*-infected Arabidopsis (Chen et al., 2007). Intriguingly, certain soil-borne microorganisms, such as rhizobia and parasitic nematodes, are able to induce the formation of new root structures by affecting processes related to auxin transport (Grunewald et al., 2009). A role of auxin as a signaling molecule in beneficial plant-microbe interactions is supported by the recent discovery of the role of the effector MiSSP7 of the ectomycorrhizal fungus *Lacaria bicolor* in promoting auxin-related gene expression in colonized roots (Plett et al., 2011). Considering that auxin signaling promotes susceptibility to microbes with a biotrophic lifestyle through the suppression of SA-dependent defenses, it seems that *L. bicolor* hijacks the auxin pathway for its own benefit. Alterations in root morphology conferred by beneficial rhizobacteria may provide plants with an advantage to explore soil niches for nutrients, while the rhizobacteria may benefit from the increased exudation that predominantly occurs in the elongation zone of newly formed roots. Another possibility to be considered is related to the role of auxin as effector molecule in suppressing defense responses in host-pathogen interactions. In this case, beneficial rhizobacteria may trigger an auxin response in roots of host plants as a mechanism to attenuate innate immune response for efficient colonization.

Iron deficiency signaling: the root of rhizobacteria-mediated ISR?

Using the plant model Arabidopsis and the PGPR strain *Pseudomonas fluorescens* WCS417, major components of the ISR signaling pathway have been uncovered (Van Wees et al., 2008; Van der Ent et al., 2009a). Evidence is also accumulating that systemic immunity triggered by soil *Trichoderma* species is regulated and manifested in a similar manner (Segarra et al., 2009). Although effective against a broad variety of pathogens and even insects, WCS417-ISR is not associated with the direct activation of defense responses. Instead, ISR-expressing plants are conditioned for accelerated JA/ET-dependent gene expression (Van Wees et al., 1999; Verhagen et al., 2004) and increased deposition of callose at the site of pathogen entry (Van der Ent et al., 2009b). This form of protection is called priming and provides plants with an energy-efficient ability to protect themselves against pathogens (Conrath et al., 2006). In Arabidopsis, establishment of WCS417-ISR in systemic tissues depends on the JA/ET and ABA signaling pathways and requires MYC2 and NPR1 (Pieterse et al., 1996; Pieterse et al., 1998; Pozo et al., 2008; Van der Ent et al., 2009b).

In roots, initiation of ISR is regulated by the root-specific MYB72 transcription factor and components of the ET-signaling pathway that locally act in the generation of a so-far unknown systemic signal (Van der Ent et al., 2008). The fact that MYB72, an essential signaling component of rhizobacteria- and *Trichoderma*-mediated ISR in Arabidopsis, is also induced under iron limited conditions (Colangelo and Guerinot, 2004; Dinneny et al., 2008; Buckhout et al., 2009), prompted us to investigate a possible link between iron-deficiency inducible mechanisms and rhizobacteria-mediated ISR. In Chapter 3, we demonstrated that volatile compounds emitted by the ISR-inducing bacterium *P. fluorescens* WCS417 are capable of activating an iron-deficiency response in the roots of Arabidopsis, evidenced by the upregulation of the iron-limitation responsive genes *FRO2* and *IRT1*. We also demonstrated that *MYB72*, upon root colonization by WCS417 bacteria, is similarly regulated as the iron uptake genes *FRO2* and *IRT1*. We further showed that bacterial volatiles function downstream of ET and auxin signaling and upstream of the central regulator FIT, known to orchestrate transcriptional responses to iron deficiency (Colangelo and Guerinot, 2004) (Figure 1). How exactly volatile signals are perceived by plant cells, remains an open question. Nitric oxide during iron-deficient conditions is known to function downstream of auxin and upstream of FIT1 (Chen et al., 2010), and thus, is a candidate signal. This is further supported by the fact that levels of nitric oxide are increased in roots exposed to WCS417-emmited volatile signals. Furthermore, we demonstrated that constitutive expression of the iron deficiency response in *opt3-2* (Stacey et al., 2008) and *frd3-1* (Rogers and Guerinot, 2002) resulted in enhanced resistance against necrotrophic pathogens. Together our findings indicate that ISR-inducing rhizobacteria hijack the iron deficiency response in plant roots, resulting in enhanced disease resistance in foliar tissues.

Hence, recruitment of iron deficiency signaling by ISR-inducing rhizobacteria emerges as an important early component in the onset of rhizobacteria-ISR. The importance of mineral nutrients in crop production has been recognized for more than 2000 years. Plant mineral nutrition is unique because green plants, the only multicellular autotrophic organism, can mine inorganic minerals from the environment without having to rely on high-energy compounds synthesized by other organisms. Not surprisingly, plants partly rely for their nutrition on probiotic functions of root microbiota (Barber, 1968; Tinker, 1984). Such roles of soil beneficial microbes in improving plant nutrition are well-documented. Arbuscular mycorrhizal fungi, which form an extensive dense hyphal network in the soil, are known for their ability to solubilize organic phosphorus from distal to root soil niches and subsequently provide sequestered phosphate to host plants, in return of fixed carbon (Parniske, 2008). Although the transcript levels of many transporters in host cells decrease with an increase in Pi status and also in the arbuscular mycorrhizal symbiosis, the expression of a small subgroup of symbiosis-specific Pht1-type transporters is specifically induced in cortical cells containing mycorrhizal arbuscules in order to facilitate phosphate transportation

(Javot et al., 2007). Likewise, rhizobia within the symbiotic organ, the root nodule, fix atmospheric nitrogen into the usable form of ammonia, which then reaches the assimilation sites in the host cell cytoplasm via plant-encoded transporters (Yokota and Hayashi, 2011). In analogy to these examples, and in the view of results presented in Chapter 3, it is tempting to speculate that the mutualistic relationship between ISR-inducing bacteria and host plants involves the provision of iron to the plant, in return of fixed carbon and photosynthates (Figure 1). This is further supported by microarray data in Chapter 4, demonstrating that almost one third of WCS417-regulated genes, are also induced under iron limited conditions.

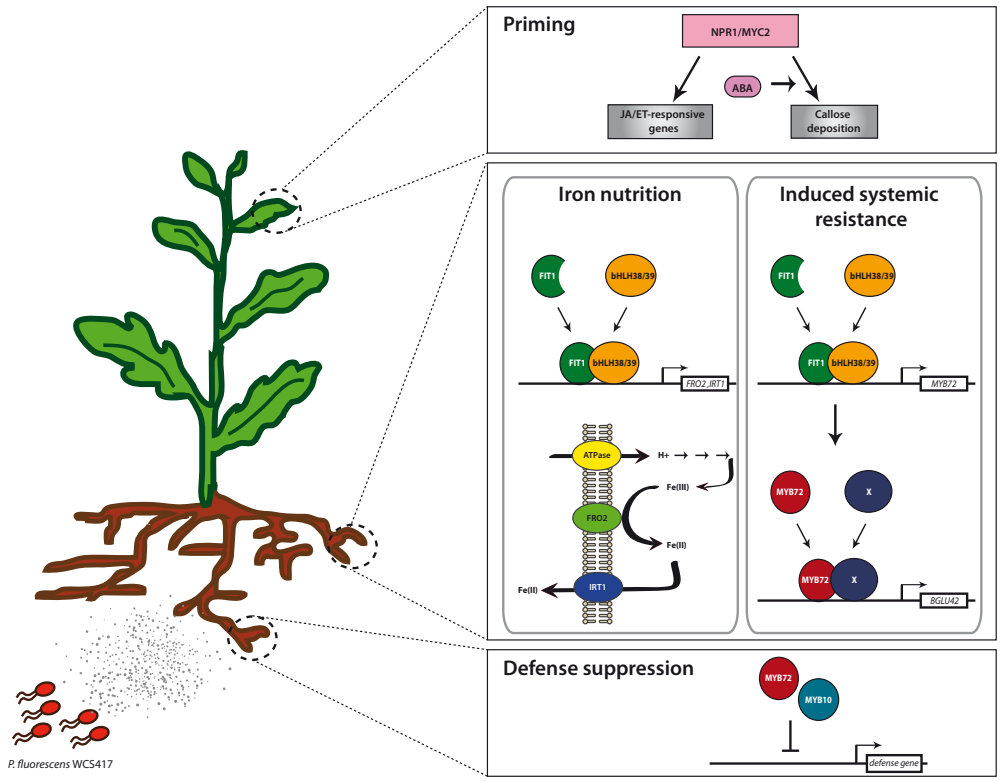


Figure 1. Molecular mechanisms involved in rhizobacteria-ISR. ISR-inducing bacteria emit volatile compounds that trigger locally in roots an iron-deficiency response resulting in improved iron nutrition and the onset of ISR. Depicted are the signaling components (FIT1, bHLH38, bHLH39, MYB72) and effector molecules (FRO2, IRT1 and BGLU42) involved in these two responses (middle panel). MYB72 redundantly with MYB10 transcription factor may function to repress the expression of defense-related genes in order to allow bacteria efficiently colonize roots of host plants (bottom panel). In systemic tissues, the onset of ISR depends on functional JA/ET and ABA signaling pathways and further requires the transcriptional regulators NPR1 and MYC2. ISR expressing plants are primed for accelerated JA/ET-responsive gene expression and increased callose deposition at the sites of pathogen or insect attack (top panel).

Systemic immune responses: the elusive systemic signals

Although ISR, systemic acquired resistance (SAR) and wound-induced resistance (WIR) are physiological different phenomena, all require the production and transport of a signaling molecule to enhance resistance in distal plant tissues. SAR is often triggered upon infection by a necrosis-inducing pathogen, although tissue necroses at the site of primary infection are

dispensable for SAR elicitation (Mishina and Zeier, 2007). A signaling cascade that occurs in the vascular tissues appears to be crucial for SAR initiation (Champigny et al., 2011). However, the identity of the systemic signal(s) that are released into the phloem stream has been a subject of controversy for many years. Despite the fact the SA accumulates in the phloem sap of SAR-expressing plants, grafting experiments with tobacco have shown that SA itself is unlikely to be the systemic signal (Vernooij et al., 1994). Instead, a wealth of genetic and biochemical data suggest a central role for lipid-based signaling in SAR. Arabidopsis DEFECTIVE in INDUCED RESISTANCE (DIR1), encodes a putative lipid transfer protein suggesting that DIR1 is likely to function as a chaperone for a lipid-related mobile signal (Maldonado et al., 2002). Further supporting a lipid-based molecule as the systemic signal of SAR is the fact that enzymes involved in the biosynthesis of plastidial glycerolipids, such as FATTY ACID DESATURASE7 (FAD7), SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1 (SFD1) and SFD2, are essential for the SAR initiation (Kachroo et al., 2001; Nandi et al., 2004; Chaturvedi et al., 2008). Recently, glycerol-3-phosphate (G3P), synthesized through the glycerolkinase NON HOST RESISTANCE 1 (NHO1) and the G3P dehydrogenase SFD1, has been demonstrated to be a critical mobile inducer of SAR (Chanda et al., 2011). Other molecules that have been proposed so far to act as long-distance molecules include indole-derived metabolites (Truman et al., 2010), the organic ester methyl salicylate (MeSA) (Park et al., 2007) and the plant hormone JA (Truman et al., 2007), although their role as phloem-mobile signals in Arabidopsis appears to be conditional (Attaran et al., 2009; Liu et al., 2011). Azaleic acid has also been identified as critical SAR-mobile signal that triggers a primed response in systemic tissues (Jung et al., 2009). In systemic tissues, the onset of SAR requires intact cuticle (Xia et al., 2009) and the function of FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) (Mishina and Zeier, 2006), possibly to transduce or amplify a long-distance signal originating from primary leaves.

Mechanical damage, such as caused by herbivorous insects, elicits a systemic defense response characterized by the production of anti-feedant enzymes and toxic metabolites, as well as the emission of volatile substances that act to attract parasitoids and predators of the attacking herbivore . In tomato, tobacco and Arabidopsis the onset of the transcriptional reprogramming in systemic tissues requires an intact JA-perception machinery and most of the systemic wound responses are mediated by COI1 (Kessler and Baldwin, 2002; Wang et al., 2008; Koo et al., 2009). In tomato, the 18-amino-acid peptide systemin was initially proposed to act as the long-distance molecule in the systemic wound response (Ryan and Pearce, 1998). Nevertheless, grafting experiments demonstrated that JA biosynthesis is required at the site of wounding but not in the remote leaves, and that systemin cooperatively with other elicitors, such as hydroxyproline-rich glycopeptides, is locally acting to trigger or amplify the JA biosynthesis in the companion cells of the vascular bundles (Narváez-Vásquez et al., 2005; Schilmiller and Howe, 2005). It is therefore possible that the pool of JA itself, or a JA derivative produced in the companion cells, is mobilized in systemic tissues via the phloem to immunize distal tissues. In Arabidopsis, rapidly transited signals trigger in the systemic tissues de novo synthesis of JA-Ile via OCP3 and JAR1, which in turn activates JA-responsive genes via the SCF^{COI1}/JAZ pathway (Koo et al., 2009).

Rhizobacteria-mediated ISR resembles WIR, since both depend on functional JA signaling pathway and are manifested in systemic tissues as priming for JA-regulated defense responses (Van Wees et al., 1999; Koo et al., 2009) (Figure 1). However, in contrast to WIR, hydraulic and physical signals are unlikely to be involved in ISR, given that root tissues remain intact upon root bacterization. In fact, results from this thesis (Chapter 3 and Chapter 4) largely support the concept that enhanced disease resistance in systemic leaves upon the onset of ISR stems from the induction of an iron-deficiency response locally in roots. It is tempting to speculate that iron deficiency generates locally in roots stress-signals that are systemically transported to aerial tissues as an early warning of fluctuations in external nutrient concentrations. This in turn may result in systemic reactions expressed by transcriptional or metabolic changes with impact on immune

responses. By employing microarray analysis and comparing transcriptional profiles of bacterized roots of wild type and ISR-impaired *myb72* plants (Chapter 4), we could identify an enzyme, the β-glucosidase BGLU42, as an important component of the ISR-signaling pathway that functions downstream of MYB72 in the initiation of ISR. *BGLU42* is expressed in the root hairs and root trichoblasts, suggesting that ISR signals are generated and modified in the outermost cell layer. The putative product that is liberated upon the enzymatic action of BGLU42 may either be the systemic signal itself, or may be perceived by the xylem parenchyma cells. The first scenario implies lateral transportation to the innermost cell layers, with at least one step involving symplastic movement towards the casparian strip, followed by direct loading into the transpiration stream. In analogy to SAR and WIR, the second scenario implies signal perception by xylem parenchyma cells for the generation of xylem mobile signals (Figure 2).

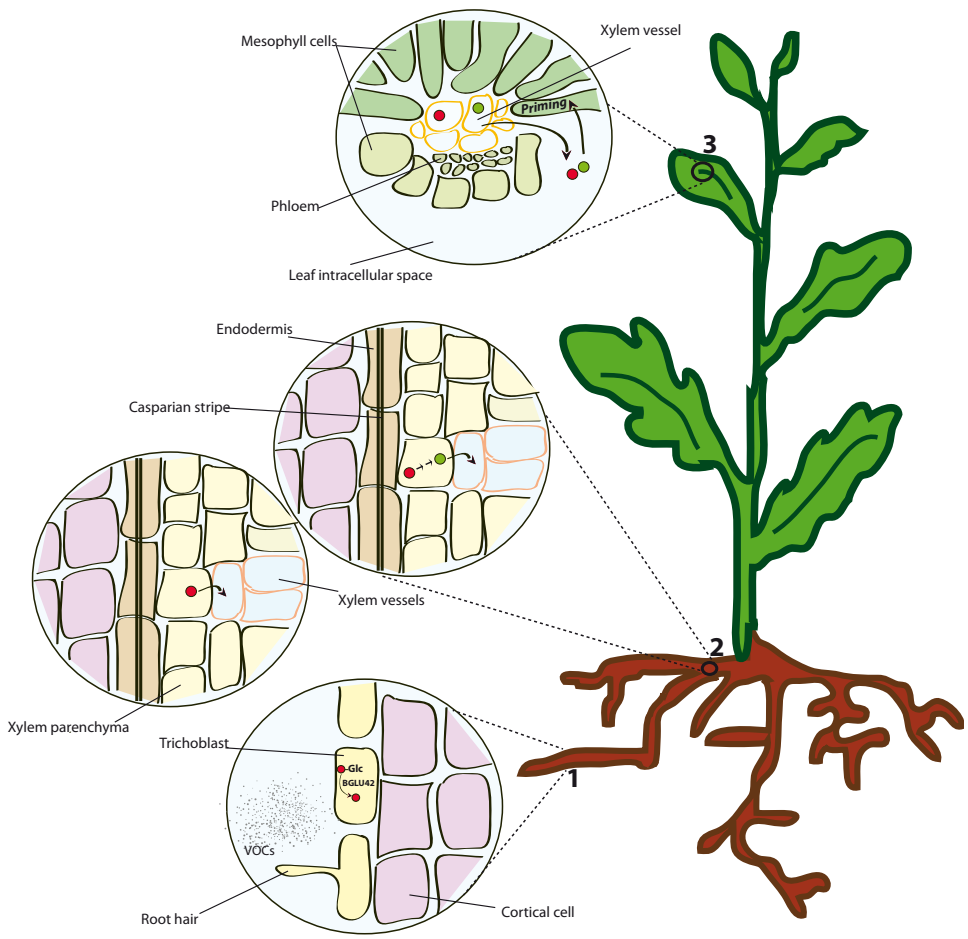


Figure 2. Proposed model for P. fluorescens WCS417-mediated ISR in Arabidopsis. (1). Volatile organic compounds (VOCs) produced by the rhizosphere bacteria activate the β-glucosidase gene *BGLU42* in a MYB72-dependent manner. Subsequently, a putative signal (red dot) is generated in root trichoblasts upon BGLU42-mediated hydrolysis of its glucoside **(2).** The signal moves to the root endodermis (either following the symplastic or apoplastic route) and subsequently, through the endodermal cells, it passes to the xylem parenchyma cells. It is then, either directly loaded to the xylem vessels or putative secondary xylem-mobile signals are generated (green dot) **(3).** The signal is transported to the shoot, moves from the xylem vessels to the apoplast surrounding the neighboring leaf cells, and is perceived by the leaf parenchyma cells, which become primed for defense.

So far, it remains unknown how individual cell types across the root layers respond to PGPR. Cell-type and tissue-specific transcriptional and metabolic changes triggered by beneficial rhizobacteria and fungi, could be studied by Fluorescence Activated Cell Sorting (FACS) of root protoplasts (Birnbaum et al., 2005). This approach has been successfully applied for the generation of temporal and spatial gene expression and metabolic profiles (digital in situ), under standard and stress conditions (Birnbaum et al., 2003; Dinneny et al., 2008; Petersson et al., 2009). Such studies may provide a molecular framework of how plant's regulatory transcriptional networks operate in response to PGPR in order to establish the transcriptional program required for systemic signaling and plant growth promotion.

Suppression of plant defenses in beneficial plant-microbe interactions

Each gram of soil contains billions of microbes. However, the microbial community on plant roots is very different from that in bulk soil. Hence, plants are able to recruit specific microbes from the soil to their roots (Mendes et al., 2011). The establishment of beneficial associations requires mutual recognition and a high degree of coordination of plant and microbial responses through a continuous molecular dialogue between the plant and the beneficial. Like leaf tissues, roots are well-capable of responding to a variety of defense elicitors, such as MAMPs produced by soil-borne microbes (Millet et al., 2010). Because beneficial microbes are recognized as alien organisms, active interference with the plant immune system is fundamental for the establishment of intimate mutualistic relationships (Zamioudis and Pieterse, 2012). For instance, the beneficial PGPF *Piriformospora indica* recruits the JA pathway to suppress both early and late defenses, including SA-mediated defenses (Jacobs et al., 2011). The recently discovered MiSSP7 effector of the ectomycorrhizal fungus *Laccaria bicolor* and the SP7 effector of the arbuscular mycorrhizal fungus *Glomus intraradices* are two examples of symbiotic effectors that promote biotrophic interactions by affecting hormonal signaling pathways involved in plant defenses (Kloppholz et al., 2011; Plett et al., 2011). The SP7 effector is the first symbiotic effector described so far to clearly possess immune-suppressive function by targeting the ET signaling pathway, which is an important component of MTI in the roots. SP7 is translocated into the nucleus of host cells, where it interacts with the defense-related transcription factor ERF19 and blocks its transcriptional program (Kloppholz et al., 2011).

Recently, Millet et al. (2010) provided evidence that the PGPR *P. fluorescens* WCS417 is able to suppress flagellin-triggered MTI responses in Arabidopsis roots via apoplastic secretion of one or more low molecular weight molecule(s). In the Chapter 5 we demonstrated that MYB72, redundantly with its closely related transcription factor MYB10, suppresses the expression of a large set of genes that encode components of MAMP-trigged immunity, upon root colonization of the roots by WCS417. Since volatiles released by WCS417 are potent activators of *MYB72* gene expression and the iron deficiency response (Chapter 3), it is tempting to speculate that bacterial volatiles, via MYB72 and MYB10 induction, function as effector molecules that contribute to the suppression of MAMP-triggered immunity locally in roots in order to facilitate root colonization by ISR-inducing bacteria (Figure 1).

Concluding remark

In conclusion, the results denoted in this thesis demonstrate that bacteria-derived molecules manipulate signaling mechanisms of host plants related to root development and mineral nutrition, ultimately resulting in growth promotion and disease resistance. Identification of the chemical identity of those components will give us the possibility to develop new strategies for improved nutrition and crop protection.

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SUMMARY

Root colonization by selected strains of beneficial soil-resident bacteria is known to improve plant growth, influence root system architecture and trigger a systemic immune response that is effective against a broad range of pathogens, known as induced systemic resistance (ISR). In this thesis we explore signaling mechanisms that are activated in the roots in response to ISR-inducing bacteria. We demonstrate that the plant growth-promoting rhizobacterium *Pseudomonas fluorescens* WCS417 secretes molecules that positively influence plant growth in Arabidopsis, and confer significant alterations on root morphology evidenced by increased number of lateral roots and root hairs. By employing confocal microscopy and genetic analysis, we highlight auxin signaling as the central hormonal pathway to mediate these effects. In Arabidopsis, WCS417-ISR requires the root-specific transcription factor MYB72 that locally operates in the generation or translocation of a thus-far unidentified systemic signal. *MYB72* expression is strongly activated by WCS417, but it is also known to be specifically induced under iron limited conditions. We demonstrate that ISR-inducing rhizobacteria upregulate the iron-deficiency response Strategy I, independently of the iron availability status, ultimately improving host's iron nutrition. We further demonstrate that rhizobacteria-induced *MYB72* expression depends on FIT1, the central transcription factor that orchestrates plant responses to iron limitation, and that *MYB72* is similarly regulated as the iron uptake genes *FRO2* and *IRT1*. A search for bacterial determinants involved in *MYB72* expression revealed that volatile organic compounds emitted by WCS417 are potent elicitors of the iron deficiency response in roots. By employing whole-genome transcript profiling we identified the β-glucosidase BGLU42 as an essential component of ISR. In addition, we demonstrate that overexpression of *BGLU42* in the absence of rhizobacteria confers a significant level of protection against various pathogens. Consistent with the fact that overexpression of *MYB72* does not lead to constitutive disease resistance, we found none of the MYB72-target genes to be constitutively upregulated in roots of plants overexpressing *MYB72*. This suggests that MYB72 may function synergistically with other cellular components for the initiation of ISR. A search for MYB72-interacting proteins revealed that MYB72 interacts with the closely-related MYB10 transcription factor, and co-operatively trigger the expression of the *AT5G55620* gene. In addition, we show that MYB72 and MYB10 function redundantly in regulating the expression of genes involved in the shikimate, the general phenylpropanoid, and the lignin biosynthetic pathway. We further show that overexpression of *MYB72* or *MYB10* suppresses the expression of a large group of defense-related genes upon root colonization by WCS417 bacteria, suggesting that these transcription factors may be targets of bacterial effectors that function during the interaction in order to suppress innate immune responses and enable bacteria to establish long-term associations with host roots. In conclusion, our results demonstrate that volatiles of root-colonizing rhizobacteria manipulate signaling mechanisms of host plants related to root development and mineral nutrition, ultimately resulting in growth promotion and disease resistance. Identification of the chemical identity of these volatiles will provide tools to develop new strategies for improved nutrition and crop protection.

SAMENVATTING

Met de zon als energiebron leggen planten CO_2 vast in de vorm van suikers. Tot 40% van deze koolhydraten wordt via het wortelstelsel weer uitgescheiden om de bodemmicroflora te voeden. Goedaardige micro-organismen in de bodem vervullen belangrijke functies, zoals stimulatie van plantengroei en activatie van het immuunsysteem van de plant. De goedaardige bodembacterie *Pseudomonas fluorescens* WCS417r (WCS417r) is in staat om beide microbiële functies te stimuleren in de modelplant Arabidopsis *thaliana* (Zandraket).

Kolonisatie van het wortelstelsel van Arabidopsis door WCS417r bleek de architectuur van het wortelstelsel drastisch te veranderen (hoofdstuk 2). WCS417r-behandelde Arabidopsiswortels vormden significant meer zijwortels en de lengte van de wortelharen verdubbelde. Hierdoor ontstaat een fijner vertakt wortelstelsel waardoor de plant meer voedingstoffen kan opnemen. Dit resulteerde in een verdubbeling van het versgewicht van de groene delen van de plant. Dit groeibevorderende effect van WCS417r kon worden toegeschreven aan het groeihormoon auxine. Auxine reporterlijnen lieten een verhoogde stimulatie van auxinesignalering zien in de door WCS417r gekoloniseerde Arabidopsiswortel. Bovendien lieten Arabidopsis mutanten die verstoord zijn in auxine signalering het effect op de wortelarchitectuur niet meer zien. Veelal wordt gedacht dat het groeibevorderende effect van goedaardige bodembacteriën zoals WCS417r gekoppeld is aan het vermogen van deze microorganismen om het immuunsysteem van de plant te stimuleren. Uit dit onderzoek bleek echter dat beide microbiële functies volledig onafhankelijk van elkaar opereren. Arabidopsis mutanten die verstoord zijn in hun vermogen om door WCS417r geïnduceerde resistentie tot expressie te brengen, bleken nog een normale verandering in de wortelarchitectuur te ondergaan na kolonisatie door WCS417r. Ook *Pseudomonas* bacteriën die geen ISR in Arabidopsis induceren bleken nog steeds groeibevorderende effecten te hebben. Uit dit onderzoek wordt dus geconcludeerd dat stimulatie van groei en activatie van het immuunsysteem van de plant door goedaardige bodembacteriën niet per definitie gelinkt zijn.

Kolonisatie van het worteloppervlak door WCS417r resulteert in de stimulatie van het immuunsysteem, waardoor bovengrondse delen van de plant beter beschermd zijn tegen plantenziekten en insectenplagen. Dit fenomeen wordt “induced systemic resistance (ISR)” genoemd. De wortel-specifieke transcriptiefactor MYB72 vormt hierbij een belangrijke schakel tussen herkenning van de bacterie door de wortel, en het doorgeven van een signaal naar bovengrondse delen van de plant, wat ter plaatse een betere bescherming tegen ziekten en plagen veroorzaakt. Confocal laser scanning microscopie liet zien dat het *MYB72* gen wordt aangeschakeld in specifieke wortelcellen in de wortelepidermis (hoofdstuk 4). Hierbij spelen vluchtige stoffen die door WCS417r worden geproduceerd een belangrijke rol. Een gedetailleerde analyse van de expressie van alle 25000 Arabidopsisgenen liet zien dat MYB72 in de wortel een groot aantal afweergenen uitschakelt, mogelijk om kolonisatie van de wortel door goedaardige microorganismen te bevorderen. Slechts een klein aantal Arabidopsisgenen werd door MYB72 aangeschakeld. Eén van deze genen codeert voor een enzym met β-glucosidase activiteit (BGLU42) dat in staat is om signaalmoleculen vrij te maken in plantencellen. Net als *MYB72* wordt *BGLU42* specifiek door WCS417r aangeschakeld in specifieke wortelcellen in de wortelepidermis. Overexpressie van het *BGLU42* gen resulteerde in een verhoogde ziekteresistentie in bovengrondse delen van de plant. Bovendien liet de knockout *bglu42* mutant geen ISR meer zien. De β-glucosidase BGLU42 is dus een belangrijke nieuwe schakel in de ISR signaaltransductieroute. Mogelijk is BGLU42 betrokken bij het genereren van het systemisch getransporteerd signaal wat in bovengrondse delen van de plant ziekteresistentie teweeg brengt.

Het *MYB72* gen wordt specifiek aangeschakeld in de plantenwortel na kolonisatie door goedaardige microorganismen in de bodem. Echter, ijzer-limiterende omstandigheden leiden ook tot activatie van het *MYB72* gen in de wortels. Ijzer is een essentieel element voor planten. Bij

een tekort aan ijzer schakelt de plant mechanismen in werking die de opname van ijzer bevordert. Een belangrijk mechanisme wat in planten tijdens ijzer-stress wordt geactiveerd is “Strategy I”. Door dit mechanisme aan te schakelen scheiden de plantenwortels H^+-ionen uit waardoor de omgeving verzuurt en er meer Fe^{3+} in oplossing wordt gebracht. Dit wordt door de plant omgezet in Fe^{2+} wat vervolgens wordt opgenomen door de plantenwortel. In hoofdstuk 3 en 5 tonen we aan dat WCS417r de plantenwortel om de tuin leidt door de ijzer-stress reaktie “Strategy I” aan te schakelen, ook al heeft de plant voldoende ijzer tot zijn beschikking. Bacteriële vluchtige stoffen die worden geproduceerd door WCS417r blijken hiervoor verantwoordelijk te zijn. Het aanschakelen van “Strategy I” is mogelijk noodzakelijk voor het genereren en/of systemisch transporteren van een tot nog toe onbekend signaal wat systemisch in bovengrondse delen van de plant ISR induceert en de plant beter beschermt tegen een breed spectrum aan pathogenen en insecten.

In dit onderzoek hebben we aangetoond dat groeibevordering en activatie van het immuunsysteem twee onafhankelijke microbiële activiteiten zijn die tegelijkertijd kunnen worden uitgeoefend door specifieke goedaardige bodembacteriën. De gedetailleerde ontrafeling van de cellulaire rol van de wortelspecifieke transcriptiefactor MYB72, de ontdekking dat vluchtige stoffen van bodembacteriën de ijzer-stress reaktie aanschakelen in gekoloniseerde plantenwortels, en de belangrijke rol van de β-glucosidase BGLU42 in de vroege interactie tussen wortelcellen en ISR-inducerende bodembacteriën, zijn belangrijke nieuwe inzichten in de moleculaire keten die in gang wordt gezet in plantenwortels na kolonisatie door goedaardige bodembacteriën. Het tot in detail begrijpen van de mechanismen waarbij planten voordeel halen uit microbiële functies die worden uitgeoefend door goedaardige bodembacteriën kan bijdragen aan de ontwikkeling van nieuwe strategieën voor duurzame gewasbescherming.

ΣΥΝΟΨΗ

Μεταγωγή σήματος στις ρίζες του φυτού *Arabidopsis thaliana* κατά την αλληλεπίδραση με ωφέλιμους μικροοργανισμούς του εδάφους

Ο αποικισμός του ριζικού συστήματος των φυτών από συγκεκριμένα γένη βακτηρίων και μυκήτων έχει ευεργετικές επιδράσεις στην ανάπτυξη των φυτών, ενώ συχνά οδηγεί στην ανάπτυξη μιας μορφής διασυστηματικής ανθεκτικότητας έναντι ασθενειών η οποία ονομάζεται επαγόμενη διασυστηματική ανθεκτικότητα (ISR; induced systemic resistance). Σκοπός της διατριβής αυτής είναι η μελέτη γεγονότων μεταγωγής σήματος που λαμβάνουν χώρα τοπικά στις ρίζες κατά τα πρώιμα στάδια της αλληλεπίδρασης των φυτών με ωφέλιμους μικροοργανισμούς του εδάφους. Στη διατριβή αυτή καταδεικνύεται ότι το ωφέλιμο ριζοβακτήριο *Pseudomonas fluorescens* WCS417 παράγει βιοχημικά σήματα τα οποία προάγουν την ανάπτυξη του πρότυπου φυτού *Arabidopsis thaliana* (Arabidopsis) ενώ ταυτόχρανα επιφέρει σημαντικές τροποποιήσεις στην αρχιτεκτονική του ριζικού συστήματος προκαλώντας επιβράδυνση στην ανάπτυξη της πρωτογενούς ρίζας, μαζική παραγωγή δευτερογενών ριζών καθώς επίσης αθρόα έκπτυξη ριζικών τριχιδίων. Με χρήση συνεστιακής μικροσκοπίας σάρωσης LASER, περιγράφουμε της τροποποιήσεις αυτές σε κυτταρικό επίπεδο και περεταίρω καταδεικνύουμε τον ουσιώδη ρόλο της μεταγωγή σήματος μέσω του μονοπατιού της αυξίνης στις αποκρίσεις αυτές. Στην Arabidopsis, η επαγόμενη απο το ριζοβακτήριο *Pseudomonas fluorescens* WCS417 διασυστηματική ανθεκτικότητα, εξαρτάται από το μεταγραφικό παράγοντα MYB72. Το γονίδιο εκφράζεται ως απόκριση των ριζών στον αποικισμό από ωφέλιμα βακτήρια και μύκητες του εδάφους, είναι όμως επίσης γνωστό ότι επάγεται ισχυρά κάτω από συνθήκες έλλειψης σιδήρου. Βασιζόμενοι στην παρατήρηση αυτή, περιγράφουμε ότι ριζοβακτήρια ικανά να επάγουν ISR στην Arabidopsis, επιπλέον ενεργοποιούν - τοπικά στη ρίζα - μηχανισμούς οι οποίοι σχετίζονται με έλλειψη σιδήρου. Η ενεργοποίηση των μηχανισμών αυτών δεν οφείλεται σε ανταγωνισμό μεταξύ ριζών και βακτηρίων για σίδηρο. Αντιθέτως, ανεξαρτήτως των διαθέσιμων επιπέδων σιδήρου, πτητικές ενώσεις οι οποίες ελευθερώνονται από τα ριζοβακτήρια επάγουν την έκφραση γονιδιακών δεικτών σχετιζόμενων με έλλειψη σιδήρου (*FRO2*, *IRT1*), προάγοντας παράλληλα την έκφραση του *MYB72*. Μια τέτοια μεταχείριση των μηχανισμών ομοιόστασης του σιδήρου από πλευράς βακτηρίων αποδεικνύεται τελικά ευεργετική για τα φυτά καθότι συμβαλει θετικά στην ανόργανη θρέψη με το ιχνοστοιχείο αυτό. Προκειμένου να βρούμε γονιδιακούς στόχους των οποίων η έκφραση ελέγχεται από τον MYB72 μεταγραφικό παράγοντα, πραγματοποιήσαμε ευρέως φάσματος γονιδιακή έκφραση χρησιμοποιώντας μικροσυστοιχίες γονιδίων (microarrays). Τα αποτελέσματα της ανάλυσης αυτής κατέδειξαν τη β-γλυκοσιδάση BGLU42 ως απαραίτητο συστατικό της επαγόμενης από WCS417 ριζοβακτήρια ISR. Μεταλλάγματα Arabidopsis ελλειμματικά στο *BGLU42* γονίδιο δεν ενεργοποιούν ISR, ενώ η υπερέκφραση του γονιδίου οδηγεί σε αυξημένη ανθεκτικότητα έναντι παθογόνων. Τέλος, στην διατριβή αυτή περιγράφονται κοινές λειτουργίες μεταξύ του MYB72 και του συγγενή MYB10 μεταγραφικού παράγοντα στην ενεργοποίηση του βιοσυνθετικού μονοπατιού της λιγνίνης καθώς επίσης στην καταστολή της άμυνας του ξενιστή τοπικά στη ρίζα. Τα πειραματικά δεδομένα της διατριβή αυτής αποτελούν ένα επιπλέον βήμα προς στην κατεύθυνση να κατανοήσουμε καλύτερα τους μοριακούς μηχανισμούς που ενεργοποιούνται στις αλληλεπιδράσεις των φυτών με ωφέλιμους μικροοργανισμούς. Πιθανές πρακτικές εφαργογές της έρευνας που περιγράφεται στη διατριβή επίσης αναλύονται.

ΣΥΝΟΨΗ **(Εκλαϊκευμένο κείμενο)**

Τα φυτά χρησιμοποιώντας φώς, διοξείδιο του άνθρακα, νερό και ιχνοστοιχεία παράγουν τα απαραίτητα για τη θρέψη τους συστατικά. Οι ρίζες των φυτών έρχονται σε επαφή με πληθώρα

μικροοργανισμών όπως μύκητες και βακτήρια. Σκεφτείτε μόνο το εξής: 1 γραμμάριο εδάφους περιέχει περισσότερο από 1 τρισεκατομμύρια μικροοργανισμούς!!! Πολλοί από τους οργανισμούς αυτούς είναι ωφέλιμοι για το φυτό. Με αυτούς το φυτό αναπτύσσει στις ρίζες του μία αγαστή σχέση συνεργασίας. Δίνει τροφή υπό τη μορφή σακχάρων ενώ ως αντάλλαγμα, ωφέλιμα βακτήρια και μύκητες βοηθούν στη θρέψη με ανόργανα συστατικά. Συχνά, ο αποικισμός της ρίζας των φυτών απο ωφέλιμα μικρόβια δημιουργεί τοπικά στη ρίζα σήματα τα οποία αφού μεταφερθούν στα φύλλα καθιστούν το φυτό πιο ανθεκτικό σε προσβολές από παθογόνα. Επιτελούν δηλαδή οι μικροοργανισμοί αυτοί παρόμοιες λειτουργίες με αυτές των ωφέλιμων μικροοργανισμών στο πεπτικό σύστημα του ανθρώπου και άλλων θηλαστικών. Στην διατριβή αυτή περιγράφουμε γονίδια του φυτού που είναι απαραίτητα στις ρίζες για τη γένεση των σημάτων της ανοσοποίησης. Επιπλέον περιγράφουμε ότι συγκεκριμένα ωφέλιμα βακτήρια που ζουν και αναπτύσσονται στη ριζόσφαιρα των φυτών παράγουν πτητικές ενώσεις με τις οποίες βελτιώνουν την ικανότητα του φυτού να προσλαμβάνει σίδηρο, ο οποίος και αποτελεί απαραίτητο συστατικό της θρέψης των φυτών.

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Στους γονείς μου Δίτσιο και Ευγενία χρωστώ τα ΠΑΝΤΑ! Ποτέ δεν σταματήσατε να πιστεύετε σε μένα και να με στηρίζετε σε όσες δυσκολίες συνάντησα. Μπαμπά και μαμά, ευχαριστώ για όλα! Αναστασία και Μάκη, ευχαριστώ πολύ για τη στήριξη!

Εύη μου, σε ευχαριστώ που είσαι στη ζωή μου!

Χρήστος

CURRICULUM VITAE

Christos Zamioudis was born the 16th of June 1979 in Pella, Greece. He received his primary and secondary education in Pella and higher education in Thessaloniki, Greece. He studied Agricultural Sciences in the Aristotle University of Thessaloniki (AUTH) with specialization in plant protection. He performed his Bachelor's internship in the Laboratory of Pesticide Science of AUTH where he was trained for a year in analytical chemistry techniques. He obtained his Bachelor Degree in 2003 with distinction. During his bachelor studies, he developed a special interest in molecular plant pathology and soon after his graduation he joined the Master Program "Applied Genetics and Biotechnology" in the Department of Biology of AUTH. He performed his Master's internship in the Laboratory of General Microbiology under the supervision of Dr. Sivropoulou on a project entitled "Secondary metabolites of aromatic plants as inducers of systemic acquired resistance". He obtained his Master Diploma in 2005 with the highest distinction. In 2007, following successful participation in national competition held by the Greek State Scholarship Foundation, he received a fellowship and moved to the Netherlands for advanced post-graduate studies. In October 2007, he joined the Plant-Microbe Interactions research group at Utrecht University and started his PhD research on early signaling events during rhizobacteria-mediated induced systemic resistance under the supervision of Prof. Corné Pieterse. The results of these studies are described in this thesis. After his graduation he will continue working in the same group as postdoctoral fellow.

LIST OF PUBLICATIONS

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