Transcriptomics and knockout mutant analysis of rhizobacteria-mediated induced systemic resistance in Arabidopsis

Bas Verhagen

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Transcriptoom- en mutantenanalyze van door rhizobacteriën geïnduceerde systemische resistentie in Arabidopsis

(met een samenvatting in het Nederlands)

Proefschrift

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Het regent zonnestralen

Op een terras ergens in Frankrijk in de zon
Zit een man die het tot gisteren nooit won
Maar zijn auto vloog hier vlakbij uit de bocht
Zonder hem, zonder Herman,
Want die had hem net verkocht
Herman in de zon op het terras
Leest in 't AD dat 'ie niet meer in leven was
Zijn auto was volledig afgebrand
En die man die hem gekocht had,
Stond onder zijn naam in de krant

O, o, o,
Even rustig ademhalen
Het lijkt alsof het regent als altijd
Maar het regent zonnestralen

Nog geen week terug, in een park in Amsterdam
Had hij zijn leven overzien en schrok zich lam
Hij was een man wiens leven nu al was bepaald
En van al zijn jongensdromen
Was alleen het oud worden gehaald

O, o, o,
Even rustig ademhalen
Want het lijkt alsof het regent als altijd
Maar het regent zonnestralen

Op een bankje in het park kwam het besluit
Noem het dapper, noem het vluchten maar ik knijp er tussenuit
Nu een week geleden en hier zat hij dan maar weer
Met meer vrijheid dan hem lief was en nu wist hij het niet meer

Herman leest wel honderd keer de krant
Het staat er echt, pagina achttien, zwartomrand
Hield 'ie vroeger al zijn meningen
En al zijn dromen stil
Nu was 'ie niks niet niemand nergens meer
Kan dus gaan waar 'ie maar wil

Herman rekent af en staat dan op
Hij heeft eindelijk de wind weer in zijn kop
'Ik heb een tweede kans gekregen
En da's meer dan ik verdien
Maar als dit het is, is dit het
En we zullen het wel zien'

Acda en de Munnik, Naar Huis '98

Voor mijn ouders
en Madelinde
Merci

Contents

Chapter 1

Science may set limits to knowledge,
but should not set limits to imagination.

Bertrand Russell (1872 - 1970)

General Introduction

Plant Disease Resistance

Non-host, basal and R-gene mediated resistance

Plants are sessile organisms, incapable of fleeing from possibly harmful organisms. In order to defend themselves against pathogen attack, plants possess a range of constitutive and inducible resistance mechanisms. The most effective type is non-host resistance. This non-host resistance is expressed when a plant comes into contact with a pathogen which is incapable of provoking any disease in this plant (Agrios, 1997).

In the absence of non-host resistance, the plant is susceptible. However, even susceptible plants are capable of reacting in a way that may slow down growth of the pathogen. The presence of such basal resistance was evidenced by the isolation of enhanced disease susceptibility (*eds*) mutants. These mutants showed increased susceptibility to moderately virulent pathogens such as *Pseudomonas syringae* pv. *maculicola* ES4326 (Glazebrook et al., 1996). Basal resistance is often dependent on the action of one or more of the plant hormones jasmonic acid (JA), ethylene (ET) and salicylic acid (SA). Upon attack the levels of these hormones are usually enhanced. Such enhancement depends on the attacker that is trying to invade the plant, and each of the hormones act in the resistance against a specific set of pathogens. For instance, basal resistance against the oomyceteous pathogen *Peronospora parasitica* or the viral pathogen turnip crinkle virus was reduced in Arabidopsis

mutants affected in pathogen-induced biosynthesis of SA. In contrast, it was not reduced in mutants impaired in JA- or ET-signaling (Delaney et al., 1994; Thomma et al., 1998; Nawrath and Métraux, 1999; Kachroo et al., 2000; Ton et al., 2002c). Conversely, JA- and ET-signaling mutants showed enhanced susceptibility to the fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea*, whereas SA-signaling mutants did not (Thomma et al., 1998; Thomma et al., 1999). Furthermore, ET-insensitive tobacco and Arabidopsis plants have been shown to be more susceptible to a range of necrotizing pathogens, e.g. *B. cinerea, Pythium* spp. and *Fusarium* spp. (Geraats et al., 2002). Some pathogens are resisted by a SA-dependent mechanism, as well as JA- and ET-dependent mechanisms, e.g. *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and *Xanthomonas campestris* pv. *armoraciae* (Delaney et al., 1994; Pieterse et al., 1998; Ton et al., 1998; Ton et al., 2002a). Certain pathogens are resisted by the hormones acting sequentially, e.g. *Xanthomonas campestris* pv. *vesicatoria* in tomato (O'Donnell et al., 2003). In this interaction, JA, ET and SA are required in succession. These results clearly demonstrate the intertwining of several signal transduction pathways in basal resistance. Moreover, it shows that the roles of different hormones vary depending on the plant-pathogen interaction.

Within plant species, individuals can vary in their level of resistance to a certain pathogen, with some individuals being fully resistant. Such resistance can usually be described by a gene-for-gene relationship. A pathogen carrying a specific avirulence (*AVR*) gene is recognized only by a plant carrying a corresponding resistance (*R*) gene. In such an incompatible interaction, the pathogen usually triggers a rapid and local defense response that leads to a hypersensitive reaction. The hypersensitive reaction is often regarded as a form of programmed cell death that is dependent on e.g. reactive oxygen species. The resulting small necrotic lesion is involved in preventing the pathogen from spreading any further (Dangl et al., 1996). Furthermore, deposition of anti-microbial compounds, strengthening of cell walls and expression of defense-related genes in the vicinity of the lesion all contribute to the restriction of the pathogen (Hammond-Kosack and Jones, 1996).

Inducible Disease Resistance

Biological and chemical induction of disease resistance

During evolution plants have developed sophisticated defensive strategies to perceive pathogen attack and to translate this perception into an appropriate adaptive response. When under attack, a plant is capable of

enhancing its resistance, and this condition is often referred to as induced, or acquired, resistance. Acquired disease resistance is thought to involve an enhancement of basal resistance (Van Loon, 2000; Ton et al., 2002a). A classic example of biologically induced disease resistance is triggered after attack by a necrotizing pathogen (Ross, 1961). This attack renders distant, uninfected plant parts more resistant towards a broad spectrum of virulent pathogens (Kuc, 1982; Ryals et al., 1996; Sticher et al., 1997). The onset of this enhanced resistance, known as systemic acquired resistance (SAR), is accompanied by a local and systemic increase in SA levels (Malamy et al., 1990; Métraux et al., 1990). Subsequently, a large set of SAR genes, including genes encoding pathogenesis-related (PR) proteins, is up-regulated (Ward et al., 1991; Van Loon, 1997; Van Loon and Van Strien, 1999). These *PR* genes are often considered as marker genes for SAR expression. Several PR proteins possess anti-microbial activity and are thought to contribute to the state of resistance attained. The PR proteins can accumulate to levels from 0.3 up to 1% of the total protein content of the leaf (Lawton et al., 1995).

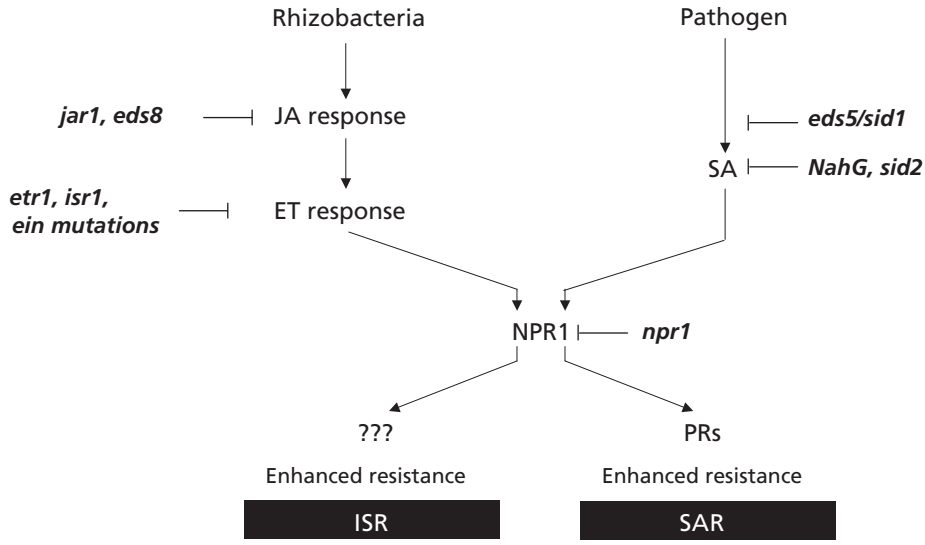

Figure 1: Schematic representation of the signal transduction pathways leading to ISR and SAR, including the putative positions of different mutants as postulated by Pieterse et al. (1998) and Ton et al. (2002c).

A large variety of chemicals have been shown to activate the SAR response. Benzothiadiazole (BTH), 2,6-dichloroisonicotinic acid (INA), and SA induce the same set of *PR* genes comparable to biologically-induced SAR (Ward et al., 1991; Uknes et al., 1992; Lawton et al., 1996). Using parsley cells as a model, SAR induction by the commercially available plant activator

BION and the biological agent Milsana, as well as a large group of related chemicals, was demonstrated (Siegrist et al., 1998).

A second, well-studied biologically-induced disease resistance occurs after root colonization by selected strains of non-pathogenic *Pseudomonas* spp. (Van Peer et al., 1991; Wei et al., 1991). This type of resistance is generally called rhizobacteria-induced systemic resistance (ISR, Pieterse et al., 1996; Pieterse et al., 1998). ISR has been demonstrated in different plant species against several pathogens under conditions where the rhizobacteria and the pathogen remained spatially separated (Van Loon et al., 1998; Pieterse et al., 2001; Pieterse et al., 2002; Pieterse et al., 2003). The expression of rhizobacteria-mediated ISR was shown to be independent of the presence of SA or enhanced *PR* gene expression. Phenotypically, rhizobacteria-mediated ISR resembles pathogen-induced SAR. Although the terms SAR and ISR are synonymous (Hammerschmidt et al., 2001), for convenience we distinguish between pathogen- and rhizobacteria-induced resistance by using the term SAR for the pathogen-induced type and ISR for the rhizobacteria-induced type of resistance. The non-protein amino acid ß-aminobutyric acid (BABA) appears to have a different mode of action. Like ISR, it induces resistance without the expression of *PR* genes (Cohen and Gisi, 1994; Zimmerli et al., 2001). However, BABA can induce resistance to *P. parasitica* in Arabidopsis independent of SA, JA or ET. On the other hand, BABA-induced resistance against the bacterial pathogen *Pst* DC3000 and the fungal pathogen *B. cinerea* is SA dependent (Zimmerli et al., 2000; Zimmerli et al., 2001).

Systemic acquired resistance

After local infection by a necrotizing pathogen leading to an HR, the enhanced state of resistance extends systemically into the uninfected plant parts. During the onset of SAR, a locally altered transcriptional response precedes the hypersensitive reaction and a second wave of transcriptional reprogramming, not apparent in a virulent attack, marks the transition from basal to induced resistance (de Torres et al., 2003). The induction of SAR is accompanied by local and systemic accumulation of endogenous levels of the plant hormone SA, followed by *PR* gene expression (Malamy et al., 1990; Métraux et al., 1990). The importance of the accumulation of SA for the expression of SAR was demonstrated by using transgenic NahG plants. These plants express the bacterial salicylate hydroxylase *nahG* gene, making them incapable of accumulating SA (Gaffney et al., 1993). NahG plants do not show a SAR response (Ryals et al., 1996). Exogenous application of SA, or one of its functional analogs BTH or INA, leads to the full expression of

SAR. Likewise, SA production-deficient mutants *sid1* (also called *eds5*) and *sid2* (also known as *eds16*) do not show a SAR response after infection with a necrotizing pathogen (Figure 1) (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999; Wildermuth et al., 2001). These results indicate that SA is necessary and sufficient for the induction of SAR. In tobacco, up to 70% of the SA accumulating in non-infected leaves may originate from the infected leaves (Shulaev et al., 1995). The remaining SA is produced *de novo* in stems and petioles in response to a mobile signal (Smith-Becker et al., 1998). Even though SA is transported, grafting experiments between tobacco wild-type and NahG plants showed that SA is not the transported signal responsible for the systemic nature of SAR. However, SA is needed in non-infected tissues to effectively express SAR (Vernooij et al., 1994; Willits and Ryals, 1998). As shown recently, ET perception is required to generate the systemic signal, but is not needed for the response to the systemic signal leading to SAR (Verberne et al., 2003). In stead, a putative apoplastic lipid transfer protein (DIR1) was characterized, which is thought to interact with a lipid-derived molecule to promote long distance signaling during SAR (Maldonado et al., 2002).

Transduction of the SA signal into *PR* gene expression and enhanced resistance requires the regulatory protein NPR1 (Cao et al., 1994). Mutant *npr1* plants accumulate normal levels of SA after pathogen attack, but are incapable of transducing this SA accumulation into a response leading to *PR* gene expression and SAR. Moreover, treatment of *npr1* with SA or its analogue INA does not lead to induction of resistance. These results indicate that NPR1 is acting downstream of SA in the SAR signal transduction pathway (Figure 1) (Cao et al., 1994). Since the discovery of the *NPR1* gene, several mutant screens based on impaired SAR expression (Delaney et al., 1995), reduced SA-induced *PR* gene expression (Shah et al., 1997), or enhanced disease susceptibility (Glazebrook et al., 1996) identified mutations allelic to the *npr1* mutation. These results further illustrate the importance of the *NPR1* gene in SAR. The *NPR1* gene encodes a protein with a BTB/POZ (for Broad-complex, Tramtrack and Bric-a-brac/Poxyvirus and Zinc fingers) (Harrison and Travers, 1990; DiBello et al., 1991; Koonin et al., 1991; Godt et al., 1993) and an ankyrin-repeat domain (Cao et al., 1997; Ryals et al., 1997; Aravind and Koonin, 1999). Both domains have previously been shown to be important in protein-protein interactions (Bork, 1993). In non-induced plants, NPR1 is present as a multimer through forming intermolecular disulfide bonds (Mou et al., 2003). During the induction of SAR, SA triggers a biphasic change in cellular redox potential that leads to reduction of the disulfide bonds. This results in conversion of NPR1 into a monomeric form. These monomers are translocated into the nucleus (Kinkema et al., 2000), where they interact with members of the TGA/OBF subclass of basic-leucine-zipper (bZIP) transcription factors. These transcription factors are involved in

SA-dependent activation of *PR* genes (Lebel et al., 1998; Zhang et al., 1999; Després et al., 2000; Niggeweg et al., 2000; Zhou et al., 2000; Subramaniam et al., 2001; Fan and Dong, 2002; Zhang et al., 2003). A direct interaction between NPR1 and specific TGA transcription factors is required for binding of the complex to elements within the promoter of the *PR* genes (Després et al., 2000; Fan and Dong, 2002). Overexpression of the *NPR1* gene leads to an enhanced resistance to pathogen attack (Cao et al., 1998; Friedrich et al., 2001).

Rhizobacteria-induced disease resistance

SA- or stress-dependent enhancement of resistance

Plants produce exudates and lysates at their root surface, where rhizobacteria are attracted in large numbers (Lynch and Whipps, 1991; Lugtenberg et al., 2001; Walker et al., 2003) . Selected strains of non-pathogenic rhizobacteria are named plant growth-promoting rhizobacteria (PGPR), because they possess the capability to stimulate plant growth (Kloepper et al., 1980; Pieterse and Van Loon, 1999; Bloemberg and Lugtenberg, 2001; Pieterse et al., 2003). *Pseudomonas* spp. are among the most effective PGPR. Moreover, they have been shown to be responsible for the reduced activity of soil-borne pathogens in disease-suppressive soils (Raaijmakers and Weller, 1998; Weller et al., 2002; Duff et al., 2003). This type of biological control can be the result of competition for nutrients, siderophore-mediated competition for iron, antibiosis or the production of lytic enzymes (Bakker et al., 1991; Van Loon and Bakker, 2003). Apart from such direct antagonistic effects on soil-borne pathogens, some PGPR strains are also capable of reducing disease incidence in above-ground plant parts through plant-mediated mechanisms. Under iron-limiting conditions, certain rhizobacterial strains produce SA as an additional siderophore (Meyer et al., 1992; Visca et al., 1993). The enhanced defensive capacity elicited by *Pseudomonas fluorescens* CHA0 in tobacco might be fully explained by the bacterial production of SA, which could elicit a SAR response. Treatment of tobacco roots with CHA0 triggers accumulation of SA-inducible PR proteins in the leaves (Maurhofer et al., 1994). Moreover, transformation of the SA-biosynthetic gene cluster of CHA0 into *Pseudomonas fluorescens* P3 improved the systemic resistance inducing-capacity of this strain (Maurhofer et al., 1998).

Another strain that has been suggested to elicit a SA-dependent enhanced defensive capacity is *Pseudomonas aeruginosa* 7NSK2. A SA-deficient mutant of this bacterium failed to induce resistance in bean and tobacco (De Meyer and Höfte, 1997). Moreover, 7NSK2 was unable to induce resistance in NahG tobacco plants against TMV (De Meyer et al., 1999). A SA overproducing

mutant of 7NSK2 was shown to trigger the SA-dependent SAR pathway by producing SA at the root surface (De Meyer and Höfte, 1997). Recently, however, Audenaert et al. (2002) showed that the secondary siderophore pyochelin and the antibiotic pyocyanin are the determinants for the enhanced resistance induced by wild-type 7NSK2. SA is an intermediate in the formation of pyochelin and the combination of pyocyanin and pyochelin is toxic to root cells, thereby setting off the SAR response.

General stresses can also induce similar responses. Cartieaux et al. (2003) performed a transcriptome analysis of Arabidopsis roots and shoots upon colonization of the roots by *Pseudomonas thivervalensis* (strain MLG45). MLG45 induced a clear growth reduction under the conditions used (Persello-Cartieaux et al., 2001; Cartieaux et al., 2003), suggesting that systemic changes in gene expression observed were due to a more general stress response. A significant increase in defense-related transcripts was detected prior to challenge inoculation, whereas very few changes in the transcriptome of roots were apparent, suggesting that the systemic changes in gene expression observed in MLG45-induced plants are primarily related to stress-induced reduction of plant growth.

Bacterial-plant interactions involved in ISR

Other strains of fluorescent *Pseudomonas* spp. have been shown to trigger ISR in an SA-independent manner (Pieterse and Van Loon, 1999). SA-independent ISR has been shown in Arabidopsis (Van Wees et al., 1997; Iavicoli et al., 2003a; Ryu et al., 2003), cucumber (Wei et al., 1991), tobacco (Press et al., 1997; Zhang et al., 2002), radish (Leeman et al., 1995a), and tomato (Yan et al., 2002). This wide range of induction of ISR indicates that the ability of these *Pseudomonas* strains to activate a SA-independent pathway controlling systemic resistance is common to a broad range of plants. ISR-inducing rhizobacteria show little specificity in their colonization of roots of different plant species (Van Loon et al., 1998). In contrast, the ability to induce ISR appears to be dependent on the bacterium/host combination. For instance, *Pseudomonas fluorescens* WCS374r is capable of inducing ISR in radish, but not in Arabidopsis (Leeman et al., 1995a; Van Wees et al., 1997). Conversely, Arabidopsis is responsive to *Pseudomonas putida* WCS358r, while radish is not (Van Peer et al., 1991; Van Peer and Schippers, 1992; Leeman et al., 1995a; Van Wees et al., 1997). *Pseudomonas fluorescens* WCS417r is capable of inducing ISR in both Arabidopsis and radish (Van Wees et al., 1997), as well as in other species, i.e. carnation (Van Peer et al., 1991), radish (Leeman et al., 1995a), tomato (Duijff et al., 1998), and bean (Bigirimana and Höfte, 2002).

Besides differences in inducibility between species, there can also be differences within species. Arabidopsis accessions Columbia (Col-0) and

Landsberg *erecta* (L*er*-0) are responsive to ISR induction by WCS417r, while accessions Wassilewskija (Ws-0) and RLD1 are not (Van Wees et al., 1997; Ton et al., 1999; Ton et al., 2001; Pieterse et al., 2002). Apparently, these accessions are compromised in a step between the recognition of the bacterium and expression of ISR. Moreover, these data indicate that ISR is genetically determined. Up until now, several compounds have been implicated in the elicitation of ISR (Van Loon et al., 1998; Bakker et al., 2003). Apart from live WCS417r bacteria, also dead bacteria are capable of inducing ISR, indicating that bacteria do not need to be metabolically active to induce ISR. Cell wall preparations, purified lipopolysaccharides, siderophores, antibiotics, and flagella all are capable of inducing systemic resistance (Van Peer and Schippers, 1992; Leeman et al., 1995b; Van Wees et al., 1997; Bakker et al., 2003; Iavicoli et al., 2003b). Because of there multiple inducing determinants, bacterial mutants lacking flagella or the O-antigenic side chain of the lipopolysaccharides were still able to elicit ISR in Arabidopsis (Van Wees et al., 1997; Bakker et al., 2003). So far, putative receptors for the bacterial cell wall preparations have not been isolated. However, a sensitive perception mechanism for bacterial flagellins has been identified (Felix et al., 1999). A receptor kinase was characterized that shares homology with known plant disease resistance genes (Gomez-Gomez and Boller, 2000), suggesting that bacteria are recognized similar to plant pathogens.

Range of ISR effectiveness

The plant-growth promoting WCS417r (Pieterse and Van Loon, 1999) has been shown to induce resistance in Arabidopsis against a broad range of pathogens (Van Loon et al., 1998; Pieterse et al., 2002; Pieterse et al., 2003). This makes ISR phenotypically comparable to pathogen-induced SAR (Kuc, 1982). Like classic pathogen-induced SAR, colonization of roots by WCS417r leads to ISR against different types of pathogens, including the fungal root pathogen *F. oxysporum* f.sp. *raphani*, the oomycetous leaf pathogen *P. parasitica*, and the bacterial leaf pathogens *X. campestris* pv. *armoraciae* and *Pst* DC3000(Pieterse et al., 1996; Van Wees et al., 1997; Ton et al., 2002a). In contrast to SAR, treatment of Arabidopsis with WCS417r does not lead to systemic resistance against turnip crinkle virus. Conversely, ISR appears to be highly effective against the fungal pathogen *A. brassicicola*, whereas SAR is not (Ton et al., 2002a). The spectra of effectiveness of ISR and SAR partly overlap, but are also clearly divergent. This indicates a different mechanism of rhizobacteria-mediated ISR compared to the classic SAR response. This different mode of action was further apparent from the simultaneous activation of the ISR and SAR pathways. This results in an enhanced level of protection against *Pst* DC3000, compared to each treatment alone (Van Wees et al., 2000).

Rhizobacteria-mediated ISR: signal transduction

The differential range of effectiveness of ISR, compared to SAR, indicates the involvement of different mechanisms. Pathogen-induced SAR is accompanied by both local and systemic increases in SA levels and the expression of *PR* genes (Van Loon and Van Strien, 1999; Métraux, 2001). Compelling evidence that rhizobacteria-mediated ISR is a SA-independent defense response came from experiments with SAR-compromised NahG plants. Upon colonization of the roots by ISR-inducing WCS417r bacteria, Arabidopsis NahG plants expressed normal levels of ISR (Pieterse et al., 1996; Van Wees et al., 1997). Furthermore, WCS417r-induced radish plants did not accumulate PR proteins in association with their enhanced defensive capacity against fusarium wilt disease (Hoffland et al., 1995). Moreover, WCS417r-induced, ISR expressing Arabidopsis plants showed enhanced resistance against *Pst* DC3000 and *F. oxysporum* f.sp. *raphani*, without activation of the SAR marker genes *PR-1, PR-2,* and *PR-5* (Pieterse et al., 1996; Van Wees et al., 1997), and analysis of SA levels revealed no changes after ISR induction, neither locally nor systemically (Pieterse et al., 2000). Taken together, it was concluded that WCS417r-mediated ISR in Arabidopsis is regulated by a SA-independent signaling pathway.

Further studies revealed that treatment of the roots with WCS417r failed to trigger ISR in JA-insensitive *jar1* plants or ET-insensitive *etr1* plants. This indicated that the JA- and ET-response pathways are essential for the establishment of ISR (Figure 1) (Pieterse et al., 1998; Pieterse et al., 2000). Another indication for the involvement of the JA-signaling pathway came from the analysis of Arabidopsis mutant *eds8*, which was previously shown to exhibit enhanced susceptibility to *P. syringae* (Glazebrook et al., 1996). This mutant is impaired both in WCS417r-mediated ISR (Ton et al., 2002c), and JA-signaling (Ton et al., 2002a; Glazebrook et al., 2003). To further elucidate the role of ET in the ISR signaling pathway, a large set of well-characterized ET-signaling mutants was analyzed. None of these mutants showed an ISR response against *Pst* DC3000 after colonization of the roots by WCS417r (Knoester et al., 1999). These results confirmed that an intact ET-signaling pathway is required for the establishment of ISR. Particularly interesting was the analysis of the *eir1* mutant, which is ET-insensitive in the roots, but not in the shoot. This *eir1* mutant is incapable of showing ISR after root colonization by WCS417r. In contrast, after leaf infiltration with WCS417r, it did show ISR indicating that responsiveness to ET is required at the site of induction (Knoester et al., 1999). However, these results do not exclude the possibility that the ET response is also needed during the expression of ISR upon challenge inoculation.

Further evidence for the involvement of the ET-response pathway came from the identification of the Arabidopsis *ISR1* locus (Ton et al., 1999). Genetic analysis of the progeny of a cross between the WCS417r-responsive ecotype Col-0 and the ISR-impaired ecotype RLD1 revealed that both the potential to express ISR and basal resistance against *Pst* DC3000 are monogenic, dominant traits that are genetically linked. The *ISR1* locus is required for effective ISR against several different pathogens (Figure 1) (Ton et al., 2002b). Interestingly, analysis of the ISR-impaired ecotype RLD1 revealed that it has a reduced sensitivity to ET, which co-segregates with the *ISR1* locus (Ton et al., 2001). These results strongly indicate that the Arabidopsis *ISR1* locus encodes a novel component in the ET-signal transduction pathway that is important in both basal resistance and ISR in Arabidopsis.

Both at the site of application of the bacteria and systemically in the leaves, the JA content and the ET evolution remained unaltered upon ISR induction (Knoester et al., 1999; Pieterse et al., 2000). Moreover, *LOX2* co-suppressed S-12 plants, which are blocked in the increase of JA after wounding (Bell et al., 1995) and pathogen infection (Pieterse et al., 2000), were normally responsive to WCS417r. Nevertheless, resistance comparable to ISR can be induced chemically by application methyl jasmonate (MeJA) or the ET precursor 1-aminocyclopropane-1-carboxylate (ACC). MeJA induction of resistance was blocked in the *jar1* and *etr1* mutants. In contrast, the ACC-induced expression of resistance was blocked in *etr1*, but not in *jar1*. Therefore, it was postulated that WCS417r-mediated ISR is regulated by a signaling pathway with the requirement for JA-signaling preceding the ET-signaling response (Figure 1) (Pieterse et al., 1998).

To determine whether ISR is associated with increased JA- or ET-responsive gene expression, the expression of a large set of well-known JA- and ET-responsive genes (e.g. *VSP2, PDF1.2, LOX1, LOX2, HEL, CHI-B,* and *PAL*) was analyzed in WCS417r-induced Arabidopsis plants. None of these genes tested showed an up-regulation, neither locally in the roots, nor systemically in the leaves (Van Wees et al., 1999). These results indicate that the enhanced defensive state during WCS417r-mediated ISR is not associated with enhanced JA- or ET-dependent gene expression. Because ISR is not associated with enhanced levels of the hormone signals JA or ET (Pieterse et al., 2000), but nevertheless requires responsiveness to JA and ET (Pieterse et al., 1998), it was suggested that ISR is based on an enhanced sensitivity to these hormones, rather then on an increase in their production (Figure 1).

Dual role for NPR1 in SAR and ISR

To investigate the possible involvement of the SAR regulatory protein NPR1 in ISR signaling, the Arabidopsis *npr1* mutant was tested in the ISR bioassay. Surprisingly, the *npr1* mutant was incapable of showing WCS417r-mediated ISR (Pieterse et al., 1998). This result clearly showed that WCS417r-mediated ISR, like SA-dependent SAR, is an NPR1-dependent defense response (Figure 1). Further analysis of the ISR signal-transduction pathway revealed that NPR1 acts downstream of the JA- and ET-response pathways (Pieterse et al., 1998). Because SAR is associated with NPR1-dependent *PR* gene expression, and ISR is not, this demonstrates that NPR1 must differentially regulate gene expression, depending on the signaling pathway that is activated upstream of it. NPR1 seems not a limiting factor since simultaneous activation of ISR and SAR leads to an enhanced defensive activity compared to that observed with either type of induced resistance alone (Van Wees et al., 2000). These results suggest that the NPR1 protein is important in regulating and intertwining different hormone-dependent defense pathways.

Apart from its role in SA-dependent gene expression, NPR1 was also shown to be involved in the negative regulation of the JA-response by SA (Spoel et al., 2003). Whereas nuclear localization of NPR1 is needed for SA-induced *PR-1* gene expression (Kinkema et al., 2000), the SA-induced, NPR1-mediated negative effect on JA-responsive gene expression is exerted through an as yet uncharacterized function in the cytosol (Spoel et al., 2003). These results indicate that NPR1 can have different functions at different locations in the cells.

Gene Expression Analysis

Northern blotting

Routinely, expression of individual genes is analyzed by northern blotting (Abraham and Eikhom, 1975). The mRNA of differently treated plants is subjected to electrophoresis and subsequently blotted onto a membrane. A cDNA or RNA probe is made based on the sequence of the gene of interest and is radioactively labeled. The probe is hybridized to the membrane and the amount of radioactivity retained after washing is a measure for the amount of mRNA of that specific gene.

Differential display reverse transcriptase PCR

In the last decade, enormous developments have occurred in the analysis of gene expression. Now it is possible to analyze the expression of many genes simultaneously. This landscape of gene expression is also referred to as an expression profile. One of the earliest techniques developed to determine transcript patterns was the differential display reverse transcriptase polymerase chain reaction technique (DDRT-PCR) (Liang and Pardee, 1992). DDRT-PCR is a method in which mRNA from differently treated plants is converted into cDNA. After a PCR amplification step with random and specific oligo-dT primers, PCR products are separated on an agarose gel. Bands present in one sample, but not in another, can be excised and sequenced. In this way, genes with specific expression patterns can be identified. A problem is the high number of false positives, resulting in the necessity to verify the expression patterns by other methods, such as northern blotting.

By using DDRT-PCR, Lange et al. (1999) isolated a receptor-like protein kinase from common bean (*Phaseolus vulgaris* L. cv. Saxa) that was induced 9 and 24 hr after infection with the virulent pathogen *Fusarium solani* f.sp. *phaseoli*. This result demonstrates the great advantage of the DDRT-PCR method, namely that new gene sequences can be identified from plant species that are genetically not very well accessible. In this example, the isolated receptor-like protein kinase (RLPK) appears to be a new type of a previously undiscovered class of kinases. The transcript levels did not increase after infection with several symbionts. These results suggest that the receptor could be important in the differential reaction between symbionts and antagonistic micro-organisms. The DDRT-PCR method was also used to identify an ABC transporter important in general defense responses of tobacco to *Pst* (Sasabe et al., 2002). This illustrates the strong potential of this method to identify genes whose expression is altered after certain treatments, such as pathogen attack.

cDNA amplified fragment length polymorphisms

In order to analyze chromosomal DNA patterns on a large scale, Vos et al. (1995) developed a PCR-based technology for mapping genomic DNA, called amplified fragment length polymorphisms (AFLP). This AFLP technique was subsequently adapted for mRNA expression analysis after conversion of the mRNA to cDNA, and was called cDNA-AFLP (Bachem et al., 1996). In principle, the technique consists of four steps. Firstly, the mRNA isolated from plants with different treatments is reversely transcribed into cDNA. Secondly, the cDNA is digested and oligonucleotide adapters are ligated. Thirdly, selective sets of restriction fragments are amplified

with specific primers. Fourthly, the PCR products are separated on agarose gels and the patterns in the different treatments are compared. Bands with specific expression patterns are then isolated and sequenced. One of the great advantages of the cDNA-AFLP method is that it is possible to analyze expression patterns of genes with unknown sequences on a large scale (Aubry et al., 2003). Another advantage is the possibility to analyze the expression patterns of low abundance mRNAs.

The cDNA-AFLP method was used in an attempt to isolate genes from the obligatory biotrophic oomycete *P. parasitica* that are expressed during infection of Arabidopsis (Van der Biezen et al., 2000). cDNA-AFLP fragments were analyzed from infected and non-infected Arabidopsis leaves. Most of the genes with altered expression in the infected leaves were of *P. parasitica* origin. A disadvantage of the technique is that low abundance mRNAs are not as easily detected as high abundance ones. This could explain why in this latter study mostly *P. parasitica* household genes were found. However, this research did show that with cDNA-AFLP it is possible to detect and study plant- and pathogen-derived genes simultaneously.

Suppression subtractive hybridization

Diatchenko et al. (1996) developed a method to discover rarely expressed transcripts. This technique is called suppression subtractive hybridization (SSH). In short, cDNA populations are made from mRNA of the treatment, called tester cDNA, and of the control treatment, referred to as driver cDNA. The cDNAs are digested, and to the tester cDNA two different adapters are ligated. Next, an excess of driver cDNA is hybridized to the tester cDNA. Transcripts that are present in the tester pool, but not in the driver pool, are then amplified exponentially by PCR. Subsequently, these are sequenced to identify the gene transcripts. Although this technique is prone to false positives, it can be used to isolate low abundance transcripts specific to a certain treatment. Drawbacks of the technique are that it is not quantitative, it cannot be done on a large scale, and confirmation by other techniques like northern blotting is still needed.

Rice seedlings treated with BTH, which induces SAR, have been compared with untreated seedlings by using the SSH method (Song and Goodman, 2002). A phospholipase C gene was discovered that was induced by the BTH treatment. Moreover, this gene appeared to be also induced by SA. Further analysis of the expression pattern of the phospholipase C gene upon treatment with JA or *Pst* revealed that phospholipid signaling might be involved in induced disease resistance in rice. Using the advantage that sequence information is not needed to perform an SSH experiment,

Chinese cabbage infected with the HR-eliciting *Pst* strain 259 was compared to control plants (Ryang et al., 2002). Infection led to the induction of several genes, including *PR1a*, chitinases, a thaumatin-like gene, and *PR-4*. These are all genes that are commonly induced after inoculation with a necrotizing pathogen. Moreover, Ryang et al. (2002) found cytochrome P_{450} genes responsible for the biosynthesis of glucosinolates, as well as several unknown genes. This shows that it is possible to investigate specific gene expression patterns after pathogen attack, without prior knowledge of the genes involved.

DNA microarray technology

Schena et al. (1995) developed a system to investigate the expression of thousands of genes simultaneously. This system is based on spotting single-stranded cDNAs onto a microscope slide, called a microarray (Figure 2). Besides using cDNA sequences as starting material, also genomic sequences can be used to create microarrays, such as the Complete Arabidopsis Transcriptome Micro Array (CATMA; http://www.catma.org) (Crowe et al., 2003). Next, mRNA from treated and control plants is isolated, and converted into cDNA. The two pools are labeled with two fluorescent tags with different excitation and emission optima. This makes it possible to differentiate between the two pools of cDNA, even when they are mixed. The two pools are simultaneously hybridized to a microarray slide. For each cDNA element on the microarray, the ratio of the fluorescence emission at two discriminative wavelengths reflects the ratio of the abundance of that sequence in the two original mRNA pools. Since two samples are hybridized to a single microarray, the outcome is always ratio-based, and indicates an up- or down-regulation in the treatment compared to the control (Figure 2). To read a spot, a minimal amount of fluorescence above background is needed, thus, low expression levels cannot be detected with this method.

A dedicated microarray with 2,375 defense-associated and regulatory genes was probed to investigate the expression profile of Arabidopsis plants infected with the incompatible fungal pathogen *A. brassicicola* (Schenk et al., 2000). An increase in the expression of 168 genes, and a decrease in expression of 39 genes was observed. Some of these were previously indicated to be involved in defense, but many had no previously described function. Because plant responses to pathogens are mostly controlled by pathways requiring the signaling molecules SA, JA, or ET, also SA-, JA-, and ET- dependent gene expressions were compared. Large numbers of genes appeared to be co-regulated in several hormone treatments. Among other observations, co-regulation of SA-dependent and JA-dependent genes was found, whereas

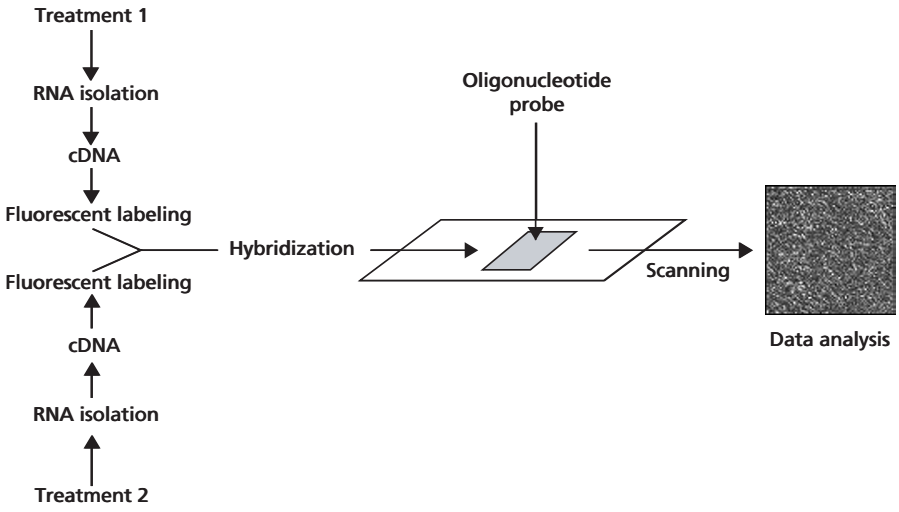

Figure 2: Schematic representation of a microarray experiment. In short, RNA is isolated from control plants (treatment 1) as well as appropriately treated plants (treatment 2). The two pools of mRNA are reverse-transcribed into cDNA and labeled with two different fluorescent labels. Subsequently, the two pools of labeled cDNA are hybridized to the microarray containing cDNA probes attached to a solid carrier. Non-hybridized cDNAs are washed off and the microarray is scanned for the presence of the two different fluorescent labels. This scanning results in a ratio of the two fluorescent labels, which represents the amounts of mRNA present in the two samples. With the Affymetrix GeneChips only one treatment is hybridized to the GeneChip. The result is not a ratio, but a level of fluorescence for each mRNA in a single treatment. The oligonucleotide probes are synthesized on the carrier directly.

previously these two hormones were thought to act only antagonistically (Doares et al., 1995; Schenk et al., 2000). Using large scale microarray analysis, other novel insights in transcriptional changes during environmental stresses such as pathogen attack, wounding and insect feeding was obtained in various research groups (Reymond et al., 2000; Stintzi et al., 2001; Ramonell and Somerville, 2002). Analysis of the expression of 150 genes in mechanically wounded and water stressed Arabidopsis leaves showed a large group of genes with comparable regulation (Reymond et al., 2000). Feeding by the larvae of *Pieris rapae* leads to wounding as well, but results in an expression pattern that is clearly distinct. These results show that Arabidopsis reacts to different treatments with a specific gene expression pattern. The wound and caterpillar-feeding responses were shown to be in part dependent on JA-signaling (Reymond et al., 2000). Further analysis of the JA-signaling pathway revealed that JA-precursors, like 12-oxo-phytodienoate (OPDA) and dinor-OPDA, are involved in regulating the expression of JA-dependent defense genes (Stintzi et al., 2001). Analysis of the JA- and coronatine-insensitive *coi1* mutant of Arabidopsis revealed that the JA response is largely dependent on COI1 but that part of the JA-response is regulated in a COI1-independent

manner (Feng et al., 2003). These results show that the induction of JA and the response to JA are fine-tuned at several stages allowing the plant to react differentially to different JA-inducing stimuli.

Affymetrix GeneChip technology

Another form of microarrays are based on oligonucleotides and these so-called GeneChips are developed by Affymetrix (http://www.Affymetrix.com). The general idea of oligonucleotides on a solid carrier, onto which the samples are hybridized, is the same as for the microarrays described above, except for some minor differences. These GeneChip arrays are produced by synthesis of oligonucleotides directly onto a solid matrix. For each mRNA to be detected, 16 probe pairs of the 25-mer oligonucleotides are synthesized. One set of 16 consists of perfect matches whereas the other set contains a mismatch of the thirteenth nucleotide in each 25-mer. These mismatches are used to assess cross-hybridization and local background signals. Because of these large-scale controls and the proven high reproducibility of these arrays, the Affymetrix GeneChips are hybridized with one fluorescently labeled sample at a time. This results in a relative fluorescence level for each gene, instead of a ratio, which allows comparison of multiple samples across experiments (Zhu et al., 2001).

To create a global view of the transcriptome during the resistance response of Arabidopsis to *P. syringae* pv. *maculicola* strain ES4326 (*Psm* ES4326), a large-scale Affymetrix GeneChip analysis was performed by Glazebrook et al. (2003). Wild-type Arabidopsis plants, as well as 12 different SA-, JA-, or ET-signaling mutants were infected with *Psm* ES4326 and the expression patterns of over 8,000 genes were compared. In addition to the co-regulation between SA- and JA-dependent gene expression observed by Schenk et al. (2000), inhibition between SA- and JA-dependent signaling was also apparent. Moreover, a subset of genes was shown to require JA, ET, as well as an unknown factor for full expression. Comparing global expression patterns of several mutants led to the conclusion that the enhanced disease susceptibility mutant *eds3* is affected in SA signaling, while *eds8* and the phytoalexin deficient *pad1* are compromised in JA-signaling. These results show that GeneChip analysis can be used not only for expression profiling, but also to predict gene function. These results led to a general scheme integrating SA-, JA-, and ET-signaling, as well as an additional signal in the resistance of Arabidopsis against *Psm* ES4326. Whereas *Psm* ES4326 is resisted through the combined action of SA, JA, and ET, resistance of Arabidopsis to *A. brassicicola* is regulated somewhat differently (Van Wees et al., 2003). Mutants affected in phytoalexin production (*pad1, pad2, pad3, pad5*) or JA

signaling (*pad1, coi1*) were found to be more susceptible than wild-type plants and mutants with a defect in SA (*pad4, sid2*) or ET signaling (*ein2*). Wildtype Arabidopsis plants showed drastic changes in 645 genes within 12 hours, which persisted past 36 hours after infection. Of these, 265 required a functional *COI1* gene for full expression. Despite the fact that *A. brassicicola* and *Psm* ES4326 are resisted by different defensive pathways, about half of the *A. brassicicola*-induced genes were also induced by *Psm* ES4326 infection (Glazebrook et al., 2003). The requirement for *COI1* was consistent in both responses. These data suggest that the regulatory effect of *COI1* in plant defenses is similar, regardless of the initial stimulus. Co-regulation between different pathogen-responsive, hormone-dependent signaling pathways was also observed in an analysis of 402 distinct transcription factor genes during several stress responses (Chen et al., 2002). Moreover, Chen et al. (2002) identified a novel highly conserved promoter motif in genes that respond to a broad set of pathogens. The different microarray experiments clearly show that disease resistance is associated with large transcriptional reprogramming and that the different hormone-dependent defense mechanisms are regulated through complex interactions. This complexity is also evident in the interaction-specific hormone signatures in Arabidopsis after attack by a broad range of pathogens and insects (Van Oosten et al., 2004).

Transcriptome analysis of SAR

All the different large-scale analyses of the transcriptome of Arabidopsis show that plants react with major changes to different pathogens, like *Psm* ES4326 and *A. brassicicola*, and stimuli, like JA, the ET precursor ACC, and SA (Schenk et al., 2000; Cheong et al., 2002; Glazebrook et al., 2003; Katagiri and Glazebrook, 2003; Schenk et al., 2003; Tao et al., 2003; Van Wees et al., 2003). Northern blot analysis confirmed a limited set of SAR marker genes (Ward et al., 1991; Ryals et al., 1996). Using a small set of Arabidopsis enhanced sequenced tags (EST's), Schena et al. (1995) made the DNA microarray technology accessible for plant research, making it possible to examine the expression of a large group of genes simultaneously. Since then, and aided by the full sequencing of the Arabidopsis genome (Kaul et al., 2000), many microarray service centers have been established, which now provide a range of different small microarrays up to complete genome arrays (Reymond, 2001). Using a DNA microarray representing about 25% of all Arabidopsis genes, Maleck et al. (2000) monitored gene expression after treatment of wild-type and different SAR-impaired mutants with several different SAR inducers. About 300 (4.3%) out of the 7,000 genes were shown to be involved in the SAR response. These results indicate that expression

of SAR leads to a much larger transcriptional reprogramming than just the changes in *PR* gene expression observed by convential methods. Moreover, Maleck and co-workers (2000) provided evidence for a common promoter element in a set of coordinately regulated genes, including *PR-1*. In addition, they showed that subtle differences in gene expression patterns occur under different SAR-inducing and repressing conditions, indicating that although every treatment leads to SAR, induction is accompanied by additional treatment-specific gene expression. Moreover, gene expression during the SAR response was found to partly overlap with responses observed during a compatible interaction. This paradox is thought to be explained by the fact that during a compatible interaction, host defense responses are turned on, but too slowly or too late to be effective. These results support the idea that acquired resistance is an enhancement of basal resistance, and involves the same resistance mechanisms (Van Loon, 2000).

Outline of this thesis

In the last 15 years, Arabidopsis has evolved into a model plant for investigating molecular and genetic aspects of plant development and plant reactions to various environmental conditions. Arabidopsis was found to be excellently suited for studying a wide variety of plant defense responses (Kunkel, 1996; Dempsey et al., 1999; Dangl and Jones, 2001; Dong, 2001; Glazebrook, 2001; Thomma et al., 2001; Conrath et al., 2002; Pieterse et al., 2002; Dicke et al., 2003). Moreover, important tools, resources and experimental approaches have been developed that have greatly stimulated plant biological research (Somerville and Koornneef, 2002). With the sequencing of the complete Arabidopsis genome in 2000 (Kaul et al., 2000) and the development of large-scale microarrays (Zhu and Wang, 2000; Reymond, 2001; Ramonell and Somerville, 2002), it has become possible to examine gene expression patterns on a large scale. Moreover, gene knockout mutant collections (Parinov et al., 1999; Sessions et al., 2002; Alonso et al., 2003) and other molecular tools have been developed to analyze gene function. Previously, an Arabidopsis-based model was developed that can be used to study rhizobacteria-mediated ISR that is effective against a broad range of pathogens, including *Pst* DC3000 (Pieterse et al., 1996; Pieterse et al., 1998; Pieterse et al., 2002). This model system is used in the work described in this thesis. In the past, the signal transduction pathway of this ISR was partly characterized, but no rhizobacteria-induced gene expression patterns could be revealed. The main goal of this work was to unravel gene expression patterns during the induction and expression of ISR.

Chapter 2 describes the screening of a large number of gene trap and enhancer trap lines for WCS417r-induced gene expression. This resulted in the isolation of an enhancer trap line with WCS417r-induced *GUS* expression in the roots. Further study revealed that this induction occurs upon colonization of the roots by different non-pathogenic *Pseudomonas* spp. strains, but not after colonization by *Escherichia coli*. Moreover, *GUS* expression was also observed after treatment with ACC, but not with JA or SA. Analysis of the flanking sequences revealed that the *GUS* gene was activated in *cis* by the thaumatin-like gene *AtTLP1*, which encodes a pathogenesis-related protein of the PR-5 family. However, analysis of an *AtTLP1* knockout mutant indicated that WCS417r-induced expression of this gene is not required for the expression of ISR against *Pst* DC3000. Moreover, overexpression of the *AtTLP1* gene did not result in a constitutive or enhanced ISR response. These results indicate that *AtTLP1* gene expression is a common response of Arabidopsis roots to non-pathogenic *Pseudomonas* bacteria, but it is unlikely that the AtTLP1 protein contributes to the enhanced defensive capacity observed in ISR-expressing plants.

In Chapter 3, the transcriptome of Arabidopsis during ISR induction and expression is analyzed using Affymetrix GeneChips containing about one third of the genes present in the Arabidopsis genome. Colonization of the roots by WCS417r resulted in changes in expression of a large group of genes locally in the roots. Part of these changes was transient and only visible at a single time point, whereas 97 genes showed consistent changes in time. This group is thought to be involved in the local onset of ISR. Systemically in ISR expressing leaves, prior to pathogen challenge, none of the ~8,000 genes tested showed consistent changes in expression. These observations indicate that the state of ISR, in contrast to SAR, is not associated with detectable changes in gene expression. Gene expression patterns were also determined after challenge inoculation with *Pst* DC3000. In non-induced, infected control plants, a large set of genes showed changes in expression after pathogen challenge. Part of this set is thought to be important for basal resistance against *Pst* DC3000. Also in ISR-expressing plants, a large group of genes showed altered expression levels. This group was slightly smaller in number, perhaps because of the enhanced resistance in ISR-expressing plants. In challenged control plants, these general *Pst* DC3000-responsive genes were shown to be predominantly dependent on JA/ET and SA signal transduction. A group of 81 of these -mainly JA/ET-dependent- genes showed augmented expression in ISR-expressing plants, indicating that these genes were primed to respond faster and/or more strongly upon pathogen attack. These results demonstrate that ISR is associated with potentiation of gene expression. Priming of pathogen-induced genes allows the plant to react more effectively

to the invader encountered, which might explain the broad-spectrum action of rhizobacteria-mediated ISR.

In Chapter 4, knockout mutant analysis of a selected set of genes that was upregulated in WCS417r-treated Arabidopsis roots is described. One knockout mutant, mutated in the *AtMYB72* transcription factor gene, was impaired in its ability to mount ISR, but showed normal responsiveness to induction of SAR. This *AtMYB72* gene was shown to be WCS417r-induced in wild-type plants and SA-deficient NahG transgenics, but not in the ET-insensitive *ein2* mutant. Moreover, expression of the *AtMYB72* gene was up-regulated by the ET precursor ACC but not by MeJA, indicating that, like ISR, up-regulation of *AtMYB72* is ET-dependent. The induction of resistance against *Pst* DC3000 by MeJA, ACC and SA was unaltered in the ISR-impaired knockout mutant. These results indicate that the *AtMYB72* gene is required locally for the onset of ISR, but not systemically for the expression of the enhanced disease resistance. With the discovery of AtMYB72, a novel factor in ISR signaling is identified.

In Chapter 5, the results from previous chapters are discussed with reference to the current knowledge on plant-pathogen interactions, the gene expression patterns involved, and the phenomenon of priming.

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Chapter 2

Nature does nothing uselessly.

Aristotle (384 BC - 322 BC)

Colonization of the Arabidopsis rhizosphere by fluorescent *Pseudomonas* bacteria activates a root-specific, ethylene-responsive *PR-5* gene in the xylem

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Abstract

Plants of which the roots are colonized by selected strains of non-pathogenic, fluorescent *Pseudomonas* bacteria develop an enhanced defensive capacity against a broad spectrum of pathogens. In *Arabidopsis thaliana*, this rhizobacteria-induced systemic resistance (ISR) functions independently of salicylic acid but requires responsiveness to the plant hormones jasmonic acid and ethylene. In contrast to pathogen-induced systemic acquired resistance (SAR), this rhizobacteria-mediated ISR is not associated with systemic accumulation of pathogenesis-related (PR) proteins. To identify genes that are specifically expressed in response to colonization of the roots by ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria, we screened a collection of Arabidopsis enhancer trap and gene trap lines. From the enhancer trap screen, we identified one gene that is specifically expressed in xylem tissue of roots upon colonization by WCS417r. The corresponding gene, *AtTLP1*, encodes a thaumatin-like protein, which belongs to the PR-5 family of pathogenesis-related proteins. Exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) mimicked rhizobacteria-induced expression of *AtTLP1* in the root xylem, suggesting that *AtTLP1* expression is regulated by ethylene. Moreover, fluorescent *Pseudomonas* spp. strains *P. fluorescens* WCS374r and *P. putida* WCS358r triggered a similar expression pattern, whereas ISR-non-inducing *Escherichia coli* bacteria did not. However, *AtTLP1* knockout mutant plants and *AtTLP1*-overexpressing 35S:*AtTLP1* plants showed normal levels of WCS417r-mediated ISR against the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000. These results suggest that activation of the ethylene-responsive *AtTLP1* gene in root xylem tissue is a general response of Arabidopsis plants to rhizosphere-colonizing fluorescent *Pseudomonas* bacteria, and that AtTLP1 is not involved in ISR against *Pseudomonas syringae* pv. *tomato* DC3000 in Arabidopsis.

Introduction

Selected strains of non-pathogenic, root-colonizing bacteria are referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1980). Plant growth promotion results mainly from suppression of soil-borne pathogens and other deleterious micro-organisms (Schippers et al., 1987), but also direct effects on plant growth have been reported (Lynch, 1976; Van Peer and Schippers, 1989). Fluorescent *Pseudomonas* spp. are among the most effective PGPR and have been shown to be responsible for the reduction of soil-borne diseases in naturally disease-suppressive soils (Raaijmakers and Weller, 1998). Selected *Pseudomonas* spp. strains have been selected as biological control

agents and are effective under field conditions (Tuzun and Kloepper, 1995; Wei et al., 1996) and in commercial greenhouses (Leeman et al., 1995b). Disease suppressive activity can be the result of competition for nutrients, siderophore-mediated competition for iron, antibiosis, or the production of lytic enzymes (Van Loon and Bakker, 2003).

Apart from direct antagonistic effects on soil-borne pathogens, some rhizobacterial strains are also able to reduce disease in above-ground plant parts through a plant-mediated mechanism that is commonly referred to as induced systemic resistance (ISR) (Van Loon et al., 1998). Rhizobacteria-mediated ISR has been demonstrated in several plant species, e.g. bean, carnation, cucumber, radish, tobacco, tomato and the model plant *Arabidopsis thaliana*, and is effective against a broad spectrum of plant pathogens, including fungi, bacteria and viruses (Van Loon et al., 1998). Phenotypically, rhizobacteria-mediated ISR resembles classic pathogen-induced resistance, in which non-infected parts of infected plants become more resistant to further infection. This latter form of induced resistance is known as systemic acquired resistance (SAR) (Ross, 1961; Ryals et al., 1996; Sticher et al., 1997).

Pseudomonas fluorescens strain WCS417r has been shown to trigger ISR in several plant species, e.g. carnation (Van Peer et al., 1991), radish (Leeman et al., 1995a), tomato (Duijff et al., 1998), bean (Bigirimana and Höfte, 2002) and Arabidopsis (Pieterse et al., 1996; Pieterse et al., 2002). Colonization of Arabidopsis roots by WCS417r protects the plant systemically against different types of pathogens, including the bacterial leaf pathogens *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Xanthomonas campestris* pv. *armoraciae*, the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani*, the fungal leaf pathogen *Alternaria brassicicola*, and the oomycetous leaf pathogen *Peronospora parasitica* (Pieterse et al., 1996; Van Wees et al., 1997; Ton et al., 2002a). The spectrum of effectiveness of WCS417r-mediated ISR and pathogen-induced SAR overlaps, but is also partly divergent. For instance, ISR is effective against *A. brassicicola*, whereas SAR is not. Conversely, SAR is effective against turnip crinkle virus (TCV), whereas SAR is not (Ton et al., 2002a). In some cases, the level of induced protection can be enhanced further when both ISR and SAR are activated simultaneously (Van Wees et al., 2000), indicating that the effects of both types of induced resistance are additive.

It has been established that the signal transduction pathways of rhizobacteria-mediated ISR and pathogen-induced SAR are distinct. The state of SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA) (Malamy et al., 1990; Métraux et al., 1990) and the concomitant activation of a set of so-called SAR genes (Ward et al., 1991). Many SAR genes encode pathogenesis-related proteins (PRs) (Van Loon and Van Strien, 1999), some of which have been shown to possess anti-fungal activity and are thought to contribute to the induced resistant state. Arabidopsis mutants affected in SA

production, and transgenic NahG plants that express the bacterial salicylate hydroxylase (*nahG*) gene, are incapable of developing SAR and do not show SAR gene activation upon pathogen infection, indicating that SA is a necessary intermediate in the SAR signaling pathway (Gaffney et al., 1993; Wildermuth et al., 2001; Nawrath et al., 2002). Transduction of the SA signal requires the function of the regulatory protein NPR1 (also known as NIM1 or SAI1) (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Interaction of NPR1 with the b-ZIP transcription factor TGA2 is required for activation of the SA-regulated gene *PR-1*, suggesting that NPR1 acts by altering the activity of transcription factors (Fan and Dong, 2002).

Some rhizobacteria trigger the SA-dependent SAR pathway by producing SA at the root surface (Maurhofer et al., 1998; De Meyer et al., 1999). However, WCS417r induces normal levels of protection against *Pst* strain DC3000 in Arabidopsis genotypes that are impaired in SA accumulation (i.e. NahG, *eds5*, *sid2*) (Pieterse et al., 1996; Pieterse et al., 2002; Ton et al., 2002b). Moreover, WCS417r-mediated ISR in wild-type Arabidopsis plants is not accompanied by activation of the SAR marker genes *PR-1*, *PR-2*, and *PR-5* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Thus, this type of induced resistance is SA-independent. Analysis of the jasmonic acid (JA)-response mutant *jar1-1*, a range of ethylene-response mutants, and the SAR-compromised mutant *npr1-1* revealed that components of the JA- and the ethylene-response are required for triggering WCS417r-mediated ISR, but that like SAR, this induced resistance response depends on NPR1 (Pieterse et al., 1998; Knoester et al., 1999). However, downstream of NPR1, the ISR and the SAR signaling pathways diverge, because unlike SAR, ISR is not accompanied by the concomitant activation of *PR* genes (Pieterse et al., 1996; Van Wees et al., 1997; Van Wees et al., 1999). Apart from WCS417r, other fluorescent *Pseudomonas* spp. strains have also been shown to induce SA-independent ISR in Arabidopsis (Van Wees et al., 1997; Iavicoli et al., 2003; Ryu et al., 2003), tobacco (Press et al., 1997; Zhang et al., 2002) and tomato (Yan et al., 2002), indicating that the ability to trigger an SA-independent pathway controlling systemic resistance is not uncommon among ISR-inducing rhizobacteria.

In Arabidopsis, both JA and ethylene activate specific sets of defense-related genes (Schenk et al., 2000) and, when applied exogenously, each of both can induce resistance (Pieterse et al., 1998; Van Wees et al., 1999). To investigate whether ISR is associated with changes in JA/ethylene-responsive gene expression, Van Wees et al. (1999) monitored the expression of a set of well-characterized JA- and/or ethylene-responsive genes (i.e. *LOX1*, *LOX2*, *VSP, PDF1.2, HEL*, *CHI-B*, and *PAL1*) in Arabidopsis plants expressing WCS417r-mediated ISR. None of these genes was up-regulated in induced plants, neither locally in the roots, nor systemically in the leaves. This suggested that the resistance attained was not associated with major increases in the levels

of either JA or ethylene. Indeed, analysis of JA and ethylene levels in leaves of ISR-expressing plants revealed no changes in the production of these signal molecules (Pieterse et al., 2000). Therefore, it had to be assumed that the JA and ethylene dependency of ISR is based on an enhanced sensitivity to these hormones, rather than on an increase in their production.

Although rhizobacteria-mediated ISR was not associated with changes in defense-related gene expression (Van Wees et al., 1999; Pieterse et al., 2002), ISR-expressing plants do possess an enhanced defensive capacity against a broad spectrum of pathogens. Therefore, plants must possess as yet undiscovered factors that are responsible for the broad-spectrum resistance associated with ISR. To identify novel defense-related genes that are potentially involved in rhizobacteria-mediated ISR, we screened a collection of gene trap and enhancer trap lines with transposable elements of the *Ac/Ds* system carrying the β-glucuronidase (*GUS*) reporter gene (Sundaresan et al., 1995; Vroemen et al., 1998), for genes that are specifically expressed in response to colonization of the roots by ISR-inducing WCS417r bacteria. Here, we identified a thaumatin-like gene (*AtTLP1*) that is specifically expressed in the root xylem tissue after colonization by fluorescent *Pseudomonas* bacteria, and after treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). The putative thaumatin-like protein shows high homology to members of the PR-5 group of pathogenesis-related proteins. The role of this *AtTLP1* gene in ISR was investigated in *AtTLP1* overexpressing transgenics and *AtTLP1* knockout mutant plants.

Results

Screening transposants for P. fluorescens *WCS417r induced* GUS *expression*

In total 200 gene trap (WGT) transposants and 400 enhancer trap (WET) transposants were examined for *GUS* expression patterns in roots and leaves after colonization of the roots by ISR-inducing WCS417r bacteria. The gene trap lines were constructed to detect expression of a chromosomal gene with a transposon insertion within the transcribed region. To this purpose, the *Ds* element contained a promoterless *GUS* gene, whose expression relies on the transcription from the inserted chromosomal gene (Sundaresan et al., 1995; Vroemen et al., 1998). The enhancer trap lines contained a *Ds* element with the *GUS* gene fused to a minimal -1 to -46 bp CaMV 35S promoter, which is not active in the absence of enhancer sequences. In these lines, expression of the *GUS* reporter gene is dependent on insertion near chromosomal enhancer sequences (Sundaresan et al., 1995; Vroemen et al., 1998). Thus, when the *Ds*

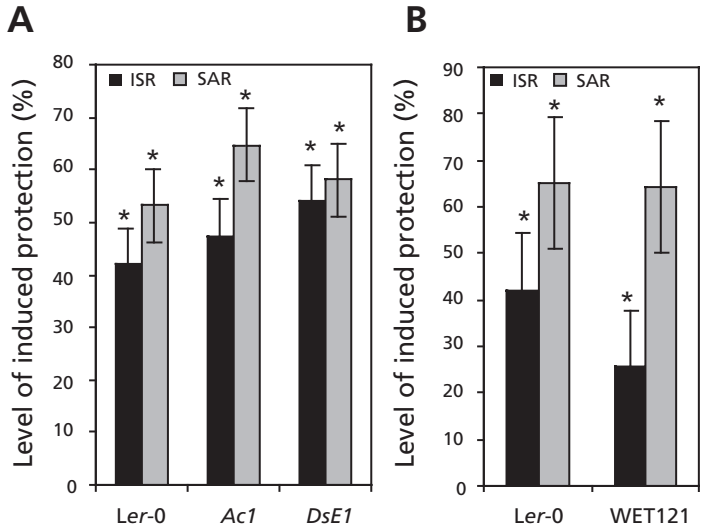

Figure 1. Levels of induced protection against *Pst* DC3000 as a result of *P. fluorescens* WCS417r-mediated ISR and *Pst* DC3000(*avrRpt2*)-induced SAR in wild-type L*er*-0 plants, the starter lines *Ac1* and *DsE1* (A) and enhancer trap line WET121 (B).

ISR was induced by growing plants for 3 weeks in soil containing ISR-inducing *P. fluorescens* WCS417r bacteria at 5 x 10^7 cfu.g^{-1}. Five-week-old plants were challenge inoculated with a bacterial suspension of virulent *Pst* DC3000 at 2.5 x 10^7 cfu.ml^{-1}. Induction of SAR was performed 3 days before challenge inoculation by pressure infiltrating three lower leaves with a suspension of *Pst* DC3000(*avrRpt2*) bacteria at 10^7 cfu.ml^{-1}. Four days after challenge inoculation, the percentage of diseased leaves was determined and the level of induced protection calculated on the basis of the reduction in the percentage of diseased leaves per plants relative to challenged control plants. Asterisks indicate statistically significant differences compared to non-induced, *Pst* DC3000-challenged control plants (students *t*-test: α =0.05; n=20). Data presented are means from a representative experiment that was repeated at least twice with similar results. Error bars represent standard errors.

element of an enhancer trap line is inserted in the proximity of a chromosomal gene, within or outside the coding sequence, *GUS* gene expression can be activated.

The WGT and WET transposants were constructed in the L*er*-0 background, an accession previously shown to develop ISR after colonization of the roots by WCS417r (Van Wees et al., 1997; Ton et al., 1999). Before screening the transposants, starter lines *Ac1* and *DsE1* (Vroemen et al., 1998) were checked for their ability to express induced resistance against the bacterial pathogen *Pst* DC3000 using standard ISR and SAR bioassays (Pieterse et al., 1996). Figure 1A demonstrates that both starter lines were able to express levels of WCS417r-mediated ISR and *Pst* DC3000(*avrRpt2*)-induced SAR that are similar to those observed in wild-type L*er*-0 plants, indicating that the ability to express induced resistance was not affected in these lines.

To screen transposants for *GUS* expression patterns, 2-week-old seedlings of the WGT and WET lines were transplanted into soil with or without

ISR-inducing WCS417r bacteria. Systemic protection in ISR-expressing plants results from the continuous stimulation of the plant by ISR-inducing rhizobacteria at the root surface. In Arabidopsis, full expression of WCS417r-mediated ISR in the leaves can be detected within 7 days after treatment of the roots with this rhizobacterial strain (Ton et al., 2002a). All lines were harvested 4 and 11 days after induction treatment and examined for *GUS* expression patterns. Of the WGT lines, approximately 20% showed *GUS* expression, whereas approximately 35% of the WET lines stained for GUS activity. This is in good agreement with the percentages found by Vroemen and co-workers (1998). Only enhancer trap line WET121 displayed a *GUS* expression pattern that was specifically induced by the WCS417r treatment. In this line, GUS activity was apparent in the roots by 4 days after colonization by WCS417r bacteria (Fig. 2A). Interference contrast microscopy revealed that WCS417r-induced GUS activity was localized in xylem tissue (Fig. 2C). No GUS activity was detected in leaves or reproductive organs (data not shown), suggesting that the observed WCS417r-induced *GUS* expression was root specific.

Specificity of rhizobacteria-induced GUS *expression in WET121*

Development of rhizobacteria-mediated ISR is dependent on the plant/rhizobacterium combination, suggesting that recognition of the rhizobacteria by the plant is involved in the induction of ISR. Previously, rhizobacterial strains *P. fluorescens* WCS374r and *Pseudomonas putida* WCS358r were demonstrated to trigger ISR in radish and Arabidopsis, respectively (Leeman et al., 1995a; Van Wees et al., 1997). To investigate whether these strains also activate *GUS* expression in WET121, they were applied to the roots of WET121 plants that were grown on vertical agar plates. ISR-non-inducing *Escherichia coli* bacteria were used as a control. Figures 2D-F show that WCS358r and WCS374r induced a similar *GUS* expression pattern in WET121 as did WCS417r. Application of *E. coli* bacteria did not result in *GUS* expression, indicating that physical colonization of the roots by bacteria is not sufficient to activate the *GUS* reporter gene in WET121. Apparently, specific components of the *Pseudomonas* bacteria are required for the activation of the gene.

Chemical induction of GUS *expression in WET121*

WCS417r-mediated ISR in Arabidopsis functions independently of SA but requires responsiveness to the plant hormones JA and ethylene (Pieterse et al., 1996; Pieterse et al., 1998). To investigate whether *GUS* expression in

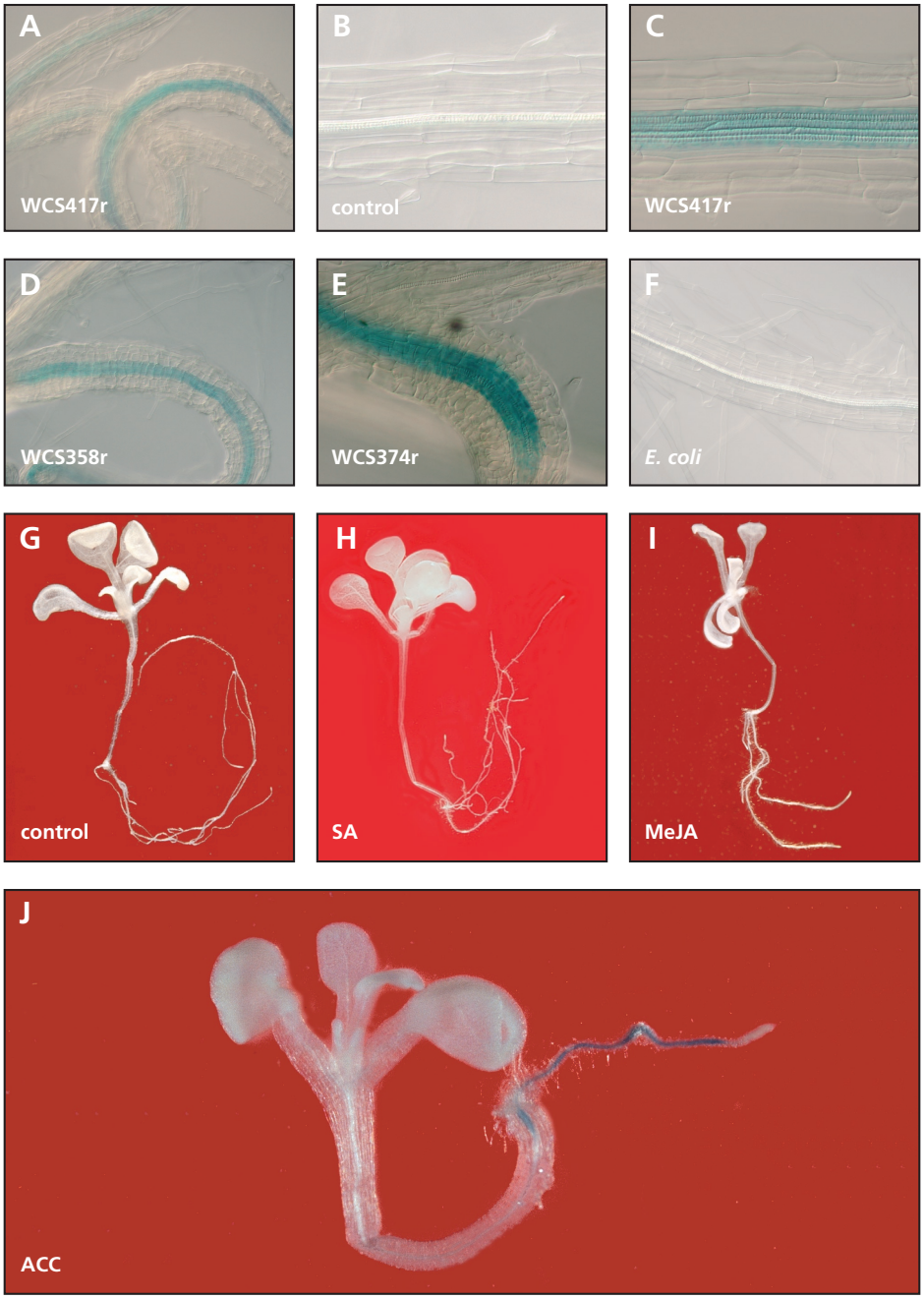

Figure 2. GUS expression patterns in enhancer trap line WET121.

Pictures show light microscopic images (A, D-F), interference contrast microscopic images (B-C), or photographs (G-J) of (A+C) roots grown in soil with *P. fluorescens* WCS417r; (B) roots grown in soil without rhizobacteria; (D) roots grown on MS-agar with *P. putida* WCS358r; (E) roots grown on MS-agar with *P. fluorescens* WCS374r; (F) roots grown on MS-agar with *E. coli* S17; (G) seedling grown on MS-agar; (H) seedling grown on MS-agar with 0.1 mM SA; (I) seedling grown on MS-agar with 0.01 mM MeJA; and (J) seedling grown on MS-agar with 0.1 mM ACC.

WET121 is regulated also by these signals, seedlings were grown on MS-agar containing either 0.01 mM methyl jasmonate (MeJA), 0.1 mM of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), or 0.1 mM SA. The concentrations used were previously demonstrated to activate JA-, ethylene-, or SA-responsive gene expression, respectively, and to induce resistance against *Pst* DC3000 (Van Wees et al., 1999). Figures 2G-I show that MeJA and SA did not induce *GUS* expression in WET121 seedlings. However, when incubated in the presence of ACC, WET121 seedlings showed a pronounced *GUS* expression pattern similar to that in rhizobacteria-treated WET121 plants (Fig. 2J). These results indicate that the *Ds* element in WET121 is inserted in the vicinity of a rhizobacteria-inducible, ethylene-responsive gene.

Molecular analysis of WET121

To identify the gene activated in response to rhizobacteria and ACC, the number of *Ds* elements in the genomic DNA of WET121 was determined. Genomic DNA of WET121 and wild-type L*er*-0 plants was digested with *Pst*I, which cuts once in the sequence of the *Ds* element outside of the *GUS* gene. Southern blot analysis using the *GUS* coding sequence as a probe indicated that WET121 carried a single copy of the *Ds* element (Fig. 3A).

To determine the location of the *Ds* element in the genome of WET121, genomic DNA flanking the *Ds* insertion was amplified by thermal asymmetric interlaced (TAIL) PCR, essentially as described previously (Liu et al., 1995; Vroemen et al., 1998). Figure 3B shows that TAIL-PCR resulted in specific PCR products in the secondary and tertiary round of TAIL-PCR. The PCR products in the tertiary round (1.5 kb with AD2 and 0.3 kb with AD3) were shorter then those in the secondary round and were conform the predicted size reductions, indicating that the differences in length correlated with the predicted size reductions and that the flanking sequences of the *Ds* element in WET121 were amplified successfully. Sequencing of the flanking DNA revealed that the *Ds* element was inserted into chromosome 4 (Fig. 3C). As is the case for most of the enhancer trap lines (Vroemen et al., 1998), the *Ds* element was not inserted into an open reading frame. Thus, the rhizobacteria- and ACC-induced *GUS* expression was the result of the activation of (an) enhancer sequence(s) in the vicinity of the *Ds* element. Candidate genes in close proximity to the *Ds* element were a gene encoding a thaumatin-like protein (*AtTLP1*; At4g24180), a gene encoding a kinesin heavy chain-like protein (*KHCLP*; At4g24170), and *SHEPHERD* (At424190) encoding a HSP90 type protein (Ishiguro et al., 2002).

Because the *Ds* element in WET121 was not inserted in, or in the immediate vicinity of an open-reading frame, it was expected that WET121 would show normal ISR in response to colonization of the roots by WCS417r. Figure 1B shows that indeed, WET121 developed normal levels of ISR against *Pst* DC3000.

GUS *and* AtTLP1 *are co-regulated in WET121*

Insertions in enhancer trap lines result in *GUS* expression when the *Ds* element is inserted either upstream or downstream of an active gene. Enhancers are known to act in an orientation-independent manner. Thus, insertions in

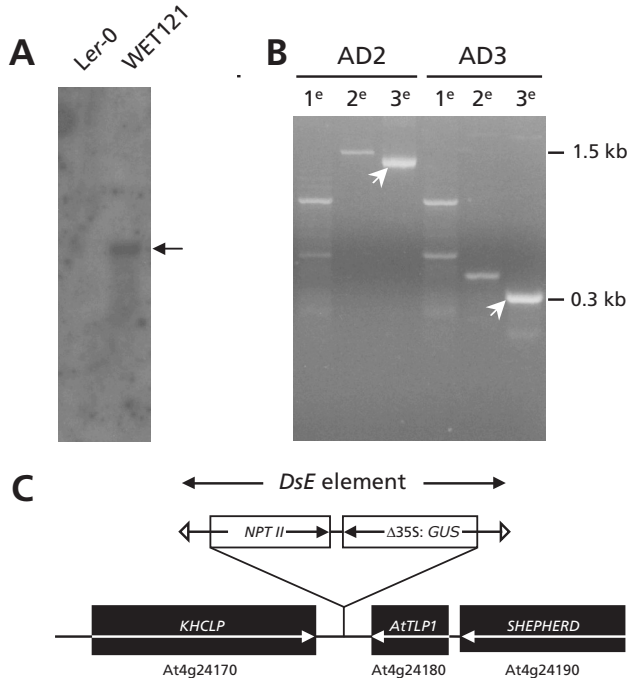

Figure 3. Molecular analysis of enhancer trap line WET121.

(A) Southern blot of genomic DNA from L*er*-0 and WET121. The DNA was digested with *Pst*I, blotted and hybridized with the entire *GUS* coding sequence. (B) Ethidium bromide-stained agarose gel showing the TAIL-PCR products after the primary (1^e), the secondary (2^e), and the tertiary (3^e) round of TAIL-PCR with arbitrary degenerate primers AD2 and AD3, and nested *Ds* specific primers Ds5-1 (1^e), Ds5-2 (2^e), and Ds5-3 (3^e). In the secondary round of TAIL-PCR a smaller PCR product appeared with both the AD2 and the AD3 primer combinations. These specific PCR products were further amplified in the tertiary round of TAIL-PCR, yielding PCR products of ca. 1.5 and 0.3 kb in length. Sequencing of these TAIL-PCR products revealed the flanking sequences of the *Ds* element insertion in WET121. (C) Schematic representation of the flanking sequences of the *Ds* element in WET121. Genes in the vicinity of the *Ds* element insertion encode a kinesin heavy chain-like protein (KHCLP), a thaumatin-like protein (AtTLP1) and the HSP90 type protein SHEPHERD.

either orientation can result in *GUS* expression (Vroemen et al., 1998). To identify the gene that is responsible for the WCS417r- and ACC-induced activation of the *GUS* gene in WET121, the expression of *SHEPHERD*, *AtTLP1*, and *KHCLP* in control and ACC-treated WET121 plants was analyzed. RNA gel blot analysis showed that *SHEPHERD* is expressed in roots and leaves of control- and ACC-treated plants (Fig. 4A). This was incompatible with the ACC-induced *GUS* expression pattern observed in WET121 and disqualified *SHEPHERD* as a candidate gene for co-regulation of the *GUS* gene.

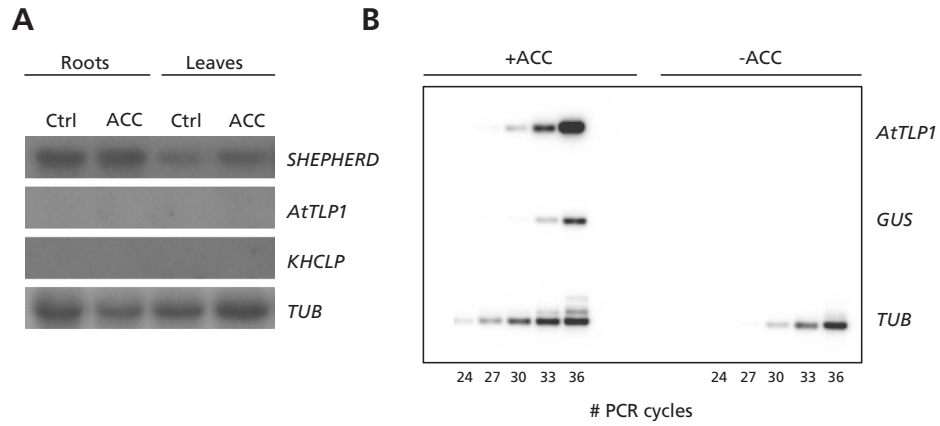

Figure 4. Expression analysis of genes flanking the *Ds* element insertion in WET121

Seedlings of WET121 were grown vertically on MS-agar plates for 12 days. Subsequently, 2 ml of water (Ctrl) or a solution containing 1 mM ACC was added onto the plates. Six days later, roots and leaves were harvested separately for RNA extraction. (A) RNA gel blot analysis of *SHEPHERD*, *AtTLP1*, and *KHCLP* mRNA in roots and leaves of WET121 plants grown with or without ACC. The blots were hybridized with gene-specific probes. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β-tubulin (*TUB*). (B) RT-PCR analysis of *AtTLP1*, *GUS*, and *TUB* transcript levels in control- and ACC-treated roots of WET121. Shown are PCR products in the RT-PCR mixture after 24 to 36 PCR cycles.

KHCLP and *AtTLP1* transcripts could not be detected by RNA blot analysis. Therefore, their expression patterns were examined by RT-PCR. Again, *KHCLP* mRNA could not be detected (data not shown). In untreated control plants, no *AtTLP1* mRNA could be detected after 36 rounds of PCR. However, *AtTLP1* transcripts accumulated in the roots upon treatment with ACC, which clearly correlated with the concomitant increase in *GUS* mRNA levels (Fig. 4B). Both *AtTLP1* and *GUS* transcripts could not be detected in control and ACC-treated leaves (data not shown). These results indicate that the induced *GUS* expression pattern observed in the xylem tissue of the roots of WET121 are regulated by an enhancer of the *AtTLP1* gene and, thus,

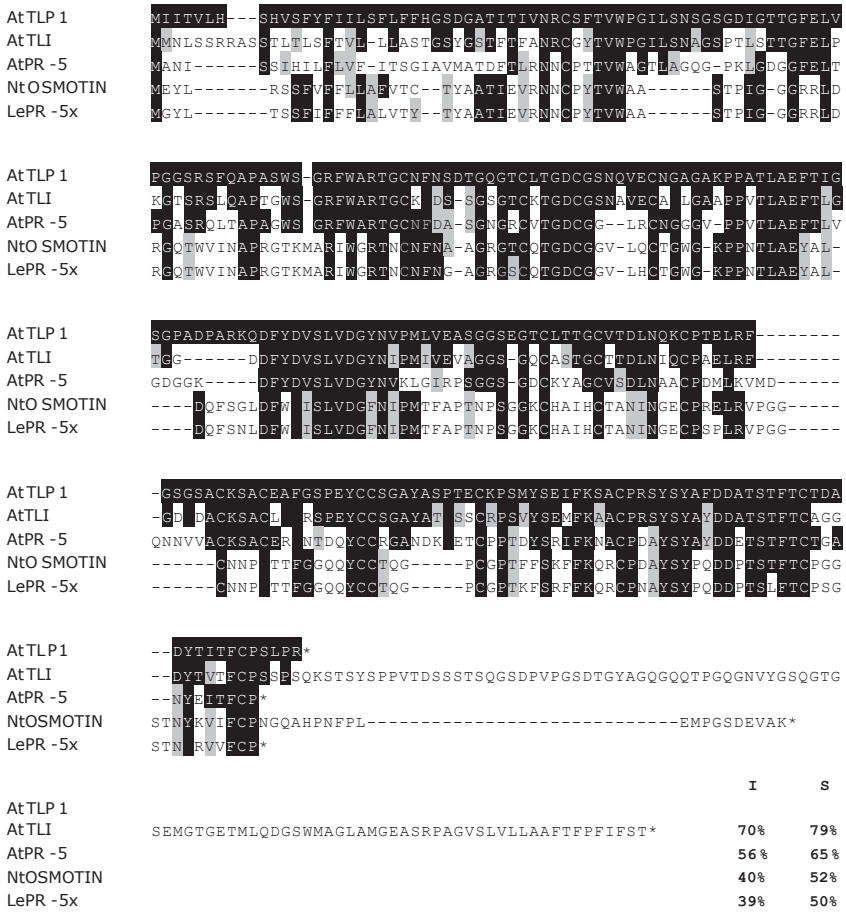

Figure 5. **Comparison of the predicted amino acid sequence of AtTLP1 with other PR-5 type proteins.**

Alignment showing the amino acid sequences of several PR-5 type proteins. Sequences shown are from Arabidopsis AtTLP1 (AGI number At4g24180), Arabidopsis AtPR-5 (AGI number At1g75040; Uknes et al. (1992), Arabidopsis AtTLI (AGI number At4g38660), tobacco NtOSMOTIN (GeneBank accession number X95308; Singh et al. (1989)), and LePR-5x (GeneBank accession number AAM23272; Rep et al. (2002)). Black shading indicates amino acids identical to the AtTLP1 template, and gray shading indicates amino acids that are similar to the AtTLP1 template.

identifies the *AtTLP1* gene as a plant gene that is activated upon colonization by fluorescent *Pseudomonas* bacteria.

AtTLP1 *encodes a PR-5 type protein*

The *AtTLP1* gene encodes a thaumatin-like protein belonging to the pathogenesis related PR-5 family. The putative AtTLP1 protein contains 255 amino acids and has a predicted molecular mass of 26,917 D, a theoretical pI of

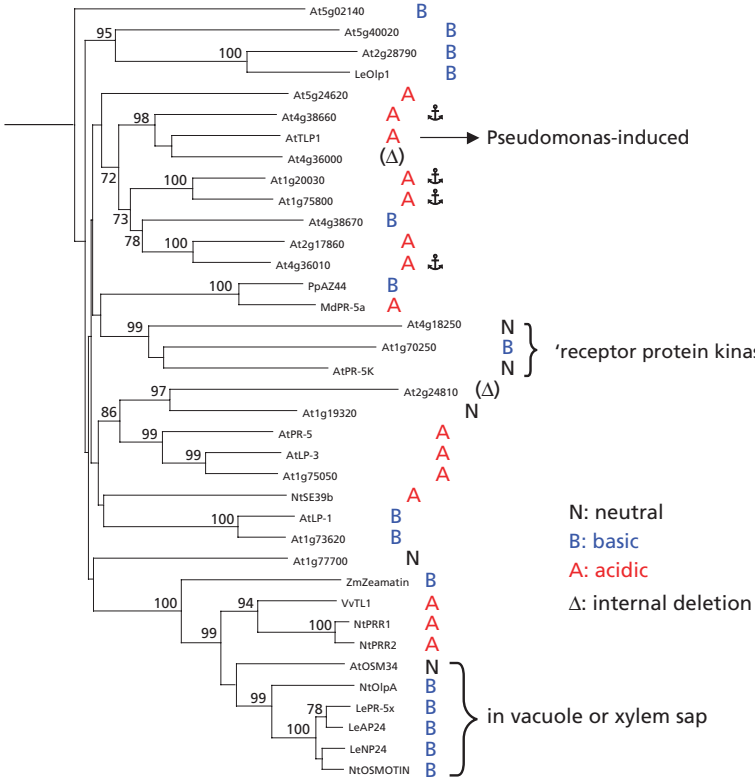

Figure 6. **Phylogenetic tree of Arabidopsis PR-5 proteins and selected PR-5 proteins from other plants.**

All 24 predicted proteins of Arabidopsis with a PR-5 ("thaumatin-like") domain present in The Arabidopsis Information Resource (www.arabidopsis.org) were aligned with selected PR-5 proteins from other plants. Additions to the core PR-5 consensus sequence at N-termini (signal sequences) and C-termini (vacuolar targeting sequences or other extensions beyond the conserved sequence "FCP") were trimmed. This alignment was used to construct a phylogenetic tree using neighbour-joining. A PR-5-like protein from *Caenorhabditis elegans* (accession AAF60831) was used to root the tree. Based on the pI of the core sequences, the proteins were classified as acidic (pI < 5.5, "A"), neutral (5.5 < pI < 7.5, "N") or basic (pI > 7.5, "B"). Two Arabidopsis PR-5 proteins, At4g36000 and At2g24810, were not classified because these have internal deletions (of about 60 residues) relative to all other PR-5 proteins (indicated with "Δ"). Four Arabisopsis PR-5 proteins had long C-terminal extensions (71-100 residues) ending with a stretch of about 20 hydrophobic residues that could serve as a membrane anchor. In the figure, these are indicated with an anchor. Three Arabidopsis proteins that have a central hydrophobic region and a C-terminal protein kinase domain characteristic of receptor protein kinases (Wang et al., 1996) group together based on their PR-5 core sequence.

Published sequences are referred to by protein names: PR-5 (Uknes et al., 1992) , PR-5K (Wang et al., 1996), OSM34 (Capelli et al., 1997), LP-1 (Hu and Reddy, 1995), LP-3 (Hu and Reddy, 1997) from Arabidopsis; Olp1 (Chen et al., 1996), NP24 (King et al., 1988), AP24 (Ruiz-Medrano et al., 1992) and PR-5x (Rep et al., 2002) from tomato; OSMOTIN (Singh et al., 1989), SE39b (Kuboyama et al., 1997), PR-R1 (Payne et al., 1988), and PR-R2 (Cornelissen et al., 1986) from tobacco; Zeamatin (Richardson et al., 1987) from maize; PWIR2 (Rebmann et al., 1991) from wheat; PpAZ44 (Ruperti et al., 2002) from peach ; MdPR-5a (Oh et al., 2000) from apple; VvTL1 (Tattersall et al., 1997) from grape. All sequence names are preceded by species abbreviations (At: Arabidopsis, Le: tomato, Md: apple, Nt: tobacco, Pp: peach, Ta: wheat, Vv: grape, Zm: maize). Bootstrap percentages are provided for branches receiving 70% or more support. Branch length reflects the extent of sequence divergence.

4.69, and one predicted transmembrane domain (http://mips.gsf.de/proj/thal/db/). Comparison of the predicted amino acid sequence with sequences in the databank revealed that it shares highest homology with the predicted amino acid sequence of the thaumatin-like isolog gene *AtTLI* (At4g38660; 70% identity) (Fig. 5). AtTLP1 shares 56% identity at the amino acid level with the previously identified Arabidopsis AtPR-5 protein that is associated with SAR (Uknes et al., 1992), and is homologous to other proteins belonging to the PR-5 family, including tobacco NtOSMOTIN (Singh et al., 1989) (40% identity) and the tomato LePR-5x protein (39% identity), which was found to accumulate in the xylem sap of *F. oxysporum*-infected tomato plants (Rep et al., 2002). The homology with the previously characterized PR-5 type proteins NtOSMOTIN and LePR-5x is relatively low. Figure 6 shows a phylogenetic tree of all 24 Arabidopsis PR-5 type proteins and selected PR-5 type proteins from other plant species. Within this tree, AtTLP1 clusters together with a number of acidic PR-5 type proteins of Arabidopsis that have not been characterized so far.

Effect of AtTLP1 *overexpression*

To investigate whether the induced expression of *AtTLP1* in response to rhizobacteria plays a role in induced resistance, we set out to construct *AtTLP1*-overexpressors with the gene under transcriptional control of the CaMV 35S promoter. Almost all of the primary 35S:*AtTLP1* transformants did not survive to full maturity. Those that did showed an abnormal phenotype in that they developed a significantly higher number of leaves (Fig. 7A). However, most of them died prematurely or didn't set seeds and subsequently got lost, suggesting that overexpression of the *AtTLP1* gene affects normal plant development. One 35S:*AtTLP1* transgenic line survived and set seed. Homozygous T3 seedlings were selected for further study. Compared to wild-type Col-0 plants, 35S:*AtTLP1* plants developed up to 40% more leaves and showed up to 80% higher fresh weight of the rosette (Fig. 7B). Southern blot analysis of 35S:*AtTLP1* genomic DNA showed that the 35S:*AtTLP1* line contained a single transgene (Fig. 7C). Overexpression of the *AtTLP1* gene was verified by RT-PCR using RNA isolated from roots and leaves of non-induced 35S:*AtTLP1* plants. Figure 7D shows that *AtTLP1* transcripts accumulated to high levels in roots and leaves of 35S:*AtTLP1* plants, but not in the non-induced wild-type Col-0 plants.

To investigate the effect of *AtTLP1* overexpression on the effectiveness of WCS417r-mediated ISR, comparative bioassays were performed with Col-0 and 35S:*AtTLP1* plants. Figure 8A shows that a lower percentage of the leaves of non-induced 35S:*AtTLP1* plants developed symptoms after

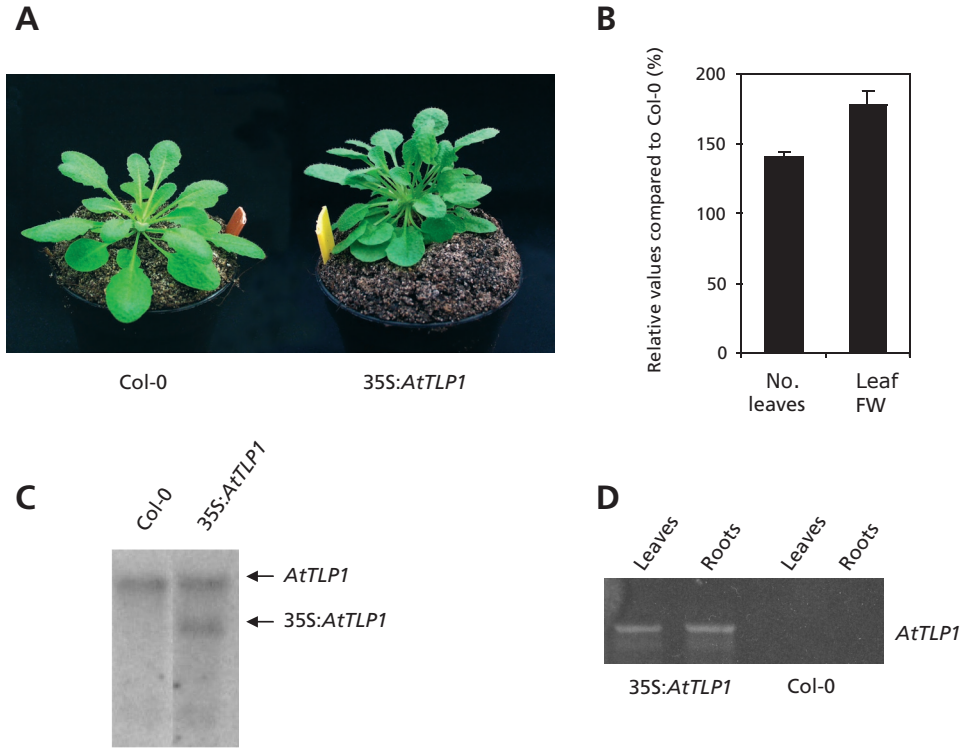

Figure 7. Analysis of the *AtTLP1*-overexpressing line 35S:*AtTLP1*.

(A) Five-week-old wild-type Col-0 and transgenic *35S*:*AtTLP1* plants grown in soil under climate chamber conditions. (B) Analysis of the number of fully-grown leaves and leaf fresh weight (FW) of 5-week-old plants. Shown are the relative values of 35S:*AtTLP1* plants compared to Col-0 plants (set at 100%). (C) Southern blot analysis of genomic DNA of Col-0 and 35S:*AtTLP1*. Genomic DNA was digested with *Hin*dIII. The blot was hybridized with a gene-specific probe of the *AtTLP1* gene, resulting in the detection of the endogenous *AtTLP1* gene (upper band in both Col-0 and 35S:*AtTLP1* genomic DNA) and the 35S:*AtTLP1* transgene (lower band in 35S:*AtTLP1* genomic DNA only). (D) RT-PCR analysis of *AtTLP1* transcript levels in leaves and roots of 35S:*AtTLP1* and Col-0 plants.

challenge inoculation with *Pst* DC3000 then did wild-type plants. However, this difference was not statistically significant and might be caused by altered morphology of the transgenic line. Nevertheless, the WCS417r-induced level of protection in 35S:*AtTLP1* was similar to that observed in Col-0 plants. It can thus be concluded that overexpression of the *AtTLP1* gene does not result in significantly enhanced resistance against *Pst* DC3000, and neither does it affect the effectiveness of WCS417r-mediated ISR against this pathogen.

In wild-type Arabidopsis, *AtTLP1* is induced only in xylem tissues of roots upon colonization by rhizobacteria, and, thus, it may have only a function locally. Therefore, we examined the level of resistance of 35S:*AtTLP1* plants to infection by the vascular wilt fungus *F. oxysporum* f.sp. *raphani*, because 1) WCS417r-mediated ISR is effective against this pathogen in Arabidopsis

(Pieterse et al., 1996), and 2) Rep and co-workers (2002) recently showed that a AtTLP1 homologue of tomato, PR-5x, accumulates in xylem vessels of *F. oxysporum*-infected tomato plants. Four weeks after challenge inoculation, the percentage of leaves with fusarium wilt symptoms progressed up to 66% in 35S:*AtTLP1* plants, whereas in control Col-0 plants only 11% of the leaves had developed symptoms by that time (Fig. 8B). The enhanced severity of fusarium wilt disease in *35S:AtTLP1* plants was reflected by a dramatic reduction in shoot fresh weight (Fig. 8C). These results indicate that overexpression of *AtTLP1* does not lead to enhanced resistance against fusarium wilt disease, but rather results in enhanced susceptibility to the fungus.

Effect of AtTLP1 *knockout mutation*

Previously, Parinov et al. (1999) generated a randomly mutagenized collection of gene trap lines carrying *Ds* element insertions throughout the Arabidopsis genome. This collection contained a knockout mutant line (SGT5141) with a *Ds* element inserted 133 bp upstream of the stop codon of the *AtTLP1* gene, leading to a truncated protein. The presence of the

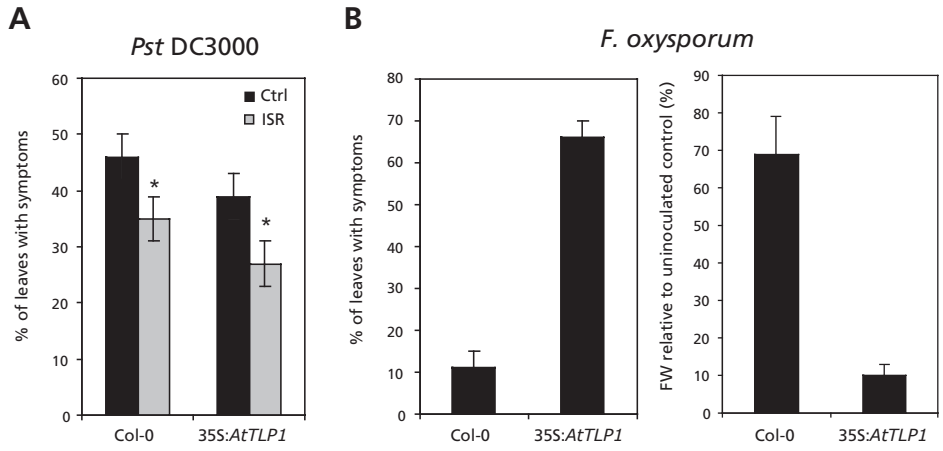

Figure 8. Resistance bioassays with wild-type Col-0 and transgenic 35S:*AtTLP1* plants and the pathogens *Pst* DC3000 and *F. oxysporum* f. sp. *raphani*.

(A) Quantification of induced resistance as a result of *P. fluorescens* WCS417r-mediated ISR in Col-0 and 35S:*AtTLP1* plants. Shown are percentages of leaves with symptoms 4 days after challenge with *Pst* DC3000. For details see legend to Figure 1. (B) Severity of fusarium wilt disease in Col-0 and 35S:*AtTLP1* plants 4 weeks after inoculation of 2-week-old seedlings with *F. oxysporum* f.sp. *raphani*, expressed as the percentage of leaves with fusarium wilt symptoms (left panel) and shoot fresh weight (FW; right panel) relative to the non-inoculated controls.

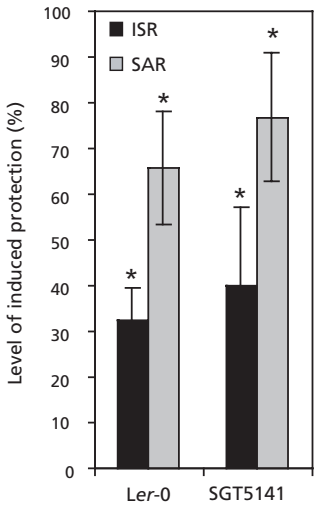

Figure 9. Levels of induced protection against *Pst* DC3000 as a result of *P. fluorescens* WCS417r-mediated ISR and pathogen-induced SAR in L*er*-0 and the *AtTLP1* knockout mutant SGT5141. For details see legend to Figure 1.

insertion was verified as described for the WET121 line (data not shown). To examine the effect of this *AtTLP1* knockout mutation on the effectiveness of WCS417r-mediated ISR, ISR bioassays were performed with wild-type L*er*-0 and mutant SGT5141 plants. For comparison, the effectiveness of pathogen-induced SAR was also examined. Figure 9 shows that SGT5141 developed levels of SAR similar to wild-type L*er*-0. Moreover, SGT5141 showed the same level of ISR after colonization of the roots by WCS417r. Thus the *Ds* insertion within the *AtTLP1* gene had no effect on the ability of SGT5141 to be induced by WCS417r and to express ISR.

Discussion

Colonization of the roots of Arabidopsis by selected strains of fluorescent *Pseudomonas* bacteria leads to an enhanced defensive capacity that is effective against a broad range of foliar pathogens (Pieterse et al., 2002). In contrast to pathogen-induced SAR, rhizobacteria-mediated ISR is not associated with induced expression of *PR* genes (Pieterse et al., 1996; Van Wees et al., 1997; Pieterse et al., 1998; Van Wees et al., 1999). In search for genes that are specifically activated during ISR, we used a *Ac*/*Ds*-based gene/enhancer trap system, developed according to Sundaresan et al. (1995) by Vroemen and co-workers (1998), to detect genes that are specifically expressed in response to colonization of the roots by ISR-inducing WCS417r bacteria. Based on GUS activity staining, we identified the thaumatin-like gene *AtTLP1* that is specifically expressed in root xylem tissue of Arabidopsis upon colonization by fluorescent *Pseudomonas* spp. strains WCS417r, WCS358r, and WCS374r, but not after colonization of the roots by ISR-non-inducing *E. coli* bacteria. Previously, WCS417r and WCS358r have been shown to trigger ISR in Arabidopsis (Van Wees et al., 1997), suggesting that induced *AtTLP1* expression is associated with ISR. However, strain WCS374r, which consistently induces ISR in radish (Leeman et al., 1995a), but shows variable

results in Arabidopsis (Van Wees et al., 1997; Ran et al., 2000), activated the *AtTLP1* gene as well. These results did not allow a conclusion concerning the importance of the *AtTLP1* gene in the onset or expression of ISR. Experiments with *AtTLP1*-overexpressing transgenics and knockout mutant plants revealed that these genotypes develop normal levels of WCS417r-induced ISR against *Pst* DC3000. Therefore, it must be concluded that rhizobacteria-induced *AtTLP1* gene expression is unlikely to play any role in ISR against *Pst* DC3000, but instead must be regarded as a common response of Arabidopsis to non-pathogenic *Pseudomonas* bacteria.

Previously, Hase et al. (2003) described another response of Arabidopsis to *Pseudomonas* bacteria that is unrelated to their ability to induce ISR. Leaves of plants of which the roots were colonized by WCS417r, WCS358r, or WCS374r bacteria showed an enhanced capacity to convert the ethylene precursor ACC to ethylene, leading to a potentiated expression of the ethylene-responsive genes *PDF1.2* and *HEL* after treatment of the leaves with 1 mM ACC, and a significantly higher level of ethylene emission after pathogen challenge. This enhanced ACC-converting capacity was also apparent in the ISR-impaired mutants *jar1-1* and *npr1-1* after treatment of the roots with *Pseudomonas* bacteria. Both increases in response to colonization of the roots by non-pathogenic *Pseudomonas* bacteria demonstrate that Arabidopsis plants perceive signals from the rhizobacteria that are present on the root surface, and translate these signals into local and systemic responses. To what extent these rhizobacteria-induced responses contribute to an enhanced defensive capacity remains to be further elucidated.

The *AtTLP1* gene encodes a thaumatin-like protein, which belongs to the evolutionarily conserved class of PR-5 proteins (Van Loon and Van Strien, 1999). A large number of PR-5 proteins from different plant species has been isolated and characterized (Anžlovar and Dermastia, 2003). The amino acid sequences of all PR-5 proteins show similarity to the sweet-tasting protein thaumatin present in the fruits of the tropical shrub *Thaumatococcus danielii* (Van der Wel and Loeve, 1972). Within the family of PR-5 proteins, several subgroups can be distinguished. Members of the acidic subclass, such as the previously identified, SAR-related AtPR-5 protein of Arabidopsis (Uknes et al., 1992), are mostly found in the extracellular space. Members of the basic subclass, such as tobacco NtOSMOTIN (Singh et al., 1987), are usually found in the plant vacuole (Van Loon and Van Strien, 1999; Anžlovar and Dermastia, 2003). Several PR-5 proteins have been shown to possess antimicrobial activity *in vitro* (Woloshuk et al., 1991; Anžlovar and Dermastia, 2003), or *in vivo* (Liu et al., 1994), or appear to be involved in osmotic adaptation (Singh et al., 1987; Anžlovar and Dermastia, 2003). Besides constitutive and tissue-specific *PR-5* genes, many *PR-5* genes are induced upon biotic or abiotic stresses (Van Loon and Van Strien, 1999). The putative AtTLP1 protein shares

significant amino acid sequence homology with other members of the PR-5 family (Figs. 5 and 6). Among the 24 putative PR-5 proteins in Arabidopsis, AtTLP1 clusters together with eight, so far uncharacterized homologues, seven of which are acidic and one basic (Fig. 6). The amino acid sequence homology with the previously characterized acidic AtPR-5 protein (Uknes et al., 1992) is relatively low (56% identity), suggesting that the two proteins may have different functions. This is supported by the fact that the expression of *AtTLP1* is regulated by ethylene (Figs. 2 and 4), whereas AtPR-5 is controlled in a SA-dependent manner (Uknes et al., 1992). *AtTLP1* is specifically activated in the xylem tissue upon colonization of the roots by *Pseudomonas* bacteria. Previously, the tomato PR-5 type protein LePR-5x was found to accumulate in xylem sap of tomato plants after root infection with *F. oxysporum* (Rep et al., 2002). However, in contrast to the putative AtTLP1 protein, LePR-5x is basic and has only low homology with AtTLP1 (39% identity). Thus, although both AtTLP1 and LePR-5x are predicted to act in the xylem, it is not known whether they have a similar function.

What might be the role of induced *AtTLP1* gene expression in roots that are colonized by *Pseudomonas* bacteria? Most of the isolated PR-5 type proteins possess antimicrobial activity in *in vitro* assays, in which they inhibit germination and cause lysis of spores of fungal and oomycete pathogens (Woloshuk et al., 1991; Abad et al., 1996). Although the specific mode of action of PR-5 type proteins is not known, it is thought that they are capable of permeabilizing fungal membranes (Anžlovar and Dermastia, 2003). Because *AtTLP1* is specifically expressed in root xylem tissue, the putative AtTLP1 protein may have a local antimicrobial function. However, experiments with *AtTLP1*-overexpressing transgenics showed that disease provoked by *F. oxysporum* in the vessels was stimulated rather than reduced. However, enhanced susceptibility may reflect the weak physiological status of the *AtTLP1* overexpressor, and is, thus, difficult to compare to wild-type plant in this respect.

Most of the primary 35S:*AtTLP1* transformants died prematurely or did not set seeds. The ones that survived showed a 'bushy' phenotype, indicating that *AtTLP1* overexpression causes morphological and developmental changes. *NtOSMOTIN*-overexpressing tobacco and potato plants did not show any morphological or developmental changes when compared to wild-type plants (Liu et al., 1994), suggesting that the aberrant phenotype of *AtTLP1* overexpressors may be AtTLP1 specific.

Transposons have been used extensively for insertional mutagenesis of several plant species in order to identify and characterize genes conferring a mutant phenotype (Ramachandran and Sundaresan, 2001). A useful aspect of the *Ac/Ds*-transposon based gene/enhancer trap system described by Sundaresan et al. (1995) is that the transposons carry a *GUS* reporter gene

that can respond to *cis*-acting transcriptional signals at the site of integration, thereby facilitating the identification of genes based on their expression pattern, rather than on their mutant phenotype. Another advantage of this system is that it may allow the identification of genes that, due their low or restricted expression patterns, remain undetected in other differential screening methods or DNA microarray analyses. An example of such a gene is described in this study. The *GUS* gene in enhancer trap line WET121, which is activated in *cis* by the *AtTLP1* gene in the vicinity of the *Ds* insertion, was expressed in a tissue-specific manner in the roots in response to colonization. Rhizobacteria-induced expression levels of *AtTLP1* were below the detection limit of Affymetrix GeneChip hybridizations when total RNA from roots was used as template for probe preparations (Chapter 3, supplementary data table). Thus, although screening of the *Ac/Ds*-based gene/enhancer trap system is laborious and time consuming, it can have an added value when compared to other modern methods for gene discovery.

Experimental procedures

Bacterial and fungal cultures

Non-pathogenic, rifampicin-resistant *Pseudomonas fluorescens* strain WCS417r was used for induction of ISR and for screening the enhancer trap and gene trap collection. WCS417r was grown for 24 hr at 28°C on Kings medium B agar plates (King et al., 1954) containing the appropriate antibiotics as described previously (Pieterse et al., 1996). Subsequently, bacteria were collected and resuspended in 10 mM $MgSO_4$ to a density of 10^9 cfu.ml^{-1} (OD_{660}=1.0) before being mixed through the soil. Growth of *Pseudomonas fluorescens* strain WCS374r, *Pseudomonas putida* strain WCS358r, and *Escherichia coli* strain S17 for analysis of GUS expression in WET121 was performed in a similar manner.

An avirulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 carrying the avirulence gene *avrRpt2* (*Pst* DC3000(*avrRpt2*)) (Kunkel et al., 1993) was used for induction of SAR. *Pst* DC3000(*avrRpt2*) bacteria were grown overnight at 28°C in liquid KB supplemented with 25 mg.ml^{-1} kanamycin to select for the plasmid. After centrifugation, the bacterial cells were resuspended in 10 mM $MgSO_4$ to a final density of 10^7 cfu.ml^{-1}. Virulent wild-type *Pst* DC3000 (Whalen et al., 1991), used for challenge inoculations, was cultivated in a similar manner in liquid KB without kanamycin. After centrifugation, the bacterial cells were resuspended in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands) to a final density of 2.5x10^7 cfu.ml^{-1}.

Fusarium oxysporum f.sp. *raphani* strain WCS600 (Leeman et al., 1995b) was grown in Czapek Dox medium on an orbital shaker at 100 to 150 rpm and 24°C for 1 week. Mycelium was removed by filtering through glass wool. The conidia-containing filtrate was centrifuged at 8,000 x *g* for 20 min. The pellet was washed twice, resuspended in 10 mM $MgSO_4$, and adjusted to a concentration of 10^6 conidia per ml.

Plant growth conditions

For induction of ISR or isolation of RNA or DNA from leaves, seeds of wild-type *Arabidopsis thaliana* accessions Landsberg *erecta* (L*er*-0) or Columbia (Col-0), enhancer trap line WET121, starter lines *Ac2* and *DsE3* (Vroemen et al., 1998), *AtTLP1* knockout mutant line SGT5141 (Parinov et al., 1999), and 35S:*AtTLP1* overexpressing plants were sown in quartz sand. WET21, SGT5141, *Ac2* and *DsE3* lines were in the L*er*-0 background, 35S:*AtTLP1* in the Col-0 background. Two-week-old seedlings were transplanted into 60-ml pots containing a sand/potting soil mixture (5:12, v/v) that had been autoclaved twice for 20 min with a 24-hr interval. Prior to transfer of the Arabidopsis seedlings, a suspension of ISR-inducing WCS417r bacteria (10^9 cfu.ml^{-1}) was mixed thoroughly through the soil to a final density of $5x10^7$ cfu.g^{-1} as described previously (Pieterse et al., 1996). Control soil was supplemented with an equal volume of 10 mM $MgSO_4$.

For isolation of RNA from roots, 2-week-old seedlings were laid out horizontally on rock-wool cubes (Rock-wool/Grodan B.V., Roermond, the Netherlands) as described previously (Pieterse et al., 1996; Van Wees et al., 1997). Subsequently, the root systems were covered with 1 ml of a 1:1 (w/v) mixture of talcum powder and either a suspension of ISR-inducing WCS417r in 10 mM $MgSO_4$ (final density $5x10^8$ cfu.g^{-1}) , or a solution of 10 mM $MgSO_4$ as a control. Plants were cultivated in a growth chamber with a 9-hr day (200 µE m^{-2} sec^{-1} at 24 °C) and a 15-hr night (20 °C) cycle at 70% relative humidity. Plants received a modified half-strength Hoagland nutrient solution once a week as described previously (Pieterse et al., 1996).

Bioassays

Induction of ISR was performed by mixing ISR-inducing WCS417r bacteria through the soil as described above. Induction of SAR was performed 3 days before challenge inoculation by pressure infiltrating three lower leaves with a suspension of avirulent *Pst* DC3000(*avrRpt2*) bacteria at 10^7 cfu.ml^{-1} as described previously (Pieterse et al., 1996). For assaying induced resistance,

plants were challenged when 5 weeks old by dipping the leaves for 2 sec in a suspension of virulent *Pst* DC3000 bacteria at $2.5x10^7$ cfu.ml^{-1} in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77. One day before challenge inoculation, the plants were placed at 100% relative humidity. Four days after challenge, 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. From the number of diseased and non-diseased leaves, the disease index was calculated for each plant (n=20). Colonization levels of the rhizosphere of the tested plants by rifampicin-resistant WCS417r bacteria were determined at the end of each ISR bioassay as described (Pieterse et al., 1996).

Bioassays with the fungal root pathogen *F. oxysporum* f.sp. *raphani* were performed essentially as described previously (Geraats et al., 2003). Briefly, 2-week-old seedlings were transferred to soil with or without the pathogen ($5x10^4$ conidia per gram soil). Subsequently, the plants were monitored for fusarium wilt symptoms over a period of 4 weeks as described earlier (Pieterse et al., 1996; Van Wees et al., 1997). At the last time point, fresh weights of the shoots were determined (Geraats et al., 2003).

Gene trap and enhancer trap screening

For the Arabidopsis gene trap and enhancer trap screening, a collection of 200 Wageningen Gene Trap (WGT) lines and 400 Wageningen Enhancer Trap (WET) lines (in L*er*-0 background) was used that was kindly provided by Dr. C.W. Vroemen (Wageningen University, The Netherlands). Construction of the gene trap and enhancer trap lines (Vroemen et al., 1998) was based on transposable elements of the *Ac/Ds* system carrying the *GUS* reporter gene (Sundaresan et al., 1995). For the selection procedure, seeds were surface sterilized with 2% (w/v) sodium hypochlorite in 50% (v/v) ethanol and sown on 0.6% (w/v) agar plates containing 0.46% (w/v) Murashige and Skoog (MS) salts (Duchefa BV, Haarlem, The Netherlands), 1.0% (w/v) sucrose and 50 mg.l^{-1} kanamycin to select for plants with the gene/enhancer trap construct among the heterozygous lines. The plates were incubated in a growth cabinet with an 9-hr day (24 °C) and 15-hr night (20 °C) cycle. After 11-12 days, the seedlings were transferred to soil with or without ISR-inducing WCS417r bacteria as described above. Four and 11 days after transfer to soil, whole seedlings were removed from the soil, rinsed with tap water to remove excess soil particles from the roots, and analyzed for GUS expression.

For histochemical localization of GUS activity, whole plants were immersed in GUS staining solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl ß-D-glucuronide) in 100 mM NaP_i buffer pH 7.0, 10 mM

EDTA, 0.1% Triton X-100 as described (Spoel et al., 2003). After overnight incubation at 37°C, the plants were destained by repeated washes in 70% ethanol and inspected under a stereomicroscope. For differential interference contrast microscopy, the GUS staining solution was complemented with 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide, and the plants were incubated at 37°C for 3 days. After destaining, the plants were cleared by incubation in clearing solution (7 g chloralhydrate, 1 ml glycerol, 2 ml water) for 2–16 hr.

GUS expression in WET121

Homozygous WET121 seeds were surface sterilized and sown on MS-agar plates supplemented with 0.1 mM SA, 0.01 mM MeJA, or 0.1 mM ACC as described (Ton et al., 2002b; Spoel et al., 2003). Plants were grown for 14 days prior to determination of GUS activity. Because ACC inhibits growth, alternatively, seeds were sown on MS-agar plates as described above and after 12 days 2 ml of a solution containing 1 mM ACC was added. GUS expression was determined 6 days after ACC application. MeJA was purchased from Serva Brunschwig (Amsterdam, the Netherlands), ACC from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands) and SA from Mallinckrodt Baker BV (Deventer, the Netherlands).

To determine the effect of bacterial treatments, seeds were sown on 0.7%-agar plates containing half-strength Hoagland nutrients (2 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, and trace elements, pH 7 (Hoagland and Arnon, 1938)) and 10 μM Sequestreen (Ciba Geigy, Basel, Switzerland). The agar plates were incubated vertically to allow development of the root system along the surface of the agar plates. After 14 days, 1 μl of a bacterial suspension containing 10^7 cfu.ml^{-1} of WCS417r was applied at the base of the hypocotyl, resulting in colonization of the whole root system within 24 hr. Four days later, GUS expression was determined as described above. Analysis of GUS expression in response to treatment of the roots with *P. fluorescens* WCS374r, *P. putida* WCS358r, or *E. coli* strain S17 was performed in a similar manner.

Analysis of flanking sequences of the Ds *insertion in WET121*

Isolation of genomic DNA and Southern blot analysis were performed as described previously (Vroemen et al., 1998). To amplify genomic sequences flanking the *Ds* insertion in WET121, TAIL-PCR was performed essentially

as described (Liu et al., 1995; Vroemen et al., 1998). In brief, two arbitrary degenerate (AD) primers, and three specific nested *Ds* primers were used to amplify the *Ds* flanking sequences in a primary, a secondary and a tertiary round of TAIL-PCR. In the consecutive rounds of TAIL-PCR, the positions of the *Ds* primers were chosen closer to the 5'-end of the *Ds* element. This resulted in consecutively smaller PCR products, indicative of a successful amplification of flanking sequences. Primers used were the nested specific primers located on the 5'-end of the *Ds* element: Ds5-1, 5'-CCG TTT ACC GTT TTG TAT ATC CCG-3'; Ds5-2, 5'-CGT TCC GTT TTC GTT TTT TAC C-3'; Ds5-3, 5'-GGT CGG TAC GGA ATT CTC CC-3'; and the arbitrary degenerate (AD) primers AD-2, 5'-NGT CGA (C/G)(A/T)G ANA (A/T)GA A-3', AD-3, 5'-(A/T)GT GNA G(A/T)A NCA NAG A-3'. The products from the tertiairy PCR reaction were separated on an agarose gel, isolated from the gel, cloned into pGEM-T-easy vector (Promega), and sequenced using universal T7 and SP6 sequencing primers.

Alignment software and phylogenetic tree construction

Sequence alignments and phylogenetic tree construction was done with MacVector (Oxford Molecular Group) as described previously (Rep et al., 2002).

RNA gel blot analysis and RT-PCR

Total RNA was extracted by homogenizing frozen tissue in extraction buffer (0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 ml per gr plant tissue). The homogenates were extracted with phenol/chloroform/isoamylalcohol (25:24:1; v/v/v) and the RNA was precipitated using LiCl, as described (Sambrook et al., 1989). Analysis of gene expression in the roots was performed by RT-PCR as described earlier (Pieterse et al., 1998). Analysis of gene expression in the leaves was performed by RNA gel blot analysis. To this end, 15 µg of RNA was denatured using glyoxal and DMSO as described (Sambrook et al., 1989). Subsequently, samples were electrophoretically separated on 1.5% agarose gels and blotted onto Hybond-N^+ membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. RNA blots were hybridized with gene-specific probes as described previously (Pieterse et al., 1998). Templates for the preparation of gene-specific probes were prepared by PCR with the primers described below.

After hybridization with (α^{32}P-dCTP-labelled probes, blots were subjected to autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, The Netherlands) with Quantity One software (BioRad, Veenendaal, the Netherlands).

For detection of the *AtTLP1* transcript, the following gene-specific primers were designed based on the annotated sequences corresponding to AGI number At4g24180: AtTLP1-FOR 5'-AGC ATT GCA TTT GGT AGG AAG TGA-3', AtTLP1-REV 5'-CTA GGC AAG GAA GGG CAA AAA GT-3'. Detection of *KHCLP*, *SHEPHERD*, and *TUB* gene expression was performed in a similar manner with primers based on the annotated sequences corresponding to AGI numbers At4g24170 (*KHCLP*), At4g24190 (*SHEPHERD)* and At5g44340 (*TUB*): SHEPHERD-FOR, 5'-CTG ATG GAT TAC GAA GAC AAA AAG-3'; SHEPHERD-REV, 5'-GTA AAC ACA AAG GAG AAA CAT CAG-3'; KHCLP-FOR, 5'-CTA AAA GCC TAA GTG GAG TGC-3'; KHCLP-REV, 5'-GCG GAG AAG AGA AGA TAT TGG-3' and TUB-FOR 5'-AAT ACG TCG GCG ATT CTC CG-3'; TUB-REV, 5'-CAC AGA CAC TGG ACT TGAC G-3'. For RT-PCR detection of *GUS* mRNA, the following primers were used: GUS-1, 5'-AGACTGTAACCACGCGTCTC-3' and GUS-2, 5'-CCGACAGCAGTTT CATCAATC-3' (Vroemen et al., 1998).

*Construction and analysis of 35S:*AtTLP1

Arabidopsis Col-0 plants were transformed using *Agrobacterium tumefaciens* strain C58 carrying the binary vector pCAMBIA1302 with the cauliflower mosaic virus (CaMV) 35S promotor driving the AtTLP1 gene. Standard molecular cloning techniques were performed essentially as described (Sambrook et al., 1989). *Agrobacterium*-mediated transformation of Arabidopsis Col-0 plants was performed using the floral dip procedure (Clough and Bent, 1998). Southern blot analysis of 35S:*AtTLP1* genomic DNA and RT-PCR of *AtTLP1* mRNA was performed as described above.

AtTLP1 *knockout mutant SGT5141*

Knockout mutant SGT5141 (in L*er*-0 background) was selected from the database of gene trap lines carrying *Ds* element insertions that was previously described (Parinov et al., 1999) and can be accessed via http://www.plantcell.org/cgi/content/full/11/12/2263/DC1/1. The *Ds* element in SGT5141 was found to be inserted 399 bp before the stop codon

of the *AtTLP1* gene, leading to a truncated protein 133 amino acids shorter than the mature AtTLP1 of 255 amino acids. Homozygous SGT5141 plants were tested for their ability to express rhizobacteria-mediated ISR as described above.

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Chapter 3

The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not 'Eureka!' (I found it!) but 'That's funny …'

Isaac Asimov (1920 - 1992)

The transcriptome of rhizobacteria-induced systemic resistance in Arabidopsis

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Abstract

Plants develop an enhanced defensive capacity against a broad spectrum of plant pathogens after colonization of the roots by selected strains of non-pathogenic, fluorescent *Pseudomonas* spp. In *Arabidopsis thaliana*, this rhizobacteria-induced systemic resistance (ISR) functions independently of salicylic acid but requires responsiveness to the plant hormones jasmonic acid and ethylene. In contrast to pathogen-induced systemic acquired resistance (SAR), rhizobacteria-mediated ISR is not associated with changes in the expression of genes encoding pathogenesis-related (PR) proteins. To identify ISR-related genes, we surveyed the transcriptional response of over 8,000 Arabidopsis genes during rhizobacteria-mediated ISR. Locally in the roots, ISR-inducing *Pseudomonas fluorescens* WCS417r (WCS417r) bacteria elicited a substantial change in the expression of 97 genes. However, systemically in the leaves, none of the ~8,000 genes tested showed a consistent change in expression in response to effective colonization of the roots by WCS417r, indicating that the onset of ISR in the leaves is not associated with major changes in gene expression. After challenge inoculation of WCS417r-induced plants with the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000, 81 genes showed an augmented expression pattern in ISR-expressing leaves, suggesting that these genes were primed to respond faster and/or more strongly upon pathogen attack. The majority of the primed genes was predicted to be regulated by jasmonic acid and/or ethylene signaling. Priming of pathogen-induced genes allows the plant to react more effectively to the invader encountered, which might explain the broad-spectrum action of rhizobacteria-mediated ISR.

Introduction

Selected strains of root-colonizing fluorescent *Pseudomonas* spp. have been shown to trigger a plant-mediated resistance response in above-ground plant parts. This type of induced resistance is referred to as rhizobacteria-mediated induced systemic resistance (ISR) (Van Loon et al., 1998). Phenotypically, rhizobacteria-mediated ISR resembles classical pathogen-induced systemic acquired resistance (SAR), in which non-infected parts of locally infected plants become more resistant to further infection (Ross, 1961). *Pseudomonas fluorescens* strain WCS417r has been shown to effectively trigger ISR in several plant species, e.g. carnation (Van Peer et al., 1991), radish (Leeman et al., 1995), tomato (Duijff et al., 1998), bean (Bigirimana and Höfte, 2002) and *Arabidopsis thaliana* (Pieterse et al., 1996; Pieterse et al., 2002). Colonization of Arabidopsis roots by WCS417r protects the plant

systemically against different types of pathogens, including the bacterial leaf pathogens *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000) and *Xanthomonas campestris* pv. *armoraciae*, the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani*, the fungal leaf pathogen *Alternaria brassicicola* and the oomycete leaf pathogen *Peronospora parasitica* (Pieterse et al., 1996; Van Wees et al., 1997; Ton et al., 2002a). The spectrum of effectiveness of WCS417r-mediated ISR and pathogen-induced SAR overlaps, but is also partly divergent. For instance, SAR is effective against turnip crinkle virus (TCV), whereas ISR is not. Conversely, ISR is effective against *A. brassicicola*, whereas SAR is not (Ton et al., 2002a). In the case of *Pst* DC3000, which is affected by both ISR and SAR, the level of induced resistance can be enhanced further when both types of induced resistance are activated simultaneously (Van Wees et al., 2000), indicating that the effects of ISR and SAR are additive.

Although both rhizobacteria-mediated ISR and pathogen-induced SAR are each effective against a broad spectrum of pathogens, their signal-transduction pathways are clearly distinct. The onset of SAR is accompanied by a local and systemic increase in the endogenous levels of salicylic acid (SA) (Malamy et al., 1990; Métraux et al., 1990) and the concomitant up-regulation of a large set of genes, including those encoding pathogenesis-related (PR) proteins (Ward et al., 1991; Maleck et al., 2000). Several PR proteins possess antimicrobial activity and are thought to contribute to the state of resistance attained (Van Loon and Van Strien, 1999). Transduction of the SA signal requires the function of the regulatory protein NPR1 (also known as NIM1) (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Interaction of NPR1 with the b-ZIP transcription factor TGA2 is required for activation of the SA-regulated gene *PR-1*, suggesting that NPR1 acts by altering the activity of transcription factors (Fan and Dong, 2002). In contrast to SAR, WCS417r-mediated ISR functions independently of SA. This was demonstrated by the observation that Arabidopsis genotypes that are impaired in SA accumulation (i.e. NahG, *eds5*, *sid2*) display levels of ISR equal to those of wild-type plants upon colonization of the roots by WCS417r (Pieterse et al., 1996; Pieterse et al., 2002; Ton et al., 2002b). Analysis of the jasmonic acid (JA)-response mutant *jar1-1*, a range of ethylene (ET)-response mutants, and the SAR-compromised mutant *npr1-1*, revealed that components of the JA- and the ET-response are required for triggering ISR and that this induced resistance response, like SAR, depends on NPR1 (Pieterse et al., 1998; Knoester et al., 1999). However, downstream of NPR1, the ISR and the SAR signaling pathways diverge because, unlike SAR, ISR is not accompanied by the concomitant activation of *PR* genes (Pieterse et al., 1996; Van Wees et al., 1997; Van Wees et al., 1999).

Apart from WCS417r, other fluorescent *Pseudomonas* spp. strains have also been shown to induce the SA-independent ISR pathway in Arabidopsis

(Van Wees et al., 1997; Iavicoli et al., 2003; Ryu et al., 2003), tobacco (Press et al., 1997; Zhang et al., 2002) and tomato (Yan et al., 2002), indicating that the ability to trigger an SA-independent pathway controlling systemic resistance is not uncommon among ISR-inducing rhizobacteria. However, not all resistance-inducing rhizobacteria trigger a SA-independent resistance. For instance, an SA-overproducing mutant of *Pseudomonas aeruginosa* 7NSK2 and the genetically modified, SA-overproducing *P. fluorescens* strain P3 have been shown to trigger the SA-dependent SAR pathway by producing SA at the root surface (De Meyer and Höfte, 1997; Maurhofer et al., 1998).

In Arabidopsis, both JA and ET activate specific sets of defense-related genes (Schenk et al., 2000) and, when applied exogenously, both can induce resistance (Pieterse et al., 1998; Van Wees et al., 1999). To investigate whether ISR is associated with changes in JA/ET-responsive gene expression, Van Wees et al. (1999) monitored the expression of a set of well-characterized JA- and/or ET-responsive genes (i.e. *LOX1*, *LOX2*, *VSP, PDF1.2, HEL*, *CHI-B*, and *PAL1*) in Arabidopsis plants expressing WCS417r-mediated ISR. None of the genes tested was up-regulated in induced plants, neither locally in the roots, nor systemically in the leaves. This suggests that the resistance attained was not associated with major increases in the levels of either JA or ET. Indeed, analysis of JA and ET levels in leaves of ISR-expressing plants revealed no changes in the production of these signal molecules (Pieterse et al., 2000). Therefore, it was assumed that the JA and ET dependency of ISR is based on enhanced sensitivity to these hormones, rather than on an increase in their production.

The onset of SAR is accompanied by substantial transcriptional reprogramming (Maleck et al., 2000), resulting in the accumulation of *PR* gene products to levels from 0.3 up to 1% of the total mRNA and protein content (Lawton et al., 1995). While resulting in a similar enhanced resistance against different types of pathogens, ISR has not been associated with changes in gene expression, but should be likewise dependent on additional defensive activity (Van Wees et al., 1999; Pieterse et al., 2002). Therefore, plants must possess as yet undiscovered defense-related genes, whose products contribute to the broad-spectrum resistance associated with ISR. To identify novel defense-related genes that are potentially involved in rhizobacteria-mediated ISR, we determined expression levels of ~8,000 genes, representing approximately one-third of the Arabidopsis genome, in ISR-expressing plants at different time points before and after challenge inoculation with the pathogen *Pst* DC3000. Analysis of the expression patterns revealed 97 genes that show a substantial change in the level of expression in the roots upon colonization by WCS417r. In contrast, in the leaves none of these ~8,000 genes showed a consistent change in expression level, indicating that the onset of ISR in the leaves is not associated with detectable changes in gene expression. However,

after pathogen challenge, a large set of pathogen-responsive genes showed a specific or augmented change in the level of expression in the ISR-expressing plants. These results indicate that ISR-expressing plants are primed to react faster to pathogen challenge.

Results

Transcript profile of Arabidopsis roots colonized by ISR-inducing P. fluorescens *WCS417r*

To determine the transcript profile of Arabidopsis roots in response to colonization by ISR-inducing WCS417r bacteria, two-week-old seedlings of Arabidopsis accession Col-0 were cultivated in a rock-wool-based system. This system was previously demonstrated to be well-suited for studying ISR in Arabidopsis (Pieterse et al., 1996; Van Wees et al., 1997) and has the advantage that clean root material can be easily collected for RNA extraction. For induction of ISR a suspension of WCS417r bacteria in 10 mM $MgSO_4$, mixed with talcum powder as a carrier, was applied to the roots. As a control 10 mM $MgSO_4$ was applied in a similar manner. Systemic protection in ISR-expressing plants results from the continuous stimulation of the plant by ISR-inducing rhizobacteria on the root surface. In Arabidopsis, full expression of WCS417r-mediated ISR in the leaves can be detected within 7 days after treatment of the roots with this rhizobacterial strain (Ton et al., 2002a). Therefore, to survey the transcript profile of roots of Arabidopsis plants during the onset of ISR, root samples were collected at 3 and 7 days after the start of WCS417r treatment.

RNA was prepared from two independent biological replicates, each consisting of approximately 75 root systems. These two replicates were pooled to reduce noise arising from variation in experimental conditions. The transcript profile of each pool was obtained by hybridization of an Affymetrix Arabidopsis GeneChip microarray representing approximately 8,000 Arabidopsis genes (Zhu and Wang, 2000). On this microarray, each gene is represented by at least one 'probe set' consisting of 16-20 25-mer oligonucleotides. After hybridization, expression values from each pooled sample were normalized globally. To validate the global normalization, the fold change in expression level of a set of ten genes previously identified as representative, constitutively expressed controls (Kreps et al., 2002), was calculated. As expected, the fold change ratio in WCS417r- over mock-treated roots was close to 1 for most of these genes (Table 1).

To identify WCS417r-responsive genes, the following conservative selection criteria were applied. First, the expression level had to be >40 in at

Table 1: Fold-change ratio of representative constitutively expressed control genes in *P. fluorescens* WCS417r- compared to mock-treated Arabidopsis roots.

	ROOTS			
Annotation	Fold Change[1] t = 3 d	Fold Change[1] t = 7 d	Probe Set No.	AGI No.
Polyubiquitin, UBQ10	0.66	0.57	12833_f_at	AT4G05320
Eukcaryotic initiation factor elF-4A1	1.66	0.98	16026_at	AT3G13920
Aquaporin, PIP-1B	2.74	1.15	15977_s_at	AT2G45960
V-type H^+-ATPase, 16 kD-subunit	2.00	0.99	15584_s_at	AT1G19910
40S ribosomal protein S16	1.17	1.05	17390_at	AT2G09990
Actin 2	1.71	1.21	16476_at	AT3G18780
Plasma membrane H^+-ATPase, AHA1	1.38	1.08	14713_s_at	AT2G18960
Tubulin, ß-4	0.72	1.33	15988_at	AT5G44340
Calmodulin-1	1.52	1.03	15173_f_at	AT5G37780
Ca-dependent protein kinase, CPK3	1.57	1.15	17058_s_at	AT4G23650
Average	1.5	1.1		

[1]Fold change ratios (WCS417r/mock) are based on gene expression profiles of roots of Col-0 plants at 3 or 7 d after treatment with ISR-inducing WCS417r bacteria or 10 mM $MgSO_4$ (mock).

least one of the data sets. Second, the change in expression level in WCS417r-treated roots compared to that in mock-treated roots had to be at least 2-fold. Under these conditions, the technical false positive rate is ~0.25%, representing ~20 genes among the 8,000 genes analyzed by the GeneChip (Zhu and Wang, 2000). A total of 1691 probe sets met these two conditions on at least one time point after treatment of the roots with WCS417r. However, to avoid false positives we required the changes to occur at both time points in the same direction. Therefore, only those probe sets were selected that met these selection criteria at both time points tested. Finally, 102 probe sets representing 97 genes met these stringent selection conditions, the majority of which were down-regulated (62%) (Table 2). Of the 97 genes that showed consistent changes, 18% are annotated as "unclassified proteins", 18% are predicted to be involved in cell rescue and defense, 14% in metabolism, 15% in regulating gene transcription, and 7% in cellular communication and signal transduction. In view of the ethylene dependency of ISR, genes encoding a putative ACC oxidase, ethylene response factor 1 (ERF1), ethylene responsive element binding factor 1 (EREBP1) and 2 (EREBP2) are of particular interest. Among all of the WCS417r-responsive genes, the transcript for a putative flavonol reductase was observed as the most strongly induced (i.e. over 14-fold).

Table 2: Fold-change ratio of *P. fluorescens* WCS417r-responsive genes in WCS417r- compared to mock-treated Arabidopsis roots[1].

	ROOTS			
Annotation[2]	Fold Change t = 3 d	Fold Change t = 7 d	Probe Set No.	AGI No.
Transcription				
Myb family transcription factor	7.4	3.4	18479_at	AT3G12820
Identical to WRKY transcription factor 31	3.0	2.4	18213_at	AT4G22070
Myb family transcription factor (MYB72)	2.8	3.1	12725_r_at	AT1G56160
GATA zinc finger protein	2.3	3.7	13168_i_at	AT2G45050
Putative chloroplast nucleoid DNA binding protein	2.2	2.1	15720_at	AT2G03200
Myb family transcription factor (MYB88)	-2.5	-2.9	14852_s_at	AT2G02820
Putative C2H2-type zinc finger protein	-2.5	-2.1	20620_g_at	AT2G37430
CONSTANS B-box zinc finger family protein	-2.7	-2.1	19855_at	AT1G78600
No apical meristem (NAM) protein family	-2.8	-2.0	18590_at	AT1G69490
Myb family transcription factor	-3.4	-2.5	19707_s_at	AT5G67300
RING-H2 finger protein RHA1a -like protein	-3.7	-2.2	16130_s_at	AT4G11370
Ethylene response factor 1	-6.0	-2.2	17514_s_at	AT3G23240
Putative MYB family transcription factor (MYB25)	-6.3	-2.4	17606_s_at	AT2G39880
Ethylene responsive element binding factor 2	-8.7	-2.3	16609_at	AT5G47220
Ethylene responsive element binding factor 2	-24.9	-3.3	12905_s_at	AT5G47220
Ethylene responsive element binding factor 1	-59.1	-2.6	12904_s_at	AT4G17500
Cell rescue and defense				
Peroxidase	4.1	3.9	19622_g_at	AT5G42180
Putative protein	3.5	2.3	13973_at	AT4G36980
Peroxidase, putative	3.3	3.1	12386_at	AT1G44970
RAS-related GTP-binding protein (ARA-1)	3.3	2.1	18195_at	AT1G05810
Drought-induced protein like (Di21)	2.2	2.1	18231_at	AT4G15910
Polyubiquitin (UBQ4)	-2.4	-2.7	12830_f_at	AT5G20620
Peroxidase ATP5a	-2.4	-2.5	19602_at	AT1G49570
Expressed protein	-2.6	-3.1	15083_at	AT4G32190
Expressed protein	-2.8	-2.0	12114_at	AT4G39680
Pathogenesis-related protein 1 precursor	-2.9	-3.1	18451_s_at	AT4G33710
Cytochrome P450	-3.0	-2.0	14248_at	AT3G26830
Polyubiquitin (UBQ4)	-3.1	-3.0	12831_f_at	AT5G20620
Peroxidase, putative	-3.6	-2.6	12475_at	AT5G06730
Zinc finger protein 5, ZFP5	-4.0	-2.8	16582_s_at	AT1G10480
Seven transmembrane MLO protein family (MLO8)	-4.8	-3.3	13687_s_at	AT2G17480
Pathogenesis-related protein 1 precursor	-5.1	-2.5	20308_s_at	AT4G33720

Heavy-metal-associated domain-containing protein	-8.3	-2.4	14485_at	AT1G01490
Germin-like protein (GLP10)	-8.7	-4.5	17037_s_at	AT3G62020
Systemic regulation of / interaction with cellular environment				
Similar to gibberellin-regulated proteins	3.9	2.9	19863_at	AT2G14900
GAST1 protein homolog	3.6	2.6	15121_s_at	AT1G75750
GAST1 protein homolog	3.5	2.7	16014_s_at	AT1G75750
Putative amino-cyclopropane-carboxylic acid oxidase	2.5	2.0	18310_at	AT1G12010
Gibberellin 3 beta-hydroxylase, putative (GA4)	-2.9	-8.2	17549_at	AT1G15550
LAX1/AUX1-like permease	-3.7	-2.1	18667_at	AT5G01240
Terpene synthase/cyclase family	-5.7	-2.2	17511_s_at	AT1G61120
Cellular communication or signal transduction				
Leucine rich repeat family	9.0	4.0	16408_at	AT4G18760
Expressed protein	2.6	2.1	12154_at	AT2G35190
Expressed protein	-2.6	-2.4	14096_at	AT1G76960
CBL-interacting protein kinase 4	-3.5	-2.0	12395_s_at	AT4G14580
Protein kinase (ADK1)	-3.9	-3.0	15692_s_at	AT1G03930
Putative protein/phospholipase C	-6.1	-2.0	12213_at	AT4G34920
Putative protein/leucine-rich-repeat protein	-9.0	-2.3	15249_at	AT4G29880
Metabolism				
Dihydroflavonol 4-reductase family	14.4	14.3	18198_at	AT2G45400
Terpene synthase/cyclase family	9.9	2.1	18127_at	AT4G20230
1-deoxy-D-xylulose5-phosphate reductoisomerase	8.8	2.5	12218_at	AT5G62790
Short chain dehydrogenase/reductase family protein	3.0	2.2	17378_at	AT1G67730
FAD-linked oxidoreductase family	2.3	2.4	13622_i_at	AT4G20820
Glycosyl hydrolase family 1	-2.2	-2.1	16778_at	AT2G44480
Glycine-rich RNA binding protein (AtGRP7)	-2.5	-2.6	15105_s_at	AT2G21660
Putative tyrosine aminotransferase	-3.0	-2.3	17008_at	AT2G24850
Copper amine oxidase like protein	-3.9	-2.0	20555_s_at	AT4G12280
Glycosyltransferase family 20	-4.7	-2.4	13706_s_at	AT2G18700
Geranylgeranyl pyrophosphate synthase (ggps6)	-5.8	-4.6	13257_s_at	AT1G49530
S-adenosylmethionine decarboxylase	-6.7	-2.3	16437_s_at	AT3G02470
Lipase (class 3) family	-6.9	-2.6	14358_s_at	AT4G16820
Ferrochelatase-I	-8.3	-3.0	12571_s_at	AT5G26030
Protein synthesis				
Translation initiation fact. eIF-2 gamma subunit	-11.1	-3.0	17941_at	AT2G18720
Translation initiation fact. eIF-2B delta subunit	-29.5	-2.5	16255_at	AT2G44070
Transport facilitation				
Unknown protein/cation transport protein	3.0	3.2	15544_at	AT4G31290
Zinc transporter (ZIP2)	2.3	2.0	15666_s_at	AT5G59520
Monooxygenase family	-3.0	-2.5	17051_s_at	AT2G29720

Glucose-6-phosphate/phosphate-translocator precursor, putative	-9.6	-2.1	17775_at	AT1G61800
DNA damage-inducible protein (EDS5/SID1)	-14.0	-3.5	17653_at	AT4G39030
Cell cycle and DNA processing				
Putative AAA-type ATPase	2.1	2.0	16345_at	AT2G03670
Unknown protein	-3.3	-2.3	14130_at	AT1G03080
Development (systemic)				
Nodulin-like protein (mtn21)	2.5	7.6	16258_at	AT2G39510
Control of cellular organization				
Actin depolymerizing factor-like protein	7.1	9.8	19684_at	AT4G34970
Small heat shock protein	2.1	2.5	13282_s_at	AT4G25200
Energy				
Expressed protein	2.9	2.1	15851_i_at	AT2G27370
Nitrate reductase 2 (NR2)	-5.8	-3.8	14242_s_at	AT1G37130
Nitrate reductase 1 (NR1)	-14.1	-11.4	14240_s_at	AT1G77760
Nitrate reductase 1 (NR1)	-41.7	-9.0	18899_s_at	AT1G77760
Subcellular localisation				
Unknown protein	-6.3	-3.2	14524_s_at	AT1G65580
Cell fate				
Expressed protein	9.5	9.8	18721_at	AT3G02040
Expressed protein	-2.5	-2.8	18346_at	AT4G35890
Protein fate (folding, modification,destination)				
DegP protease	-2.1	-2.3	16067_at	AT3G27925
Putative DnaJ protein	-11.7	-6.2	15367_at	AT1G76700
Serine carboxypeptidase -related	-27.8	-3.4	18132_at	AT4G15100
Unclassified proteins				
Lateral organ boundaries (LOB) domain family	9.2	2.4	15808_at	AT2G30130
Auxin-induced protein-related	4.3	4.1	15017_at	AT2G24400
Auxin-induced protein-related	4.2	3.2	16751_at	AT4G34750
Putative OBP32pep protein	3.5	9.7	13855_at	AT1G23590
Putative protein	3.5	3.0	20487_at	AT4G34810
Glucose-methanol-choline oxidoreductase family	2.7	3.8	19068_i_at	AT1G14185
Expressed proteins	2.5	3.1	14436_at	AT3G50750
Unknown protein	2.3	2.1	15861_at	AT2G35850
Expressed protein	2.3	2.2	15918_at	AT1G30750
Expressed protein	2.1	2.4	12163_at	AT2G42780
Putative protein	-2.0	-9.5	13963_at	AT4G18890
RWP-RK domain containing protein	-2.1	-2.5	14521_at	AT4G38340
Kelch repeat containing F-box protein family	-2.2	-2.1	12695_at	AT4G38940
Cytochrome p450 family	-2.7	-3.0	18951_at	AT4G15330
Expressed protein	-3.9	-2.6	15933_at	AT1G21830

Expressed protein	-5.0	-2.6	18160_at	AT4G16745
Putative protein	-5.3	-2.0	20179_at	AT4G38060
Putative tropinone reductase	-73.1	-2.6	20370_at	AT2G29150

[1] Fold-change ratios (WCS417r/mock) are based on gene expression profiles of roots of Col-0 plants, 3 and 7 d after treatment with 10 mM $MgSO_4$ (mock) or ISR-inducing WCS417r bacteria.

[2] Annotations are as predicted by the MIPS Arabidopsis thaliana Genome Database (MatDB; http://mips.gsf.de/proj/thal/db/index.html).

To verify the GeneChip results we selected an up-regulated gene encoding a MYB72 transcription factor (probe set 12725_r_at), a down-regulated gene encoding a MLO8 protein (probe set 13687_s_at), and a gene with unchanged expression encoding vegetative storage protein 2 (VSP2; probe set 14675_s_at), and analyzed their transcript levels in WCS417r- and mock-treated Arabidopsis roots by RT-PCR. To this end, in an independent experiment, RNA was isolated from roots of 3-week-old Col-0 plants that were grown for 7 days in the presence or absence of WCS417r bacteria in the rockwool system. Figure 1 shows that the transcript level of the selected up-regulated *MYB72* transcription factor gene was clearly increased in WCS417r-treated roots, whereas the mRNA level of the selected down-regulated *MLO8* gene was clearly lower in WCS417r-treated roots. As expected, transcript levels of the selected *VSP2* gene remained unchanged in the roots. These results confirm the data from the GeneChip experiments.

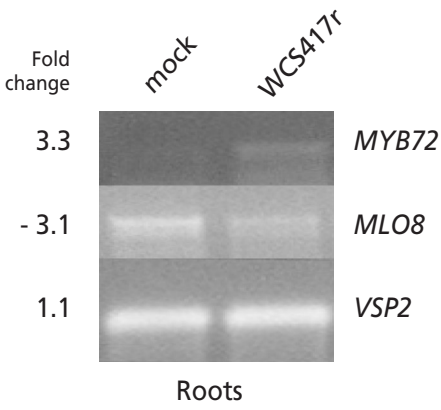

Figure 1. Verification of Root GeneChip Data.

Transcript levels of genes that were selected in the GeneChip analysis as being up-regulated, down-regulated, or unchanged (see fold change) in WCS417r-treated roots were analyzed by RT-PCR. Shown are ethidium bromide-stained agarose gels with RT-PCR products obtained after amplification of equal portions of first-strand cDNA using gene-specific primers of the genes indicated. First-strand cDNA was synthesized on mRNA that was isolated from roots of Arabidopsis Col-0 plants, 7 days after treatment of the roots with 10 mM $MgSO_4$ (mock) or ISR-inducing WCS417r bacteria.

ISR is not associated with systemic changes in gene expression in the absence of pathogen infection

Colonization of the roots of Arabidopsis accession Col-0 by WCS417r results in a systemic resistance in the leaves that is effective against different types of pathogens (Pieterse et al., 2002). To identify genes that show a specific change in expression in the leaves in response to treatment of the roots with ISR-inducing rhizobacteria, two-week-old Col-0 seedlings were transplanted into soil with or without WCS417r. To examine the expression profile of leaves of induced and non-induced plants, leaf samples were collected 3 and 7 days after induction. Expression of ISR was routinely verified in parallel using our standard bioassay (Pieterse et al., 1996) with the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 as the challenging pathogen (data not shown). RNA was prepared from 3 independent biological replicates, each consisting of approximately 25 rosettes, that were pooled to reduce noise arising from variation in experimental conditions. Transcript profiling was performed for two independent experiments on data sets that were normalized globally. Global normalization was again validated by calculating the fold change in expression level of the 10 representative constitutively expressed control genes. The fold change ratio in leaves of WCS417r- over mock-treated plants was close to 1 for most of these genes (Table 3).

To identify genes that respond systemically in the leaves to colonization of the roots by WCS417r, we selected probe sets that had an expression level of >40 and that showed a >2-fold change in ISR-expressing leaves compared to non-induced leaves. The number of probe sets that met these criteria on the single time points varied between 20 and 23, which is close to the technical false positive rate of ~0.25% (Zhu and Wang, 2000). To reduce false positives we required the changes to be consistent at both time points tested or to show reproducibility between experiments. Interestingly, none of the probe sets met the selection criteria. These results indicate that, although ISR-expressing leaves possess an enhanced defensive capacity, the state of ISR is not associated with major changes in the expression of the ~8,000 genes represented on the GeneChip.

Selection of Pst *DC3000-responsive genes in control and ISR-expressing plants*

The observed lack of changes in gene expression in leaves of WCS417r-induced plants suggests that the broad-spectrum effectiveness of ISR might be based on processes that are only apparent after pathogen attack. Previously,

Table 3: Fold-change ratio of representative constitutively expressed control genes in leaves of *P. fluorescens* WCS417r- compared to mock-treated Arabidopsis plants, before and after challenge inoculation with Pst DC3000.

	LEAVES				
	Before Challenge[2]		After Challenge[3]		
Annotation[1]	Fold Change t = 3 d	Fold Change t = 7 d	Fold Change t = 0 h	Fold Change t = 6 h	Fold Change t = 24 h
Polyubiquitin, UBQ10	0.79	1.08	1.05	1.39	0.85
Eukcaryotic initiation factor elF-4A1	1.01	1.20	1.08	1.02	0.88
Aquaporin, PIP-1B	1.03	1.12	0.94	0.98	0.94
V-type H^+-ATPase, 16 kD-subunit	0.97	0.99	0.98	1.05	1.01
40S ribosomal protein S16	1.07	1.03	1.15	1.02	1.01
Actin 2	0.98	1.02	1.14	1.29	1.27
Plasma membrane H^+-ATPase, AHA1	1.43	0.79	0.96	0.99	1.23
Tubulin, ß-4	1.24	1.35	1.04	0.98	0.87
Calmodulin-1	1.04	1.13	0.93	0.92	1.03
Ca-dependent protein kinase, CPK3	1.08	0.64	1.04	1.06	1.01
Average	1.1	1.0	1.0	1.1	1.0

1 For details on probe set and AGI numbers see Table 1.
2 Fold-change ratios (WCS417r/mock) are based on gene expression profiles of leaves of Col-0 plants at 3 and 7 d after treatment of the roots with ISR-inducing WCS417r bacteria or 10 mM $MgSO_4$ (mock).
3 Fold-change ratios (WCS417r/mock) are based on gene expression profiles of leaves of Col-0 plants, grown in the presence or absence of WCS417r bacteria, at 0, 6 and 24 h after challenge inoculation with *Pst* DC3000.

RNA blot analyses of the JA-responsive, pathogen-inducible gene *VSP2* revealed that ISR is associated with enhanced expression of this gene after infection of the leaves by *Pst* DC3000 (Van Wees et al., 1999), suggesting that ISR-expressing plants are primed to express specific pathogen-inducible genes at a higher level after challenge. To investigate the transcript profile of pathogen-responsive genes in ISR-expressing plants, Arabidopsis Col-0 plants grown in soil with or without ISR-inducing WCS417r bacteria were challenge inoculated with *Pst* DC3000 and checked for expression of ISR (data not shown). Leaf samples were collected from control- and WCS417r-induced plants at 0, 6 and 24 h after challenge inoculation. RNA was prepared from 3 independent biological replicates, each consisting of approximately 7 rosettes, that were pooled to reduce noise arising from variation in experimental conditions. To identify *Pst* DC3000-responsive genes in non-induced and

in ISR-expressing plants, probe sets were selected that showed an expression level of >40 and a >2-fold change at 6 and/or 24 h after pathogen inoculation compared to non-challenged plants. A total of 1661 probe sets satisfied this condition in non-induced plants, whereas 1507 probe sets met the selection criteria in WCS417r-induced plants. The lower number of selected probe sets from the data set of WCS417r-induced plants correlated with the observed reduction of disease symptoms in the ISR-expressing plants (data not shown). Only those probe sets were selected that met the above-mentioned selection criteria at both 6 and 24 h after challenge inoculation, resulting in a total of 523 probe sets representing 469 genes in non-induced plants, and 479 probe sets representing 425 genes in WCS417-induced plants. A list of the selected probe sets is provided in Table S1 of the supplementary material available from http://www.bio.uu.nl/~fytopath/Table_S1.htm.

ISR-expressing plants are primed for augmented pathogen-responsive gene expression

Having identified *Pst* DC3000-responsive genes, we tested our hypothesis that ISR-expressing plants are primed to respond faster and/or with a greater magnitude to pathogen infection. To this end we compared the expression levels of the *Pst* DC3000-responsive genes in non-induced and in ISR-expressing plants. To identify *Pst* DC3000-responsive genes that show an augmented expression pattern in ISR-expressing plants (so-called ISR-primed genes), we required the change in expression level of the *Pst* DC3000-responsive genes to be >1.5-fold higher in the WCS417r-treated plants. This latter criterion was based on quantitative expression data of the *VSP2* gene, which was previously demonstrated to be primed in ISR-expressing plants (Van Wees et al., 1999). In several independent experiments, *VSP2* transcripts consistently accumulated to a ~1.5-fold higher level in *Pst* DC3000-inoculated, ISR-expressing plants than in similarly inoculated control plants (data not shown). Therefore, to select for ISR-primed genes we required their expression levels to be at least 1.5-fold higher in WCS417r-treated plants. Note that in addition to this selection criterion, the *Pst* DC3000-responsive genes were already pre-selected to show an expression level of >40 and a >2-fold change at both 6 and 24 h after infection.

A comparison of the changes in transcript levels of the ISR-primed, *Pst* DC3000-responsive genes in control and ISR-expressing Col-0 plants is depicted in Figure 2. A total of 52 probe sets representing 51 genes met the selection conditions and are listed in Table 4. The previously identified ISR-primed, *Pst* DC3000-responsive gene *VSP2* was among those in this group, illustrating the validity of this analysis. Other ISR-primed genes of particular

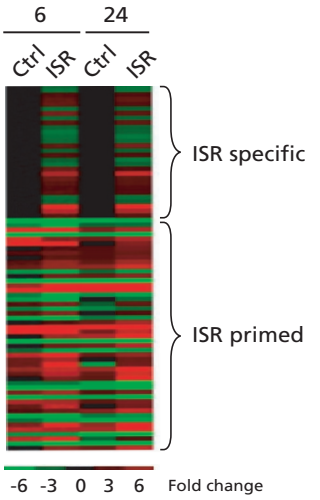

Figure 2. Augmented Expression of *Pst* DC3000-Responsive Genes in Arabidopsis Plants Expressing *P. fluorescens* WCS417r-Mediated ISR.

Comparison of the changes in transcript levels of ISR-specific and ISR-primed genes in control and ISR-expressing Col-0 plants at 6 and 24 h after inoculation with *Pst* DC3000. ISR-specific genes show a consistent >2-fold change in the level of expression at 6 and 24 h after inoculation, whereas these genes remained unchanged in *Pst* DC3000-inoculated control plants. ISR-primed genes display a consistent >2-fold change in both control and ISR-expressing plants at 6 and 24 h after inoculation. In addition, the magnitude of this change is >1.5-fold stronger in challenged ISR-expressing plants as compared to *Pst* DC3000-inoculated control plants. The values are visualized by TreeView software.

interest are the JA/ET-responsive gene *PDF1.2*, a thaumatin-like gene, a chitinase gene and a gene encoding ethylene responsive element binding factor 2 (EREBP2). Besides the 51 selected ISR-primed genes, 30 genes showed a *Pst* DC3000-induced change in WCS417r-treated plants only; in non-induced plants the mRNA levels of the corresponding genes remained unchanged after pathogen infection (Figure 2; Table 5). Apparently, these genes respond to *Pst* DC3000 infection only in ISR-induced plants. This latter group is further referred to as ISR-specific. Of the 30 ISR-specific genes, 6 are annotated as "unclassified proteins", 5 are predicted to be involved in metabolism, and 4 genes are likely to be involved in regulating gene transcription (Table 5).

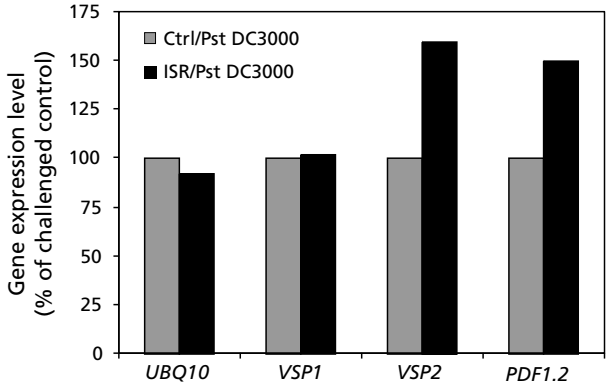

Figure 3. Verification of Leaf GeneChip Data.

Transcript levels of the *Pst* DC3000-responsive gene *VSP1*, the ISR-primed, *Pst* DC3000-responsive genes *VSP2* and *PDF1.2*, and the constitutively expressed gene *UBQ10* were analyzed in control and ISR-expressing plants at 24 h after inoculation with *Pst* DC3000. Hybridization signals obtained with the RNA gel blot analysis were quantified using a Phosphor Imager. Shown are relative transcript levels at 24 h after inoculation (mRNA levels in *Pst* DC3000-inoculated control plants were set at 100%).

Moreover, 5 genes are predicted to be involved in signal transduction. One of those shows homology to *ERF1*, which encodes a transcription factor that acts downstream of ET and JA signaling and has been implicated in the defense response of Arabidopsis against various pathogens (Solano et al., 1998; Berrocal-Lobo et al., 2002; Lorenzo et al., 2003).

To verify the GeneChip data we examined the expression levels of the *Pst* DC3000-responsive gene *VSP1* (probe set 15125_f_at), the ISR-primed, *Pst* DC3000-responsive genes *PDF1.2* (probe set 14621_at) and *VSP2* (probe set 14675_s_at), and the constitutively expressed ubiquitin gene *UBQ10* (probe set 12835_f_at) in an independent experiment. To this end, RNA gel blot analysis was performed using RNA from leaves of 5-week-old control and ISR-expressing plants that were harvested 0 and 24 h after inoculation with *Pst* DC3000. Transcript levels of *VSP1, VSP2,* and *PDF1.2* were clearly increased in response to *Pst* DC3000 infection, whereas the expression of *UBQ10* remained unchanged (Data not shown). Moreover, *VSP2* and *PDF1.2* showed a clearly enhanced level of expression in challenged ISR-expressing plants over challenged control plants, whereas the *Pst* DC3000-induced expression level of *VSP1* remained unchanged (Figure 3). The level of enhanced expression of *VSP2* and *PDF1.2* in challenged ISR-expressing plants over *Pst* DC3000-inoculated control plants was similar to that observed in the GeneChip data analysis ("ratio" in Table 4). These results fully agree with and confirm the corresponding data of the GeneChip data analysis.

Table 4: Fold-change ratios of ISR-primed, Pst DC3000-responsive genes in leaves of *P. fluorescens* WCS417r-treated plants[1].

	CHALLENGED LEAVES							
	t = 6 h			t = 24 h				
	Fold Change			Fold Change				
Annotation[2]	Ctrl	ISR	**Ratio**	Ctrl	ISR	**Ratio**	Probe Set No.	AGI No.
Transcription								
Ethylene responsive element binding factor 2	12.2	19.8	**1.6**	1.0	3.8	**3.8**	12905_s_at	AT5G47220
WRKY family transcription factor	2.0	3.1	**1.5**	2.2	2.9	**1.3**	13115_at	AT1G62300
Putative YABBY3 axial regulator	-2.2	-4.1	**-1.9**	-5.5	-3.8	**1.4**	17530_at	AT4G00180
Putative heat shock transcription factor	-11.8	-23.9	**-2.0**	-4.1	-6.6	**-1.6**	12431_at	AT2G26150
Cell rescue and defense								
Cytochrome P450 family	4.9	14.2	**2.9**	9.8	28.0	**2.8**	14609_at	AT2G30770

Thaumatin-like protein	6.3	17.0	**2.7**	-2.1	2.2	**4.6**	20384_at	AT4G36010
Antifungal protein PDF1.2	3.2	7.9	**2.5**	13.4	31.9	**2.4**	14621_at	AT5G44420
Glycosyl hydrolase fam. 19 (chitinase)	2.2	4.3	**2.0**	2.5	4.9	**1.9**	13153_r_at	AT2G43590
Glycosyl hydrolase fam. 19 (chitinase)	2.9	4.5	**1.6**	4.4	7.0	**1.6**	13154_s_at	AT2G43590
Expressed protein	-22.2	-25.2	**-1.1**	-13.9	-25.2	**-1.8**	16637_s_at	AT4G14690
Gamma-glutamyltransferase-related	-3.8	-4.5	**-1.2**	-2.2	-3.9	**-1.8**	13255_i_at	AT4G39640
Heat shock factor protein 7 (HSF7)	-6.7	-10.4	**-1.5**	-2.8	-3.7	**-1.3**	19629_at	AT4G11660
Cellular communication or signal transduction mechanism								
Receptor-related serine/threonine protein kinase ARK3	1.8	4.7	**2.5**	5.7	12.7	**2.2**	16360_at	AT4G21380
Protein kinase-like protein	3.7	8.4	**2.3**	4.4	12.3	**2.8**	20232_s_at	AT4G23130
PP1/PP2A phosphatases pleiotropic regulator PRL2	3.0	5.2	**1.7**	1.2	2.9	**2.5**	17954_s_at	AT3G16650
Ras-related GTP-binding protein (Rab7)	-3.6	-3.7	**-1.0**	-8.7	-14.2	**-1.6**	20330_at	AT1G22740
Metabolism								
Short-chain alcohol dehydrogenase-like protein	1.8	4.2	**2.3**	3.0	6.4	**2.1**	20685_at	AT4G13180
2-oxoglutarate-dependent dioxygenase (AOP2)	2.4	3.9	**1.6**	2.0	2.1	**1.1**	15238_at	AT4G03060
Putative cytochrome P450	2.1	3.0	**1.5**	4.0	6.5	**1.6**	19549_s_at	AT2G22330
Anthranilate synthase component I-1 precursor	6.4	6.0	**-1.1**	5.4	8.3	**1.5**	20291_at	AT5G05730
Arabinogalactan-protein (AGP4)	-2.9	-4.8	**-1.6**	-3.2	-2.4	**1.3**	15107_s_at	AT5G10430
Storage protein								
Vegetative storage protein VSP2	15.1	23.0	**1.5**	143.4	178.6	**1.2**	14675_s_at	AT5G24770
Putative protein/storage protein	18.0	25.7	**1.4**	10.6	18.7	**1.8**	15886_at	AT4G24350
Protein activity regulation								
Serpin, putative	2.7	6.4	**2.4**	-1.3	3.2	**4.0**	19322_at	AT1G47710
Energy								
Phosphoadenylyl-sulfate reductase (thioredoxin) (PAPS reductase)	-7.5	-13.7	**-1.8**	-4.4	-5.3	**-1.2**	18696_s_at	AT1G62180
Expressed protein	-3.0	-5.4	**-1.8**	-3.6	-4.3	**-1.2**	14917_at	AT2G35760
Glutaredoxin protein family	-2.0	-4.4	**-2.1**	-17.6	-17.4	**1.0**	13258_s_at	AT2G47880
Development (systemic)								
Aldose 1-epimerase family	1.8	3.2	**1.8**	2.7	4.1	**1.5**	13880_s_at	AT4G25900
No apical meristem (NAM) protein family	4.5	6.5	**1.4**	4.9	8.7	**1.8**	18591_at	AT5G08790

Protein with binding function or cofactor requirement								
DEAD box RNA helicase, putative	3.0	12.0	**4.1**	-3.9	3.1	**12.2**	18016_r_at	AT5G08610
DEAD/DEAH box RNA helicase protein, putative	8.9	13.8	**1.6**	3.4	6.5	**1.9**	15906_at	AT1G59990
Zinc finger protein Zat12	1.5	2.1	**1.4**	2.1	3.3	**1.6**	13015_s_at	AT5G59820
Putative zinc-finger protein	-9.6	-15.8	**-1.6**	-8.6	-7.9	**1.1**	14504_s_at	AT2G28200
Subcellular localisation								
Unknown protein	-1.4	3.6	**4.9**	2.4	11.4	**4.7**	18625_at	AT1G03290
Gamma glutamyl hydrolase, putative	1.4	2.4	**1.7**	2.1	3.2	**1.5**	13118_f_at	AT1G78660
E3 ubiquitin ligase (RMA1)	6.3	9.2	**1.5**	2.7	4.4	**1.6**	17552_s_at	AT4G03510
Thioredoxin family	-2.7	-2.7	**1.0**	-4.2	-7.9	**-1.9**	18637_at	AT2G42580
Transport facilitation								
Proline transporter 2 (ProT2)	5.5	5.2	**-1.1**	2.3	3.5	**1.5**	19158_at	AT3G55740
Unclassified proteins								
Expressed protein	1.2	2.9	**2.4**	3.2	5.9	**1.9**	17010_s_at	AT2G20670
ACT domain-containing protein	2.7	6.1	**2.3**	2.8	7.0	**2.5**	18624_at	AT2G39570
Expressed protein	1.1	2.1	**1.9**	3.0	5.2	**1.7**	15479_at	AT4G26060
Expressed protein	2.2	3.6	**1.6**	-2.0	2.7	**5.4**	15063_at	AT4G12070
Protease inhibitor/seed storage/lipid transfer protein	-7.4	-4.9	**1.5**	-4.3	-7.3	**-1.7**	18983_s_at	AT4G12510
Subunit 1 of NADH dehydrogenase	3.6	5.1	**1.4**	2.2	3.4	**1.6**	18709_at	ATNADH
Expressed protein	8.0	11.6	**1.4**	3.6	5.6	**1.6**	12212_at	AT3G52070
Expressed protein	-4.0	-6.3	**-1.6**	-1.8	-2.4	**-1.4**	14923_at	AT2G28320
Expressed protein	-3.3	-5.1	**-1.6**	-2.1	-2.4	**-1.2**	12128_at	AT2G41010
Expressed protein	-2.6	-4.1	**-1.6**	-5.3	-4.5	**1.2**	19952_at	AT1G12020
Expressed protein	-16.2	-28.0	**-1.7**	-5.4	-4.5	**1.2**	12027_at	AT4G20170
Expressed protein	-5.0	-8.9	**-1.8**	-2.7	-3.6	**-1.3**	15476_at	AT2G21560
Cathepsin B-like cysteine protease, putative	7.4	3.8	**-1.9**	3.0	5.6	**1.8**	12757_at	AT1G02300
Expressed protein	-5.0	-9.9	**-2.0**	-7.0	-5.3	**1.3**	20678_at	AT1G11700

[1] Fold-change ratios (t=6 h/t=0h or t=24 h/t=0 h) are based on transcript profiles of leaves of control (Ctrl) and ISR-expressing Col-0 plants at 0, 6 and 24 h after challenge inoculation with *Pst* DC3000. Bold-printed ratios express the level of augmented expression of the ISR-primed, *Pst* DC3000-responsive genes (fold-change ratio ISR/fold-change ratio Ctrl).

[2] Annotations are as predicted by the MIPS *Arabidopsis thaliana* Genome Database (MatDB; http://mips.gsf.de/proj/thal/db/index.html).

The role of JA and ET in priming

Arabidopsis mutants affected in their ability to respond to either JA or ET are compromised in WCS417r-mediated ISR, indicating that the expression of ISR requires an intact JA and ET signaling pathway (Pieterse et al., 1998; Pieterse et al., 2002). To investigate the possible role of JA and ET in the regulation of ISR-primed genes, we made use of a microarray data set previously published by Glazebrook et al. (2003). This data set consists of global expression patterns of *P. syringae* pv. *maculicola* (*Psm*) ES4326-induced genes in wild-type Arabidopsis Col-0 and various signaling-defective mutant plants. Previously, Tao et al. (2003) demonstrated that the expression profile of wild-type Col-0 plants inoculated with either *Pst* DC3000 or *Psm* ES4326 is very similar. Therefore, we assumed that the global expression phenotype of *Psm* ES4326-responsive genes is very similar to that of *Pst* DC3000-responsive genes. To validate this assumption we compared the group of *Pst* DC3000-responsive genes in control plants (Table S1) with the group of *Psm* ES4326-responsive genes that showed an expression level of >40 and a >2-fold change compared to mock-inoculated plants. Of the 523 *Pst* DC3000-responsive probe sets that showed a consistent change in expression in non-induced, *Pst* DC3000-infected Col-0 plants, a large group of 278 probe sets (53%) showed a similar expression pattern in *Psm* ES4326-infected Col-0 plants. This group of *Pst*/*Psm*-responsive genes was used in the analysis of the data sets that were obtained with the signaling-defective genotypes (see below). The expression level of the remaining *Pst* DC3000-responsive genes did not appear to change substantially in *Psm* ES4326-inoculated plants. This might be due to differences in experimental set-up, as the expression profile of the *Psm* ES4326-responsive genes was determined at 30 h after pressure-infiltrating of *Psm* ES4326 into the leaves, whereas our data were collected at 6 and 24 hours after dipping the leaves in a suspension of *Pst* DC3000.

To determine the involvement of JA, ET and SA in the regulation of the ISR-primed, *Pst* DC3000-responsive genes, we compared their expression profile in *Psm* ES4326-infected wild-type Col-0 plants and the various signaling-defective transgenic or mutant genotypes in the Col-0 background. These genotypes were *coi1-1*, which blocks JA signaling (Feys et al., 1994; Xie et al., 1998); *ein2-1*, which blocks ET signaling (Guzmán and Ecker, 1990; Alonso et al., 1999), and *eds5-1* and NahG, which are affected in SA signaling (Gaffney *et al*., 1993; Nawrath *et al*., 2002). Of the 51 ISR-primed, *Pst* DC3000-responsive genes, 29 genes (57%) were also responsive to *Psm* ES4326. This set of ISR-primed, *Pst*/*Psm*-responsive genes was further analyzed in the signaling-defective genotypes.

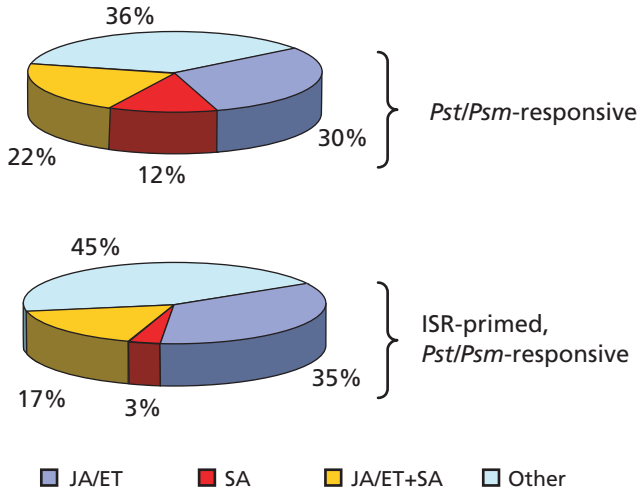

Figure 4. The group of ISR-primed, pathogen-responsive genes is enriched for genes that are affected by JA and ET signaling.

The effect of JA, ET and SA on pathogen-responsive gene expression was assessed for 254 Arabidopsis genes that showed a similar expression pattern in Col-0 plants in response to infection by *Pst* DC3000 and *Psm* ES4326. Shown are the frequency distributions of the pathogen-responsive genes over the classes JA and/or ET-responsive (JA/ET), SA-responsive (SA), SA- and JA/ET-responsive (JA/ET+SA), and JA-, ET-, and SA-independent (other) for all the *Pst*/*Psm*-responsive genes and the group of pathogen-responsive genes that shows an augmented expression pattern in challenged ISR-expressing plants (ISR-primed, *Pst*/*Psm*-responsive genes).

To analyze the role of JA, ET and SA in the regulation of the *Pst*/*Psm*-responsive genes we compared their expression levels in wild-type Col-0 plants with those in the various signaling-defective genotypes. A gene was scored as JA-, ET-, or SA-responsive when an altered *Psm* ES4326-response was observed in the respective mutant or transgenic compared to the wild type, or when the change in expression differed at least 1.5-fold compared to that observed in *Psm* ES4326-infected Col-0 plants. Of all the 278 *Pst*/*Psm*-responsive probe sets, 12% were dependent on SA signaling only, 30% were regulated by JA- and/or ET signaling, and 22% were affected by a combination of JA/ET- and SA-signaling (Figure 4, top panel). The remaining 36% of the *Pst*/*Psm*-responsive genes showed a similar expression pattern in all genotypes tested, indicating that their expression was not affected by either of these signals. Analysis of the ISR-primed, *Pst*/*Psm*-responsive genes revealed that only 3% was solely dependent on SA signaling, 35% of the genes were regulated by JA/ET-signaling, and 17% of the genes were affected by a combination of JA/ET and SA (Figure 4, bottom panel). These results suggest that the group of ISR-primed genes is enriched for JA/ET-responsive genes.

Table 5: Fold-change ratios of ISR-specific, *Pst* DC3000-responsive genes in leaves of *P. fluorescens* WCS417r-treated plants[1].

	CHALLENGED LEAVES			
Annotation[2]	Fold Change[1] t = 6 h	Fold Change[1] t = 24 h	Probe Set No.	AGI No.
Transcription				
Hypothetical protein	3.4	2.5	13517_g_at	AT4G18690
WRKY family transcription factor	2.4	2.4	20382_s_at	AT2G30250
Similar to ethylene response factor 1	2.0	2.0	19755_at	AT2G31230
Squamosa promoter binding protein-related 2	-2.0	-2.8	18029_g_at	AT5G43270
Cell rescue and defense				
Putative thaumatin	2.3	2.0	19839_at	AT2G28790
Expressed protein	-2.0	-2.6	11995_at	AT2G29970
Regulation of and interaction with cellular environment				
TAT-binding protein, putative	-2.2	-2.1	14052_at	AT1G10070
Gluthatione reductase	-2.5	-2.0	13262_s_at	AT3G54660
Cellular communication or signal transduction				
Serine/threonine protein phosphatase type one(PP1)	6.1	5.5	20333_at	AT5G27840
Similar to receptor kinase 1	2.6	3.5	16348_at	AT1G65790
Calcium-dependent protein kinase (CDPK)(AK1)	-2.3	-2.4	16088_f_at	AT5G04870
Protein kinase 6-like	-2.5	-2.5	19917_at	AT5G58950
Protein serine/threonine kinase, putative	-2.6	-2.2	18316_at	AT1G01540
Metabolism				
NADC homolog	5.5	6.6	18657_at	AT2G01350
UDP-glycosyltransferase family	3.0	2.0	18512_at	AT1G24100
Cytochrome P450-like protein	2.4	2.6	12526_at	AT4G27710
Aspartate kinase-homoserine dehydrogenase	2.3	2.3	19749_at	AT1G31230
Pectinesterase-related	-2.3	-2.2	13635_at	AT4G12390
Energie				
Glyceraldehyde-3-phosphate dehydrogenase	-2.2	-2.5	20640_s_at	AT1G42970
Amine oxidase family	-2.6	-2.7	12241_at	AT4G29720

Protein with binding function or cofactor requirement				
Flowering time control protein (FCA)	2.7	2.1	13250_s_at	AT4G16280
26S protease regulatory subunit 6A	2.3	2.5	14026_at	AT1G09100
Transport facilitation				
CLC-c chloride channel protein	2.0	2.1	12493_g_at	AT5G49890
Protein fate (folding, modification,destination)				
Putative leucine aminopeptidase	2.1	2.1	17956_i_at	AT2G24200
Unclassified proteins				
Expressed protein	10.2	8.6	20165_at	AT1G23150
Expressed protein	2.9	2.4	19218_at	AT1G54520
Expressed protein	-2.0	-2.1	16499_at	AT4G32020
Expressed protein	-2.2	-2.1	19984_at	AT1G61900
Arabinogalactan-protein (AGP3)	-2.5	-2.9	15208_s_at	AT4G40090
Expressed protein	-2.8	-2.2	14450_at	AT1G79160

[1] Fold-change ratios (t=6 h/t=0h or t=24 h/t=0 h) are based on transcript profiles of leaves of ISR-expressing Col-0 plants at 0, 6 and 24 h after challenge inoculation with *Pst* DC3000. None of the selected genes showed a substantial change in expression in *Pst* DC3000-inoculated control plants.

[2] Annotations are as predicted by the MIPS *Arabidopsis thaliana* Genome Database (MatDB; http://mips.gsf.de/proj/thal/db/index.html).

Discussion

Rhizobacteria-mediated ISR and pathogen-induced SAR are two inducible defense responses that are controlled by distinct signaling pathways (Pieterse et al., 1998). Expression profiling of Arabidopsis plants expressing SAR revealed that this type of induced resistance is accompanied by substantial transcriptional reprogramming (Maleck et al., 2000), resulting in the accumulation of SAR-gene products to levels from 0.3 up to 1% of the total mRNA and protein content (Lawton et al., 1995). In this study, we analyzed the transcript profile of roots and leaves of Arabidopsis plants expressing WCS417r-mediated ISR, using a GeneChip representing approximately one-third of the Arabidopsis genome. A consistent shift in the expression of 102 probe sets (representing 97 genes) was observed in roots after colonization by WCS417r (Table 2), indicating that Arabidopsis roots respond to external

stimuli perceived from this non-pathogenic rhizobacterium. These changes may be related to the roots being colonized by the bacterial strain and may be involved in the expression of localized and/or systemically induced resistance. The large quantity of data makes it impossible to discuss all possible functions of the identified WCS417r-responsive genes in ISR. However, in view of the ethylene-dependency of ISR, it is worth noting that genes encoding ERF1, EREBP1, EREBP2 and a putative ACC oxidase are strongly down-regulated in roots that are colonized by ISR-inducing WCS417r bacteria. ERF1, EREBP1 and EREBP2 are positive regulators of ET-dependent plant processes, including defense-related gene expression (Wang et al., 2002). Down regulation of the corresponding genes, as observed in WCS417r-treated roots suggests, therefore, that the onset of ISR is associated with a reduction in ET signaling. Among all of the WCS417r-responsive genes, the transcript for a flavonol reductase was observed as the most strongly induced. Flavonol reductases have been implicated in e.g. lignin and anthocyanin biosynthesis (Ostergaard et al., 2001).

While the roots clearly responded to colonization by WCS417r, we were unable to identify genes that showed a >2-fold change in expression in the leaves of ISR-expressing plants. None of the ~8,000 genes tested showed a consistent change in the level of expression in the above-ground plant parts, indicating that, in contrast to SAR, the onset of WCS417r-mediated ISR in the leaves is not associated with detectable changes in gene expression. Nevertheless, leaves from induced plants display a clearly enhanced resistance against a broad range of pathogens. In our experiments, the effectiveness of ISR was checked for *Pst* DC3000, but under similar conditions ISR was demonstrated to be also effective against *X. campestris* pv. *armoraciae*, *A. brassicicola*, *F. oxysporum* f.sp. *raphani*, and *P. parasitica* (Pieterse et al., 1996; Ton et al., 2002a). To investigate the possibility that ISR is associated with transcriptional changes that are only apparent after pathogen attack, we analyzed the expression profile of the ~8,000 Arabidopsis genes in control and ISR-expressing plants upon challenge inoculation with *Pst* DC3000. Of the 425 *Pst* DC3000-responsive genes, 81 (19%) showed an augmented change in ISR-expressing leaves. Of this set of ISR-primed, *Pst* DC3000-responsive genes, 63% showed an enhanced expression, whereas 37% was expressed exclusively in WCS417r-treated plants upon pathogen challenge. These results indicate that ISR-expressing plants are primed for augmented expression of a specific set of pathogen-responsive genes.

Among the ISR-primed, pathogen-responsive genes, the majority of the genes were predicted to be influenced by JA and/or ET signaling, suggesting that both signals play an important role. These observations can explain our previous findings that, on the one hand, responsiveness to both JA and ET is required for full expression of ISR, while on the other hand ISR is not

associated with enhanced production of these hormones prior to pathogen attack (Pieterse et al., 1998; Pieterse et al., 2000). Moreover, the expression profile data are in line with previous findings that WCS417r-treated plants are primed for enhanced expression of the JA-responsive *VSP2* gene and for augmented production of ET after challenge with *Pst* DC3000 (Van Wees et al., 1999; Hase et al., 2003). Hence, we postulate that colonization of the roots by ISR-inducing rhizobacteria triggers a primed state in systemic tissues, resulting in the augmented expression of specific JA- and ET-dependent defense responses upon pathogen challenge.

If this hypothesis is correct, one would expect that ISR-expressing plants exhibit an enhanced defensive capacity against pathogens that trigger, and are sensitive to, JA/ET-dependent defenses. In a study in which the spectrum of effectiveness of rhizobacteria-mediated ISR was investigated, Ton et al. (2002a) recently demonstrated that, indeed, ISR is effective only against pathogens that are sensitive to JA/ET-dependent basal defenses. It was postulated that ISR constitutes a reinforcement of extant JA/ET-dependent basal defense responses, but the nature of this sensitizing effect remained unclear. Since priming of JA/ET-responsive genes allows ISR-expressing plants to respond faster and/or more strongly to pathogens that trigger JA/ET-dependent defense responses, it is likely that this process plays an important role in the enhancement of JA/ET-dependent basal defense responses during ISR.

Priming is a process that provides the plant with an enhanced capacity for rapid and effective activation of cellular defense responses that are induced only after contact with a pathogen. In the past ten years, priming has been associated with several types of induced resistance (Conrath et al., 2002). For instance, pretreatment of parsley cell cultures with low doses of the SAR activator SA, or its functional analogues 2,6-dichloroisonicotinic acid (INA) or benzothiodiazole (BTH), was demonstrated to prime the cells for potentiated activation of various cellular defense responses, in response to otherwise non-inducing doses of a cell-wall elicitor from *Phytophthora sojae*. These potentiated responses included the early oxidative burst (Kauss and Jeblick, 1995), the incorporation of various phenolics into the cell wall (Kauss et al., 1992), the secretion of antimicrobial phytoalexins (Kauss et al., 1992; Katz et al., 1998), and the potentiated expression of several defense-related genes (Thulke and Conrath, 1998). In Arabidopsis and tobacco, SAR has also been shown to be associated with priming for potentiated expression of SA-responsive *PR* genes (Mur et al., 1996; Cameron et al., 1999; Van Wees et al., 1999; Kohler et al., 2002). Other types of induced resistance in which priming plays an important role are those triggered by the non-protein amino acid *ß*-aminobutyric acid (BABA) (Zimmerli et al., 2000) and bacterial lipopolysaccharide (LPS) (Newman et al., 2002). In Arabidopsis, BABA

pretreatment was shown to result in a more rapid and stronger deposition of callose-containing papillae at the site of infection by the oomycetous pathogen *Peronospora parasitica*, or to a strong potentiation of *PR-1* gene expression in response to infection by *Pst* DC3000 (Zimmerli et al., 2000). In pepper plants, pretreatment with LPS resulted in accelerated synthesis of two anti-microbial hydroxycinnamoyl-tyramine conjugates and the potentiated expression of an acidic *PR-2* gene in response to infection by the bacterial pathogen *Xanthomonas campestris* pv. *campestris* (Newman et al., 2002). A common feature of the different types of induced disease resistance is that they display effectiveness against different plant pathogens. It is tempting to speculate that the broad-spectrum characteristic of induced resistance is based on the conditioning of the tissue to react more effectively to an invading pathogen.

Recently, Cartieaux et al. (2003) performed a transcriptome analysis of Arabidopsis upon colonization of the roots by the rhizobacterium *Pseudomonas thivervalensis* (strain MLG45). Like WCS417r, MLG45 induced a systemic resistance that was effective against *Pst* DC3000. However, in contrast to WCS417r, MLG45 induced very few changes in the transcriptome of roots, whereas systemically in the leaves an increase in defense-related transcripts was detected prior to challenge inoculation. These results clearly contrast with ours but can be readily explained. First of all, Cartieaux et al. (2003) performed their study in Arabidopsis accession Ws-0, which is blocked in its ability to express JA/ET-dependent ISR, such as triggered by WCS417r, due to a defect at the *ISR1* locus (Ton et al., 1999; Ton et al., 2001). By inference, WCS417r and MLG45 must induce different defense signaling pathways in Arabidopsis. Secondly, colonization of the roots by WCS417r stimulates plant growth in the absence of pathogen infection (Pieterse and Van Loon, 1999). In contrast, MLG45 induced a clear growth reduction under such conditions (Persello-Cartieaux et al., 2001; Cartieaux et al., 2003), suggesting that the systemic changes in gene expression observed in MLG45-induced plants are likely to be caused by a more general stress response. One must conclude, therefore, that WCS417r and MLG45 induce different defense responses in Arabidopsis.

In conclusion, our study showed that the onset of WCS417r-mediated ISR is not associated with detectable changes in gene expression in leaves, but rather results from the induction of a primed state, allowing augmented expression of pathogen-responsive genes. The molecular mechanism of priming is still unclear. It is hypothesized that induction of the primed state results in an increase in the amount or activity of cellular components with important roles in defense signaling (Conrath et al., 2002). By itself, the increased presence or activity of these cellular signaling components have no major effect, but provide the plant with an enhanced capacity to respond to an invading pathogen. In terms of energy costs for the plant, priming might

prove to be highly beneficial. On the one hand, the defense responses are only expressed when they are really needed, i.e. upon attack by a pathogen. On the other hand, only those defense responses are recruited that are triggered by the pathogen encountered. So what is the nature of the primed state of ISR? The lack of systemic changes in the expression of the ~8,000 Arabidopsis genes tested suggests that priming is not regulated at the transcriptional level, although crucial changes in gene expression might occur either below the level of detection or within the group of genes that was not present on the GeneChip. Alternatively, priming may be regulated post-translationally. Either way, future research on the mechanism of priming will provide novel insights into how plants are able to defend themselves against harmful organisms.

Experimental procedures

Growth conditions of rhizobacteria and plants

Non-pathogenic *Pseudomonas fluorescens* WCS417r bacteria were used for the induction of ISR. WCS417r was grown for 24 hours at 28 °C on King's medium B agar plates (King et al., 1954) containing the appropriate antibiotics as described previously (Pieterse et al., 1996). Subsequently, bacteria were collected and resuspended in 10 mM $MgSO_4$ to a density of 10^9 cfu/ml (OD_{660}=1.0) before being mixed through soil.

Seeds of *Arabidopsis thaliana* accession Col-0 were sown in quartz sand. For isolation of RNA from roots, two-week-old seedlings were transferred to a system of rock-wool cubes (Rockwool/Grodan B.V., Roermond, the Netherlands), as described previously (Pieterse et al., 1996; Van Wees et al., 1997). Subsequently, the root systems were covered with 1 ml of a 1:1 (w/v) mixture of talcum powder and either a suspension of ISR-inducing WCS417r bacteria in 10 mM $MgSO_4$ (final density $5x10^8$ cfu/g), or a solution of 10 mM $MgSO_4$ as a control. For isolation of RNA from leaves, two-week-old seedlings were transferred to 60-ml pots containing a sand/potting soil mixture that had been autoclaved twice for 20 min with a 24-h interval. Before transfer of the seedlings, a suspension of ISR-inducing WCS417r bacteria (10^9 cfu/ml) was mixed through the soil to a final density of $5x10^7$ cfu/g as described previously (Pieterse et al., 1996). Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. Plants were cultivated in a growth chamber with a 9-hr day (200 μE m^{-2} sec^{-1} at 24 °C) and a 15-hr night (20 °C) cycle at 70% relative humidity. Plants were watered once a week with water and once a week with modified half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938).

Pathogen inoculation and ISR bioassay

The virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Whalen et al., 1991) was used for challenge inoculation. *Pst* DC3000 was grown overnight in liquid King's medium B at 28°C. Subsequently, bacterial cells were collected by centrifugation and resuspended to a final concentration of $2.5x10^7$ cfu/ml in 10 mM $MgSO_4$ containing 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands). To confirm expression of ISR in WCS417r-treated plants, ISR bioassays were performed as described previously (Pieterse et al., 1996; Van Wees et al., 1997), using a subset of plants that were grown in parallel with the plants that were used for extraction of RNA. Briefly, 5-week-old plants were placed at 100% relative humidity one day prior to challenge inoculation. Subsequently, plants were inoculated by dipping the leaves for 2 sec in a suspension of *Pst* DC3000 at $2.5x10^7$ cfu/ml in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77. Four days later, 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 for each plant (n=20), based on the percentage of diseased leaves.

Sample preparation and microarray data collection

For isolation of RNA from roots and non-inoculated leaf tissues, whole root systems or entire rosettes were harvested 3 and 7 days after the start of the induction treatment (see above) and immediately frozen in liquid nitrogen. For extraction of RNA from *Pst* DC3000-inoculated leaves, whole rosettes from 5-week-old, soil-grown plants were harvested at 0, 6 and 24 h after challenge inoculation. RNA was prepared from frozen tissue using RNAwiz (Ambion, Huntington, United Kingdom). Subsequently, cRNA probe synthesis, hybridization to a GeneChip, and collection of data from the hybridized GeneChip was performed as described previously (Zhu et al., 2001). Hybridizations with labeled cRNAs were conducted with Arabidopsis GeneChip Microarrays (Affymetrix, Santa Clara, USA) containing probe sets for approximately 8,000 Arabidopsis genes (Zhu and Wang, 2000).

Expression profiling

Expression data were normalized globally to the average value of 100 before data analysis. Genes with accurately detectable transcript levels were defined

by probe sets showing averaged expression levels greater than 40, as described previously (Zhu and Wang, 2000). For probe sets showing an expression value of <5, it was adjusted to 5. The fold changes between induction treatments and the respective controls were calculated by dividing the induction-treated expression values by the control expression values. Using these selection criteria for the identification of genes displaying greater than 2-fold change, we expected less than 0.25% false changes resulting from inaccuracies of hybridization and detection (Zhu and Wang, 2000). To avoid false positives due to noise arising from variation in experimental conditions, we required the changes to be consistent in time and/or between experiments.

RT-PCR and RNA gel blot analysis

Total RNA was extracted by homogenizing frozen leaf tissue in extraction buffer (0.35 M glycine/0.048 M NaOH/0.34 M NaCl/0.04 M EDTA/4% (w/v) SDS; 1 ml per gram of plant tissue). The homogenates were extracted with phenol/chloroform/isoamylalcohol (25:24:1) and the RNA was precipitated using LiCl, as described by Sambrook *et al*. (1989). Analysis of gene expression in the roots was performed by RT-PCR, as described previously (Pieterse et al., 1998). Gene-specific primer pairs for the detection of *MYB72*, *MLO8*, and *VSP2* transcripts in the roots were designed based on the annotated sequences corresponding to AGI numbers At1G56160 (probe set 12725_r_at), At2G17480 (probe set 13687_s_at), and At5G24770 (probe set 14675_s_at). Analysis of gene expression in the leaves was performed by RNA gel blot analysis. To this end, 15 μg of RNA was denatured using glyoxal and DMSO, as described previously (Sambrook et al., 1989). Subsequently, samples were electrophoretically separated on 1.5% agarose gels and blotted onto Hybond-N^+ membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. RNA blots were hybridized with gene-specific probes as described previously (Pieterse et al., 1998). Templates for the preparation of gene-specific probes were prepared by PCR with primers based on the annotated sequences corresponding to AGI numbers AT4G05320 (*UBQ10*), AT5G24780 (*VSP1*), AT5G24770 (*VSP2*), and AT5G44420 (*PDF1.2*), respectively. After hybridization with α-^{32}P-dCTP-labelled probes, blots were exposed for autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, the Netherlands) with Quantity One software (BioRad, Veenendaal, the Netherlands).

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Supplementary Material

Table S1: MS Exel file with probe set numbers, normalized expression levels, fold-change information, AGI numbers and TIGR description of the selected *Pst* DC3000-responsive genes in control and ISR-expressing Arabidopsis plants. This table is available at http://www.bio.uu.nl/~fytopath/Table_S1.htm

Chapter 4

Always aim for the sun, if you miss,
you're still amongst the stars

Unknown

Rhizobacteria-induced systemic resistance in Arabidopsis is controlled by a MYB transcription factor in the roots

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Abstract

Colonization of roots of *Arabidopsis thaliana* by non-pathogenic *Pseudomonas fluorescens* WCS417r bacteria triggers a jasmonate- and ethylene-dependent induced systemic resistance (ISR) response that is effective against a broad range of foliar pathogens. Transcriptome analysis revealed that ISR-expressing leaves are primed for augmented expression of predominantly jasmonate- and ethylene-responsive genes, which may allow the plant to react more effectively to the invader encountered. Locally in the roots, a large number of genes is up or down regulated upon induction of ISR by WCS417r. To investigate the role of WCS417r-responsive, root-specific genes in ISR signaling, we analyzed knockout mutants of a subset of these genes. Analysis of selected T-DNA insertion lines revealed that AtMYB72, a R2R3-MYB-like transcription factor protein, is essential for activation of ISR. *AtMYB72* is specifically activated upon colonization of the roots by WCS417r. Disruption of the gene by T-DNA insertion 15 base pairs downstream from the start codon rendered the knockout mutant plants incapable of mounting WCS417r-mediated ISR against the challenging pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Elicitation of ISR by crude cell wall material of WCS417r or by live cells of rhizobacterial strain *Pseudomonas putida* WCS358r was also blocked in the *myb72* knockout mutant. WCS417r-induced expression of *AtMYB72* was abolished in the ethylene-insensitive, ISR-nonresponsive *ein2-1*, but not in the ISR-responsive, salicylic acid-defective transformant NahG. Moreover, AtMYB72 was found to physically interact *in vitro* with the ethylene-regulatory protein EIL3. These results indicate that induction of ISR by root-colonizing WCS417r-bacteria is controlled by the AtMYB72 transcription factor in the roots, and that AtMYB72 is an intrinsic part of local, ethylene-dependent signaling events that eventually lead to systemic expression of ISR in the leaves.

Introduction

In order to defend themselves against pathogen attack, plants have evolved sophisticated defense mechanisms in which the signal molecules salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) often play crucial roles. Clarification of signal-transduction pathways controlling disease resistance is a major objective in research on plant-pathogen interactions. The capacity of a plant to develop a broad-spectrum, systemic acquired resistance (SAR) after primary infection with a necrotizing pathogen is well-known, and its SA-dependent signal transduction pathway extensively studied (reviewed in Dong, 2001; Métraux, 2001). Plants of which the roots have been colonized by specific strains of non-pathogenic, fluorescent *Pseudomonas* spp. develop a phenotypically similar

form of systemic protection, referred to as rhizobacteria-induced systemic resistance (ISR) (Van Loon et al., 1998). Rhizobacteria-mediated ISR has been demonstrated in many plant species, e.g. bean, carnation, cucumber, radish, tobacco, tomato and the model plant *Arabidopsis thaliana*, and is effective against a broad spectrum of plant pathogens, including fungi, bacteria, and viruses (Van Loon et al., 1998)..

The ability of a plant to develop ISR in response to selected strains of root-colonizing bacteria depends on the host/rhizobacterium combination (Van Loon et al., 1998; Pieterse et al., 2002). Such specificity indicates that recognition between the plant and the ISR-inducing rhizobacterium is required for the induction of ISR, and that ISR is genetically determined. The non-pathogenic, rhizobacterial strain *Pseudomonas fluorescens* WCS417r has been shown to trigger ISR in several plant species (Pieterse et al., 2002). In Arabidopsis, colonization of the roots by WCS417r was shown to protect the plant against the bacterial leaf pathogens *Xanthomonas campestris* pv. *armoraciae* and *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, the fungal leaf pathogen *Alternaria brassicicola*, the oomyceteous leaf pathogen *Peronospora parasitica* and the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani* (Pieterse et al., 1996; Van Wees et al., 1997; Ton et al., 2002a). Protection against these pathogens is typically manifested as a reduction in disease severity as well as an inhibition of pathogen growth.

Although both rhizobacteria-mediated ISR and pathogen-induced SAR are effective against a broad spectrum of pathogens, their signal transduction pathways are clearly distinct. The onset of SAR is accompanied by local and systemic increases in endogenous levels of salicylic acid (SA) (Malamy et al., 1990; Métraux et al., 1990) and the transcriptional reprogramming of a large set of genes (Ward et al., 1991; Maleck et al., 2000), including genes encoding pathogenesis-related (PR) proteins (Van Loon and Van Strien, 1999). Several PR proteins possess *in vitro* anti-microbial activity and are thought to contribute to the enhanced state of resistance attained. Transduction of the SA signal requires the function of NPR1, a regulatory protein that was identified in Arabidopsis through genetic screens for SAR-compromised mutants (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Mutant *npr1* plants accumulate normal levels of SA after pathogen infection, but are impaired in their ability to enhance the expression of *PR* genes and to mount a SAR response. In contrast to SAR, WCS417r-mediated ISR functions independently of SA. This was demonstrated by observations that Arabidopsis genotypes that are impaired in SA accumulation (i.e. NahG, *eds5*, *sid2*) display levels of ISR equal to those of wild-type plants upon colonization of the roots by WCS417r (Pieterse et al., 1996; Pieterse et al., 2002; Ton et al., 2002b). Analysis of the jasmonic acid (JA)-response mutant *jar1-1*, a range of ethylene-response mutants, and the SAR-compromised mutant *npr1-1*, revealed that components of the JA- and the ET-response are required for triggering ISR and that this induced resistance response, like SAR, depends on NPR1 (Pieterse et al., 1998; Knoester

et al., 1999). However, downstream of NPR1, the ISR and the SAR signaling pathways diverge because, unlike SAR, ISR is not accompanied by the activation of *PR* genes (Pieterse et al., 1996; Van Wees et al., 1997; Van Wees et al., 1999).

To identify ISR-related genes, we recently surveyed the transcriptional response of over 8,000 Arabidopsis genes during rhizobacteria-mediated ISR using Affymetrix GeneChip Arabidopsis Genome Arrays (Chapter 3). Systemically in the leaves, none of the ~8,000 genes tested showed a consistent change in expression in response to effective colonization of the roots by WCS417r, indicating that the onset of ISR in the leaves was not associated with detectable changes in gene expression. After challenge inoculation of the induced plants with *Pst* DC3000, 81 genes showed an augmented expression in the leaves, suggesting that these genes were primed to respond faster and/or more strongly upon pathogen attack. The majority of the primed genes appeared to be regulated by JA and/or ethylene signaling. Priming of pathogen-induced genes will allow the plant to react more effectively to a subsequent invader, which might explain the broad-spectrum action of rhizobacteria-mediated ISR (Conrath et al., 2002: Chapter 3).

Whereas in the leaves no changes in gene expression were evident before challenge inoculation, roots responded to the colonization by ISR-inducing WCS417r bacteria with significant changes in the expression of 97 genes (Chapter 3). To investigate the role of these WCS417r-responsive, root-specific genes in ISR signaling, we analyzed knockout mutants of a subset of these genes. Analysis of selected T-DNA insertion lines revealed that the *AtMYB72* gene, encoding a R2R3-MYB-like transcription factor protein, is essential for the systemic activation of ISR. *AtMYB72* is a member of the large *R2R3-MYB* gene familiy of which 125 members have been identified in Arabidopsis (Kranz et al., 1998; Stracke et al., 2001). R2R3-MYB transcription factors are implicated in the regulation of specific plant processes, although the function of most of them is still unknown (Stracke et al., 2001).

Results

Knockout mutant analysis of root-specific, WCS417r-responsive genes

Previously, analysis of changes in the transcriptome of Arabidopsis roots in response to colonization by ISR-inducing WCS417r bacteria revealed 97 genes that showed a locally altered expression in the roots (Chapter 3). To investigate the possible involvement of these WCS417r-responsive genes in ISR signaling, we started to systematically analyze knockout mutants of these genes for their ability to express WCS417r-mediated ISR against *Pst* DC3000. Out of 11 knockout mutants tested, one knockout mutant with a T-DNA

insertion in the *AtMYB72* gene, which is significantly up-regulated in the roots upon colonization by WCS417r (Chapter 3), was identified as being ISR-non-responsive and was subjected to further detailed studies. Figure 1A shows that the *myb72* mutant was unable to mount ISR against *Pst* DC3000 in response to colonization of the roots by WCS417r. As a control we used a knockout mutant with a T-DNA insertion in a gene encoding a nodulin-like protein, which was also found to be up-regulated in the roots in response to WCS417r (Chapter 3). The nodulin-like protein knockout mutant showed wild-type levels of ISR (Fig. 1A), indicateing that the presence of a T-DNA construct by itself had no effect on the capacity of Arabidopsis to express ISR.

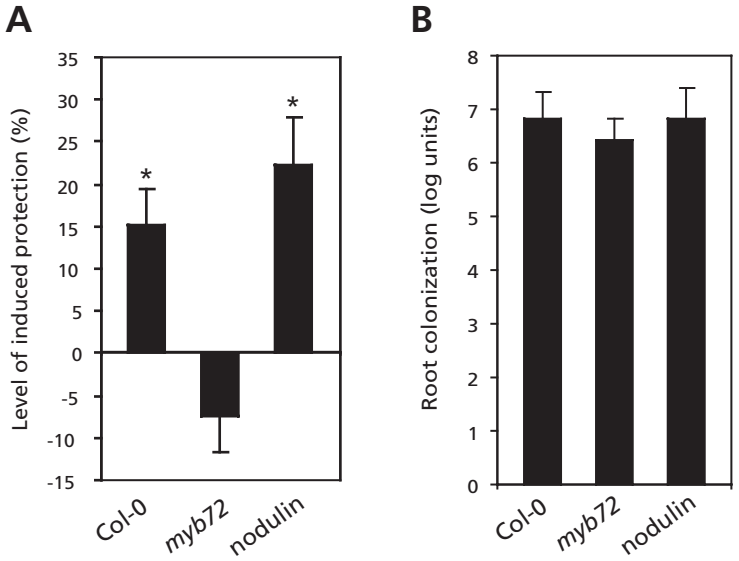

Figure 1. Quantification of *P. fluorescens* WCS417r-mediated ISR against *Pst* DC3000.

(A) Level of induced protection against *Pst* DC3000 in wild-type Col-0 and knockout mutant plants containing a T-DNA insertion in the *AtMYB72* (*myb72*) gene or in a gene encoding a nodulin-like protein (nodulin). ISR was induced by growing the plants for 3 weeks in soil containing ISR-inducing *P. fluorescens* WCS417r bacteria at 5 x 10^7 cfu.g-1 or 10 mM $MgSO_4$ as a control. Five-week-old plants were challenge inoculated with a bacterial suspension of virulent *Pst* DC3000 at 2.5 x 10^7 cfu.ml-1. Four days after challenge inoculation, the percentage of diseased leaves was assessed and the level of induced protection calculated on the basis of the reduction in disease symptoms relative to challenged, non-induced plants. Asterisks indicate statistically significant differences compared to non-induced control plants (Students *t*-test: α=0.05; n=20). (B) Number of rifampicin-resistant WCS417r bacteria (log10 of the number of cfu.g-1 FW) in the rhizospere of the plants at the end of the ISR bioassay. In the rhizosphere of non-induced plants, no rifampicin-resistant bacteria were detected (detection limit 10^3 cfu.g-1 root FW). Data presented are means from a representative experiment that was repeated at least twice with similar results. Error bars represents standard errors.

To investigate whether the inability to express ISR in *myb72* was caused by insufficient colonization of the rhizosphere by WCS417r, the number of rifampicin-resistant WCS417r bacteria per gram of root fresh weight was

determined at the end of the bioassay. Figure 1B shows that WCS417r colonized the rhizosphere of Col-0 plants and the knockout mutant lines to similar levels. Thus, the loss of the capacity to express WCS417r-mediated ISR in *myb72* was not caused by changes in bacterial root colonization, but must be the result of the disruption of the *AtMYB72* gene.

Knockout mutant myb72 *is specifically blocked in rhizobacteria-mediated ISR*

Previously, rhizobacterial strain *Pseudomonas putida* WCS358r and a crude cell wall preparation of WCS417r were demonstrated to trigger the ISR signaling pathway in Arabidopsis, resulting in a similar level of induced protection against *Pst* DC3000 as ISR induced by live WCS417r bacteria (Van Wees et al., 1997). To investigate whether ISR triggered by these inducers is also blocked in *myb72*, roots of Col-0 and *myb72* plants were treated with killed WCS417r cells, with living WCS417r or WCS358r bacteria, and tested for the expression of ISR. Col-0 plants treated with crude WCS417r cell wall material, living WCS417r or WCS358r bacteria all showed comparable levels of protection against *Pst* DC3000 (Fig. 2A). Knockout mutant *myb72* was unable to mount ISR in response to all three inducers, confirming that *AtMYB72* is required for ISR signaling. Again, the lack of ISR expression in *myb72* could not be attributed to changes in bacterial root colonization, because the roots of Col-0 and *myb72* were colonized equally well by live WCS417r and WCS358r (Fig. 2A).

To investigate the effect of the *myb72* mutation on pathogen-induced SAR, we compared the levels of rhizobacteria-mediated ISR and pathogen-induced SAR in this mutant. SAR was induced 3 days prior to challenge inoculation with virulent *Pst* DC3000 by infiltrating three lower leaves with avirulent *Pst* DC3000(*avrRpt2*). Wild-type Col-0 plants developed significant levels of protection against *Pst* DC3000 in response to induction of ISR and SAR (Fig. 2B). In contrast to ISR, SAR was expressed to wild-type levels in *myb72*, indicating that the ability to develop SAR was not altered in the mutant.

Like rhizobacteria-mediated ISR and pathogen-induced SAR, exogenous application of methyl jasmonate (MeJA), the ethylene precursor 1-amino-cyclopropane-1-carboxylate (ACC), or SA triggers an enhanced level of resistance against *Pst* DC3000 (Van Wees et al., 1999). To examine the effect of the *myb72* mutation on resistance induced by these chemicals, Col-0 plants were pre-treated with MeJA, ACC, or SA at 7 and 4 days before challenge inoculation with virulent *Pst* DC3000. Figure 2C shows that *myb72* developed wild-type levels of protection against *Pst* DC3000 in response to all three chemicals, indicating the *myb72* mutation has no effect on the ability to express enhanced resistance in response to MeJA, ACC, and SA.

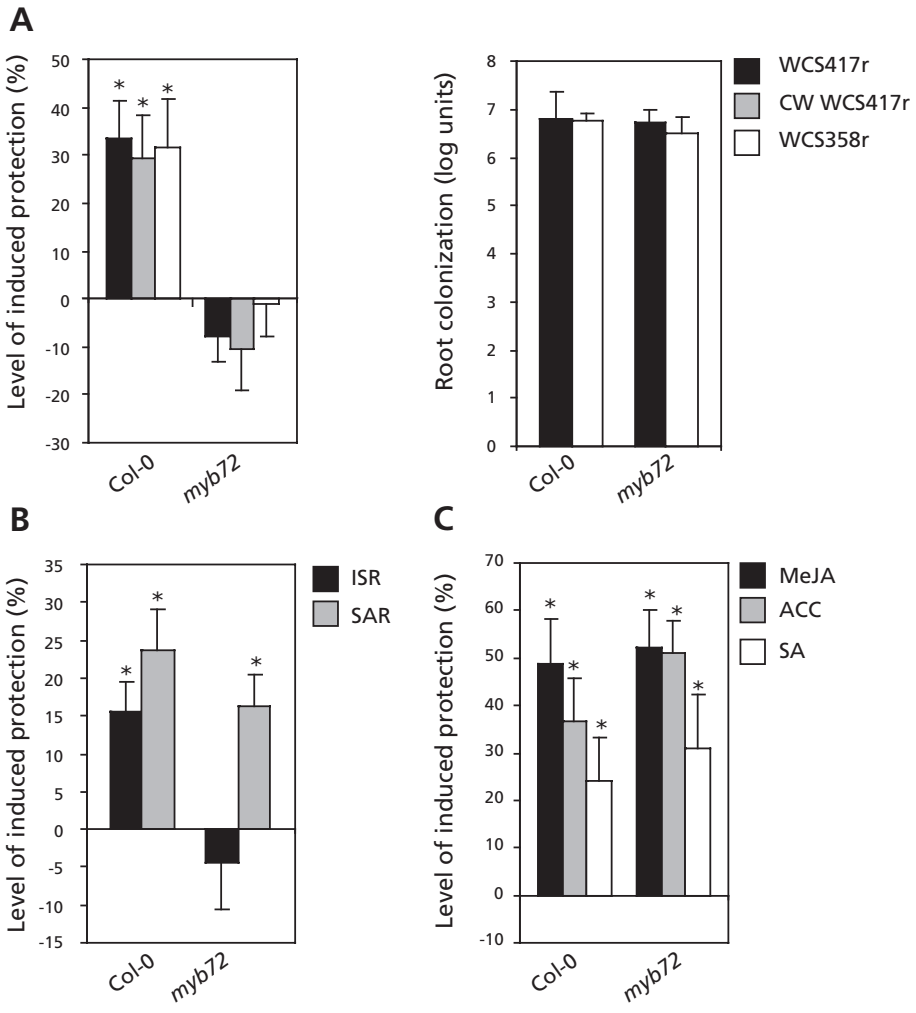

Figure 2. **Levels of induced protection against *Pst* DC3000 in wild-type Col-0 and knockout mutant *myb72*.**

Quantification of induced protection against *Pst* DC3000 in wild-type Col-0 and knockout mutant *myb72* plants after (A) treatment of the roots living *P. fluorescens* WCS417r or *P. putida* WCS358r bacteria, or with crude cell wall material of WCS417r (CW WCS417r); (B) elicitation of pathogen-induced SAR in comparison to WCS417r-mediated ISR; and (C) induction of resistance by exogenous application of either MeJA, ACC, or SA. For details on the ISR bioassay and root colonization (A and B), see the legend to Figure 1. Induction of SAR was performed 3 days before challenge inoculation by pressure infiltrating three lower leaves with a suspension of *Pst* DC3000(*avrRpt2*) bacteria at 10^7 cfu.ml-1. Chemical inductions were performed by applying 10 ml of either 0.1 mM MeJA, 1 mM ACC, or 1 mM SA as a soil drench 7 and 4 days prior to challenge inoculation with *Pst* DC3000. Three days after challenge inoculation, the percentage of diseased leaves was assessed, and the level of induced protection calculated on the basis of the reduction in disease symptoms relative to challenged control plants. Asterisks indicate statistically significant differences compared to non-induced control plants (Students *t*-test: α=0.05; n=20). Data presented are means from a representative experiment that was repeated at least twice with similar results. Error bars represents standard errors.

Molecular analysis of knockout mutant myb72

According to the SAIL table (www.tmri.org), knockout mutant *myb72* (insertion line SAIL_713G10) contains a single T-DNA insertion 15 bp downstream of the start codon of the *AtMYB72* gene (Fig. 3A). To verify the predicted insertion of the T-DNA in the *myb72* knockout mutant, genomic DNA flanking the T-DNA insertion was amplified using the PCR procedure described by Sessions et al. (2002). Amplification of a PCR product from genomic DNA of Col-0 with *AtMYB72*-specific forward and reverse primers that were predicted to anneal left and right of the T-DNA insertion in *myb72*, resulted in a PCR product of an expected size of 547 bp (Fig. 3B). Amplification on genomic DNA of the *myb72* mutant did not result in a PCR product, suggesting that the *AtMYB72* gene was indeed disrupted by the large 4762 bp T-DNA insertion, resulting in a fragment that is too large to be amplified under the PCR conditions used. PCR analysis using the *AtMYB72*-

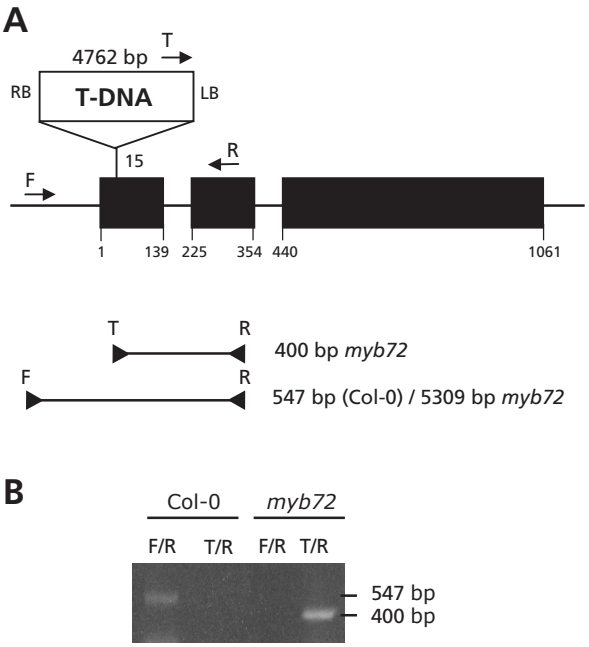

Figure 3. Genomic structure of the *AtMYB72* gene and verification of the position of the T-DNA in knockout mutant *myb72*.

(A) Structure of the *AtMYB72* gene and position of the T-DNA insertion in the *myb72* mutant allele. Exons are indicated as black boxes. The nucleotide numbers underneath indicate the start and the end of the exons. The 4762-bp T-DNA insertion in *myb72* is located 15 bp downstream of the predicted start codon of the *AtMYB72* open reading frame. The primers used for the verification of the position of T-DNA insertion are indicated by arrows. The expected size of the PCR products for Col-0 and *myb72* are indicated underneath the schematic structure of the *AtMYB72* gene. LB, left border of the T-DNA; RB, right border of the T-DNA; F, *AtMYB72* forward primer; R, *AtMYB72* reverse primer; T, T-DNA left border primer. (B) PCR amplification of genomic DNA of Col-0 and *myb72* plants using the T-DNA left border primer and the *AtMYB72* forward and reverse primers.

specific reverse primer and the T-DNA left border primer yielded a product when genomic DNA from the *myb72* knockout mutant was used as template, and not when genomic DNA from Col-0 was used. These results confirm that *myb72* contains a T-DNA insertion at the predicted location in the *AtMYB72* gene.

AtMYB72 *encodes a R2R3-MYB transcription factor protein*

AtMYB72 encodes a putative R2R3-MYB protein in Arabidopsis. Previously, the entire Arabidopsis R2R3-MYB gene family was analyzed (Kranz et al., 1998; Stracke et al., 2001). The conserved R2R3 domain of AtMYB72 shares highest sequence homology with the subgroup 3 R2R3-MYB proteins AtMYB58 (At1g16490; 83% identity) and AtMYB63 (At1g79180; 80% identity) (Fig. 4). No functional data exist for these R2R3-MYB transcription factor proteins. However, a high homology was found also with Arabidopsis AtMYB15 (74% identity; AGI number At3g23250). This gene was shown to be active in the roots and is strongly induced within 0.5 hr upon wounding (Cheong et al., 2002). Alignment of the R2R3 domain of AtMYB72 with amino acid sequences in the databases further revealed high homology with the MYB-like transcription factor proteins OsLTR1 from rice (75% identity; GeneBank accession AAP92750) and ZmMRP1 from maize (75% identity; GeneBank accession S04898). The OsLTR1 protein is thought to be involved in JA-induced resistance (NCBI database locus information).

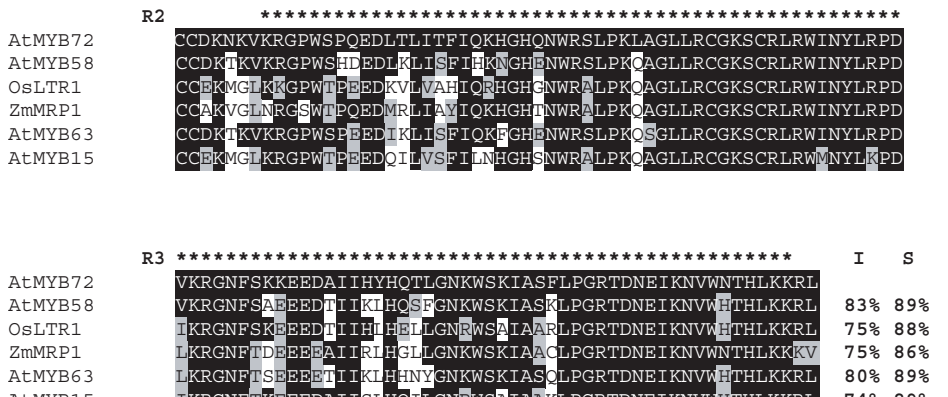

Figure 4. **Alignment of AtMYB72 with other R2R3 MYB transcription factor proteins.**

Alignments show the amino acid sequences of the R2 and R3 repeats and their flanking regions. The repeats are indicated by asterisks. Amino acids in black are identical, whereas those in grey are similar to the amino acids in AtMYB72. Percentages of identity (I) and similarity (S) of the aligned amio acid sequences to the AtMYB72 sequence are indicated. Sequences are from Arabidopsis (AtMYB72, AtMYB58, ATMYB63, AtMYB15), *Oryza sativa* (OsLTR1) and *Zea mays* (ZmMRP1)(AGI and GeneBank numbers At1g56160, At1g16490, At1g79180, At3g23250, AAP92750 and S04898 respectively).

WCS417r-induced AtMYB72 *expression in the roots requires ethylene signaling*

Analysis of the transcriptome of Arabidopsis roots revealed that the *AtMYB72* gene is specifically activated in roots upon colonization by WCS417r (Chapter 3). Analysis of *AtMYB72* transcript levels by RT-PCR confirmed that *AtMYB72* is up-regulated in WCS417r-treated roots of wild-type Col-0 plants (Fig. 5A). Systemically in the leaves, *AtMYB72* mRNA was not detectable (data not shown), indicating that WCS417r-induced expression of *AtMYB72* is confined to the roots. SA-non-accumulating NahG plants that are blocked in SAR (Gaffney et al., 1993), but develop normal levels of rhizobacteria-mediated ISR (Pieterse et al., 1996), showed wild-type levels of WCS417r-induced *AtMYB72* gene expression in the roots. This result confirms that *AtMYB72* gene expression is not associated with SAR (Fig. 2B). As a control, transcript levels of the *VSP2* gene were determined, because *VSP2* gene expression was shown to remain unchanged in WCS417r-colonized roots (Chapter 3).

Previously, Knoester et al. (1999) demonstrated that induction of rhizobacteria-mediated ISR requires ethylene signaling at the site of application of the inducer. To investigate whether rhizobacteria-induced *AtMYB72* gene expression in the roots is regulated by ethylene, *AtMYB72*

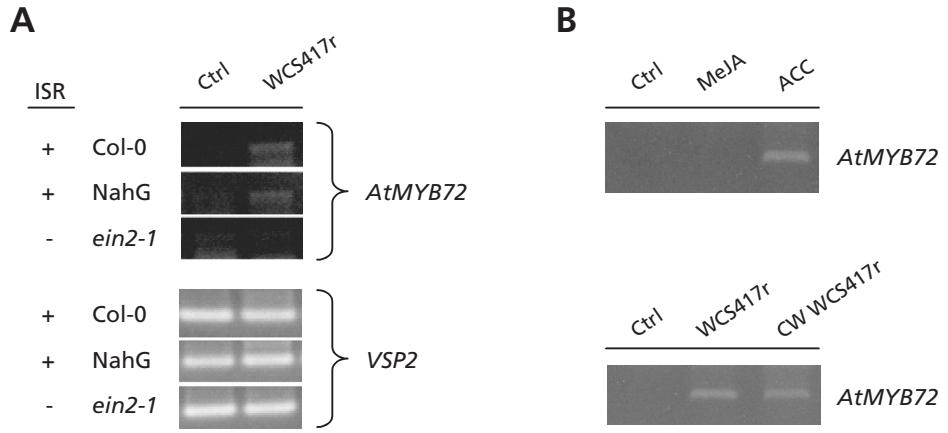

Figure 5. RT-PCR analysis of *AtMYB72* gene expression in roots of wild-type Col-0, transgenic NahG, and mutant *ein2-1* plants.

Shown are ethidium bromide-stained agarose gels with RT-PCR products obtained after amplification of equal portions of first-strand cDNA using gene-specific primers for the *P. fluorescens* WCS417r-responsive *AtMYB72* gene or the *VSP2* gene, which is constitutively expressed in the roots. (A) RT-PCR analysis of *AtMYB72* and *VSP2* gene expression in roots of Arabidopsis Col-0, NahG and *ein2-1* plants 7 days after treatment of the roots with $MgSO_4$ (Ctrl) or WCS417r bacteria. WCS417r is capable of triggering ISR in wildtype Col-0 and NahG plants (+) but not in *ein2-1* plants (-). (B) RT-PCR analysis of *AtMYB72* gene expression in the roots of Col-0 plants after treatment of the roots with with 10 mM $MgSO_4$ (Ctrl), 0.1 mM MeJA, 1 mM ACC, living WCS417r bacteria, or crude cell wall material of WCS417r (CW WCS417r).

transcript accumulation was examined in the ethylene-insensitive mutant *ein2-1* (Guzmán and Ecker, 1990), which is blocked in the expression of WCS417r-mediated ISR (Knoester et al., 1999). Figure 5A shows that WCS417r-induced expression of *AtMYB72* was abolished in *ein2-1*, indicating that rhizobacteria-induced expression of *AtMYB72* in the roots is dependent on ethylene signaling. To further investigate the signals involved in the induction of *AtMYB72* gene expression, *AtMYB72* transcript levels were analyzed in roots that were treated with either crude cell wall material of WCS417r, 1 mM ACC, or 0.1 mM MeJA. Figure 5B shows that MeJA did not activate *AtMYB72* gene expression. However, treatment of the roots with crude cell wall material of WCS417r or ACC resulted in the accumulation of *AtMYB72* transcripts, suggesting that WCS417r triggers *AtMYB72* gene expression in an ethylene-dependent, but JA-independent manner.

Ethylene sensitivity of myb72 *plants*

In Arabidopsis, loss of sensitivity to ethylene results in the inability to develop WCS417r-mediated ISR (Pieterse et al., 1998; Knoester et al., 1999; Ton et al., 2001). The observation that WCS417r-induced *AtMYB72* expression requires ethylene signaling prompted us to investigate whether the inability of the *myb72* knockout mutant to express ISR is based on a reduced sensitivity to ethylene. The "triple response" is a reaction of etiolated seedlings to ethylene, and is commonly used as a reliable marker for ethylene sensitivity (Guzmán and Ecker, 1990). Etiolated Col-0, *ein2-1* and *myb72* seedlings were grown in the dark on MS agar plates with or without ACC. Ten days after germination, Col-0 seedlings grown on ACC showed a typical ethylene-induced growth inhibition of the hypocotyl and root, both characteristics of the triple response (Fig. 6). As expected, the triple response was not apparent in the ethylene-insensitive *ein2-1* seedlings. In contrast, mutant *myb72* seedlings reacted in the same way as wild-type plants. These results demonstrate that knockout mutant *myb72* is not impaired in general ethylene sensitivity.

AtMYB72 physically interacts with EIL3 in vitro

Transcription factors usually exert their action in a complex with other proteins. Therefore, a systematic search for proteins that physically interact with AtMYB transcription factors has been initiated by members of the EU-funded REGIA (Regulatory Gene Initiative in Arabidopsis) consortium (http://www.cordis.lu/en/home.html). In a systematic screen using the ProQuest two-hybrid system (Invitrogen), AtMYB72 was found to physically interact with the ETHYLENE INSENSITIVE3 (EIN3)-like protein EIL3 (M. Proveniers, personal communication). EIN3 and its paralogs, the EIN3-like proteins EIL1, EIL2 and EIL3, are nuclear transcription factors that bind to the promoters of ethylene-responsive genes, such as *ETHYLENE RESPONSE FACTOR1*

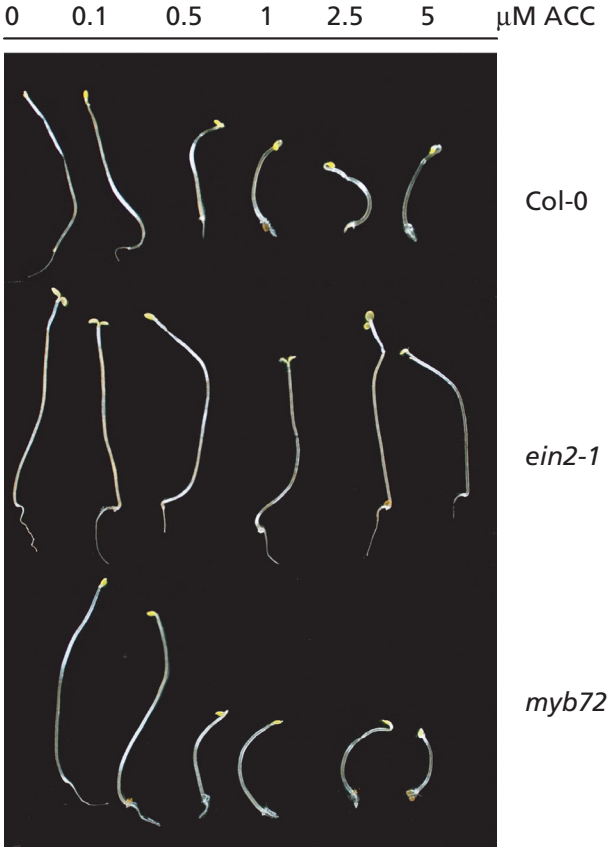

Figure 6. Triple response test of Col-0, *ein2-1* and knockout mutant *myb72*.

Seeds were germinated on agar containing 0, 0.1, 0.5, 1, 2.5 or 5 µM ACC for 3 days at 4°C in the dark and grown further in darkness at 20°C. Photographs were taken after growth for 7 days.

(ERF1), and initiate a transcriptional cascade leading to the regulation of ethylene target genes (Chao et al., 1997; Solano et al., 1998). These results again suggest a role of the ethylene signaling pathway in the regulation of AtMYB72 function..

Discussion

Colonization of the roots of Arabidopsis by non-pathogenic fluorescent *Pseudomonas* bacteria, such as WCS417r and WCS358r, leads to an enhanced level of resistance against a broad spectrum of pathogens in foliar tissues (Pieterse et al., 2002). Genes that show root-specific changes in expression in response to colonization by ISR-inducing rhizobacteria are potentially

involved in the onset of ISR. In a previous study, we identified 97 genes that show this characteristic (Chapter 3). Here, we demonstrate the role of one of these genes, *AtMYB72,* in the onset of rhizobacteria-mediated ISR in the roots of Arabidopsis. A T-DNA insertion in the *AtMYB72* gene resulted in a loss to express rhizobacteria-mediated ISR in the leaves against *Pst* DC3000 (Fig. 1). Pathogen-induced SAR and resistance induced by the chemicals MeJA, ACC and SA was not affected in this mutant (Fig. 2), indicating that the *myb72* mutation specifically affects rhizobacteria-mediated induction of resistance. These findings indicate that AtMYB72 plays a role in the onset of ISR in the roots and is required for systemic activation of ISR in the leaves.

AtMYB72 is a member of a large class of genes that contains one or more MYB domains (Stracke et al., 2001). *MYB* genes were first identified as oncogenes derived from retroviruses in animal cells (Klempnauer et al., 1982). *MYB* genes encode transcription factor proteins that share the conserved MYB DNA-binding domain (Jin and Martin, 1999). MYB proteins are categorized into subfamilies depending on the number of conserved repeats of the MYB domain. MYB proteins from animals generally contain three MYB repeats, which are referred to as R1, R2 and R3. Most of the *MYB*-like genes in plants have only the R2 and R3 repeats (Kranz et al., 1998). An inventory of the Arabidopsis genome revealed that this plant species contains approximately 125 *R2R3-MYB* genes (Stracke et al., 2001). R2R3-MYB proteins in plants have been implicated in a range of activities, such as in plant secondary metabolism, regulation of cell death, stress tolerance (reviewed in Stracke et al., 2001), and pathogen resistance (Mengiste et al., 2003), but the functions of most of them have not been determined. The AtMYB72 protein was found to possess high homology with AtMYB58, AtMYB63 and AtMYB15, and with the MYB transcription factor proteins ZmMRP1 from maize and OsLTR1 from rice (Fig. 4). Rice OsLTR1 has been implicated in JA-dependent defense responses, whereas AtMYB15 is implicated in the wound response (Cheong et al., 2002). Previously, Mengiste et al. (2003) identified R2R3 MYB transcription factor protein BOS1 (AtMYB108) and demonstrated a role for this protein in resistance against necrotrophic pathogens. Outside the conserved R2R3 domain, the amino acid sequence of AtMYB72 has no significant homology with that BOS1, suggesting that both MYB transcription factors are not functionally related.

Previously, Kranz et al. (1998) analyzed the expression patterns of a large set of Arabidopsis *R2R3-MYB* genes in different plant organs and under various conditions, such as various hormone treatments, different forms of abiotic stress, and infection by *P. syringae*. *AtMYB72* transcripts were not detected in any of the organs or conditions tested (Kranz et al., 1998). This agrees with our findings that *AtMYB72* transcripts cannot be detected in roots or leaves of non-induced or *Pst* DC3000-infected plants (Fig. 5; Chapter 3).

These results suggests that WCS417r-induced *AtMYB72* gene expression, as observed in roots of ISR-expressing Col-0 and NahG plants (Fig. 5), may be a specific response of the roots to ISR-inducing WCS417r bacteria. The observation that ACC activates the expression of *AtMYB72*, and that the ISR-non-expressing mutant *ein2-1* is unable to respond to WCS417r-mediated induction of *AtMYB72*, suggests that *AtMYB72* gene expression is dependent on ethylene signaling in the roots.

Interestingly, yeast two-hybrid experiments revealed that AtMYB72 physically interacts with EIL3, a member of the EIN3 family of transcription factors. EIN3 and its paralogs, the EIN3-like proteins EIL1, EIL2 and EIL3, are key transcription factors of ethylene-regulated gene expression and act as positive regulators of ethylene signaling (Stepanova and Ecker, 2000). They bind to promoters of ethylene-responsive genes, such as *ERF1*, and initiate a transcriptional cascade leading to the expression of ethylene-targeted genes (Chao et al., 1997; Solano et al., 1998). Recently, EIN3 transcription factors were shown to physically interact with the F-box proteins EBF1 and EBF2, which target EIN3 for degradation through the ubiquitin/proteasome pathway (Guo and Ecker, 2003; Potuschak et al., 2003). Thus, regulation of EIN3 protein stability appears to be a key step in certain responses of plants to ethylene. Like all ethylene-insensitive mutants tested, mutant *ein3-1* is blocked in WCS417r-mediated ISR (Knoester et al., 1999; Pieterse et al., 2002). Whether a mutation in the *EIL3* gene compromises ISR as well is currently investigated.

A requirement for ethylene-dependent signaling in the roots for the induction of ISR has been coined earlier by Knoester et al. (1999). It was shown that mutant *eir1-1*, which is insensitive to ethylene in the roots only, develops no ISR when WCS417r bacteria were applied to the roots, but showed normal levels of ISR when WCS417r bacteria were infiltrated in the leaves. If ethylene signaling were required only for expression of ISR at the site of challenge inoculation, *eir1-1* plants would develop normal levels of ISR in the leaves after application of WCS417r to the roots. However, this was not the case. Thus, ethylene signaling is required at the site of application of the inducer, and may be involved in the generation or translocation of the systemically transported signal. This conclusion is significant because recently it was demonstrated that ethylene is also required for the generation or the translocation of the systemically transported signal in SAR (Verberne et al., 2003). Yet, the two signals must differ, because ISR and SAR are different types of induced resistance that are effective against partly dissimilar ranges of pathogens.

Although AtMYB72 is essential for the onset of ISR, it does not affect general ethylene sensitivity, because the ethylene-induced triple response was unaltered in knockout mutant *myb72* (Fig. 6). The requirement of ethylene

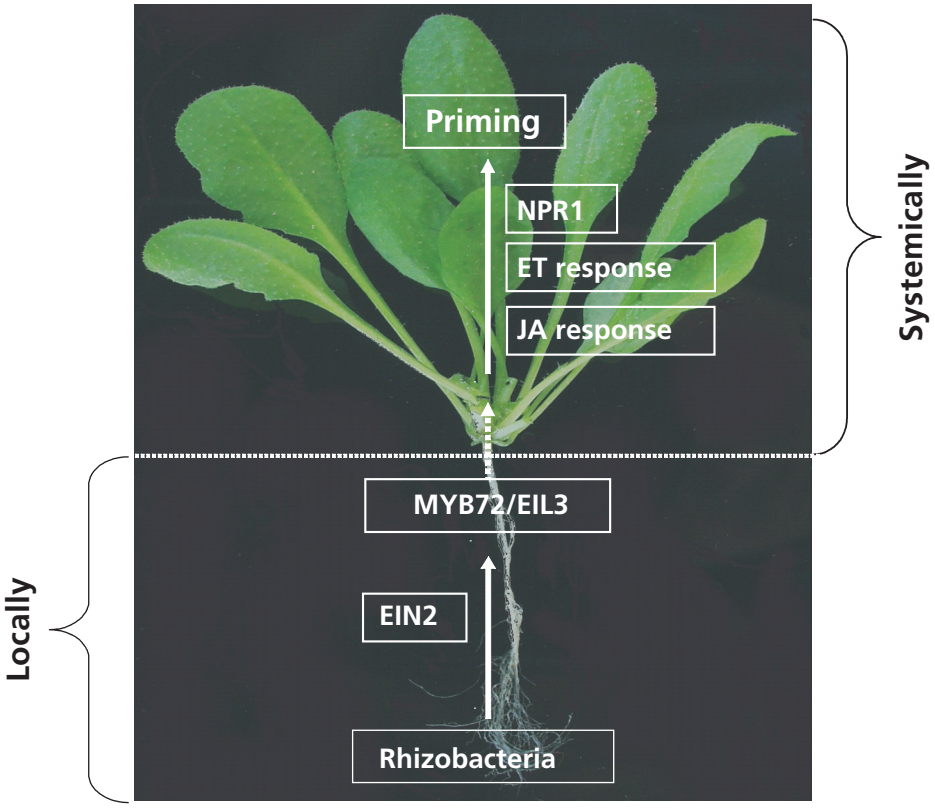

Figure 7. **Proposed model for the role of AtMYB72 in the signal transduction pathway controlling rhizobacteria-mediated ISR.**

Colonization of the roots by ISR-inducing *P.fluorescens* WCS417r leads to a local, EIN2-dependent activation of the *AtMYB72* gene. Subsequently, AtMYB72 interacts physically with the EIL3 protein, resulting in the onset of an as yet unknown ethylene-dependent process, which eventually leads to the systemic expression of ISR. Systemically in the leaves, the ISR signal transduction cascade requires responsiveness to both JA and ethylene, and is dependent on NPR1. Finally, induction of ISR is associated with priming of a large set of JA- and ethylene-dependent genes for augmented expression after pathogen attack. This allows the plant to react more effectively to an invading pathogen, which may explain the broad-spectrum characteristic of rhizobacteria-mediated ISR.

at the site of ISR induction, however, does not rule out the possibility that ethylene responsiveness is also required for the systemic expression of ISR in tissues distant from the site of induction. This is supported by the fact that endogenous application of ACC to *myb72* knockout mutant plants leads to enhanced resistance against *Pst* DC3000 (Fig. 2C). Apparently, *myb72* is only blocked in its ability to induce WCS417r-mediated ISR, but not in the capacity to mount defense responses that are directly induced by ethylene.

The identification of AtMYB72 as an important signaling component in the local induction of ISR adds a new factor to ISR signal transduction.

Figure 7 summarizes our current understanding of the ISR signaling pathway. The local onset of WCS417r-mediated ISR is associated with the activation of the *AtMYB72* gene, which is required for the systemic expression of ISR. WCS417r-induced *AtMYB72* expression in the roots is EIN2-dependent. How the observed physical interaction between AtMYB72 and EIL3 is involved in this process is currently unclear. Local AtMYB72 activity precedes systemic activation of ISR in the leaves. In the leaves, expression of ISR requires responsiveness to JA and ethylene and is dependent on NPR1 (Pieterse et al., 1998). Systemic expression of WCS417r-mediated ISR in Arabidopsis has been shown to be predominantly effective against pathogens that are resisted through JA- and ethylene-dependent defenses, and that ISR constitutes an enhancement of these basal defenses responses (Ton et al., 2002a). Indeed, upon pathogen challenge of ISR-expressing plants, JA/ethylene-dependent defenses are primed (Chapter 3). This state of enhanced defensive capacity allows the plants to respond faster and/or more strongly to pathogens that trigger JA/ethylene-dependent defense responses.

Apart from WCS417r and WCS358r, other non-pathogenic rhizobacteria have also been shown to induce a SA-independent systemic induced resistance pathway in Arabidopsis (Iavicoli et al., 2003; Ryu et al., 2003), tobacco (Press et al., 1997; Zhang et al., 2002) and tomato (Yan et al., 2002). Thus, the ability to trigger an SA-independent pathway controlling systemic resistance is not uncommon among ISR-inducing rhizobacteria. However, not all resistance-inducing rhizobacteria appear to trigger a SA-independent resistance. For instance, an SA-overproducing mutant of *Pseudomonas aeruginosa* 7NSK2 and a genetically modified, SA-overproducing derivative *P. fluorescens* strain CHA0 have been shown to trigger the SA-dependent SAR pathway by producing SA at the root surface (De Meyer and Höfte, 1997; Maurhofer et al., 1998). In most SA-independent cases of rhizobacteria-mediated ISR, JA and ethylene seem to play major roles. Whether MYB72-like transcription factors generally play a role in the local onset of ISR is currently unknown. Addressing this question would provide insight in the signaling events associated with rhizobacteria-mediated ISR. Moreover, understanding the role of the physical interaction between AtMYB72 and EIL3 in ethylene-dependent processes that are locally induced by WCS417r and which are required for systemic activation of ISR will be an exciting challenge for future ISR research.

Experimental procedures

Bacterial cultures

Non-pathogenic, rifampicin-resistant *Pseudomonas fluorescens* WCS417r and *Pseudomonas putida* WCS358r bacteria were used for induction of

ISR (Van Wees et al., 1997). Both strains were grown for 24 hr at 28°C on Kings medium B agar plates (King et al., 1954) containing the appropriate antibiotics as described previously (Pieterse et al., 1996). An avirulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 carrying the avirulence gene *avrRpt2* (*Pst* DC3000 (*avrRpt2*) (Kunkel et al., 1993) was used for SAR induction. *Pst* DC3000(*avrRpt2*) bacteria were grown overnight at 28°C in liquid KB medium supplemented with 25 mg.ml^{-1} kanamycin to select for the plasmid. Virulent *Pst* DC3000 (Whalen et al., 1991) was used for challenge inoculations and cultivated in a similar manner in liquid KB medium without kanamycin. After centrifugation for 10 min. at 5,000 x *g*, the bacterial cells were resuspended in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands) to a final density of 2.5x107 cfu.ml^{-1}.

Preparation of crude cell wall material of WCS417r

To check the effect of killed rhizobacterial cells, upon collection and centrifugation cells were resuspended in 50 mM Tris-HCl, 2 mM EDTA (pH8.5). The cells were then sonicated eight times for 15 sec on ice at resonance amplitude. Still intact cells were removed by centrifugation at 600 x *g* for 20 min. After centrifugation of the supernatant at 8000 x *g* for 60 min, the pellet of crude cell wall material was resuspended in 10 mM phosphate buffered saline (pH7.2), 0.01% sodium azide, and stored at -80°C until further use.

Plant growth conditions

Seeds of wild-type *Arabidopsis thaliana* Col-0, transgenic NahG plants harboring the bacterial *nahG* gene (Gaffney et al., 1993), mutant *ein2-1* (Guzmán and Ecker, 1990), and the various knockout mutants (see below), were sown in quartz sand. For bioassays and isolation of RNA from the leaves, 2-week-old Arabidopsis seedlings were transferred into a sand/potting soil mixture that had been autoclaved twice for 20 min with a 24-h interval. Prior to transfer of the seedlings to the pots, a suspension of ISR-inducing WCS417r or WCS358r bacteria (10^9 cfu.ml^{-1}) was mixed thoroughly through the soil to a final density of 5x10^7 cfu.g^{-1}, as described previously (Pieterse et al., 1996). Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. For isolation of RNA from roots, 2-week-old seedlings were transferred to a system of rock-wool cubes (Rock-wool/Grodan B.V., Roermond, The Netherlands), as described previously (Pieterse et al., 1996; Van Wees et al., 1997). Subsequently, the root systems were covered with 1 ml of a 1:1 (w/v) mixture of talcum powder and either a suspension of ISR-inducing rhizobacteria in 10 mM $MgSO_4$ (final density 5x10^8 cfu.g^{-1}), or a solution of 10 mM $MgSO_4$ (final density 5x10^8 cfu.g^{-1}), or a solution of 10 mM $MgSO_4$ as a control. Plants were cultivated in a growth chamber with a 9-hr day (200 µE m^{-2} sec^{-1} at 24°C) and a 15-hr night (20°C) cycle at 70% relative humidity.

Plants were supplied with modified half-strength Hoagland nutrient solution once a week, as described (Hoagland and Arnon, 1938; Pieterse et al., 1996).

Knockout mutants

Homozygous knockout mutants SAIL713G10 (Sessions et al., 2002) and SALK037123 (Alonso et al., 2003), containing a T-DNA insertion in At1g56160 (*AtMYB72*), and At2g39510 (encoding a nodulin-like protein), respectively, were grown as described above. Confirmation of the T-DNA insert in SAIL713G10 was obtained by PCR on genomic DNA with a T-DNA left border primer and a gene-specific forward and reverse primer as described previously (Sessions et al., 2002). The following primers were used: 5'-TTCATAACCAATCTCGATACAC-3' (T-DNA left border); 5'-TGCCGTTAACTTCCGTACGTT-3' (*AtMYB72* forward); 5'-CTTCACGTCCGGTCTCAGAT-3' (*AtMYB72* reverse).

Induction treatments

Induction of ISR with living rhizobacteria was performed by mixing ISR-inducing rhizobacteria through the soil as described above. For tests with killed cells, a crude cell wall preparation of WCS417r bacteria (in 10 mM $MgSO_4$) was mixed through the soil in a similar manner, using the equivalent of the number of live bacteria introduced in the soil ($5x10^7$ cfu.g^{-1}). Seven days before the challenge inoculation, a similar amount of the crude cell wall material was applied to each plant as a soil drench as described previously (Van Wees et al., 1997).

Induction of SAR was performed 3 days before challenge inoculation by pressure infiltrating 3 lower leaves with a suspension of avirulent *Pst* DC3000(*avrRpt2*) bacteria at 10^7 cfu.ml^{-1}, as described previously (Pieterse et al., 1996).

Chemical treatments were performed 7 and 4 days prior to challenge inoculation with *Pst* DC3000 by applying 10 ml of either 100 μM methyl jasmonate (MeJA), 1 mM 1-aminocyclopropane-1-carboxylate (ACC), or 1 mM SA as a soil drench. Control plants were treated with an equal volume of water. MeJA was purchased from Serva Brunschwig (Amsterdam, the Netherlands), ACC from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands) and SA from Malinckrodt Baker BV (Deventer, the Netherlands).

Challenge inoculation and disease assessment

For assaying induced resistance, plants were challenged when 5 weeks old by dipping the leaves for 2 sec in a suspension of virulent *Pst* DC3000 bacteria at $2.5x10^7$ cfu.ml^{-1} in 10 mM $MgSO_4$, 0.01% (v/v) Silwet L-77. One day before challenge inoculation, the plants were placed at 100% relative humidity. Four days after challenge, 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. Based on the number of diseased and non-diseased leaves, the disease index was calculated for each plant (n=20) as described (Pieterse et al., 1996).

Root colonization

Colonization of the rhizosphere of wild-type and mutant plants by rifampicin-resistant WCS417r and WCS358r bacteria was examined at the end of the ISR bioassay. In quadruplicate, roots of five plants per treatment were harvested, weighed, and shaken vigorously for 1 min in 5 ml of 10 mM $MgSO_4$ containing 0.5 g of glass beads (0.17 mm). Appropriate dilutions were plated onto King's medium B agar supplemented with cycloheximide (100 $mg.l^{-1}$), ampicillin (50 $mg.l^{-1}$), chloramphenicol (13 $mg.l^{-1}$), and rifampicin (150 $mg.l^{-1}$), which is selective for rifampicin-resistant, fluorescent *Pseudomonas* spp. (Geels and Schippers, 1983). After overnight incubation at 28°C, the number of rifampicin-resistant colony-forming units per gram of root fresh weight was determined.

RT-PCR and RNA Gel Blot Analysis

Total RNA was extracted by homogenizing frozen tissue in extraction buffer (0.35 M glycine/0.048 M NaOH/0.34 M NaCl/0.04 M EDTA/4% (w/v) SDS; 1 ml per gram plant tissue). The homogenates were extracted with phenol/chloroform/isoamylalcohol (25:24:1) and the RNA was precipitated using LiCl, as described previously (Sambrook et al., 1989). Analysis of gene expression in the roots was performed by RT-PCR, as described previously (Pieterse et al., 1998). The following gene-specific primers for detection of the *AtMYB72* transcript were designed based on the annotated sequences corresponding to AGI number At1g56160: 5'-GACGGGGCTCAAGAAGAAATACAT-3' and 5'-AATCTAGC CGAAAAACGAACCAATA-3'.

Analysis of gene expression in the leaves was performed by RNA gel blot analysis. To this end, 15 μg of RNA was denatured using glyoxal and DMSO as described previously (Sambrook et al., 1989). Subsequently, samples were electrophoretically separated on 1.5% agarose gels and blotted onto Hybond-N^+ membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. RNA blots were hybridized with gene-specific probes as described previously (Pieterse et al., 1998). Templates for the preparation of gene-specific probes were prepared by PCR with the primers described above. As a control a probe to detect the 18S rRNA was used as described previously (Pruitt and Meyerowitz, 1986). After hybridization with α-^{32}P-dCTP-labelled probes, blots were subjected to autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, The Netherlands) with Quantity One software (Biorad, Veenendaal, The Netherlands).

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Chapter 5

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

Sir William Bragg (1862 - 1942)

General discussion

Rhizobacteria-mediated induced systemic resistance

Because plants are sessile organisms, they had to evolve highly sophisticated defensive strategies to withstand all kinds of attackers. These strategies can be based on constitutive or induced defense mechanisms. A classic example of an induced response is triggered after attack by a necrotizing pathogen and called systemic acquired resistance (SAR) (Ross, 1961; Ryals et al., 1996). Another type of biologically-induced systemic resistance occurs after root colonization by certain non-pathogenic, plant-growth promoting rhizobacteria, such as *Pseudomonas fluorescens* WCS417r. This response is often referred to as rhizobacteria-mediated induced systemic resistance (ISR) (Pieterse et al., 1996; Van Loon et al., 1998). Both SAR and ISR are effective against a broad spectrum of pathogens. SAR is regulated by a salicylic acid (SA)-dependent signaling pathway and is associated with the accumulation of pathogenesis-related (PR) proteins. In the model plant Arabidopsis, the signal-transduction pathway leading to ISR was shown to function independently of SA and *PR* gene expression (Pieterse et al., 1996; Van Wees et al., 1997; Pieterse and Van Loon, 1999). Instead, ISR requires responsiveness to the hormonal signals jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 1998; Knoester et al., 1999; Pieterse et al., 2000; Ton et al., 2002b). However, ISR is neither associated with enhanced levels of JA or ET, nor with increased expression of JA- or ET-responsive gene expression (Van Wees et al., 1999; Pieterse et al., 2000). Therefore, it was suggested that ISR is based on an enhanced sensitivity to these hormones, rather than on an increase in their production.

Previously, genetic studies on the molecular basis of rhizobacteria-mediated ISR, employing Arabidopsis SA-, JA-, and ET-defective signaling mutants and the natural variation in ISR inducibility among Arabidopsis accessions, revealed crucial components of the ISR signaling pathway (Van Wees, 1999; Ton, 2001; Pieterse et al., 2002). Besides components from the

JA and ET signaling pathway, the SAR regulatory protein NPR1 was shown to be required for full expression of ISR (Pieterse et al., 1998). Because NPR1 is required for both SA-dependent SAR and JA/ET-dependent ISR, it is likely that NPR1 is able to differentially regulate defense-related gene expression, depending on the signaling pathway that is activated upstream of it. Downstream of NPR1, SAR signaling is associated with the activation of a large set of defense-related genes (Ward et al., 1991; Maleck et al., 2000). However, several attempts to identify genes that are specifically expressed during ISR failed (Van Wees, 1999; Van Wees et al., 1999). Therefore, the work described in this thesis was started with the following question:

* What mechanism, and which genes are important for the local onset and systemic expression of rhizobacteria-mediated ISR in Arabidopsis?

In order to answer this question, a large collection of gene trap and enhancer trap lines was screened to identify genes that are specifically activated in response to colonization of the roots by ISR-inducing rhizobacteria. Moreover, the transcriptome of Arabidopsis during the onset of ISR in the roots and systemic expression of ISR in the leaves was investigated using microarray analysis. Other techniques, such as cDNA-AFLP and suppression subtractive hybridization were also considered, because these methods are particularly suited for studying low abundance mRNAs. However, these techniques were not pursued in this research but might be of advantage addition in future research.

Local onset of rhizobacteria-mediated ISR

Transcriptome of roots upon colonization by ISR-inducing rhizobacteria

Root colonization by *P. fluorescens* WCS417r leads to enhanced resistance in the leaves against a broad range of pathogens, e.g. the bacteria *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and *Xanthomonas campestris* pv. *armoraciae* (*Xca*), the fungus *Alternaria brassicicola,* and the oomycete *Peronospora parasitica* (Pieterse et al., 1996; Van Wees et al., 1997; Ton et al., 2002a). Moreover, colonization by WCS417r leads to enhanced resistance against the soil-borne fungus *Fusarium oxysporum* f.sp. *raphani* (Pieterse et al., 1996; Van Wees et al., 1997). This indicates that upon perception of rhizobacteria by the plant a signal is generated that is transported to non-colonized tissues, where it renders those plant parts more resistant to pathogen attack. To identify genes that are activated locally in the

roots upon colonization by ISR-inducing rhizobacteria, we screened gene trap and enhancer trap lines for WCS417r-induced gene expression (Chapter 2) and analyzed the transcriptome of WCS417r-treated roots (Chapter 3). Colonization by WCS417r led to many changes in gene expression in Arabidopsis roots, some transient, some more persistent (Chapters 2 and 3). Seven days after application of WCS417r bacteria to the roots, a much smaller number of genes showed altered expression than at four days, indicating that roots adapt to the presence of the rhizobacteria.

Recently, Cartieaux et al. (2003) performed a transcriptome analysis of Arabidopsis upon colonization of the roots by the rhizobacterium *Pseudomonas thivervalensis* (strain MLG45). Like WCS417r, MLG45 induced a systemic resistance that was effective against *Pst* DC3000. However, in contrast to WCS417r, MLG45 induced very few changes in the transcriptome of roots, while in the leaves many stress-related genes were activated. This clearly contrasts with the findings described in Chapter 3. Firstly, this difference must, at least in part, be related to the use of Arabidopsis accession Ws-0, which is incapable of expressing ISR due to a defect at the *ISR1* locus (Ton et al., 1999; Ton et al., 2001b). By inference, WCS417r and MLG45 must induce different defense signaling pathways in Arabidopsis. Secondly, colonization of the roots by WCS417r stimulates plant growth in the absence of pathogen infection (Pieterse and Van Loon, 1999). In contrast, MLG45 induced a clear growth reduction under such conditions (Persello-Cartieaux et al., 2001; Cartieaux et al., 2003), suggesting that the systemic changes in gene expression observed in MLG45-induced plants caused by a more general stress response. Large changes in gene expression are commonly observed upon stress treatments, such as cold, drought and high-salinity (Cheong et al., 2002; Kreps et al., 2002; Seki et al., 2002; Oono et al., 2003).

Besides the thaumatin-like gene *AtTLP1*, identified in Chapter 2, transcript profiling revealed 97 additional genes showing consistent changes in the roots upon colonization by WCS417r. Of these 97 genes, 18% are predicted to be involved in cell rescue and defense, 14% in metabolism, 15% in regulating gene transcription, and 7% in cellular communication and signal transduction (Chapter 3). To what extent these root genes are essential for the onset of ISR was further investigated using knockout mutants.

Activation of *AtTLP1* is a common response to non-pathogenic rhizobacteria, but is unrelated to ISR

AtTLP1, which was found to be specifically activated in root xylem tissue in response to fluorescent *Pseudomonas* spp. strains and the ET precursor

1-aminocyclopropane-1-carboxylate (ACC) (Chapter 2), encodes a protein that belongs to the class of PR-5 proteins (Van Loon and Van Strien, 1999; An_lovar and Dermastia, 2003). A large number of PR-5 proteins from different plant species has been isolated and characterized (Anžlovar and Dermastia, 2003). Several PR-5 proteins have been shown to possess antimicrobial activity in *in vitro* assays (Woloshuk et al., 1991; Anžlovar and Dermastia, 2003), or *in vivo* (Liu et al., 1994), suggesting a role for AtTLP1 in the enhanced resistance observed during ISR. However, knockout mutant and overexpressor analyses revealed that WCS417r-induced expression of *AtTLP1* is unlikely to contribute to the induced resistance observed during ISR. Thus, *AtTLP1* activation must be a common response of Arabidopsis to non-pathogenic rhizobacteria.

Previously, another ISR-unrelated response of Arabidopsis to non-pathogenic rhizobacteria was described. Hase et al. (2003) showed that leaves of plants of which the roots are colonized by WCS417r bacteria have an enhanced capacity to convert ACC to ET. This enhanced ACC-converting capacity led to a potentiated expression of the ET-responsive genes *PDF1.2* and *HEL* after treatment of the leaves with 1 mM ACC, and a significantly higher level of ET emission after challenge inoculation with *Pst* DC3000. However, *Pseudomonas fluorescens* WCS374r bacteria that were unable to induce ISR against *Pst* DC3000 likewise enhanced the *in vivo* ACC oxidase activity in wild-type plants. Moreover, the ISR-compromised mutants *jar1* and *npr1* also showed a significant increase in their ability to convert ACC to ET after treatment of the roots with WCS417r. These results suggested that the induction of an enhanced ACC-converting capacity is a general response of plants to *P. fluorescens* bacteria and that this response does not contribute to ISR against *Pst* DC3000 in *Arabidopsis*.

Nevertheless, both WCS417r-induced responses clearly demonstrate that plants perceive the rhizobacteria present on the roots and react in ways that may, or may not, involve ISR. Plants are actively secreting exudates and releasing lysates at their root surface (Lynch and Whipps, 1991; Walker et al., 2003) that can be used as a food source by rhizobacteria. The large group of genes with altered expression after root colonization by WCS417r that are related to metabolism, protein synthesis and export (Chapter 3) could be involved in nutrient acquisition. Root colonization by rhizobacteria is a common mechanism, while the induction of ISR is plant-bacteria specific (Van Loon et al., 1998; Ton et al., 1999; Ton et al., 2001a; Pieterse et al., 2002). This can explain why WCS417r affects the expression of many different genes in the roots, while only some of them appear to be important for ISR induction.

AtMYB72: a novel critical component in the onset of ISR in the roots

Although part of the genes with an altered expression after root colonization by WCS417r do not seem to be essential for the expression of ISR (Chapters 2 and 4), it cannot be excluded that some of these genes have a modulating influence. In contrast, the *AtMYB72* gene, which was specifically activated in the roots upon colonization by WCS417r, appeared to be essential for the induction of ISR. Knockout analysis revealed that a mutation in this MYB-like transcription factor gene leads to a complete loss of the ability to mount rhizobacteria-mediated ISR (Chapter 4). This gene does not appear to be needed for other types of acquired disease resistance, since induction of SAR by SA or avirulent *Pst* DC3000 was unimpaired. Like *AtTLP1*, the expression of *AtMYB72* appeared to be regulated in an ET-dependent manner (Chapter 4). Moreover, yeast-two hybrid screens revealed that AtMYB72 physically interacts with the ET-regulatory, EIN3-like protein EIL3 *in vitro*. EIN3 and its paralogs, the EIN3-like proteins EIL1, EIL2 and EIL3, are nuclear transcription factors that bind to the promoters of ET-responsive genes, such as *ETHYLENE RESPONSE FACTOR 1* (*ERF1*), and initiate a transcriptional cascade leading to the regulation of ET target genes (Chao et al., 1997; Solano et al., 1998). These results strongly suggest a role of the ET signaling pathway in the regulation of AtMYB72 function in ISR. This is in agreement with the results by Knoester et al. (1999), who showed that ET signaling is needed at the site of ISR induction. In contrast, JA did not appear to affect the expression of *AtMYB72*, suggesting that JA is not required for the induction of ISR in the roots. Knoester et al. (1999) showed that the induction or ISR is impaired after root colonization of the *eir1* mutant, which is ET insensitive in the roots, but not in the shoot. The role of JA during the local onset of ISR could be investigated if a mutant were be available that is insensitive to JA in the roots, but not in the shoot. Unfortunately, such a mutant has not been described yet.

AtMYB72 is a member of a large class of R2R3-MYB-like transcription factor genes of which more than 125 members have been identified in the Arabidopsis genome (Kranz et al., 1998; Stracke et al., 2001). R2R3-MYB transcription factors have been implicated in a range of processes, including plant secondary metabolism, regulation of cell death, stress tolerance (reviewed in Stracke et al., 2001), and pathogen resistance (Mengiste et al., 2003). However the functions of most of them have not been determined. The implication of AtMYB72 in ISR signaling adds a novel function of MYB transcription factors to those known to date.

Down-regulation of ET-responsive genes during the onset of ISR in the roots

In contrast to the WCS417r-induced, ET-responsive genes *AtTLP1* and *AtMYB72,* analysis of the transcriptome of WCS417r-treated Arabidopsis roots revealed that locally in the roots , a set of ET-related genes was significantly down-regulated. Among those down-regulated genes were the genes encoding ERF1, ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 1 (EREBP1), ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 2 (EREBP2), and a putative ACC oxidase (Chapter 3). All these gene products are involved in ET signaling and are thought to function downstream of the AtMYB72-interacting EIL3 protein, suggesting a role for AtMYB72 in this down-regulation. All together, these results suggest that the onset of ISR is accompanied by the down-regulation of the ET signaling pathway. Down-regulation of ET signaling by plant-growth promoting bacteria was also observed during treatment of tomatoes with *Achromobacter piechaudii* ARV8 (Mayak et al., 2004). This rhizobacterial strain shows ACC deaminase activity and reduced the production of ET by tomato seedlings. Colonization by ARV8 significantly improved the recovery after rehydration of plants pretreated by a period of drought.

Interestingly, after root colonization of Arabidopsis by WCS417r, two genes with a function in the SA-dependent resistance response, *SID1* and a *PR-1* homologue, are down-regulated as well (Chapter 3). This could indicate that the onset of ISR is also accompanied by the down-regulation of the SA-dependent pathway. ET has been shown to influence the expression of the *PR-1* gene by sensitizing the tissue (Lawton et al., 1994). Moreover, ethylene and SA signaling may both play a role in the expression of defense-related genes, such as the glutathione S-transferases *AtGSTF2* and *AtGSTF6* (Lieberherr et al., 2003). The down-regulation of *SID1* and the *PR-1* homologue suggests that down-regulation of ET signaling after WCS417r treatment results in the decrease in expression of these genes. Further data are needed to test this hypothesis.

The state of ISR in the leaves

Upon root colonization with WCS417r, ISR can be demonstrated in the leaves seven days later (Ton et al., 2002a). We analyzed gene expression profiles of ISR-expressing leaves at 4 and 7 days after the onset of WCS417r treatment, and compared them to non-induced control plants (Chapter 3). No genes were found that showed consistently altered expression levels during this time period. Those that showed an altered expression in the roots,

were not altered in the leaves of ISR-expressing plants prior to pathogen challenge. These results demonstrate that the onset of ISR is not associated with detectable changes in gene expression in the leaves. It is still possible that genes with an altered expression were not among the 8,000 genes represented on the microarray, that levels of expression were below the background level of 40 fluorescent units needed for detection, or that the changes were less than two-fold and, therefore, too small to pass the stringent selection criteria. These limitations were obvious for the *AtTLP1* gene in the roots, whose expression was below the detection limit on the microarray. The results in Chapter 3 at least show that the expression of ISR is not associated with readily apparent changes in gene expression, which is in strong contrast to plants showing SAR (Maleck et al., 2000; Schenk et al., 2000). This finding corroborates the results of Van Wees et al. (1999), that a large group of well-known defense-related genes showed no difference in ISR-expressing plants compared to control plants. These data leave open the possibility that ISR is regulated post-translationally.

Expression of isr in the leaves

ISR is accompanied by priming of JA/ET-dependent, pathogen-inducible genes

Even though ISR-expressing leaves do not show detectable changes in gene expression (Chapter 3), these leaves are clearly more resistant to subsequent attack by different pathogens (Pieterse et al., 1996; Ton et al., 2002a). The molecular mechanism responsible for this state of ISR in the leaves is as yet unknown. However, Van Wees et al. (1999) demonstrated that in ISR-expressing plants, the JA-responsive gene *AtVSP2* shows a potentiated expression pattern after challenge inoculation with *Pst* DC3000. Other JA-responsive, pathogen-induced genes did not show enhanced transcript levels in ISR-expressing plants. These results suggested that ISR-expressing plants are primed for augmented expression of a specific set of JA-responsive genes. To investigate this in detail, the transcriptome of ISR-expressing Arabidopsis plants was analyzed at different time points after challenge inoculation with *Pst* DC3000. In Chapter 3, it was shown that a subset of pathogen-induced genes is primed during ISR and that this set is enriched in ET- and JA-dependent genes. This suggests that, indeed, ISR is dependent on the priming of JA/ET-dependent genes, including *AtVSP2*, confirming previous findings (Van Wees et al., 1999). Earlier, Ton et al. (2002a) demonstrated that ISR is effective only against pathogens that are sensitive to JA/ET-dependent basal defenses. Together, these results can explain why ISR is largely non-specific, and

effective against all pathogen resisted through JA/ET-dependent mechanisms, but not through SA-dependent mechanisms (Ton et al., 2002a). Indeed, the SA-dependent signal transduction pathway was not primed during ISR. SAR is effective against pathogens that are resisted in a SA-dependent manner, such as turnip crinkle virus, which is sensitive to SAR, but not to ISR. In contrast, pathogens that are resisted by JA/ET-dependent basal resistance, such as *A. brassicicola*, are sensitive to ISR, but not to SAR (Ton et al., 2002a). Pathogens that are resisted in both a SA- and a JA/ET-dependent manner, e.g. *Pst* DC3000, *Xca* and to a lesser extent *P. parasitica*, are sensitive to both SAR and ISR. These latter pathogens were resisted more strongly after induction of ISR and SAR simultaneously than after each separately (Van Wees et al., 2000, Van Pelt and Pieterse, unpublished data).

Priming in plant-pathogen interactions

Priming has emerged as a common feature of different types of induced resistance (Conrath et al., 2002). For instance, SAR-non-inducing doses of the synthetic SAR inducer benzothiadiazole (BTH) were shown to enhance two cellular defense responses after *Pst* infection, namely *PAL* gene expression and callose deposition, without inducing these responses by itself. Moreover, priming for potentiated defense gene expression was also found during *Pst* DC3000(*avrRpt2*)-induced SAR against virulent *Pst* DC3000 (Kohler et al., 2002). Priming was also shown to be important in the enhancement of resistance by the non-protein amino acid ß-aminobutyric acid (BABA). BABA potentiated the accumulation of *PR-1* mRNA after attack by *Pst* (Zimmerli et al., 2000), but BABA treatment of mutants impaired in the SAR pathway did not result in this potentiation (Zimmerli et al., 2000; Zimmerli et al., 2001). Recently, BABA-induced priming of defense responses was shown to be regulated through yet another signaling pathway, that depends on abscisic acid (Ton and Mauch-Mani, 2004). Moreover, in bean, colonization of the roots by the rhizobacterium *Bacillus pumilus* SE34 did not induce morphological alterations of root tissue. However, upon challenge inoculation with *F. oxysporum* f.sp. *pisi*, root cell walls were strengthened more rapidly at the site of attempted fungal penetration by appositions containing large amounts of callose and phenolic materials, effectively preventing fungal attack. This latter result indicated that not only WCS417r can prime resistance responses (Benhamou et al., 1996).

Priming can explain, on the one hand, the lack of changes in leaves of ISR-expressing plants prior to pathogen challenge and, on the other hand, why the plant is able to react more effectively when subsequently attacked. Plants in

a primed state do not have the metabolic costs of a constantly activated defense response, in contrast to plants in the state of SAR (Heil, 2002; Heil and Baldwin, 2002). The costs of constant activation of the SAR pathway are apparent in mutant *cpr1* (for constitutive *PR* gene expressor), which constitutively expresses SAR and is much smaller compared to wild-type Arabidopsis plants (Bowling et al., 1994). The mechanism behind the phenomenon of priming is not yet clear. Recently, it was shown that priming is not only regulated in a positive manner, but can also involve negative regulators. This was demonstrated by the isolation of mutant *edr1* (for enhanced disease resistance), which renders Arabidopsis more resistant to *Pst* and the fungus *Erysiphe cichoracearum* (Frye and Innes, 1998; Frye et al., 2001). The SA-dependent defense pathway was shown not to be constitutively active in this mutant, but to be primed. The *EDR1* gene was shown to encode a MAPKKK similar to CTR1, a negative regulator of the ET pathway (Frye et al., 2001). Because of this primed state, the *EDR1* gene appears to encode a negative regulator of SAR-associated priming. These results indicate that priming is not simply an up-regulation of one single factor, but can be regulated at several levels.

Rhizobacteria-mediated ISR: the working model

Previous research and the research described in this thesis shed light on the mechanism and signal transduction pathway leading to the enhanced state of defensive activity in rhizobacteria-mediated ISR. All the results and indications lead to the following working model. First of all, colonization by WCS417r does not lead to visible symptoms on the roots, but leads to considerable changes in gene expression in these roots. Part of these changes are likely to be needed for the onset of ISR, while other changes, such as the up-regulation of the *AtTLP1* gene, are not, but may be related to acquisition of nutrients. One of the genes that is essential for the onset of ISR is the transcription factor gene *AtMYB72*, which is up-regulated in the roots after treatment with WCS417r (Fig.1). This gene is activated in an ET-dependent, but JA- and SA-independent manner, corroborating previous findings that the local onset of ISR is dependent on responsiveness to ET (Knoester et al., 1999). Whether AtMYB72 and its putative interaction with EIL3 is involved in the generation of the systemically transported ISR signal is currently unknown.

After the up-regulation of *AtMYB72* in the roots, a signal is generated and transported to other plant parts. This AtMYB72 up-regulation does not seem to play any further role after transfer of the signal, because systemically in the leaves no *AtMYB72* gene expression was observed. Systemically, ISR-

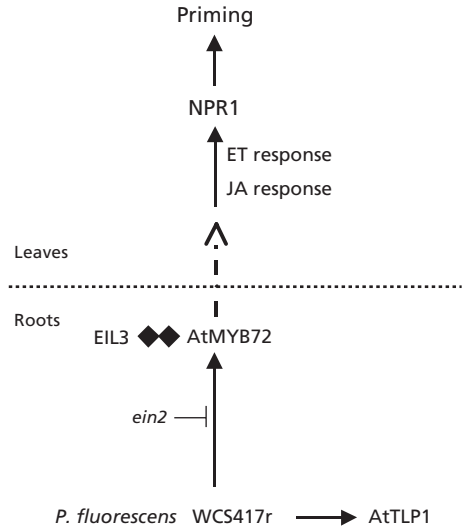

Figure 1: **Proposed model for the signal-transduction pathway controlling rhizobacteria-mediated ISR. Colonization of the roots by *P. fluorescens* WCS417r leads to an *EIN2*-dependent local up-regulation of the *AtMYB72* gene involved in ISR, as well as an up-regulation of the *AtTLP1* gene independent of ISR. The AtMYB72 protein physically interacts with the EIL3 protein. This interaction leads to the generation and transport of an as yet unknown signal to other tissues. Systemically, responsiveness to JA and ET are required in that order. Expression of ISR is also dependent on the NPR1 protein and involves the priming of a set of JA- and ET-dependent genes for augmented expression upon pathogen challenge.**

induced leaves do not show detectable changes in gene expression. Thus, ISR is not characterized by continuous activation of defense responses, such as observed during pathogen-induced SAR. After subsequent pathogen attack, ISR-expressing plants are clearly more resistant to a broad range of pathogens. This enhanced defensive capacity is then accompanied by priming of JA- and ET-dependent defense responses. Among the primed genes were the previously identified genes *AtVSP2* and *PDF1.2*, that are regulated by JA and/or ET, indicative of the involvement of JA/ET in this process. The priming and expression of this enhanced defensive capacity in the leaves is reached through a signal-transduction pathway that includes both the JA- and the ET-response, as well as NPR1, as described previously (Fig.1) (Pieterse et al., 1998).

In conclusion, locally, the onset of ISR is dependent on ET signaling and AtMYB72 function. Systemically, the expression of enhanced resistance is accompanied by priming of JA- and ET-dependent gene expression, leading to a defense response that is dependent on the regulatory NPR1 protein. The state of priming can explain, on the one hand, the lack of detectable changes in gene expression in ISR-expressing tissue prior to pathogen attack, and, on the other hand, the broad-spectrum effectiveness against pathogens that can be resisted by JA/ET-dependent defenses.

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Summary

In science one tries to tell people, in such a way as to be understood by everyone, something that no one ever knew before. But in poetry, it's the exact opposite.

Paul Dirac (1902 - 1984)

Summary

Plants are continuously exposed to harmful organisms. When under attack, plants are capable of enhancing their resistance. A classic example of induced resistance is triggered after infection by a necrotizing pathogen, rendering uninfected, distal parts more resistant to subsequent pathogen attack, and is often referred to as systemic acquired resistance (SAR). The expression of this enhanced resistance is dependent on the accumulation of salicylic acid (SA) and subsequent upregulation of a large set of genes, including ones encoding pathogenesis-related (PR) proteins. A phenotypically comparable type of induced resistance is triggered after root colonization of plants by selected strains of non-pathogenic *Pseudomonas* spp., and is often called induced systemic resistance (ISR). In contrast to pathogen-induced SAR, rhizobacteria-mediated ISR is not associated with accumulation of SA. Instead, rhizobacteria-mediated ISR requires the hormonal signals jasmonic acid (JA) and ethylene (ET). Whereas pathogen-induced SAR is accompanied by an increase in PR gene expression throughout the plant, rhizobacteria-mediated ISR in Arabidopsis is not associated with a systemic increase in the expression of any known defense-related genes.

To identify ISR-related genes that are specifically induced after treatment of the roots with ISR-triggering *Pseudomonas fluorescens* WCS417r bacteria, a large collection of gene trap and enhancer trap lines of Arabidopsis was screened on the basis of inducible expression of the ß-glucuronidase (*GUS*) gene (chapter 2). This resulted in the identification of the *AtTLP1* gene, encoding a thaumatin-like protein that belongs to the PR-5 family of PR proteins. Further analysis revealed that this gene is specifically expressed in xylem tissue of WCS417r-colonized roots. The expression of the *AtTLP1* gene was also induced after treatment of the roots with resistance inducing *Pseudomonas putida* WCS358r and *P. fluorescens* WCS374r, but not after treatment with *Escherichia coli*. Moreover, the *AtTLP1* gene was also responsive to the ET precursor 1-aminocyclopropane-1-carboxylate (ACC) but not to SA or JA, indicating that the expression of this gene is regulated by ET. *AtTLP1* over-expressing and knockout mutant plants showed normal levels of WCS417r-mediated ISR against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Hence, it must be concluded that WCS417r-induced expression of *AtTLP1* in the roots is a common response of Arabidopsis to colonization of the roots by *Pseudomonas* bacteria and is not related to the induction of ISR.

In another attempt to identify ISR-related genes, the transcriptional profile of over 8,000 Arabidopsis genes during the local onset of ISR in the roots and the systemic state and expression of ISR in the leaves was surveyed using Affymetrix Arabidopsis GeneChips (chapter 3). Root colonization by WCS417r elicited a significant change in the local expression of 97 genes. Among these were genes predicted to be involved in cell rescue and defense (18%), metabolism (14%), regulation of gene transcription (15%), and cellular communication and signal transduction (7%). Systemically in the leaves, prior to challenge inoculation, none of the 8,000 genes tested showed a consistent change in expression in response to colonization of the roots by the ISR-inducing WCS417r bacteria. This indicates that the state of ISR in the leaves is not associated with detectable changes in gene expression, even though these leaves are clearly more resistant to subsequent pathogen attack. However, after challenge inoculation with *Pst* DC3000, 81 genes showed an augmented expression pattern in leaves expressing ISR, suggesting that these genes were primed to respond faster and/or more strongly to pathogen attack. Among these primed genes were genes involved in transcription, e.g. *EREBP2* and a WRKY family transcription factor gene, as well as genes involved in defense, such as *PDF1.2* and a gene encoding a thaumatin-like protein. The majority of the primed genes were shown to be regulated in a JA- and/or ET-dependent manner. Priming of pathogen-induced genes allows the plant to react more effectively to an invading pathogen, which might explain the broad-spectrum action of rhizobacteria-mediated ISR. Moreover, since mainly JA/ET-dependent genes were primed, this can explain why ISR is predominantly effective against pathogens that are resisted by JA/ET-dependent defense responses in Arabidopsis.

To investigate the role of some of the genes that were specifically induced in the roots upon colonization by WCS417r, knockout mutant analysis of a subset of these genes was performed (chapter 4). This revealed that most of the genes with altered expression patterns in the roots were not of major importance for the onset of ISR. However, a knockout mutant with a T-DNA insertion 15 basepairs downstream of the start codon of the R2R3-MYB-like transcription factor gene *AtMYB72*, appeared to be blocked in its ability to express ISR against *Pst* DC3000. This block in ISR induction in this *myb72* mutant was apparent after treatment with live WCS417r bacteria, crude cell walls of WCS417r, as well as live WCS358r bacteria. Analysis of the expression of *AtMYB72* revealed that it is not only induced by ISR-inducing WCS417r, but also by ACC. WCS417r-induced expression of *AtMYB72* was blocked in the ET-insensitive, ISR-defective mutant *ein2-1*, but not in the ISR-responsive, SA-defective transformant NahG. Moreover, the AtMYB72 protein was found to physically interact *in vitro* with the ET-regulatory protein EIL3. Together, these results indicate that AtMYB72 plays an essential

role in the local onset of ISR in the roots and that its expression is regulated by the ET signaling pathway.

Collectively, the results described in this thesis lead to the following model of ISR induction and expression. Root colonization by WCS417r leads to several changes in gene expression locally in the roots. Part of these changes is not involved in the onset of ISR, as was shown for *AtTLP1*. In contrast, the induction of the MYB-related transcription factor gene *AtMYB72* is critical for the onset of ISR in the roots and is regulated by components of the ET signaling pathway. The latter is in agreement with previous findings that WCS417r-mediated ISR requires ET signaling at the site of application of the ISR-inducing rhizobacteria. Systemically in the leaves, the state of ISR is not associated with detectable changes in the transcriptome. However, after challenge inoculation, a large set of pathogen-responsive genes shows an augmented expression pattern, indicating that the expression of ISR is associated with priming of pathogen-responsive gene expression. The majority of these primed genes are regulated by a JA- and/or ET-dependent signaling pathway. Priming of pathogen-induced genes allows the plant to react more effectively to the invader encountered, which might explain the broad-spectrum action of rhizobacteria-mediated ISR.

Samenvatting

The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact.

Thomas H. Huxley (1825 - 1895)

Samenvatting

Planten worden voortdurend bedreigd door pathogene micro-organismen, zoals virussen, bacteriën en schimmels. Als een plant geïnfecteerd wordt door een virulent pathogeen worden in het aangetaste weefsel diverse afweermechanismen geactiveerd die erop gericht zijn de ziekteontwikkeling te vertragen. Dit verschijnsel wordt geïnduceerde resistentie genoemd. Als een plant in staat is door een lokale necrose het pathogeen in te perken, verwerft hij niet alleen lokaal, maar ook systemisch (in alle plantendelen) een verhoogde weerbaarheid. Deze vorm van resistentie wordt systemische verworven resistentie genoemd (afgekort SAR) en is effectief tegen verschillende typen pathogenen. Een resistentie die fenotypisch vergelijkbaar is met SAR kan worden geïnduceerd door bepaalde niet-pathogene, wortelkoloniserende *Pseudomonas* bacteriën. Deze vorm van geïnduceerde resistentie wordt aangeduid als geïnduceerde systemische resistentie (afgekort ISR). SAR en ISR uiten zich beide in een verminderde kolonisatie van de plant door een infecterend pathogeen, met als gevolg dat zich minder ziektesymptomen ontwikkelen dan in een niet geïnduceerde plant. De ziekteverwekkers waartegen ISR en SAR effectief zijn, komen gedeeltelijk overeen. Toch zijn er grote verschillen tussen beide vormen van geïnduceerde resistentie. Inductie van SAR gaat gepaard met ophoping van het hormoon salicylzuur (SA) in de plantencel. Dit SA activeert een signaal-transductie cascade die leidt tot de activatie van een groep van genen die coderen voor zogenaamde "pathogenesis-related proteins" (PR eiwitten). De signaal-transductie van ISR verloopt onafhankelijk van SA, maar vereist gevoeligheid voor de hormonen jasmonzuur (JA) en ethyleen (ET). Waar SAR gepaard gaat met een verhoogde expressie van een grote groep van *PR* genen, laat bij ISR geen van de bekende afweer-gerelateerde genen een verandering in expressie zien. De zoektocht naar genen die een belangrijke rol spelen in de inductie en expressie van ISR staat centraal in het onderzoek dat beschreven is in dit proefschrift.

In dit onderzoek is gebruik gemaakt van de modelplant *Arabidopsis thaliana* (zandraket). Behandeling van de wortels van Arabidopsis met de bacterie *Pseudomonas fluorescens* stam WCS417r leidt tot ISR tegen een groot aantal bladpathogenen. In een poging om genen te vinden die geïnduceerd worden door WCS417r werd een collectie "gene trap" en "enhancer trap" lijnen gescreend (hoofdstuk 2). De gebruikte "gene trap" en "enhancer trap" lijnen bevatten een insertie van een *Ds* transposon op een willekeurige plaats in het genoom. Het *Ds* transposon bevat het *ß-glucuronidase* (*GUS*) markergen

met een minimale promoter. Wanneer het *Ds* transposon direct in, of in de buurt van, een actief gen is geinserteerd, wordt het *GUS* gen geactiveerd. Na toevoeging van een substraat wordt dit zichtbaar door een blauwe kleur. Eén "enhancer trap" lijn liet specifiek *GUS* expressie zien in de vaatbundels van door WCS417r gekoloniseerde wortels. Bij toediening van de precursor van ET, 1-aminocyclopropaan-1-carboxylzuur (ACC), aan de wortels werd eenzelfde expressiepatroon waargenomen. Het *AtTLP1* gen bleek verantwoordelijk voor dit door WCS417r en ACC geïnduceerde expressiepatroon. *AtTLP1* codeert voor een thaumatine-achtig eiwit dat behoort tot de PR-5 familie van PR eiwitten. Sommige PR-5 eiwitten hebben een anti-microbiële werking. Daarom werd onderzocht of *AtTLP1* een rol speelt bij ISR. *AtTLP1* werd eveneens geactiveerd door *Pseudomonas putida* WCS358r en *P. fluorescens* WCS374r, maar niet door de niet ISR inducerende bacterie *Escherichia coli*. Voorts resulteerde overexpressie van *AtTLP1* in transgene planten niet in een verandering in het vermogen ISR tot expressie te brengen tegen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Ook had de uitschakeling van *AtTLP1* in een "knockout" mutant geen effect op ISR. Deze resultaten leiden tot de conclusie dat de verhoogde expressie van *AtTLP1* een algemene reactie van Arabidopsis is op de aanwezigheid van de *Pseudomonas* bacteriën en dat deze niet van groot belang is voor de inductie van resistentie tegen *Pst* DC3000.

Om op een andere manier ISR-gerelateerde genen te identificeren, werd het transcriptoom van Arabidopsis onderzocht tijdens de inductie van ISR in de wortels en tijdens de expressie van ISR in de bladeren (hoofdstuk 3). Hierbij werd gebruik gemaakt van zgn. Affymetrix GeneChips met daarop probes voor meer dan 8000 genen. Lokaal resulteerde wortelkolonisatie door WCS417r in een significante verandering in de expressie van 97 genen. Een aantal van deze genen coderen voor transcriptiefactoren, andere voor eiwitten betrokken bij afweer of ET signaal-transductie. In niet geïnoculeerde bladeren van door WCS417r gekoloniseerde planten werden echter geen significante veranderingen in genexpressie waargenomen. Deze resultaten betekenen dat de staat van ISR in systemisch weefsel niet gepaard gaat met waarneembare veranderingen in genexpressie, ondanks het feit dat deze bladeren aanzienlijk resistenter zijn tegen infectie door verschillende pathogenen. Om de reactie van de plant verder te analyseren werd de expressie van de 8000 genen onderzocht na "challenge" inoculatie van geïnduceerde planten met *Pst* DC3000. In planten die gekoloniseerd waren met WCS417r bleken na *Pst* DC3000 infectie 81 genen een significant sterkere en/of snellere verandering in expressie te vertonen in vergelijking met de met *Pst* DC3000 geïnoculeerde controle planten.

Het in geïnduceerde planten sneller of in sterkere mate tot expressie komen van genen na infectie door een pathogeen in vergelijking met niet-geïnduceerde planten wordt "potentiëring" of "priming" genoemd. Een groot deel van deze gepotentiëerde genen bleek gereguleerd te worden door de plantenhormonen JA en ET. Deze potentiëring stelt de plant in staat effectiever te reageren op infectie door een pathogeen, hetgeen deels het brede spectrum van de effectiviteit van ISR kan verklaren. Het feit dat met name door JA en ET gereguleerde genen betrokken zijn bij "priming" in planten die ISR tot expressie brengen, zou kunnen verklaren waarom ISR hoofdzakelijk werkzaam is tegen ziekteverwekkers die gevoelig zijn voor door JA en/of ET gereguleerde afweermechanismen in Arabidopsis.

Om de mogelijke betrokkenheid bij ISR te onderzoeken van de genen die door WCS417r specifiek in de wortels worden aangeschakeld werden zgn. "knockout" mutanten getoetst. Het grootste deel van de "knockout" mutanten bleek niet gestoord te zijn in het vermogen ISR tot expressie te brengen na kolonisatie van de wortels door WCS417r en dus niet van essentieel belang te zijn voor de inductie van ISR. Echter, een "knockout" mutant in het door WCS417r geïnduceerde gen *AtMYB72* bleek niet meer in staat tot expressie van ISR tegen *Pst* DC3000. *AtMYB72* codeert voor een R2R3 MYB-type transcriptiefactor. Naast het verlies van ISR inductie door WCS417r, bleek de *myb72* mutant ook niet meer in staat om ISR tot expressie te brengen na toediening van een celwandpreparaat van WCS417r of na kolonisatie van de wortels door *P. putida* WCS358r. Blijkbaar is AtMYB72 een essentiële factor voor de inductie van ISR in de wortels. Evenals ISR-inducerende rhizobacteriën bleek de ET precursor ACC de expressie van *AtMYB72* aan te schakelen in de wortels, terwijl JA geen effect had. Omgekeerd bleek de door WCS417r geïnduceerde expressie van *AtMYB72* geblokkeerd te zijn in de ET ongevoelige mutant *ein2-1*. Deze resultaten duiden erop dat de waargenomen expressie van *AtMYB72* na kolonisatie van de wortels door WCS417r wordt gereguleerd door ET.

Op grond van de in dit proefschrift beschreven resultaten werd het volgende model opgesteld voor de signaal-transductieroute van ISR. Kolonisatie van de wortels door WCS417r leidt lokaal tot significante veranderingen in de expressie van een groot aantal genen. Sommige van deze genen, zoals *AtMYB72*, zijn essentieel voor de inductie van ISR, terwijl andere, zoals *AtTLP1*, waarschijnlijk bij andere processen betrokken zijn. ET signaal-transductie speelt een belangrijke rol bij de inductie van *AtMYB72* door WCS417r. Omdat *AtMYB72* alleen lokaal in de wortels wordt aangeschakeld en niet systemisch in het blad, is AtMYB72 mogelijk betrokken bij productie of transport van een signaal dat systemisch door de plant wordt verspreid. Perceptie van dit signaal in het blad leidt niet direct

tot waarneembare veranderingen in genexpressie. Echter, na infectie door *Pst* DC3000 laten ISR planten gepotentiëerde veranderingen zien in het expressiepatroon van een groot aantal genen. Het grootste deel van deze genen wordt gereguleerd door ET en/of JA. Dit kan verklaren waarom ISR vooral effectief is tegen pathogenen die gevoelig zijn voor door ET/JA gereguleerde afweerreakties.

Nawoord

The power of accurate observation is
commonly called cynicism by those who have not got it.

George Bernard Shaw (1856 - 1950)

Nawoord

Het wetenschappelijke deel is af, en daarmee zijn we gekomen aan het moeilijkst te schrijven deel en dit is net dat deel wat iedereen als eerste leest (jij toch ook??:)). Om te beginnen wil ik al diegene bedanken die ik per ongeluk verder vergeet. Ondanks dat ik jullie niet noem, hebben jullie wel bijgedragen aan mijn werk of de tijd in utrecht, en daar bedank ik jullie voor.

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gemotiveerd was, en goed en veel werk gedaan hebt. Als je je verstand nu ook eens zou gebruiken terwijl je naar het voetbal kijkt/gaat (leuke canal-plus beelden ☺) dan zou je inderdaad moeten toegeven dat je beter voor PSV kan zijn en dat Sparta DE club van Rotterdam is. Hierna is het "zooitje ongeregeld" over dat er op het einde van de vier jaar bij waren; Frank, Mareike, Vivian, Bart, Martin, Sjoerd en Annemart. Nu kan ik eens zonder tegenspraak vanalles over jullie zeggen, maar ik weet niet of dat allemaal op papier blijft plakken. Verder wil ik jullie nog bedanken voor alle gezelligheid, de nuttige, maar vooral ook (volop aanwezige) nutteloze dingen die jullie hebben ingebracht de afgelopen tijd. Een extra bedankje voor Sjoerd vanwege het lezen van alle ruwe hoofdstukken en zeker naar "mijn para's" Bart en Martin, voor het zijn van goede klankborden, vele late gesprekjes over werk en andere zaken, het Hazes gevoel (jazeker, Hazesfans hebben het hoogste IQ!) en hopelijk voor de goede steun op de derde. Daarnaast wil ik natuurlijk alle mensen bedanken die ervoor zorgen dat alles op de vierde enigszins loopt, alsmede alle mensen van de kas voor de hulp en het gezellige praatje. Uiteraard gaat mijn dank ook uit naar de mensen van B&V voor de layout van mijn boekje.

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Dan blijft er natuurlijk het "home team" over, uit dat mooie Brabant. Laat ik daarbij eens beginnen met de "schoonfamilie". Ik wil jullie bedanken voor alle gezelligheid, interesse, leuke avonden en weekenden, dus eigenlijk "gewoon" voor het zorgen voor een extra thuis; Ton, Marijke, Marloes en Erik, merci voor alles. Hierna blijven mijn twee echte "thuizen" over. Ten eerste de "Liempde gang". Hier wil ik voor alles mijn opa's en oma's bedanken. Nu dit gezegd is, zal ik eens verder gaan met Peggy en Robert (plus natuurlijk de deugnieten die me aan het niezen brengen, super, ondanks de loopneus). Ik wil jullie bedanken voor de extra gezelligheid die jullie komst altijd brengt, de extra levendigheid en de vele verhalen. Ik geniet er altijd erg van, laat ik het maar eens zo zeggen, jullie zijn een prima zus en "schoonbroer" (staat nu zwart op wit, dus jullie eerst! ☺). Verder natuurlijk bedankt voor de kaft, ja, ga allemaal nog maar eens kijken en raad maar wat het is! Nu we toch bij de kaft zijn is het een kleine stap naar "ons pap en ons mam". Ma, Pa: bedankt voor alle steun! Door jullie, of beter dankzij jullie ben ik nu zoals ik ben (en ja, voordat iemand het zegt, daar ben ik blij mee ☺). Jullie hebben mij altijd en overal 100% gesteund en ervoor gezorgd dat ik een echt thuis heb gehad, waar alles altijd kon volgens en niks te gek was. Jullie hebben ervoor gezorgd dat ik alles goed kan relativeren, niet bang ben en me niet gek laat maken. Ik zeg het vast te weinig, maar ik ben erg blij met jullie als ouders. Daarom is dit boekkie dan ook zeker net zoveel jullie verdienste als de mijne: goed "gewerkt" luitjes.

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En da ge bedankt zèt dè witte!

List of publications

In science the credit goes to the man who convinces the world, not the man to whom the idea first occurs.

Sir Francis Darwin (1848 - 1925),

List of publications

Pieterse, C.M.J., Van Pelt, J.A., Van Wees, S.C.M., Ton, J., Léon-Kloosterziel, K.M., Keurentjes, J.J.B., Verhagen, B.W.M., Knoester, M., Van der Sluis, I., Bakker, P.A.H.M. and Van Loon, L.C. (2001). Rhizobacteria-mediated induced systemic resistance: triggering, signalling, and expression. *European Journal of Plant Pathology* 107: 51-61.

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Curriculum vitae

The wisest mind has something yet to learn.

George Santayana (1863 - 1952)

Curriculum vitae

Bas Verhagen werd geboren op 3 december 1975 te Liempde. In 1994 behaalde hij zijn VWO diploma aan het Jacob-Roelands Lyceum te Boxtel. In datzelfde jaar werd aangevangen met de studie Biologie aan de Universiteit Utrecht. Tijdens de doctoraalfase werden twee onderzoeksstages vervuld: Moleculaire Genetica onder begeleiding van Dr. Stefan Turk (onderzoek naar fructaan transport) en Fytopathologie onder begeleiding van Dr. Peter Bakker (onderzoek naar de rol van flagellen bij geïnduceerde resistentie). Verder werd een literatuurstudie gedaan bij de leerstoelgroep Fytopathologie onder begeleiding van Dr.ir. Corné Pieterse (onderzoek naar de signaaltransductieroute van ethyleen in Arabidopsis). In maart 2000 studeerde hij af. Van maart 2000 tot en met februari 2004 was hij werkzaam als AIO bij de leerstoelgroep Fytopathologie. Daar werd onder begeleiding van Prof.dr.ir. L.C. van Loon en Dr.ir. C.M.J. Pieterse het onderzoek uitgevoerd dat in dit proefschrift is beschreven.