Rhizobacteria-mediated induced systemic resistance in Arabidopsis

molecular-genetic basis of induced resistance in relation to basal resistance

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Door rhizobacteriën geïnduceerde systemische resistentie in Arabidopsis

moleculair-genetische basis van geïnduceerde resistentie in relatie tot basisresistentie

(met een samenvatting in het Nederlands)

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CHAPTER I

General Introduction

Disease resistance upon primary infection

Primary disease resistance in plants: non-host, race-specific, and basal resistance

Plants are constantly exposed to potentially pathogenic micro-organisms. However, plants possess an extensive array of passive and active defense mechanisms, and only a small proportion of micro-organisms is capable of infecting the plant and causing disease. Plant resistance can be broadly defined as the plant's ability to suppress or retard the damaging activity of the pathogen. The most effective type of resistance is non-host resistance. This type of resistance protects the plant entirely from infection by the pathogen, and is manifested when the pathogen comes in contact with a plant species on which it cannot cause disease (Agrios, 1997). In such an interaction, the pathogen is non-pathogenic on the plant.

If certain varieties of a plant species are susceptible to some races of a pathogen, while they are resistant against other races of the same pathogen, the interaction usually follows a gene-for-gene relationship. In such an incompatible interaction, the pathogen is capable of initiating infection, but is immediately arrested at the site of infection. The resulting race-specific or vertical resistance is generally controlled by a single dominant resistance (*R*) gene in the host, which encodes a product that either directly or indirectly recognizes the product of a matching dominant avirulence (*avr*) gene expressed by the pathogen. This early recognition of the so-called avirulent pathogen gives rise to a hypersensitive response (HR). The HR involves a wide range of active defense mechanisms, including a form of programmed cell death at the site of infection. Deposition of anti-microbial compounds, fortification of the cell walls, and expression of defense-related genes in the surrounding tissue all contribute to inhibiting further colonization of the plant tissue by the pathogen (Hammond-Kosack and Jones, 1996). If the invading pathogen does not carry an *avr* gene that is recognized by the host, the plant fails to activate a HR. In such a compatible interaction, the plant reacts inefficiently or too late to halt the pathogen. Although resistance against these so-called virulent pathogens is considerably less than that against avirulent pathogens, the plant still has strategies to restrain colonization by these pathogens. This type of resistance is not well defined, but is sometimes referred to as polygenic, horizontal, or basal resistance, and acts in slowing down the rate of disease development.

Signals involved in primary disease resistance

The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene have repeatedly been implicated in the regulation of resistance responses. In many cases, infection by both avirulent and virulent pathogens is associated with enhanced production of these hormones, and exogenous application of these compounds often results in an enhanced level of resistance (Boller, 1991; Dempsey et al., 1999; Pieterse et al., 1996, 1998, 2000; Thomma

et al., 2000). Moreover, blocking the response to either of these signals can render plants more susceptible to certain pathogens and even insects (Delaney et al., 1994; Knoester et al., 1998; McConn et al., 1997; Staswick et al., 1998; Stout et al., 1999; Thomma et al., 1998; Van Wees et al., 1999; Ton et al., 2001).

A central role for SA became apparent with the use of NahG transformants. NahG plants constitutively express the bacterial *NahG* gene, encoding salicylate hydroxylase, which converts SA into catechol. Tobacco and Arabidopsis NahG plants show enhanced disease susceptibility to a broad range of oomycetous, fungal, bacterial and viral pathogens (Delaney et al., 1994; Kachroo et al., 2000). Recently, a screen based on impaired accumulation of SA after pathogen infection resulted in the identification of two Arabidopsis mutants affected in pathogen-induced biosynthesis of SA (Nawrath and Métraux, 1999). Both mutants, *sid1* and *sid2*, displayed enhanced susceptibility to the virulent pathogens *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica*, demonstrating the importance of SA in basal resistance against both the bacterial and the oomycetous pathogen. In addition, mutant *sid1* is allelic with enhanced disease susceptibility mutant *eds5* (Nawrath and Métraux, 1999). The latter mutant was identified as being more susceptible to a virulent strain of the bacterial pathogen *Xanthomonas campestris* pv. *raphani* (Rogers and Ausubel, 1997).

Evidence for the role of JA in pathogen resistance came predominantly from analyses of Arabidopsis mutants affected in biosynthesis or responsiveness to JA. The JA-response mutant *coi1* has been documented as displaying enhanced susceptibility to the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* (Thomma et al., 1998), and the bacterial leaf pathogen *Erwinia carotovora* (Norman-Setterblad et al., 2000). Another JA-insensitive mutant of Arabidopsis, *jar1*, allows enhanced levels of growth of virulent *P. syringae* pv. *tomato* in the leaves (Pieterse et al., 1998). This clearly demonstrates that JA-dependent defenses contribute to basal resistance against these pathogens. Furthermore, both the *jar1* mutant and the *fad3*, *fad7*, *fad8* triple mutant, which is defective in JA biosynthesis, exhibit susceptibility to normally nonpathogenic soil-borne oomycetes of the genus *Pythium* (Staswick et al., 1998; Vijayan et al., 1998), indicating that JA plays a role in non-host resistance against this type of pathogens. Besides involvement in resistance responses against microbial pathogens, JA also contributes to basal resistance against insects (McConn et al., 1997).

The role of ethylene in plant resistance seems more ambiguous. In some cases ethylene is involved in disease development, whereas in other cases it is associated with disease resistance. For instance, ethylene-insensitive tomato genotypes allowed wild-type levels of growth of virulent *P. syringae* pv. *tomato* and *X. campestris* pv. *vesicatoria*, but developed less disease symptoms (Ciardi et al., 2000; Lund et al., 1998). In these interactions, ethylene clearly regulates symptom development, rather than disease resistance. Conversely, Knoester et al. (1998) reported that ethylene-insensitive tobacco transformed with the mutant

ethylene receptor gene *etr1-1* from Arabidopsis, displayed susceptibility to the normally non-pathogenic oomycete *Pythium sylvaticum*, indicating that, like JA, ethylene plays a role in non-host resistance against *Pythium*. Furthermore, several ethylene-insensitive mutants of Arabidopsis have been reported to exhibit enhanced disease susceptibility to *B. cinerea* (Thomma et al., 1999a), *P. syringae* pv. *tomato* (Pieterse et al., 1998) and *E. carotovora* (Norman-Setterblad et al., 2000). These results indicate that ethylene-dependent defenses contribute to basal resistance against these pathogens.

Depending on the host-pathogen interaction, SA, JA and ethylene appear to be differentially involved in basal resistance. In Arabidopsis, some pathogens are resisted predominantly through SA-dependent pathways, i.e. *P. parasitica* and turnip crinkle virus (TCV), whereas others are resisted predominantly through JA- and ethylene-dependent resistance mechanisms, i.e. *A. brassicicola*, *B. cinerea*, and *E. carotovora* pv. *carotovora*. Table 1 summarizes the data demonstrating differential involvement of SA, JA and ethylene in basal resistance. The information presented is based on enhanced susceptibility phenotypes of transgenics or mutants of Arabidopsis and tobacco that are impaired either in the accumulation of, or in the responsiveness to, one of the hormones.

Table 1: Differential involvement of salicylic acid (SA), jasmonic acid (JA), and ethylene in the regulation of basal resistance in different plant-pathogen interactions.

Plant Species	Pathogen	Signals involved in basal resistance[a]			Reference
		SA	**JA**	**Ethylene**	
Arabidopsis	*Peronospora parasitica*	+	0	0	Delaney et al., 1994; Thomma et al., 1998
Arabidopsis	Turnip crinkle virus	+	0	0	Kachroo et al., 2000
Tobacco	Tobacco mosaic virus	+	n.d.	0	Delaney et al., 1994; Knoester et al., 1998
Tobacco	*Phytophthora infectans*	+	n.d.	n.d.	Delaney et al., 1994
Tobacco	*Cercospora nicotianae*	+	n.d.	n.d.	Delaney et al., 1994
Arabidopsis	*Xanthomonas campestris*	+	n.d.	n.d.	Rogers and Ausubel, 1997
Arabidopsis	*Pseudomonas syringae*	+	+	+	Delaney et al., 1994; Pieterse et al., 1998
Arabidopsis	*Pythium irregulare*	n.d.	+	n.d.	Staswick et al., 1998
Arabidopsis	*Pythium mastophurum*	n.d.	+	n.d.	Vijayan et al., 1998
Tobacco	*Pythium sylvaticum*	n.d.	n.d.	+	Knoester et al., 1998
Arabidopsis	*Alternaria brassicicola*	0	+	+	Thomma et al., 1998; Thomma et al., 1999a
Arabidopsis	*Botrytis cinerea*	0	+	+	Thomma et al., 1998; Thomma et al., 1999a
Arabidopsis	*Erwinia carotovora*	0	+	+	Norman-Setterblad et al., 1998

[a] Based on the enhanced susceptibility of transgenics/mutants of Arabidopsis and tobacco, impaired in the accumulation of, or responsiveness to, a particular hormone.
+ : transgenic/mutant displaying enhanced disease susceptibility compared to wild-type plants.
0 : transgenic/mutant displaying the same level of basal resistance as wild-type plants.
n.d. : not determined.

Induced disease resistance

Biologically and chemically induced resistance

Upon appropriate stimulation, plants are capable of developing an enhanced defensive capacity, commonly referred to as induced resistance. The state of induced resistance depends either on defensive compounds that are produced as a result of the induction treatment, and/or on a quicker and intensified activation of extant defense mechanisms after challenge inoculation with a pathogen. The latter mechanism is referred to as "priming", "sensitization", or "potentiation". In either case, the resistance-inducing agent can predispose the plant to resist further pathogen attack. Induced resistance is effective against a wide range of pathogens, and is typically characterized by a restriction of pathogen growth and a suppression of disease development compared to non-induced plants infected by the same pathogen (Hammerschmidt, 1999). Induced resistance triggered by biological agents can be subdivided into two broad categories. The classical type of biologically induced resistance is often referred to as systemic acquired resistance (SAR), and occurs in distal plant parts after localized infection by a necrosis-inducing pathogen. Ross (1961) was the first who provided a detailed description of the SAR phenomenon. He demonstrated that hypersensitively reacting tobacco developed enhanced resistance in non-inoculated leaves against subsequent infection by tobacco mosaic virus (TMV). Following these initial studies, SAR was documented as an effective defense response in a variety of plant species against a broad range of pathogens (Kuć, 1982; Ryals et al., 1996; Sticher et al., 1997). The expression of SAR is associated with the transcriptional activation of genes encoding pathogenesis-related proteins (PRs; Van Loon, 1997). Therefore, PRs, or expression of the genes encoding these proteins, are generally taken as markers for the induced state of SAR.

The second type of biologically induced resistance develops systemically in response to colonization of plant roots by selected strains of non-pathogenic rhizobacteria. In 1991, two research groups independently demonstrated that rhizosphere-colonizing *Pseudomonas* spp. have the potential to enhance the resistance of the host plant (Van Peer et al., 1991; Wei et al., 1991). This type of induced resistance, generally called rhizobacteria-mediated induced systemic resistance (ISR; Pieterse et al., 1996), has been demonstrated in different plant species under conditions in which the rhizobacteria remained spatially separated from the challenging pathogen (Van Loon et al., 1998).

A variety of chemicals have been shown to induce resistance as well. Most of these compounds are activators of the SAR response. For instance, SA, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) induce the same set of PR genes that is induced upon biological induction of SAR. Moreover, their action often involves signaling steps that are also required for the expression of SAR (Lawton et al., 1996; Uknes et al., 1992; Ward et al., 1991). However, the nonprotein amino acid β-aminobutryric acid (BABA) seems to

act differently, as this compound has been reported to induce resistance without concomitant expression of PR genes (Cohen and Gisi, 1994; Zimmerli et al., 2000). The mode of action of this chemical is probably based on potentiation of pathogen-specific resistance mechanisms (Zimmerli et al., 2000).

SAR: triggering and signaling

SA was first suggested to be involved in SAR signaling based on the observation that exogenously applied SA induced resistance associated with the accumulation of PRs (Uknes et al., 1992; Ward et al., 1991; White, 1979). Furthermore, both Malamy et al. (1990) and Métraux et al. (1990) observed a strong accumulation of SA in hypersensitively reacting tobacco and cucumber, respectively, as well as a lesser rise in noninfected plant parts concomitant with SAR development. Conclusive evidence for a key role of SA in SAR came from the analysis of SA-nonaccumulating NahG plants. Both tobacco and Arabidopsis plants expressing the *NahG* gene were found to be blocked in the expression of pathogen-induced SAR, indicating that endogenous accumulation is an essential prerequisite for SAR-signaling (Gaffney et al., 1993: Lawton et al., 1995; Figure 1). Observations that mutants *sid1* and *sid2* of Arabidopsis, which are both affected in pathogen-induced biosynthesis of SA, are impaired in the expression of SAR against *P. parasitica* (Nawrath and Métraux, 1999) support this conclusion. Initially, SA was also considered a candidate for the systemically transported SAR signal. Besides the earlier observation that accumulation of SA precedes the expression of SAR and *PR* gene expression in non-incoculated plant parts (Malamy et al., 1990; Métraux et al., 1990), Shulaev et al. (1995) reported that ^{18}O-containing SA molecules that had been synthesized in the infected leaf, were transported systemically throughout the plant. In contrast to these findings, grafting experiments with tobacco suggested that SA is not the systemically transported signal. Vernooij et al. (1994) demonstrated that a non-transformed scion grafted on a TMV-infected SA-nonaccumulating NahG rootstock expressed SAR, whereas a NahG scion grafted on an TMV-infected non-transformed rootstock failed to develop SAR. Similar results were obtained with grafting experiments between non-transformed tobacco plants and transgenics exhibiting epigenetic cosuppression of the *Pal* gene, in which biosynthesis of SA is blocked (Pallas et al., 1996). Indeed, Smith-Becker et al. (1998) demonstrated that upon primary infection of a single cucumber leaf, the accumulation of SA in phloem fluids was preceded by a transient increase in PAL activity in the stems and petioles. These results suggested that SA is synthesized *de novo* in stems and petioles in response to an early mobile signal from the inoculated leaf. These data suggest that even though SA is transported within the plant, it is not the systemically transported SAR signal. Therefore, the precise location of SA in the SAR signaling pathway remains uncertain.

Another essential mediator of the SAR signaling pathway is the defense regulatory protein NPR1. A screen for mutants that failed to exhibit increased expression of a

BGL2(PR-2)-β-glucuronidase reporter gene in response to SA treatment yielded the *npr1* mutant (Cao et al., 1994). Since then, several mutant screens based on impaired SAR expression (Delaney et al., 1995), reduced SA-induced *PR* gene expression (Shah et al., 1997), and enhanced disease susceptibility (Glazebrook et al., 1996) resulted in the identification of mutations allelic to the *npr1* mutation, illustrating the broad involvement of NPR1 in plant defense. In *npr1* plants, no induced resistance was evident after pretreatment

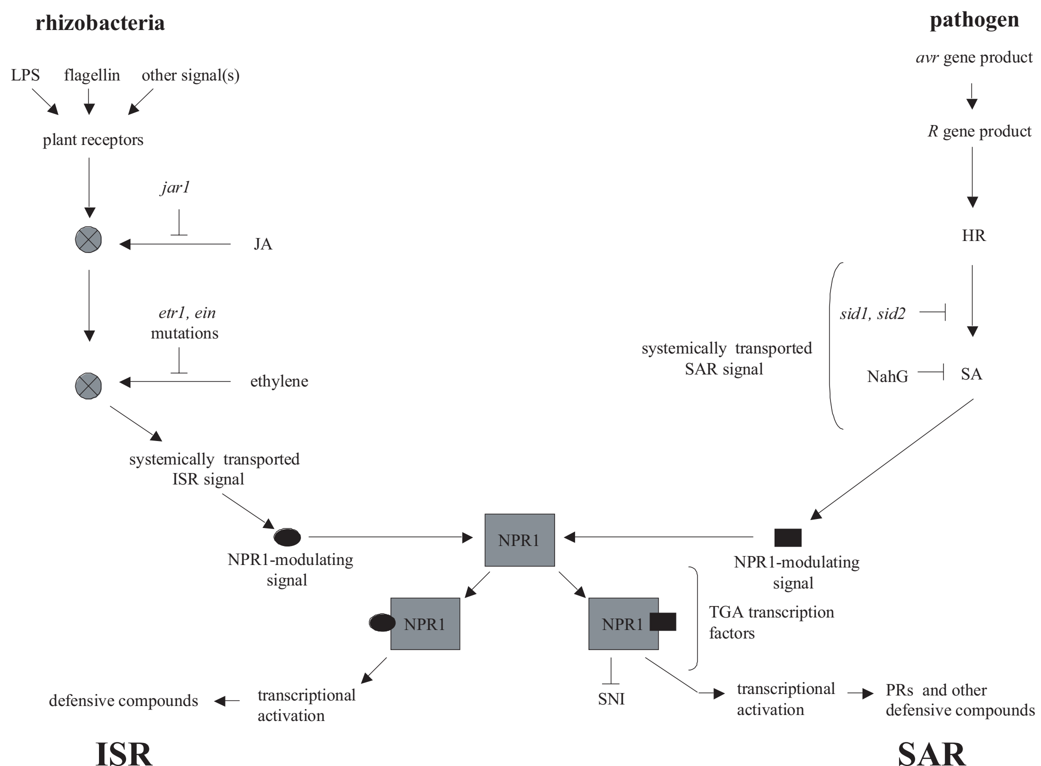

Figure 1: Proposed model for the signal transduction network controlling rhizobacteria-mediated induced systemic resistance (ISR) and pathogen-induced systemic acquired resistance (SAR) in *Arabidopsis thaliana. P. fluorescens* WCS417r-mediated ISR is controlled by a pathway that is dependent on responsiveness to jasmonic acid (JA) and ethylene, whereas pathogen-induced SAR is controlled by a pathway that depends on accumulation of salicylic acid (SA). Both pathways require the defense regulatory protein NPR1 that differentially regulates SA- and JA/ethylene dependent defense mechanisms, depending on the pathway that is activated upstream of it (Pieterse et al., 1998). LPS: lipopolysaccharides; NahG: salicylate hydroxylase; TGA transcription factors: family of transcription factors interacting with SA-induced NPR1 (Deprés et al., 2000); SNI: transcriptional repressor of SAR genes (Li et al., 1999). PRs: pathogenesis-related proteins

with SA or its functional analogue INA, indicating that NPR1 functions downstream the accumulation of SA in the SAR signaling pathway (Cao et al., 1994; Figure 1). Clues to the molecular basis of NPR1 function came from analysis of its predicted protein sequence, showing the presence of ankyrin repeats, a protein motif that is known to mediate protein-protein interactions (Cao et al., 1997; Ryals et al., 1997). By use of the yeast two-hybrid system for identifying protein-protein interactions, the NPR1 protein was recently demonstrated to interact with members of the TGA family of transcription factors (Deprés et al., 2000; Zhang et al., 1999; Zhou et al., 2000; Figure 1). Some of these transcription factors showed specific binding to a promotor element within the *PR-1* gene, suggesting a link between NPR1 and the transcriptional activation of *PR-1* during the onset of SAR.

Another factor implicated in the regulation of SAR is the SNI1 protein. This factor was identified by a mutant screen for genetic suppressors of the *npr1* mutation (Li et al., 1999). The resulting recessive *sni1* mutant showed restored SAR expression and *PR-1* transcription in response to INA treatment, indicating that SNI1 functions as a negative regulator in the establishment of SAR. It was proposed that SNI1 acts as a transcriptional repressor of SAR that can be counteracted by NPR1 after activation of the SA-dependent SAR pathway. Thereupon, the transcription factors of the TGA family would be allowed to activate the expression of *PR-1* and other genes involved in the establishment of SAR (Figure 1).

Rhizobacteria-mediated ISR: bacterial determinants

Rhizobacteria are present in large numbers on the root surface, where plant exudates and lysates provide nutrients (Lynch and Whipps, 1991). Many rhizobacterial strains have been reported to directly antagonize soil-borne pathogens (Bakker et al., 1991; Wei et al., 1996). Thus, in order to prove experimentally that resistance is induced by rhizobacteria, the pathogen and the rhizobacteria must remain spatially separated to prevent direct antagonistic interactions. During the early interaction between the ISR-inducing rhizobacteria and the host plant, the rhizobacteria must produce one or more ISR-eliciting compounds that are readily perceived by a matching receptor at the root surface. Under iron-limiting conditions, certain rhizobacterial strains produce SA as an additional siderophore (Meyer et al., 1992; Visca et al., 1993). Elicitation of ISR by strain *Pseudomonas fluorescens* CHA0 might be fully explained by the bacterial production of SA. Treatment of tobacco roots with CHA0 bacteria triggered accumulation of SA-inducible PRs in leaves (Maurhofer et al., 1994). Furthermore, transformation of *Pseudomonas fluorescens* strain P3 with SA-biosynthetic genes of CHA0 strongly improved the ISR-inducing capacity of strain P3 (Maurhofer et al., 1998). Another strain that has been suggested to elicit ISR by production of SA, is *Pseudomonas aeruginosa* 7NSK2. A SA-deficient mutant of 7NSK2 failed to induce resistance in bean and tobacco, whereas two mutants affected in other siderophores were still capable of inducing resistance (De Meyer and Höfte, 1997). Furthermore, bacterization of

NahG tobacco plants with either the wild-type strain failed to induce resistance against TMV, suggesting that 7NSK2-mediated ISR is dependent on bacterially-produced SA (De Meyer et al., 1999a). Although these examples demonstrate that rhizobacteria-mediated ISR can be elicited by bacterially produced SA resulting in the activation of the SA-dependent SAR pathway, other ISR-inducing rhizobacteria have been demonstrated to activate an SA-independent pathway (Pieterse et al., 1996, 1998; Press et al., 1997). This suggests the involvement of other ISR-eliciting determinants (Figure 1).

So far, several structural and metabolic compounds have been implicated in the elicitation of rhizobacteria-mediated ISR (Van Loon et al., 1998). Purified lipopolysaccharides (LPS) and flagella of some non-pathogenic *Pseudomonas* strains have been shown to induce systemic resistance as well (Leeman et al., 1995a; Van Peer and Schippers, 1992; Van Wees et al., 1997, P.A.H.M. Bakker, unpublished results). So far, putative receptors for the bacterial LPS have not been characterized in plants. Therefore, the molecular mechanisms behind LPS perception as related to ISR signaling remain unclear. In contrast, plants have been shown to possess a sensitive perception system for bacterial flagellins (Felix et al., 1999). Recently, a flagellin receptor of Arabidopsis was characterized as a receptor kinase sharing structural and functional homology with known plant resistance genes (Gomez-Gomez and Boller, 2000). These results suggest that the perception of bacterial flagella can result directly in elicitation of a defense-signaling pathway. Although exogenous application of purified LPS or flagella can induce systemic resistance (Leeman et al., 1995a; Van Peer and Schippers, 1992; Van Wees et al. 1997), bacterial mutants lacking flagella or the O-antigenic side chain of the LPS were still able to elicit ISR in Arabidopsis (Van Wees et al., 1997; P.A.H.M. Bakker, unpublished results). This indicates that, besides LPS and flagella, more determinants are involved in the elicitation of rhizobacteria-mediated ISR (Figure 1).

Rhizobacteria-mediated ISR: a genetic interaction between the rhizobacterium and the host

ISR-inducing rhizobacteria show little specificity in their colonization of roots of different plant species (Van Loon et al., 1998). However, elicitation of ISR appears to be highly specific with regard to both the host species and the rhizobacterial strain. The ISR-inducing rhizobacterial strains *P. putida* WCS358r and *P. fluorescens* WCS374r act differentially on different plant species: Arabidopsis is responsive to WCS358r, whereas radish and carnation are not (Leeman et al., 1995b; Van Peer, 1990; Van Peer and Schippers 1992; Van Wees et al., 1997). Conversely, radish is responsive to WCS374r, whereas Arabidopsis is not. *P. fluorescens* strain WCS417r has the ability to elicit ISR in both plant species. Van Wees et al. (1997) reported that the Arabidopsis ecotypes Columbia (Col-0) and Landsberg *erecta* (L*er*) are responsive to ISR-induction by WCS417r, whereas ecotype RLD1 is not. Root colonization

of RLD1 by WCS417r bacteria was of the same order as on Col-0 and L*er*. Apparently, ISR-inducibility in Arabidopsis is ecotype-dependent. These findings indicate that ISR requires a specific interaction between the plant and the nonpathogenic rhizobacterium, which must depend on specific genetic traits of both the rhizobacterium and the host plant.

SA-independent ISR requires responsiveness to jasmonic acid and ethylene

The existence of an SA-independent pathway controlling ISR was first demonstrated in Arabidopsis. Using *P. fluorescens* strain WCS417r as the ISR-inducing agent and the bacterial pathogen *P. syringae* pv. *tomato* DC3000 as the challenging pathogen, Pieterse et al. (1996) demonstrated that WCS417r-mediated ISR was fully maintained in NahG plants, and not associated with the transcriptional activation of genes encoding SA-inducible PRs. Further study on this model system revealed that treatment of the roots with WCS417r bacteria failed to trigger ISR in the JA-insensitive *jar1* plants or the ethylene-insensitive *etr1* plants, indicating that the JA and ethylene response pathways are essential for the establishment of ISR (Pieterse et al., 1998). Furthermore, using methyl jasmonate (MeJA) and the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) as activators of the ISR pathway, it was demonstrated that JA signaling functions upstream of ethylene signaling in the ISR signaling pathway (Pieterse et al., 1998; Figure 1).

To further investigate the roles of JA and ethylene in ISR signaling, the levels of these signaling molecules were determined in plants upon root bacterization. Both systemically and at the site of application of the WCS417r bacteria, JA content and the level of ethylene evolution remained unaltered upon ISR induction (Knoester et al., 1999; Pieterse et al., 2000). Also, *Lox2*-co-suppressed S-12 plants that are blocked in the production of JA after wounding (Bell et al., 1995) and pathogen infection (Pieterse et al., 2000), were normally responsive to induction treatments (Pieterse et al., 2000), indicating that ISR can be expressed in the absence of increased JA levels. These data suggest that the JA- and ethylene-dependency of ISR is not based on an enhancement of JA and ethylene production, but rather on enhanced sensitivity to these hormones. Since modulation of ethylene sensitivity in ethylene-response mutants of Arabidopsis results in an altered level of basal expression of ethylene-responsive genes (Knoester et al., 1999), increased expression would be expected if ISR-expressing plants have enhanced sensitivity to JA and ethylene. However, Van Wees et al. (1999) found that induction of WCS417r-mediated ISR is not associated with changes in the expression of well-characterized JA- and/or ethylene responsive genes. An alternative explanation for the JA- and ethylene-dependency of ISR could be that basal levels of both hormones are required for priming the plant to be conducive to ISR signaling (Figure 1).

To further elucidate the role of ethylene in the ISR signaling pathway, Knoester et al. (1999) tested several well-characterized Arabidopsis mutants that are disturbed in different

steps of the ethylene-response pathway. None of these mutants expressed ISR upon treatment of the roots with WCS417r, demonstrating that the entire ethylene-signaling pathway is required for the expression of ISR. However, mutant *eir1*, that is insensitive to ethylene in the roots only, developed wild-type levels of ISR after application of WCS417r to the leaves. Therefore, it was postulated that ethylene signaling is required at the site of application of the inducer, suggesting that, as in SAR in tobacco (Knoester, 1998), ethylene is involved in the generation or translocation of the systemically transported signal (Knoester et al., 1999; Figure 1). The finding that JA signaling functions upstream of ethylene signaling in the ISR pathway (Pieterse et al., 1998), implies that JA signaling is required at the site of WCS417r application as well (Figure 1). However, these findings do not rule out the possibility that components of the JA and ethylene response are also required for the expression of ISR in tissues distant from the site of application of the inducing bacterium.

The dual role of NPR1 in induced resistance

Although the signaling pathways controlling WCS417r-mediated ISR and pathogen-induced SAR clearly differ, both pathways share at least one common signaling component. Pieterse et al. (1998) reported that the *npr1* mutant of Arabidopsis is not only impaired in the expression of SAR, but also fails to express ISR after treatment of the roots with WCS417r bacteria. This demonstrated that NPR1 is required for the establishment of both SA-dependent SAR and JA- and ethylene-dependent ISR. Elucidation of the sequence of ISR-signaling events revealed that NPR1 functions downstream of the JA and ethylene response in the ISR pathway, indicating that NPR1 regulates the activation of both SA-dependent defense-related genes and so far unidentified JA- and ethylene-dependent defense components (Pieterse et al., 1998). Thus, NPR1 differentially regulates either SA- or JA/ethylene-dependent defense responses, depending on the pathway that is activated upstream of it (Figure 1). Recently, Van Wees et al. (2000) demonstrated that simultaneous activation of SAR and ISR results in an enhanced level of protection against *P. syringae* pv. *tomato*. In addition, it was demonstrated that simultaneous activation of both responses is not associated with enhanced levels of *Npr1* transcription. Thus, the constitutive level of NPR1 is sufficient for the expression of both defense responses.

Further evidence suggesting a regulatory function of NPR1 in SA-independent defense responses, came from a genetic study performed by Clarke et al. (1998). A screen for mutants in transgenic Arabidopsis constitutively expressing a *BGL2*-β-glucuronidase reporter gene yielded the identification of the dominant *cpr6* mutant. This mutant possessed enhanced levels of SA in combination with enhanced pathogen resistance and increased constitutive expression of both SA- and JA-responsive genes. The enhanced resistance of *cpr6* against *P. syringae* pv. *maculicola* was abolished in the *cpr6 npr1* double mutant, despite unaltered constitutive expression of SA-inducible *PR* genes. This not only indicates that *PR* genes can

be controlled in a NPR1-independent manner, but also illustrates that *cpr6*-mediated resistance, like WCS417r-mediated ISR, is controlled through an NPR1-dependent pathway that is not associated with SA-inducible *PR* gene expression.

Induced resistance: an enhancement of extant basal resistance?

Plants expressing induced resistance display a generally enhanced defensive capacity. This enhanced defensive capacity can result from a biochemical or physiological alteration that renders the plant more resistant. Certain PRs that are synthesized *de novo* upon SAR induction have antifungal activity (reviewed by Van Loon, 1997). Alternatively, the plant may become sensitized to activate appropriate defense mechanisms faster and more strongly upon infection with a challenging pathogen. Several mechanisms of potentiated defense have been observed in induced plants. Notably, these mechanisms also operate in non-induced plants, but they occur at lower frequency, intensity, or at a later stage during pathogen attack (Hammerschmidt, 1999). For example, non-induced bean plants infected with *Colletotrichum lagenarium* develop papillae at the sites of penetration of the pathogen. These papillae contain callose and lignin, which are thought to act as a barrier to pathogen penetration. In induced plants, the enhanced resistance is associated with a faster formation of papillae than in non-induced plants (Hammerschmidt and Kuć, 1982). Moreover, the papillae in induced plants contained higher amounts of callose and lignin (Hammerschmidt and Kuc, 1982; Kovats et al., 1991). This suggests that the induced resistance against *C. lagenarium* is realized through a potentiated expression of papilla formation, a mechanism that also determines the level of basal resistance against this pathogen. A similar situation was observed for other defense mechanisms, such as accumulation of hydroxyproline-rich glycoproteins (HRPGs) and increased peroxidase activity (Hammerschmidt, 1999). These findings suggest that the enhanced defensive capacity of plants expressing induced resistance is largely based on enhanced expression of extant basal defense mechanisms.

If induced resistance is an enhancement of extant basal resistance, one would expect that plant genotypes differing in genetically determined basal resistance also differ in the extent to which induced resistance can be expressed. Indeed, there have been several reports suggestive of such a relationship. Van Peer et al. (1991) reported that *P. fluorescens* WCS417-mediated ISR in carnation against *Fusarium oxysporum* f.sp. *dianthi* is considerably more effective in the moderately resistant cultivar Pallas than in the susceptible cultivar Lena. An opposite relationship was reported by Liu et al. (1995), who reported that *P. putida* 89B-27-mediated ISR in cucumber against *C. orbiculare* was only expressed in three susceptible cultivars, and not in a resistant cultivar. This result can be interpreted in the sense that in the already highly resistant cultivar, defenses could not be further enhanced upon induction of

ISR. However, a correlation between induced resistance and basal resistance is not always apparent. For example, both susceptible and moderately resistant radish cultivars were capable of expressing rhizobacteria-mediated ISR against Fusarium wilt (Leeman et al., 1995b).

Analysis of mutants and transgenics, particularly in Arabidopsis and tobacco, revealed that signaling pathways controlling basal resistance, are often involved also in induced resistance responses. For instance, as described above, SA-nonaccumulating NahG plants of both Arabidopsis and tobacco exhibit enhanced susceptibility to a variety of pathogens (Delaney et al., 1994). At the same time, they are affected in the expression of pathogen-induced SAR (Gaffney et al., 1993; Lawton et al., 1995). A similar correlation was found for Arabidopsis plants mutated in the *Npr1* gene. These plants not only exhibit reduced basal resistance against *P. syringae* and *P. parasitica* (Delaney et al., 1995; Glazebrook et al., 1996), but are also blocked in the expression of both pathogen-induced SAR (Cao et al., 1994; Delaney et al., 1995) and WCS417r-mediated ISR (Pieterse et al., 1998). Furthermore, the JA-insensitive *jar1* mutant and the ethylene-insensitive *etr1* mutant of Arabidopsis allowed 10-fold higher levels of growth of *P. syringae* pv. *tomato* in the leaves, and were concurrently affected in the expression of WCS417r-mediated ISR (Pieterse et al., 1998). Thus, in many cases, there seems to be a correlation between the presence of a certain level of basal resistance, and the capacity of a plant to develop induced resistance.

Outline of this thesis

The main goal of the work described in this thesis was to unravel the genetic and molecular basis of induced resistance in relation to basal resistance. In the past decade, the introduction of Arabidopsis as a model plant has provided many new tools for investigating molecular and genetic aspects of plant-pathogen interactions. The experiments described in this thesis were performed with an Arabidopsis-based model system that was previously developed by Pieterse et al. (1996). In this model system, *P. fluorescens* WCS417r was used as the ISR-inducing agent, and an HR-eliciting strain of *P. syringae* pv. *tomato* DC3000 carrying the avirulence gene *avrRpt2*, was used to elicit pathogen-induced SAR.

In chapter 2, 10 different Arabidopsis ecotypes were tested for their ability to express rhizobacteria-mediated ISR and pathogen-induced SAR. All 10 ecotypes developed normal levels of pathogen-induced SAR. However, only 8 ecotypes were capable of expressing rhizobacteria-mediated ISR, whereas two ecotypes, RLD1 and Ws-0, were not. The ISR non-inducibility of RLD1 and Ws-0 was correlated with a remarkably low level of basal resistance against *P. syringae* pv. *tomato* DC3000 This naturally occurring variation was used to initiate a genetic approach that resulted in the identification of a novel locus, designated *ISR1*, that controls both rhizobacteria-mediated ISR and basal resistance against *P. syringae*

pv. *tomato* DC3000.

In chapter 3, the physiological characterization of the *ISR1* locus is described. Based on the striking resemblance between ecotypes RLD1 and Ws-0, on the one hand, and the JA-insensitive mutant *jar1* and the ethylene-insensitive mutant *etr1*, on the other hand, it was investigated whether the *ISR1* locus is involved in either JA or ethylene signaling. This study revealed that the *ISR1* locus is involved in ethylene signaling, indicating that it encodes a novel component of the ethylene-response pathway that plays an important role in disease resistance. In chapter 4, the association between induced resistance and basal resistance against pathogenic *P. syringae* was further explored, in order to identify novel components in the ISR and SAR pathway. A collection of 11 Arabidopsis mutants with enhanced disease susceptibility to pathogenic *P. syringae* was tested for their potential to express WCS417r-mediated ISR and pathogen-induced SAR. This characterization resulted in the identification of two *eds* mutants impaired in the expression of SAR, and three *eds* mutants impaired in the expression of WCS417r-mediated ISR. The ISR- and SAR-impaired mutants were further characterized for their responsiveness to JA, ethylene and SA.

In Chapter 5 the effectiveness of WCS417r-mediated ISR and SAR against different types of pathogens is described. This research revealed that ISR is predominantly effective against pathogens that are resisted through JA/ethylene-dependent basal resistance, whereas SAR is predominantly effective against pathogens that are resisted through SA-dependent basal resistance. In chapter 6, we demonstrate that the *ISR1* locus is required for the establishment of WCS417r-mediated ISR against several pathogens. Finally, in chapter 7 the relationship between induced resistance and basal resistance is discussed with reference to the current knowledge about plant-pathogen interactions.

CHAPTER 2

Identification of a locus in Arabidopsis controlling both the expression of rhizobacteria-mediated induced systemic resistance (ISR) and basal resistance against *Pseudomonas syringae* pv. *tomato*.

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Abstract

Selected nonpathogenic rhizobacteria with biological disease control activity are able to elicit an induced systemic resistance (ISR) response that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). Ten ecotypes of *Arabidopsis thaliana* were screened for their potential to express rhizobacteria-mediated ISR and pathogen-induced SAR against the leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). All ecotypes expressed SAR. However, of the ten ecotypes tested, ecotypes RLD1 and Wassilewskija (Ws-0) did not develop ISR after treatment of the roots with nonpathogenic *Pseudomonas fluorescens* WCS417r bacteria. This nonresponsive phenotype was associated with relatively high susceptibility to *Pst* infection. The F_1 progeny of crosses between the nonresponsive ecotypes RLD1 and Ws-0 on the one hand, and the responsive ecotypes Columbia (Col-0) and Landsberg *erecta* (L*er*) on the other hand, were fully capable of expressing ISR and exhibited a relatively high level of basal resistance, similar to that of their WCS417r-responsive parent. This indicates that the potential to express ISR and the relatively high level of basal resistance against *Pst* are both inherited as dominant traits. Analysis of the F_2 and F_3 progeny of a Col-0 x RLD1 cross revealed that the potential to express ISR and basal resistance against *Pst* cosegregate in a 3 : 1 fashion, suggesting that both resistance mechanisms are monogenically determined and genetically linked. Neither the responsiveness to WCS417r, nor the relatively high level of basal resistance against *Pst* were complemented in the F_1 progeny of crosses between RLD1 and Ws-0, indicating that RLD1 and Ws-0 are both affected in the same locus, necessary for the expression of ISR and basal resistance against *Pst*. The corresponding locus, designated *ISR1*, was mapped between markers *B4* and *GL1* on chromosome III. The observed association between ISR and basal resistance against *Pst* suggests that rhizobacteria-mediated ISR against *Pst* in *Arabidopsis* requires the presence of a single dominant gene that functions in the basal resistance response against *Pst* infection.

Introduction

Induced disease resistance is the phenomenon that plants develop an enhanced defensive capacity upon appropriate stimulation. This resistance response is expressed systemically throughout the plant and is effective against a broad spectrum of pathogens (for reviews see: Hammerschmidt and Kuć 1995). The classical way of inducing systemic resistance is by predisposal infection with a necrotizing pathogen. The resulting elevated state of resistance in plant parts distant from the site of primary infection is defined as systemic acquired resistance (SAR) (Ross 1961; reviewed by Ryals et al. 1996). Pathogen-induced SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA) (Malamy et al. 1990, Métraux et al. 1990). SA appears to be an essential signaling molecule in the SAR pathway, because transgenic plants unable to accumulate SA are incapable of developing SAR (Gaffney et al. 1993). Furthermore, SAR is associated with the systemic activation of so-called SAR genes. These include genes that encode pathogenesis-related (PR) proteins (Van Loon 1985; Ward et al. 1991), some of which have *in vivo* antifungal activity and are therefore thought to contribute to the state of SAR (Ryals et al. 1996; Van Loon 1997).

Besides necrotizing pathogens, nonpathogenic rhizosphere-colonizing bacteria have been shown to induce systemic resistance as well (for review see: Van Loon et al. 1998). To differentiate this type of induced resistance from pathogen-induced SAR, the term rhizobacteria-mediated induced systemic resistance (ISR) is used. In *Arabidopsis*, ISR has been studied using *Pseudomonas fluorescens* strain WCS417r as the inducing agent. Using this model system, it was demonstrated that WCS417r-mediated ISR is effective against the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*), the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani* (Pieterse et al. 1996, Van Wees et al. 1997), and the fungal leaf pathogen *Peronospora parasitica* (Chapter 5), indicating that this type of biologically-induced resistance is also effective against different types of pathogens. In contrast to pathogen-induced SAR, WCS417r-mediated ISR is independent of SA accumulation and *PR*-gene activation (Pieterse et al. 1996). Instead, WCS417r-mediated ISR requires an intact response to the plant hormones ethylene and jasmonic acid (Pieterse et al. 1998). Yet, it is still unknown what defensive compounds contribute to the WCS417r-mediated state of enhanced resistance.

Recently, it was demonstrated that the capacity to express WCS417r-mediated ISR in *Arabidopsis* is dependent on the plant genotype. Ecotypes Col-0 and L*er* were found to be responsive to induction of ISR by WCS417r, whereas ecotype RLD1 was not (Van Wees et al. 1997). In carnation, cultivar specificity with regard to expression of rhizobacteria-mediated ISR has been reported as well. ISR induced by WCS417r against fusarium wilt, caused by *Fusarium oxysporum* f. sp. *dianthi*, was clearly expressed in the moderately resistant cultivar Pallas, but less consistently in the susceptible cultivar Lena (Van Peer et al. 1991). This

suggests that the level of genetic resistance influences the extent to which ISR is expressed. However, several cases have been reported in which a clear correlation between the capacity to express ISR and basal resistance against the challenging pathogen was absent. For instance, in cucumber it was found that two susceptible cultivars were able to express rhizobacteria-mediated ISR, whereas a resistant cultivar did not (Liu et al. 1995). Moreover, in radish it was demonstrated that both susceptible and resistant cultivars were capable of expressing rhizobacteria-mediated ISR against fusarium wilt (Leeman et al. 1995b).

In this study, the genetic basis underlying ecotype specificity of rhizobacteria-mediated ISR in *Arabidopsis* was studied in relation to the level of ecotype-specific basal resistance against *Pst*. Ten *Arabidopsis* ecotypes were tested for their potential to express WCS417r-mediated ISR and their level of basal resistance against *Pst*. We demonstrate that the WCS417r-nonresponsive phenotype correlates with a relatively low level of basal resistance against *Pst*. Furthermore, crosses were made between WCS417r-responsive and WCS417r-nonresponsive ecotypes, after which the resulting F_1, F_2, and F_3 progenies were tested for inducibility of ISR by WCS417r, as well as basal resistance against *Pst*. We provide evidence that the potential to express WCS417r-mediated ISR and the relatively high level of basal resistance against *Pst* are both dominant traits, which are monogenically inherited and genetically linked.

Results

Arabidopsis ecotypes unable to express WCS417r-mediated ISR exhibit relatively low levels of basal resistance against *Pst*.

Ten *Arabidopsis* ecotypes were tested for their potential to express pathogen-induced SAR and WCS417r-mediated ISR against virulent *Pst*. All ecotypes developed an hypersensitive response in leaves that were injected with avirulent *Pst*(*avrRpt2*) (data not shown), and subsequently expressed SAR against virulent *Pst* in the remaining noninfiltrated leaves (Figure 1). Ecotypes Col-0, L*er*, Cvi, Sha, Kas-1, C24, Wei-0, and Ren-0 developed ISR upon treatment of the roots with WCS417r bacteria, indicating that both types of biologically-induced disease resistance can be triggered in these ecotypes (Figure 1). ISR-expressing plants showed a reduction of disease symptoms ranging from 15 to 40% compared to the control treatments, whereas SAR-expressing plants consistently showed a stronger reduction of disease symptoms (40 to 60%). Ecotypes RLD1 and Ws-0 developed SAR to the same extent as the other ecotypes. However, they did not develop ISR after treatment of the roots with WCS417r. This nonresponsive phenotype was manifest irrespective of the level of disease incidence (data not shown), ruling out the possibility that ISR was masked by the high susceptibility of these ecotypes to *Pst* (see below).

To determine whether the nonresponsiveness to WCS417r in ecotypes RLD1 and Ws-0 was due to poor colonization of the rhizosphere, the population density of WCS417r in the rhizosphere of all ecotypes was determined. Table 1 shows that at the end of the bioassays the number of rifampicin-resistant bacteria isolated from the rhizosphere of all ecotypes tested was well above 10^6 per gram of root fresh weight, indicating that the inability of RLD1 and Ws-0 to express ISR was not the result of reduced root colonization by WCS417r.

As evident in non-induced plants, disease symptoms caused by *Pst* infection on the leaves of both RLD1 and Ws-0 were characterized by many large necrotic or water-soaked spots, surrounded by extensive chlorosis, whereas disease symptoms on Col-0 plants were much less severe (Figure 2). Moreover, when inoculated with *Pst* at 2.5 x 10^7 colony forming units (CFU) per mL, RLD1 and Ws-0 showed a higher proportion of leaves with symptoms than control-treated WCS417r-responsive ecotypes (within one representative experiment: RLD1, 66.3% and Ws-0, 75.7%, versus Col-0, 51.8% and L*er*, 49.3%). To quantify basal resistance against *Pst* infection, the proliferation of *Pst* in the leaves of non-induced plants was determined by 3 days after inoculation. In accordance with their more pronounced symptoms, the WCS417r-nonresponsive ecotypes RLD1 and Ws-0 allowed significantly more growth of *Pst* compared to the WCS417r-responsive ecotypes (Table 2). In the

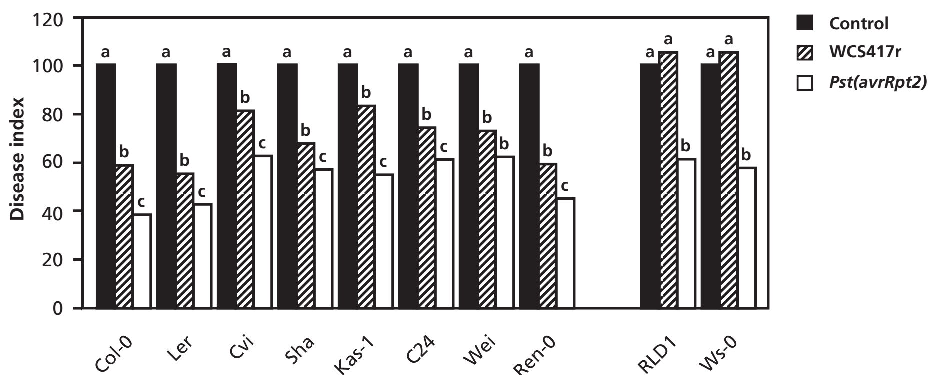

Figure 1: Quantification of ISR and SAR against *P. syringae* pv. DC3000 *(Pst) tomato* in different *Arabidopsis* ecotypes. ISR was induced by growing plants in soil containing *P. fluorescens* WCS417r bacteria at 5 x 10^7 CFU.g^{-1}. SAR was induced by pressure-infiltrating a suspension of avirulent *Pst* (*Pst(avrRpt2)*) at 10^7 CFU.mL^{-1} into the first two leaves, 4 days prior to challenge inoculation of the upper leaves. Challenge inoculation was performed with a bacterial suspension of virulent *Pst* at 2.5 x 10^7 CFU.mL^{-1} (ecotypes Col-0, L*er*, Sha, Kas-1, Cvi, C24, Wei-0, Ren-0, Ws-0) or 10^7 CFU.mL^{-1} (RLD1). Disease symptoms were scored at 3 or 4 days after challenge. The disease index is the proportion of leaves with symptoms relative to the control treatment, which was set at 100%. For each ecotype, different letters indicate statistically significant differences between treatments (Fisher's LSD test; α = 0.05; n = 20-25). The data presented are from a representative experiment that was performed at least twice for each ecotype, yielding similar results.

Table 1. Population densities of *P. fluorescens* WCS417r bacteria on roots of different *Arabidopsis* ecotypes[a].

Ecotype	CFU.g^{-1} of root fresh weight (log)[b]
WCS417r-responsive ecotypes	
Col-0	6.35 ± 0.39
L*er*	6.10 ± 0.68
Cvi	6.28 ± 0.19
Sha	6.88 ± 0.08
Kas-1	6.65 ± 0.05
C24	6.38 ± 0.26
Wei-0	6.76 ± 0.05
Ren-0	6.54 ± 0.21
WCS417r-nonresponsive ecotypes	
RLD1	6.32 ± 0.23
Ws-0	6.33 ± 0.25

[a] Values presented are means with standard deviations of the log of the average population densities from data of multiple bioassays.

[b] Roots were harvested at the end of the bioassays. In the rhizosphere of nontreated plants, no rifampicin-resistant bacteria were detected (detection limit = 10^3 CFU.g^{-1}).

WCS417r-responsive ecotypes *Pst* multiplied up to 3.1 log units over the 3-day time interval, whereas proliferation in RLD1 and Ws-0 was 4- to 5-fold higher. These results illustrate that the inability to express ISR is associated with a relatively low level of basal resistance against the challenging pathogen, suggesting that these two resistance mechanisms are related.

The potential to express ISR and the relatively high level of basal resistance against *Pst* are inherited as dominant traits.

To elucidate the genetic basis underlying the relationship between ISR and basal resistance against *Pst*, the WCS417r-nonresponsive *Arabidopsis* ecotypes RLD1 and Ws-0 were crossed with the WCS417r-responsive ecotypes Col-0 and L*er*. F_1 progenies of crosses between RLD1 x Col-0, Ws-0 x Col-0, and Ws-0 x L*er* were tested for their ability to express ISR and their level of basal resistance against *Pst*. All WCS417r-treated F_1 plants developed a level of ISR comparable to that of the WCS417r-responsive parent (Figure 3), indicating that the potential to express ISR is inherited as a dominant trait. Also the severity of disease symptoms caused by *Pst* infection (Figure 3) and the extent of proliferation of *Pst*

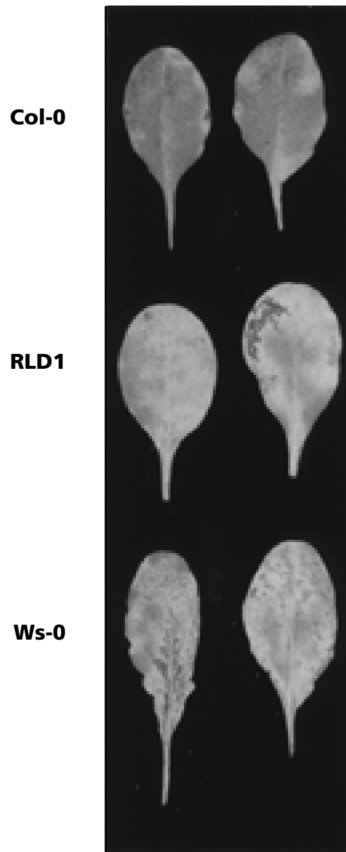

Figure 2: Symptoms of bacterial speck disease caused by *P. syringae* pv. *tomato* on leaves of *Arabidopsis* ecotypes Col-0, RLD1 and Ws-0, 4 days after inoculation. Plants were inoculated with *P. syringae* pv. *tomato* by dipping the leaves into a bacterial suspension at 2.5 x 10^7 CFU.mL^{-1}.

(Table 2) in control-treated F_1 plants were always similar to those of their control-treated Col-0 or L*er* parent, indicating that the relatively high level of basal resistance against *Pst* is inherited likewise as a dominant trait. F_1 plants derived from reciprocal crosses behaved similarly (data not shown). Thus, no maternal factors are involved in the potential to express ISR and in the relatively high level of basal resistance against *Pst*.

The potential to express ISR and the relatively high level of basal resistance against *Pst* are monogenically determined and genetically linked.

The inheritance of the potential to express ISR and basal resistance against *Pst* was further investigated by studying the segregation patterns of both traits in F_2 plants and F_3-families from the Col-0 x RLD1 cross. In two independent experiments, the percentage of leaves with disease symptoms in control- and WCS417r-treated Col-0, RLD1 and F_2 plants was determined at 4 days after challenge inoculation with *Pst*. Based on the percentage of diseased leaves, each plant was assigned to one of the 10 disease severity classes. Control-

treated Col-0 plants showed a frequency distribution centering the >50-60% disease severity class (Figure 4). Due to the expression of ISR, WCS417r-treated Col-0 plants showed less symptoms, resulting in a frequency distribution around the >30-40% and >40-50% disease severity classes. WCS417r-nonresponsiveness and the relatively low level of basal resistance of RLD1 were reflected by a normal frequency distribution around the >70-80% disease severity class in the control- and WCS417r-treated plants. The F_2 of the Col-0 x RLD1 cross showed a broad range of disease severities in control-treated plants. Treatment of the F_2 plants with WCS417r resulted in a 3 : 1 segregation: 70 F_2 plants exhibited a normal frequency distribution over the same disease severity classes as their WCS417r-treated Col-0

Table 2. Proliferation of *P. syringae* pv. *tomato* DC3000 (*Pst*) in the leaves of different *Arabidopsis* ecotypes and F_1 plants over a 3-day time interval.

Genotype	Proliferation of *Pst* (in log units)[a]
WCS417r-responsive genotypes	
Col-0	2.36 ± 0.18
RLD1 x Col-0	2.24 ± 0.15
Ws-0 x Col-0	2.35 ± 0.07
L*er*	2.83 ± 0.08
Ws-0 x L*er*	2.80 ± 0.08
Cvi	3.06 ± 0.17
Sha	2.71 ± 0.35
Kas-1	2.17 ± 0.23
C24	2.12 ± 0.09
Wei-0	2.96 ± 0.22
Ren-0	2.77 ± 0.18
WCS417r-nonresponsive genotypes	
RLD1	3.61 ± 0.09
Ws-0	3.71 ± 0.21
RLD1 x Ws-0	3.51 ± 0.24

[a] Values presented are means with standard deviations of the log of the proliferation of *Pst* over a 3-day time interval. Plants were infected by pressure-infiltrating a suspension of virulent *Pst* at 5 x 10^5 CFU.mL^{-1} into the leaves. Immediately after pressure infiltration and 3 days later, the number of *Pst* bacteria per gram of leaf fresh weight was determined and proliferation over a 3-day time-interval was calculated. The data presented are from a representative experiment that was performed at least twice with similar results.

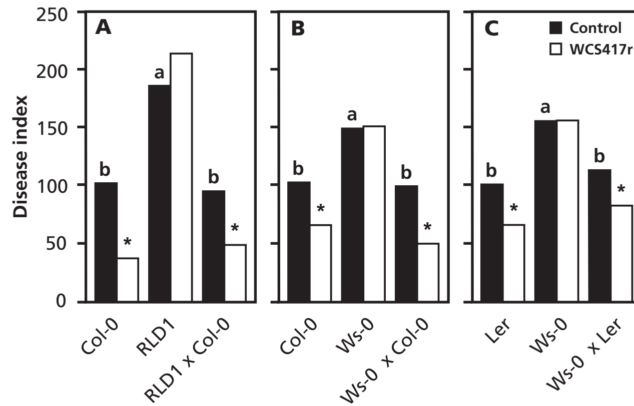

Figure 3: Quantification of *P. fluorescens* WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000 in F_1 plants of crosses between (**A**) RLD1 x Col-0, (**B**) Ws-0 x Col-0, and (**C**), Ws-0 x L*er*, and their respective parents. Plants were grown in soil with (WCS417r) or without (Control) bacteria. Challenge inoculation was performed with a bacterial suspension of virulent *Pst* at 2.5 x 10^7 CFU.mL^{-1}. Disease symptoms were scored 4 days after challenge inoculation. The disease index is the proportion of leaves with symptoms relative to the proportion of leaves with symptoms in the control-treated Col-parent (**A**, and **B**), or the control-treated L*er*-parent (**C**). The proportion of leaves with symptoms in the control treatments was set at 100%. For each panel, different letters above "control" bars indicate statistically significant differences in disease severity (Fisher's least significant difference test; $\alpha = 0.05$, n = 20-25). Asterisks indicate statistically significant differences between control- and WCS417r-treated plants within each pair (Student's *t* test; $\alpha = 0.05$; n = 20-25). This experiment was also performed with F_1 plants of the reciprocal cross, yielding similar results.

parents, suggesting that this fraction of the F_2 population expressed ISR in association with a relatively high level of basal resistance. In contrast, 28 F_2 plants exhibited a normal frequency distribution over the same disease severity classes as their WCS417r-nonresponsive RLD1 parents, indicating that these plants were nonresponsive to WCS417r and possessed a relatively low level of basal resistance against *Pst* (Figure 4). These 28 plants were clearly marked by typical RLD-like disease symptoms with many large necrotic or water-soaked spots surrounded by extensive chlorosis (Figure 2). The cosegregation of responsiveness to WCS417r with a relatively high level of basal resistance on the one hand, and nonresponsiveness to WCS417r with a relatively low level of basal resistance on the other hand, suggests that the potential to express ISR and relatively high basal resistance are genetically linked. In the second experiment a similar cosegregation pattern was observed in the WCS417r-treated F_2 population: 36 plants showed a Col-like phenotype, whereas 11 plants showed a RLD-like phenotype (data not shown). The segregation patterns in both experiments fit a statistically significant 3 : 1 segregation (Table 3), indicating that the potential to express WCS417r-mediated ISR and the relatively high level of basal resistance against *Pst* are dominant traits that are monogenically determined.

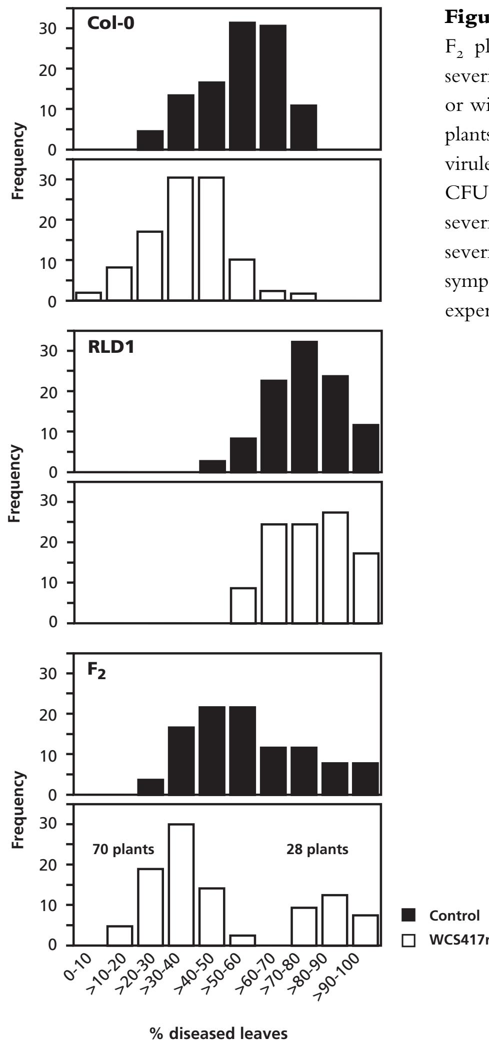

Figure 4: Frequency distributions of Col-0, RLD1, and F_2 plants of the RLD1 x Col-0 cross over 10 disease severity classes (n = 95-100). Plants were grown in soil with or without *P. fluorescens* WCS417r bacteria. Five-week-old plants were challenge inoculated with a suspension of virulent *P. syringae* pv. *tomato* DC3000 at 2.5 x 10^7 CFU.mL^{-1}. Four days later, plants were scored for disease severity and each plant was assigned to one of the 10 disease severity classes, ranging from 0-10% of the leaves with symptoms, to >90-100% of the leaves with symptoms. The experiment was performed twice with similar results.

To further prove that the potential to express ISR is genetically linked to relatively high basal resistance against *Pst*, 74 individual F_2 plants of the Col-0 x RLD1 cross were selfed, resulting in 74 F_3 families. Subsequently, sets of 16 F_3 plants of each family were challenge-inoculated with *Pst*, after which disease symptoms were monitored at 3, 4 and 5 days after inoculation. Evaluation of the disease symptoms revealed that 17 F_3 families were homozygous for Col-like disease symptoms, 40 F_3 families were heterozygous and showed both Col- and RLD-like disease symptoms, and 17 F_3 families were homozygous for RLD-like disease symptoms. This segregation pattern statistically fits a 1 : 2 : 1 segregation (χ^2 = 0.486; P = 0.784), confirming the monogenic inheritance of basal resistance against *Pst*.

Table 3. Genetic analysis of responsiveness to *P. fluorescens* WCS417r and basal resistance against *P. syringae* pv. *tomato* DC3000 (*Pst)* in WCS417r-treated F_2 plants of the RLD1 x Col-0 cross.

Experiment no.[a]	total	Col-like[b]	RLD-like[c]	expected ratio	χ^2-value[d]	*P*
1	98	70	28	3 : 1	0.667	0.414
2	47	36	11	3 : 1	0.064	0.801

[a] In two independent experiments, the cosegregation pattern of responsiveness to WCS417r and high basal resistance was determined in F_2 plants. The proportion of leaves with symptoms was determined per plant at 5 days after challenge inoculation with *Pst* and each plant was assigned to one of the 10 disease severity classes (see also Figure 4).

[b] The number of WCS417r-treated F_2 plants that exhibited a normal frequency distribution around the same disease severity classes as WCS417r-responsive Col-0 plants (Col-like).

[c] The number of WCS417r-treated F_2 plants that exhibited a normal frequency distribution around the same disease severity classes as control- and WCS417r-treated RLD1 plants (RLD-like).

[d] The numbers of Col- and RLD-like plants were tested for the expected 3 : 1 ratio, using the Chi-square test.

Subsequently, 5 randomly selected RLD- and Col-like families were further tested for proliferation of *Pst* in the leaves and responsiveness to induction of ISR by WCS417r. The 5 F_3 families selected for Col-like disease symptoms were fully capable to express WCS417r-mediated ISR and allowed relatively low levels of *Pst* proliferation, comparable with ecotype Col-0 (Table 4). In contrast, 5 F_3-families selected for RLD-like disease symptoms, did not respond to ISR-treatment with WCS417r, and exhibited relatively high levels of *Pst* proliferation, comparable with ecotype RLD1 (Table 4). The observed cosegregation in the F_3-families of responsiveness to WCS417r and relatively high basal resistance on the one hand, and nonresponsiveness to WCS417r and relatively low basal resistance on the other hand demonstrates that both defence mechanisms are genetically linked. We designate the corresponding locus *ISR1*.

RLD and Ws are both affected in the *ISR1* locus.

To investigate whether the recessive alleles of RLD1 and Ws-0 can complement each other for ISR and basal resistance against *Pst*, the F_1 progeny was tested. F_1 plants of the RLD1 x Ws-0 cross failed to express WCS417r-mediated ISR (Figure 5). In addition, the percentage of leaves with disease symptoms (Figure 5) and the extent of *Pst* proliferation (Table 2) in leaves of control-treated F_1 plants was similar to those observed in RLD1 and Ws-0. These observations demonstrate that ecotypes RLD1 and Ws-0 are unable to complement each other for the potential to express ISR and the relatively high level of basal

Table 4. Genetic linkage between responsiveness to induction of ISR by *P. fluorescens* WCS417r and basal resistance against *P. syringae* pv. *tomato* DC3000 (*Pst*).

Ecotype/ F_3-family	Test for ISR (% of induced protection)[a]		Test for basal resistance (Proliferation of *Pst* in log units)[b]	
	exp.1	exp. 2	exp. 1	exp. 2
Col-0	46.3 (*)	27.1 (*)	2.67 ± 0.17	3.1 ± 0.15
Col-fam. #1[c]	58.2 (*)		2.97 ± 0.12	
Col-fam. #2		26.5 (*)	2.57 ± 0.30	
Col-fam. #3	42.1 (*)		2.80 ± 0.32	
Col-fam. #4	49.4 (*)		2.71 ± 0.32	
Col-fam. #5		28.2 (*)	2.74 ± 0.20	
RLD1	3.8	- 4.3	3.54 ± 0.08	4.17 ± 0.09
RLD-fam. #1[d]		- 1.2		4.38 ± 0.10
RLD-fam. #2		- 6.3		3.94 ± 0.42
RLD-fam. #3	1.9			4.49 ± 0.15
RLD-fam. #4	3.4			3.95 ± 0.30
RLD-fam. #5	- 0.1			4.31 ± 0.19

[a] In two independent experiments the responsiveness to induction of ISR by *P. fluorescens* WCS417r was tested for 10 F_3 families of the RLD1 x Col-0 cross and compared to RLD1 and Col-0. Five F_3 families showing Col-like symptoms and 5 F_3 families showing RLD-like symptoms after infection with *Pst* were used. Plants were grown in soil with or without WCS417r bacteria at 5 x 10^7 CFU.g^{-1}. Challenge inoculation was performed with a bacterial suspension of virulent *Pst* at 2.5 x 10^7 CFU.mL^{-1}. Disease symptoms were scored 3 days after challenge inoculation. Values presented are the reductions in disease severity after WCS417r treatment relative to the corresponding control treatment (% of induced protection). Asterisks indicate statistically significant differences between control- and WCS417r-treated plants within a plant genotype (Student's *t* test; $\alpha = 0.05$; n = 20-25). The experiment was performed twice with similar results.

[b] Proliferation of *Pst* over a 3-day time interval in the leaves of the same plant genotypes as described in [a]. Plants were infected by pressure-infiltrating a suspension of virulent *Pst* pv. *tomato* at 5 x 10^5 CFU.mL^{-1} into the leaves. Immediately afterwards and 3 days later, the number of *Pst* pv. *tomato* bacteria per gram fresh weight was determined and proliferation calculated. Values presented are means and standard deviations of the log of the proliferation of *Pst*.

[c] F_3 families homozygous for Col-like disease symptoms caused by *Pst* infection.

[d] F_3 families homozygous for RLD-like disease symptoms caused by *Pst* infection.

resistance against *Pst*. Therefore, they both must be affected in the *ISR1* locus controlling both resistance mechanisms.

Genetic mapping of the *ISR1* locus.

Thirty-two homozygous F_3-families showing either Col- or RLD-like disease symptoms after infection with *Pst* were used to determine an approximate map position of the *ISR1* locus by cleaved amplified polymorphistic sequence (CAPS) analysis. The Col- and RLD-like phenotypes of the F_3-families showed cosegregation with markers *B4* ($\chi^2 = 13.5$; $P = 0.001$), *GL1* ($\chi^2 = 18.0$; $P < 0.001$), and *BGL1* ($\chi^2 = 5.5$; $P = 0.064$) on chromosome III: Of the 64 chromosomes tested, 15 chromosomes were recombinant with marker *B4*, yielding a recombination frequency of 23%, 14 chromosomes were recombinant with marker *GL1*, yielding a recombination frequency of 22%, and 23 chromosomes were recombinant with marker *BGL1*, yielding a recombination frequency of 36%. These frequencies indicate that the map position of the *ISR1* locus is located between markers *B4* and *GL1* on chromosome III.

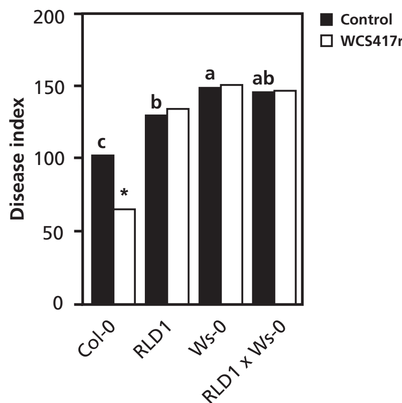

Figure 5: Quantification of *P. fluorescens* WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000 in Col, RLD1, Ws-0, and F_1 plants of a cross between RLD1 x Ws-0. For details see legend to Figure 3. The experiment was performed three times with similar results.

Discussion

Pathogen-induced SAR and WCS417r-mediated ISR are two induced resistance responses that are controlled by different signaling pathways (Pieterse et al. 1996; Van Wees et al. 1997; Pieterse et al. 1998). Here we have shown that out of the 10 Arabidopsis ecotypes tested, 8 have the ability to express both types of biologically-induced disease resistance. The two other ecotypes, RLD1 and Ws-0, are able to express SAR, but lack the ability to develop ISR upon treatment of the roots with WCS417r, confirming that ISR and SAR are distinct plant resistance responses.

A first prerequisite for the establishment of rhizobacteria-mediated ISR is an efficient colonization of the rhizosphere by the resistance-inducing strain, enabling the rhizobacteria to reach a threshold population density necessary for the initiation of ISR (Raaijmakers et al. 1995). Insufficient rhizosphere colonization by WCS417r could not explain the WCS417r-nonresponsive phenotype of ecotypes RLD1 and Ws-0, as WCS417r colonized WCS417r-responsive and WCS417r-nonresponsive ecotypes equally well (Table 1). Thus, the lack of WCS417r responsiveness in RLD1 and Ws-0 is not caused by poor root colonization, but must result from the absence of (a) genetic determinant(s) essential for induction and expression of ISR. As the recessive alleles of RLD1 and Ws-0 failed to complement each other in their F_1 progeny for responsiveness to WCS417r (Figure 5), it is likely that both ecotypes lack the same genetic determinant(s) controlling WCS417r-mediated ISR.

In *Arabidopsis*, ecotype specificity of WCS417r-mediated ISR against *Pst* was clearly associated with ecotype-specific basal resistance against *Pst*. The observed phenomenon that *Arabidopsis* ecotypes capable of expressing ISR exhibit a substantially higher level of basal resistance to *Pst* than ecotypes impaired in their ISR response, strongly suggests that WCS417r-mediated ISR in *Arabidopsis* utilizes components of the basal resistance pathway. Our results are consistent with previously obtained results of Van Peer et al. (1991), who demonstrated that WCS417r-mediated ISR in carnation was less consistently expressed in a susceptible cultivar than in a resistant cultivar, and support the hypothesis that induced resistance constitutes an enhancement of basal resistance responses (Van Loon, 1997). In this study, we further showed that the plant's potential to express ISR and basal resistance against *Pst* are both dominant traits that are monogenically inherited and genetically linked. The corresponding *ISR1* locus was mapped at chromosome III between the markers *GL1* and *B4*. We postulate that the *ISR1* locus controls a basal resistance response against *Pst* that is enhanced under conditions leading to WCS417r-mediated ISR.

Previously, Glazebrook et al. (1996) reported the isolation of *Arabidopsis* mutants that exhibit enhanced disease susceptibility to the moderately virulent *Pseudomonas syringae* pv. *maculicola* strain ES4326 (*Psm*). These *eds* mutants showed significantly enhanced growth of *Psm* in the leaves, which is proportional to the differences in growth of *Pst* observed in the leaves of the WCS417r-reponsive ecotypes and ecotypes RLD1 and Ws-0. The 12 *eds* mutants isolated included two alleles of the recently identified defense regulator gene *NPR1*, two genes encoding proteins that are involved in the phytoalexin biosynthesis, and seven so far unidentified genes. An affected *NPR1* gene seems an unlikely explanation for the WCS417r-nonresponsive and highly susceptible phenotype of RLD1 and Ws-0, as both ecotypes are capable of expressing pathogen-induced SAR (Figure 1), unlike the *npr1-1* mutant (Cao et al. 1994). Also a disturbance in phytoalexin biosynthesis seems unlikely, because three phytoalexin deficient mutants, *pad1*, *pad2* and *pad3* were found to be responsive

to ISR treatment with WCS417r (C.M.J. Pieterse, unpublished results).

Rogers and Ausubel (1997) reported that four of the previously isolated *eds* mutants, with so far unknown gene function, showed enhanced susceptibility to a subset of taxonomically-related bacterial phytopathogens, including *Pst*. In addition, the four *eds* mutants were found to be unaffected in their hypersensitive reaction to *Psm* strain ES4326, carrying the avirulence gene *avrRpt2*, and were still capable of expressing SAR. This phenotype resembles the phenotype of ecotypes RLD1 and Ws-0, with respect to the loss of basal resistance against *Pst*, on the one hand (Table 2), and the unaffected hypersensitive reaction to *Pst*(*avrRpt2*) and the SAR response (Figure 1) on the other hand. Whether the *eds* mutations affect the WCS417r-induced ISR response as well, needs to be elucidated.

Another set of mutations shown to affect pathogen resistance are mutations that cause insensitivity to the plant stress hormones ethylene and jasmonic acid. In tobacco the *etr1-1* mutation, conferring ethylene insensitivity, was demonstrated to cause susceptibility to a normally non-pathogenic soil-borne fungus (Knoester et al. 1998), indicating that ethylene controls defense mechanisms essential for non-host resistance. Likewise, the jasmonic acid response mutant *jar 1-1* of *Arabidopsis* was demonstrated to be highly susceptible to the soil-borne fungus *Pythium irregulare*, indicating that jasmonate signaling plays an important role in the resistance response to soil mico-organisms (Staswick et al. 1998). Moreover, growth of *Pst* in the leaves of the *Arabidopsis* mutants *etr1-1* and *jar1-1* has been shown to be increased ten-fold compared to wild type plants (Pieterse et al. 1998). This indicates that insensitivity to ethylene and jasmonic acid causes at least a partial loss of basal resistance against *Pst*. Development of SAR against *Pst* appears normal in *etr1-1* and *jar1-1* mutants (Pieterse et al. 1998). In contrast, both mutants are impaired in their WCS417r-mediated ISR response against *Pst*. Apparently, insensitivity to ethylene and jasmonic acid in *Arabidopsis* causes a phenotype that resembles the phenotype of ecotypes RLD1 and Ws-0 with respect to the lack of WCS417r responsiveness and the relatively low level of basal resistance against *Pst*. To our knowledge, the area on chromosome III, in which the *ISR1* locus is located, does not contain mapped genes with functions related to ethylene and jasmonate signaling, indicating that *ISR1* represents a novel locus involved in disease resistance.

Materials and Methods

Cultivation of rhizobacteria, pathogens and plants.

For treatment of the roots with ISR-inducing rhizobacteria, rifampicin-resistant *Pseudomonas fluorescens* strain WCS417r (Pieterse et al. 1996) was grown on King's medium B agar plates (King et al. 1954) for 24 hours at 28 °C. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 colony-forming units (CFU) per mL. For induction of SAR, rifampicin-resistant *Pseudomonas syringae* pv. *tomato* strain DC3000 carrying the avirulence gene *avrRpt2* (*Pst*(*avrRpt2*); Whalen et al. 1991) was cultured overnight in liquid King's medium B at 28 °C, collected by centrifugation, and resuspended in 10 mM $MgSO_4$ to a final density of 10^7 $CFU.mL^{-1}$. The virulent pathogen *P. syringae* pv. *tomato* strain DC3000 (*Pst*; Whalen et al. 1991), used for challenge inoculations, was cultured in a similar manner.

In this study, the *Arabidopsis* ecotypes, Columbia (Col-0), Landsberg *erecta* (L*er*), Shahdara (Sha), Kashmir (Kas-1), Cape Verdi islands (Cvi), Weiningen (Wei), C24, Wassilewskija (Ws-0), RLD1 and Renkum (Ren-0) were used. Ecotype Ren-0 was selected from a natural population in Renkum, The Netherlands. Seedlings were grown in quartz sand for two weeks. Subsequently, the seedlings were transferred to 60 mL pots, containing a sand/potting soil mixture that had been autoclaved twice for one hour. Plants were cultivated in a growth chamber with a 9-hour day (≈200 $\mu E.s^{-1}.m^{-2}.sec$ at 24 °C) and 15-hour night (20 °C) cycle and 65% relative humidity. For the duration of the experiments, all ecotypes remained vegetative and developed at least 10-15 mature leaves. Plants were watered on alternate days and once a week supplied with modified half strength Hoagland solution: 2 mM KNO_3, 5 mM $Ca[NO]_3$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, and trace elements, pH 7 (Hoagland and Arnon, 1938), containing 10 µM sequestreen (Fe-ethylenediamide-di[*o*-hydroxyphenylacetic acid]; Novartis, Basel, Switzerland).

Induced resistance bioassays

Prior to transfer of the *Arabidopsis* seedlings to the pots, a suspension of ISR-inducing WCS417r bacteria (10^9 $CFU.mL^{-1}$) was mixed thoroughly through the sand/potting soil mixture, to a final density of 5 x 10^7 $CFU.g^{-1}$. Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. Induction of SAR was performed 4 days before challenge inoculation by pressure-infiltrating two lower leaves with a suspension of *Pst(avrRpt2)* at 10^7 $CFU.mL^{-1}$ in 10 mM $MgSO_4$. One day before challenge inoculation, the plants were placed at 100% relative humidity. Plants were challenged when 5 weeks old by dipping the leaves in a suspension of virulent *Pst* bacteria, containing 10^7 or 2.5 x 10^7 $CFU.mL^{-1}$ in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands). Three, 4 or 5 days after challenge inoculation, the percentage of leaves with symptoms was determined per plant (n = 20-25). Leaves showing necrotic or water-soaked lesions surrounded by chlorosis, were scored as diseased.

Basal resistance bioassay

Leaves of 5-week-old plants were inoculated by pressure infiltration with a suspension of *Pst* at 5 x 10^5 CFU.mL^{-1} in 10 mM $MgSO_4$. Immediately afterwards and 3 days later, replicate leaf samples from five plants per ecotype were collected, weighed, and homogenized in 10 mM $MgSO_4$. Serial dilutions were plated on selective King's medium B agar supplemented with 100 mg.L^{-1} cycloheximide and 50 mg.L^{-1} rifampicin. After incubation at 28 °C for 2 days, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined and bacterial proliferation over the 3-day time interval was calculated.

Root colonization

Root colonization by WCS417r was determined at the end of the bioassays. Roots were harvested, weighed, and shaken vigorously in 5 mL 10 mM $MgSO_4$ containing 0.5 g of 0.17 mm glass beads. Appropriate dilutions were plated on King's medium B agar, supplemented with 100 mg.L^{-1} cycloheximide and 150 mg.L^{-1} rifampicin. After incubation at 28 °C for 2 days, the number of rifampicin-resistant colony-forming units per gram of root fresh weight was determined.

Genetic analysis

F_1 plants were obtained by reciprocally crossing ecotypes RLD1 and Col-0, Ws-0 and Col-0, Ws-0 and Ler, and RLD1 and Ws-0. The resulting F_1 progenies were tested for ISR and basal resistance against *Pst* using the induced resistance and basal resistance bioassay respectively. F_2 plants, obtained by selfing F_1 plants of the RLD1 x Col-0 cross, were tested for ISR using the induced resistance bioassay with approximately 50 or 100 plants per treatment. F_3 families were collected by selfing individual F_2 plants of the RLD1 x Col-0 cross. Sets of 16 plants per F_3 family were tested for symptom development by dipping control-treated plants in a suspension of *Pst* at 2.5 x 10^7 CFU.mL^{-1}, after which disease symptoms were monitored at 3, 4 and 5 days after challenge. For testing genetic linkage between ISR and basal resistance, 10 randomly selected F_3 families displaying either Col- or RLD-like disease symptoms were tested for ISR and basal resistance against *Pst*. Mapping the *ISR1* locus was performed with 16 homozygous F_3 families showing Col-like disease symptoms and 16 homozygous F_3 families showing RLD-like disease symptoms, using cleaved amplified polymorphic sequence (CAPS) analysis as described by Konieczny and Ausubel (1993).

ACKNOWLEDGMENTS

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CHAPTER 3

The Arabidopsis *ISR1* locus controlling rhizobacteria-mediated induced systemic resistance is involved in ethylene signaling

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Abstract

In Arabidopsis, the rhizobacterial strain *Pseudomonas fluorescens* WCS417r triggers an induced systemic resistance (ISR) response that is effective against different types of pathogens. The ISR signaling pathway functions independent of salicylic acid but requires responsiveness to both jasmonate and the ethylene. Using the genetic variability of ISR inducibility between Arabidopsis ecotypes, we recently identified a locus (*ISR1*) on chromosome III that is involved in ISR signaling. Ecotypes RLD1 and Wassilewskija (Ws-0) are recessive at the *ISR1* locus and are therefore unable to develop ISR. Here, we investigated whether the *ISR1* locus is involved in jasmonate or ethylene signaling. Compared to the ISR-inducible ecotype Columbia (Col-0), ecotypes RLD1 and Ws-0 were not affected in jasmonate-induced inhibition of root growth and expression of the jasmonate-responsive gene *Atvsp*, suggesting that the *ISR1* locus is not involved in jasmonate signaling. However, RLD1 and Ws-0 showed an affected expression of the triple response and a reduced expression of the ethylene responsive genes *Hel* and *Pdf1.2* after exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). Moreover, in contrast to Col-0, both RLD1 and Ws-0 did not develop resistance against *P. syringae* pv. *tomato* DC3000 after treatment of the leaves with ACC. Analysis of the F_2 and F_3 progeny of a cross between Col-0 (*ISR1/ISR1*) and RLD1 (*isr1/isr1*) revealed that reduced sensitivity to ethylene cosegregates with the recessive alleles of the *ISR1* locus. These results indicate that the *ISR1* locus encodes a component of the ethylene response, which is required for the expression of rhizobacteria-mediated ISR.

Introduction

Localized treatment of plants with specific biotic or abiotic agents can result in the development of enhanced resistance against pathogens in distal plant parts. Resistance induced by such treatments is generally characterized by a restriction of pathogen growth and a reduction of disease severity (Hammerschmidt, 1999). Induced resistance against pathogens can be subdivided into two categories. The classical way to induce disease resistance is by predisposal infection with a necrotizing pathogen, resulting in a systemic resistance in distal plant parts. This form of induced resistance is generally referred to as systemic acquired resistance (SAR; Ryals et al., 1996; Sticher et al., 1997). SAR is characterized by the endogenous accumulation of salicylic acid (Malamy et al., 1990; SA; Métraux et al., 1990) and a concomitant expression of genes encoding pathogenesis-related proteins (PR's reviewed by Van Loon, 1997). The second type of induced resistance develops in response to colonization of plant roots by selected strains of nonpathogenic rhizobacteria, and is often referred to as induced systemic resistance (ISR; Van Loon et al., 1998). Rhizobacteria-mediated ISR has been demonstrated to be effective in a variety of plant species under conditions in which the rhizobacteria remained spatially separated from the challenging pathogen. Both pathogen-induced SAR and rhizobacteria-mediated ISR confer an enhanced defensive capacity that is effective against a broad spectrum of plant pathogens (Ryals et al., 1996; Van Loon et al., 1998). In Arabidopsis, 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 SAR and ISR are additive and constitute two different mechanisms of induced resistance.

Rhizobacteria-mediated ISR has been studied extensively in Arabidopsis, using the nonpathogenic rhizobacterial strain *Pseudomonas fluorescens* WCS417r as the inducing agent and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) as the challenging pathogen (Pieterse et al., 2000). In this combination, the ISR signaling pathway clearly differs from the one that controls pathogen-induced SAR. SA-nonaccumulating *NahG* plants expressing the bacterial salicylate hydroxylase gene (*NahG*), fail to express SAR (Gaffney et al., 1993; Lawton et al., 1995), but show normal levels of ISR after treatment of the roots with WCS417r bacteria (Pieterse et al., 1996). This indicates that SA is a necessary signal for the SAR response, but is not required for ISR signaling. Arabidopsis mutants that are impaired in their response to the plant hormones jasmonate (JA) or ethylene, develop normal levels of SAR (Lawton et al., 1995; Pieterse et al., 1998), but are unable to express WCS417r-mediated ISR (Knoester et al., 1999; Pieterse et al., 1998). This demonstrates that, in contrast to SAR, ISR signaling requires components of the JA and the ethylene response. Despite these differences, the SAR and the ISR pathway are both controlled by the regulatory protein NPR1/NIM1 (Cao et al., 1994; Delaney et al., 1995; Pieterse et al., 1998). Downstream of NPR1/NIM1 both

pathways diverge, indicating that NPR1/NIM1 differentially regulates defense responses depending on the pathway that is activated upstream of it (Pieterse et al., 1998).

Recently, we identified a novel factor in the ISR signaling pathway by screening ten Arabidopsis ecotypes for their ability to express ISR. Two ecotypes, RLD1 and Wassilewskija (Ws-0) failed to develop ISR after treatment of the roots with WCS417r bacteria, whereas they expressed normal levels of pathogen-induced SAR (Chapter 2; Ton et al., 1999). This WCS417r-nonresponsive phenotype could not be attributed to poor root colonization by the ISR-inducing rhizobacteria, since colonization of the roots of both ecotypes was unaffected. Furthermore, the WCS417r-nonresponsive phenotype of RLD1 and Ws-0 was associated with a relatively high level of susceptibility to *Pst*. Genetic analysis of progeny of crosses between inducible and non-inducible ecotypes revealed that the potential to express ISR, as well as the relatively high level of basal resistance against *Pst*, are controlled by a single dominant locus (*ISR1*) that maps on chromosome III between CAPS markers *B4* and *GL1*. This suggested that the *ISR1* locus encodes a factor that is involved in both ISR and basal resistance against *Pst*.

Not only are the Arabidopsis mutants *etr1-1* and *jar1-1* affected in their ability to express WCS417r-mediated ISR against *Pst*, upon primary infection with *Pst*, they also develop more severe disease symptoms and allow significantly more bacterial growth compared to wild-type Col-0 plants (Pieterse et al., 1998). This strikingly resembles the *isr1* phenotype of ecotypes RLD1 and Ws-0, which are similarly affected in both the expression of ISR and basal resistance against *Pst*. The involvement of JA and ethylene in basal defense responses has repeatedly been demonstrated. In many cases, blocking the response to either of these signals can render plants more susceptible to certain pathogens and even insects. For instance, mutants that are affected in JA biosynthesis or signaling are more susceptible to pathogens such as *Pythium mastophorum* (Vijayan et al., 1998) and *Pythium irregulare* (Staswick et al., 1998), as well as to insect herbivory (McConn et al., 1997; Stout et al., 1999). Similarly, ethylene-insensitive tobacco plants transformed with the mutant *etr1-1* gene from Arabidopsis lost their ability to resist the soil-borne pathogen *Pythium sylvaticum* (Knoester et al., 1998). Furthermore, the ethylene-insensitive Arabidopsis mutant *ein2-1* gained enhanced susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea* (Thomma et al., 1999a) and the bacterial leaf pathogen *Erwinia carotovora* pv. *carotovora* (Norman-Setterblad *et al.,* 2000). Although these examples demonstrate the importance of JA and ethylene in specific basal resistance responses, other pathogens seem to be resisted predominantly through a SA-dependent pathway (Thomma et al., 1998).

The *isr1* phenotype, i.e. inability to express ISR and enhanced susceptibility to *Pst* infection, of ecotypes RLD1 and Ws-0 on the one hand, and that of mutants *etr1-1* and *jar1-1* on the other hand, prompted us to investigate whether the *ISR1* locus is involved in either JA or ethylene signaling. Arabidopsis ecotypes Col-0 (*ISR1/ISR1*), RLD1 (*isr1/isr1*) and Ws-

0 (*isr1/isr1*) were tested for their ability to respond to JA and ethylene by examining JA- and ethylene-induced inhibition of root growth and by studying JA- and ethylene-responsive gene expression. Here, we show that the *isr1* phenotype of ecotypes RLD1 and Ws-0 is caused by a reduced sensitivity to ethylene, indicating that the *ISR1* locus is involved in ethylene signaling.

Results

The *isr1* phenotype is not associated with reduced sensitivity to MeJA.

Ecotypes RLD1 and Ws-0 resemble the JA insensitive mutant *jar1-1* in that they are blocked in the ISR signaling pathway and exhibit enhanced susceptibility to *Pst* (Pieterse et al., 1998; Ton et al., 1999). Therefore, we investigated whether the *isr1* phenotype of ecotypes RLD1 and Ws is based on reduced sensitivity to JA. Previously, it was demonstrated that MeJA-induced inhibition of primary root growth and MeJA-induced expression of the *Atvsp* gene is substantially decreased in the *jar1-1* mutant (Staswick et al., 1992; 1998). Both characteristics were examined in Col-0, *jar1-1*, RLD1 and Ws-0 plants. Five days after germination, ecotype Col showed a 42% inhibition of primary root growth on MS-agar plates with 2 µM MeJA (Figure 1A). Growth of primary roots of mutant *jar1-1* was not inhibited, whereas RLD1 and Ws-0 showed a similar response to MeJA as ecotype Col-0, resulting in 38% and 45% inhibition of primary root growth, respectively. Moreover, exogenous application of 100 µM MeJA to leaves of Col-0 resulted in a strong activation of the JA-responsive gene *Atvsp*, whereas in *jar1-1* plants *Atvsp* transcripts accumulated to a much lower level (Figure 2). RLD1 and Ws-0 showed similar responses to MeJA as Col-0. These results demonstrate that the *isr1* phenotype of RLD1 and Ws-0 cannot be attributed to reduced responsiveness to JA.

RLD1 and Ws-0 show reduced sensitivity to ethylene

Like *jar1-1*, the ethylene insensitive mutant *etr1-1* is blocked in the expression of ISR and exhibits enhanced disease susceptibility to *Pst* (Pieterse et al., 1998; Knoester et al., 1999). Therefore, we investigated whether the *isr1* phenotype of ecotypes RLD1 and Ws-0 is based on reduced sensitivity to ethylene. The "triple response" is a reaction of etiolated seedlings to ethylene and has been shown to be a reliable marker for ethylene sensitivity (Guzmán and Ecker, 1990). To assess ethylene sensitivity, we first examined ethylene-induced root length inhibition of etiolating seedlings, which is one of the characteristics of the triple response. Col-0, *etr1-1*, RLD1 and Ws-0 seedlings were grown on MS-agar plates containing 0.5 µM of the ethylene precursor ACC, as this concentration was found to differentiate best for root length inhibition between Col-0 and *etr1-1* (data not shown). At 0.5 µM ACC, Col-0 plants

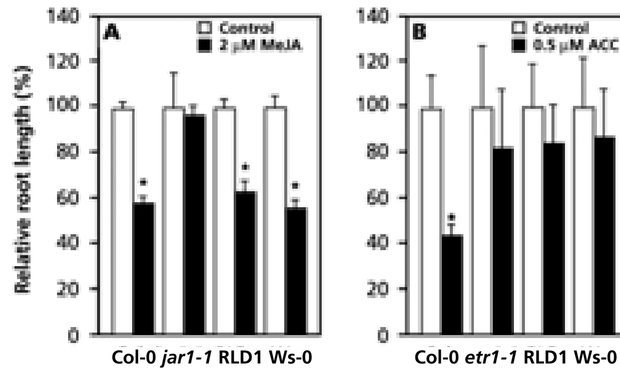


Figure 1: MeJA- and ACC-induced inhibition of primary root growth.
(**A**) MeJA-induced inhibition of primary root growth in Col-0, *jar1-1*, RLD1, and Ws-0. Seeds were surface-sterilized, distributed on MS-agar plates containing 0 or 2 µM MeJA, and germinated for 2 days at 4 °C in the dark. After an additional growth period of 5 days at 20 °C with an 8-hour photoperiod, the length of the primary roots was measured. Data are means (± SD; n = 15-25) of the relative root length compared to control plants (0 µM MeJA), which was set at 100 %. Asterisks indicate statistically significant differences compared to the control plants (Student's *t* test; $\alpha = 0.05$).
(**B**) ACC-induced inhibition of primary root length in Col-0, *etr1-1*, RLD1, and Ws-0. Seeds were germinated on MS-agar plates containing 0 or 0.5 µM ACC for 2 days at 4 °C in the dark. After an additional growth period of 3-7 days in the dark at 20 °C, the length of the primary root was measured. Data are means (± SD; n = 15-25) of the relative root length compared to control plants (0 µM ACC), which was set at 100 %. Asterisks indicate statistically significant differences compared to the control plants (Student's *t* test; $\alpha = 0.05$).

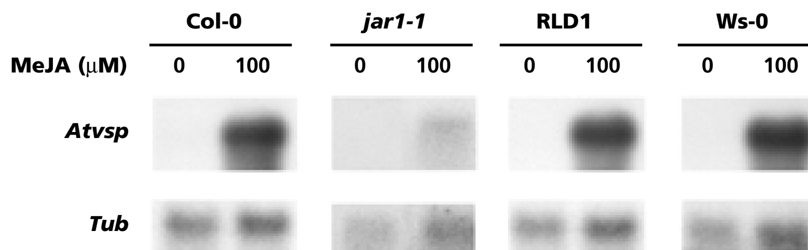


Figure 2: RNA gel blot analysis of MeJA-induced *Atvsp* gene expression in leaves of Col-0, *jar1-1*, RLD1, and Ws-0. Five-week-old plants were treated by dipping the leaves in a 0.015% Silwet L-77 solution containing 0 or 100 µM MeJA. Two days after MeJA treatment, the leaves were harvested. An Arabidopsis *Atvsp* gene-specific probe was used for the RNA gel blot hybridization. To check for equal loading, the blot was stripped and hybridized with an Arabidopsis gene-specific probe for β-tubulin (*Tub*). The experiment was repeated with similar results.

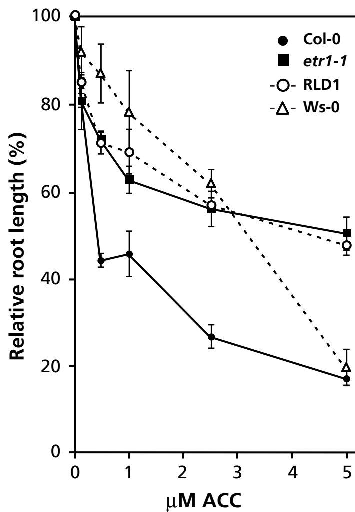


Figure 3: Dose-response curves of ACC-induced inhibition of primary root growth in Col-0, *etr1-1*, RLD1, and Ws-0. Seeds were germinated on MS-agar plates containing 0, 0.1, 0.5, 1.0, 2.5, or 5.0 μM ACC for 2 days at 4°C in the dark. After an additional growth period of 3-7 days in the dark at 20°C, the length of the primary root was measured. Data are means (± SD; n = 15-25) of the relative root length compared to that of control-treated plants (0 μM ACC), which was set at 100%.

showed a statistically significant inhibition of root growth, whereas mutant *etr1-1,* RLD1 and Ws responded only weakly (Figure 1B). In *etr1-1*, RLD1, and Ws-0, the weak inhibition of root growth was never statistically significant, indicating that RLD1 and Ws-0 exhibit a certain degree of insensitivity to ACC. To determine the extent of this insensitivity, the effect of increasing concentrations of ACC on root length inhibition was examined. Figure 3 shows that primary root length of etiolated Col seedlings was reduced by 55% to 80% when grown on 0.5 to 5 μM ACC respectively. In this concentration range, the inhibition of root elongation of both *etr1-1* and RLD1 was significantly less dramatic (ranging between 25% to 45 %). Up to 2.5 μM ACC, the effect of ACC on root elongation in ecotype Ws-0 was even less evident than in RLD1 and *etr1-1* (ranging between 10% to 35 %). However, at 5 μM ACC Ws-0 showed the same level of inhibition of root length as Col-0. Other characteristics of the triple response, i.e. inhibition of hypocotyl elongation and exaggeration of the apical hook, were clearly apparent at 0.5 μM of ACC in Col-0. In *etr1-1*, RLD1 and Ws-0 these characteristics were absent at 0.5 μM and 1 μM (Figure 4) and only occurred consistently at concentrations above 2.5 μM (data not shown).

To further investigate the apparent differences in ACC sensitivity, the expression patterns of the ethylene-inducible, defense-related genes *Hel* (Potter et al., 1993) and *Pdf1.2* (Penninckx et al., 1996) were analyzed after application of 0, 1.0 or 2.5 mM ACC to the leaves of Col-0, *etr1-1*, RLD1 and Ws-0 (Figure 5). In Col-0, both *Hel* and *Pdf1.2* transcripts were evident at 1.0 mM ACC and accumulated to a relatively high level at 2.5 mM ACC. In contrast, at increasing ACC concentrations *etr1-1* showed no increase in the steady-state *Hel* mRNA level. Moreover, only a relatively weak induction of *Pdf1.2* gene expression was apparent in *etr1-1*, which was significantly less pronounced than that observed in Col-0 plants.

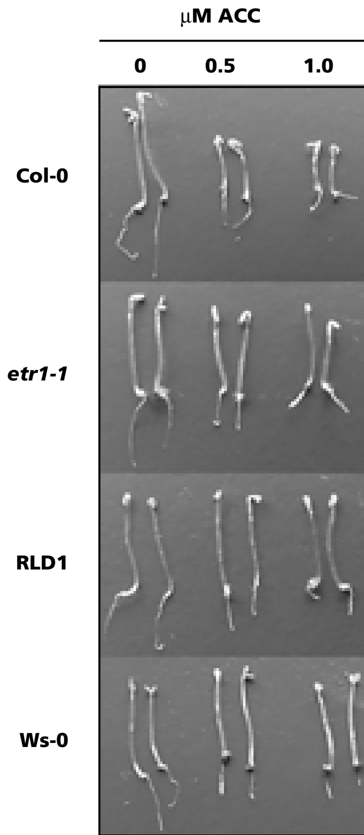
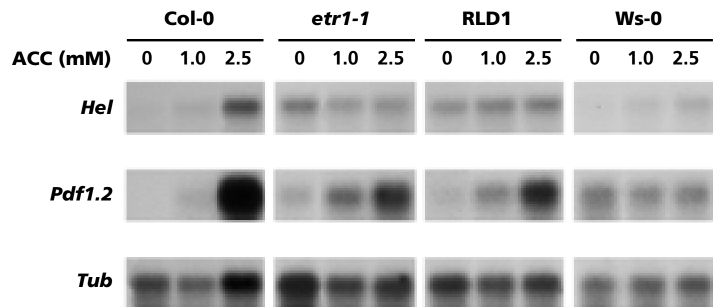


Figure 4: Triple response expression of Col-0, *etr1-1*, RLD1, and Ws-0. Seeds were germinated on MS-agar plates containing 0, 0.5, or 1.0 μM ACC for 2 days at 4°C in the dark. Photographs were taken after an additional growth period of 4 days in the dark at 20°C.

Figure 5: RNA gel blot analysis of ACC-induced *Hel* and *Pdf1.2* gene expression in the leaves of Col-0, *etr1-1*, RLD1, and Ws-0. Five-week-old plants were treated by dipping the leaves in a 0.015% Silwet L-77 solution containing 0, 1.0, or 2.5 mM ACC. Two days after ACC-treatment, the leaves were harvested. Arabidopsis *Hel* and *Pdf1.2* gene-specific probes were used for RNA gel blot hybridizations. To check for equal loading, the blots were stripped and hybridized with an Arabidopsis gene-specific probe for *β*-tubulin (*Tub*). The experiment was repeated with similar results.



RLD1 showed *Hel* and *Pdf1.2* expressions patterns similar to that observed in *etr1-1*. In Ws-0, steady-state levels of *Pdf1.2* transcripts did not increase at all after ACC treatment, whereas the *Hel* gene was weakly induced. These results confirm that ecotypes RLD1 and Ws-0 show reduced sensitivity to ACC, with RLD1 resembling *etr1-1* more than Ws-0.

After application of 1 mM ACC, all four genotypes showed similar time kinetics in the emission of ethylene (data not shown), indicating that ACC uptake and ACC converting capacity was similar for all four genotypes. It can thus be concluded that the observed differences in responsiveness to ACC are the result of differences in ethylene sensitivity.

RLD1 and Ws-0 are affected in ethylene-induced protection against *Pst*

Comparison of mutant *etr1-1* and ecotypes RLD1 and Ws-0 revealed that they are phenotypically similar in that they are nonresponsive to WCS417r-mediated induction of ISR (Figure 6A) and allow statistically significant higher levels of growth of *Pst* compared to Col-0 (Figure 6B). Both characteristics are likely to be caused by the common reduced sensitivity to ethylene. Previously, we demonstrated that exogenous application of ACC to the leaves induces protection against *Pst* in Col-0 but not in *etr1-1* (Pieterse et al., 1998). To investigate whether RLD1 and Ws-0 are similarly affected in ACC-induced protection, we determined the level of resistance against *Pst* after exogenous application of increasing concentrations of ACC (Figure 6C). In Col-0, all ACC concentrations tested induced a statistically significant level of protection against *Pst* compared to water-treated plants. In contrast, *etr1-1* and RLD1 failed to develop resistance after treatment with the three lowest ACC concentrations (50, 100 and 250 μM), whereas application of 1 mM ACC induced protection in *etr1-1* and RLD1 only. In Ws-0, all ACC concentrations tested failed to induce protection against *Pst*. It can thus be concluded that, like *etr1-1*, ecotypes RLD1 and Ws-0 are impaired in ethylene-induced resistance against *Pst*.

Reduced sensitivity to ethylene cosegregates with the *isr1* phenotype

To investigate whether the reduced sensitivity to ethylene observed in RLD1 and Ws-0 is associated with the recessive alleles of the *ISR1* locus, we tested the F_2 progeny of the RLD1 x Col-0 cross for ethylene sensitivity. The triple response was tested in the F_2 seedlings and in the RLD1 and Col-0 parents at 0 and 0.5 μM ACC. In the F_2 population, responsiveness to 0.5 μM ACC segregated in a statistically significant 3 : 1 ratio ($\chi^2 = 0.16$, $P = 0.69$; Table 1), indicating that the reduced sensitivity to ethylene is caused by a single recessive locus. This experiment was repeated twice, yielding comparable segregation ratios ($\chi^2 = 0.04$ and 0.19; $P = 0.83$ and 0.66, respectively). Subsequently, five F_3 families homozygous at the *ISR1* locus (*ISR1/ISR1*), five F_3 families homozygous at the *isr1* locus (*isr1/isr1*), and the corresponding Col-0 and RLD1 parent were tested for triple response expression at 0, 0.5, 1 and 5 μM ACC. On MS-agar plates without ACC, none of the genotypes showed triple response expression (Table 2). On 0.5 μM ACC, only the Col-0 parent and the five *ISR1/ISR1* F_3 families exhibited consistent triple response expression, whereas the RLD1 parent and the *isr1/isr1* F_3 families did not (Table 2). At higher concentrations of ACC, both RLD1 and the *isr1/isr1* F_3 families showed triple response

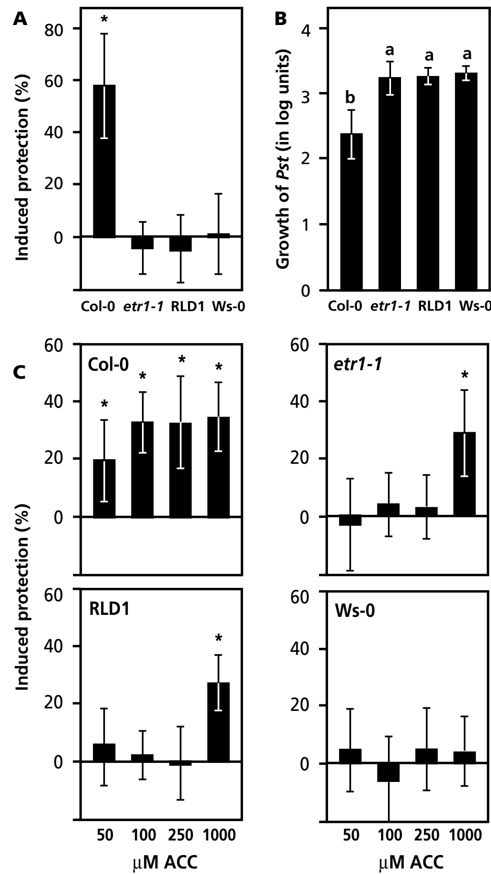


Figure 6: Level of WCS417r-induced protection, basal resistance, and ACC-induced protection against *P. syringae* pv. *tomato* DC3000 in Col-0, *etr1-1*, RLD1 and Ws-0.

(**A**) Quantification of WCS417r-mediated ISR. ISR was triggered by growing plants 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* at 2.5 x 10^7 CFU.mL^{-1}. Three days after challenge inoculation, the percentage of diseased leaves was assessed and the level of induced protection calculated. Induced protection is presented as a reduction of disease symptoms relative to challenged control plants. Asterisks indicate statistically significant differences compared to non-induced control plants (Student's *t* test; α = 0.05; n = 20-25). Data presented are means (± SD) from representative experiments that were performed at least twice with similar results.

(**B**) Growth of *Pst* over a 3-day time interval. Five-week-old plants were infected by pressure infiltrating a suspension of virulent *Pst* at 5 x 10^5 CFU.mL^{-1} into the leaves. Immediately after pressure infiltration and 3 days later, the number of *Pst* bacteria per gram of leaf fresh weight was determined and the proliferation over a 3-day time interval was calculated. Data presented are the means (± SD) of the proliferation values (log CFU.g^{-1}) of a representative experiment that was repeated twice with similar results. Different letters indicate statistically significant differences between genotypes (Fisher's LSD test; α = 0.05; n = 6)

(**C**) Quantification of ACC-induced protection against *Pst*. Plants were dipped in a solution containing different concentrations of ACC, 3 days before challenge inoculation with *Pst*. Three days after challenge, the level of induced protection was assessed as described above.

expression, although in many cases this was inconsistent (Table 2). It can thus be concluded that the reduced ethylene sensitivity observed in RLD1 is a recessive trait that cosegregates with the *isr1* phenotype.

Table 1: Genetic segregation of triple response expression at 0.5 μM ACC in Col-0, RLD1, and F_2 plants of the RLD1 x Col-0 cross[a].

Genotype	Treatment (μM ACC)	Total number of seedlings	Triple response[b]	No triple response[c]	Expected ratio	χ^2-value	*P*
Col-0	0	48	0	48	0 : 1		
	0.5	56	53	3	1 : 0		
RLD1	0	52	3	49	0 : 1		
	0.5	53	4	49	0 : 1		
F_2	0	52	4	48	0 : 1		
	0.5	74	54	20	3 : 1	0.16	0.69

[a] Surface-sterilized seeds were plated onto MS-agar plates with or without 0.5 μM ACC. After germination for 2 days at 4 °C, seedlings were grown for 5 days at 20 °C in the dark and examined for triple response expression. The experiment was repeated twice, yielding similar results.
[b] Number of seedlings exhibiting triple response characteristics.
[c] Number of seedlings showing no triple response characteristics.

The *ISR1* locus is not allelic with *Ein3*

Previously, the *ISR1* locus was mapped at chromosome III between CAPS markers *GL1* and *B4* (Chapter 2; Ton et al., 1999). The *Ein3* gene, encoding an activator of the ethylene response pathway (Chao et al., 1997), maps also in this region. Moreover, the *ein3* mutant is unable to express WCS417r-mediated ISR (Knoester et al., 1999). Therefore, we investigated whether *ISR1* and *Ein3* are allelic. For this purpose, we designed a CAPS marker based on the nucleotide sequence of the *Ein3* gene (Chao et al., 1997), and performed a CAPS marker analysis on 32 homozygous F_3 families of the RLD1 x Col-0 cross (16 *ISR1/ISR1* F_3 families and 16 *isr1/isr1* F_3 families). Of the 64 chromosomes tested, 13 were recombinant with the *Ein3* CAPS marker, yielding a recombination frequency of 20.3%. Moreover, the F_1 progeny of a complementation cross between RLD1 and the *ein3* mutant was fully capable of expressing ISR and exhibited the relatively high level of basal resistance against *Pst* of the Col-0 parent (data not shown). It can thus be concluded that the *ISR1* locus is not allelic with the *Ein3* gene.

Table 2: Triple response expression in ecotypes Col and RLD, and in F_3 families of the RLD x Col cross that are homozygous at the *ISR1* locus.

Ecotypes/ F_3-families[a]	Triple response assay[b] 0 μM ACC	0.5 μM ACC	1 μM ACC	5 μM ACC
Col-0 (*ISR1/ISR1*)	-	+	+	+
ISR1/ISR1 fam 1	-	+	+	+
ISR1/ISR1 fam 2	-	+	+	+
ISR1/ISR1 fam 3	-	+	+	+
ISR1/ISR1 fam 4	-	+		+
ISR1/ISR1 fam 5	-	+	+	+
RLD1 (*isr1/isr1*)	-	-	+	+
isr1/isr1 fam 1	-	-	+/-	+/-
isr1/isr1 fam 2	-	-	+	+
isr1/isr1 fam 3	-	-	+	+
isr1/isr1 fam 4	-	-	+/-	+/-
isr1/isr1 fam 5	-	-	-	+/-

[a] The F_3 families homozygous for the dominant *ISR1* alleles of Col-0 are capable of expressing ISR after treatment of the roots with *P. fluorescens* WCS417r and exhibit relatively high basal resistance against *Pst*. The F_3 families homozygous for the recessive *isr1* alleles of RLD1 fail to express ISR after treatment of the roots with WCS417r and exhibit relatively low levels of basal resistance against *Pst* (Chapter 2; Ton et al., 1999).

[b] Genotypes were assayed for sensitivity to ACC using the triple response assay. Surface-sterilized seeds (50-100) were plated onto MS-agar plates containing different concentrations of ACC. After germination for 2 days at 4 °C, seedlings were grown for 3-7 days at 20 °C in darkness before being examined for triple response expression. +: consistent triple response in all plants; +/-: inconsistent triple response; -: none of the plants expressed the triple response.

Discussion

Previously, we demonstrated that the *ISR1* locus on chromosome III of Arabidopsis controls the expression of both WCS417r-mediated ISR and basal resistance against *Pst* (Chapter 2; Ton et al., 1999). This study aimed at elucidating the physiological role of the *ISR1* locus in the ISR signaling pathway. In Arabidopsis, the ISR pathway requires an intact response to the plant hormones JA and ethylene (Pieterse et al., 1998). Analysis of MeJA-induced inhibition of primary root growth and *Atvsp* gene expression in the WCS417r-nonresponsive ecotypes RLD1 (*isr1/isr1*), Ws-0 (*isr1/isr1*) and the ISR-inducible ecotype Col-0 (*ISR1/ISR1*) revealed that RLD1 and Ws-0 are not affected in their response to MeJA (Figures 1 and 2),

demonstrating that the *ISR1* locus is not involved in JA signaling. In contrast, analysis of the triple response and ethylene-inducible gene expression in these genotypes demonstrated that sensitivity to ethylene is significantly reduced in both RLD1 and Ws-0 in comparison to Col-0 (Figures 3 to 5). Moreover, RLD1 and Ws-0 were impaired in their ability to express ethylene-induced resistance against *Pst* (Figure 6). Genetic analysis of the F_2 and F_3 progeny of an RLD1 x Col-0 cross revealed that the reduced sensitivity to ethylene is monogenically inherited as a recessive trait and genetically linked to the recessive alleles of the *ISR1* locus. These results indicate that the *ISR1* locus contains a gene encoding a component involved in ethylene signaling. Using a large set of ethylene response mutants, we previously demonstrated that insensitivity to ethylene causes non-responsiveness to WCS417r (Pieterse et al., 1998; Knoester et al., 1999). Therefore, it is likely that ethylene responsiveness and ISR inducibility are determined by a single gene on the *ISR1* locus. However, the possibility of close linkage between two different genes cannot be eliminated completely.

The *ISR1* locus maps on chromosome III between CAPS markers *B4* and *GL1* (Chapter 2; Ton et al., 1999). Two genes from the ethylene signaling pathway, *Ein3* and *Ein4*, map in the vicinity of the *ISR1* locus. The possibility that the *ISR1* locus is allelic with *Ein4* can be ruled out by the observation that ecotypes RLD1 and Ws-0 exhibit recessive phenotypes (Chapter 2; Ton et al., 1999, this study), whereas the *ein4* mutation is dominant (Roman et al., 1995). The considerable recombination between the *ISR1* locus and the *Ein3* gene in F_3 families of the RLD1 x Col-0 cross also rules out the possibility that *ISR1* is allelic with *Ein3*. Moreover, ecotype RLD1 and mutant *ein3* showed full complementation of ISR-inducibility and basal resistance against *Pst* in their F_1 progeny. Therefore, we hypothesize that the Arabidopsis *ISR1* locus encodes a novel component of the ethylene response pathway that plays an important role in disease resistance signaling.

Although both RLD1 and Ws-0 showed reduced sensitivity to ethylene in comparison to Col-0, the magnitude of the reduced ethylene response differed between both ecotypes. For instance, like *etr1-1*, RLD1 was affected in the inhibition of ethylene-induced primary root growth at all ACC concentrations tested (0.5 to 5.0 μM), whereas for Ws-0 this was only apparent at ACC concentrations up to 2.5 μM. At 5 μM, Ws-0 displayed a normal triple response. Analysis of ethylene-responsive gene expression revealed a similar pattern, with RLD1 resembling *etr1-1* more than Ws-0. The apparent differences in ethylene responsiveness between RLD1 and Ws-0 likely result from the considerable genetic diversity between both ecotypes (Erschadi et al., 2000). Alternatively, RLD1 and Ws-0 might be affected at different sites in the *ISR1* locus.

In Arabidopsis, considerable genetic variation between ecotypes has been reported for several developmental, physiological and biochemical traits (Alonso-Blanco and Koornneef, 2000). This naturally occurring variation of Arabidopsis has contributed to the identification of a large number of loci conferring resistance to viral, bacterial or fungal

pathogens (Kunkel, 1996). In this study, we showed that reduced sensitivity to ethylene in RLD1 and Ws-0 is causing their *isr1* phenotype. Naturally occurring variation in ethylene sensitivity is known to occur in various plant species. For instance, Voesenek et al. (1996) reported significant differences in ethylene sensitivity between three species of *Rumex*, of which one showed exceptionally high responsiveness to ethylene. This phenotype appeared to be a necessary adaptation for escaping water submergence by mediation of ethylene-induced shoot elongation. Moreover, Emery et al. (1996) reported considerable differences in ethylene responsiveness between ecotypes of *Stellaria longipes*. The enhanced ethylene responsiveness of one ecotype was explained as a critical adaptation to wind stress.

Arabidopsis plants carrying the recessive alleles of the *ISR1* locus show an enhanced susceptibility to *Pst* infection (Chapter 2; Ton et al., 1999), suggesting that ethylene signaling is involved in basal resistance against *Pst*. This is supported by the observation that the ethylene response mutant *etr1-1* allows ten-fold higher levels of growth of *Pst* than wild-type plants (Pieterse et al., 1998; this study). Moreover, treatment of Arabidopsis with the ethylene precursor ACC induces resistance against *Pst* (Pieterse et al., 1998; Van Wees et al., 1999). These findings seem to contradict those reported by Bent et al. (1992), who showed that the ethylene response mutant *ein2* allow similar levels of growth of *Pst*, and develop fewer symptoms compared to wild-type plants. However, Bent et al. (1992) used a 5-fold lower *Pst* inoculum density, suggesting that the ethylene-dependent basal resistance that we observed is only apparent when the initial inoculum density is above a certain threshold level. The plant hormones SA and JA are also involved in basal resistance against *Pst*, because genotypes that are impaired in their response to these signaling molecules are more susceptible to *Pst* infection (Delaney et al., 1994; Pieterse et al., 1998). Apparently, the mechanisms contributing to basal resistance against *Pst* are controlled by a coordinated action of SA-, JA- and ethylene-dependent signaling pathways.

Previously, Knoester et al. (1999) tested eight ethylene response mutants on their ability to express WCS417r-mediated ISR. None of these mutants were able to express ISR after application of WCS417r to the roots, demonstrating that an intact ethylene-signaling pathway is required for the expression of ISR. Mutant *eir1-1* that is insensitive to ethylene in the roots only, was capable to mount ISR after application of WCS417r to the leaves, but not after application of WCS417r to the roots, indicating that ISR signaling pathway requires ethylene sensitivity at the site of WCS417r application. From these results it was hypothesized that ethylene signaling is involved in the generation or translocation of the systemically transported ISR signal. Therefore, the position of the *ISR1* gene product in the ISR signaling pathway should be placed before the systemically transported signal, although it is still unclear whether ethylene sensitivity is also involved in the later stages of the ISR signaling pathway. Future research will be focussed on cloning of the *ISR1* gene. This will open the way to study the role of the ISR1 protein in both ISR and ethylene signaling.

Materials and Methods

Cultivation of rhizobacteria, pathogens and plant

Nonpathogenic, ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria (Pieterse et al., 1996) were grown on King's medium B (KB) agar plates (King et al., 1954) for 24 hours at 28 °C. Bacterial cells were collected and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 colony-forming units (CFU) per mL. The virulent pathogen *P. syringae* pv. *tomato* strain DC3000 (*Pst*; Whalen et al., 1991) used for challenge inoculations was grown overnight at 28 °C in liquid KB. After centrifugation, the bacterial cells were resuspended in 10 mM $MgSO_4$ with 0.015% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands) to a final density of 2.5 x 10^7 $CFU.mL^{-1}$.

Seedlings of wild-type *Arabidopsis thaliana* accessions Columbia (Col-0), Wassilewskija (Ws-0), and RLD1, and the Col-0 mutants *etr1-1* (Bleecker et al., 1988) and *jar1-1* (Staswick et al., 1992) were grown in quartz sand for two weeks. For transfer of the seedlings, a sand/potting soil mixture (5 : 12 v/v) that had been autoclaved twice for one hour with a 24 hours interval was supplemented with either a suspension of ISR-inducing WCS417r bacteria, or an equal volume of 10 mM $MgSO_4$. Seedlings were then transferred into 60-mL pots containing the sand/potting soil mixture with or without WCS417r. Plants were cultivated in a growth chamber with an 9-hour day (≈200 $\mu E.m^{-2}.sec^{-1}$ at 24 °C) and 15-hour night (20 °C) cycle at 65% relative humidity. For the duration of the experiments, all genotypes remained vegetative and developed at least 10-15 mature leaves. Plants were watered on alternate days, and once a week supplied with modified half strength Hoagland solution: 2 mM KNO_3, 5 mM $Ca[NO]_3$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, and trace elements, pH 7 (Hoagland and Arnon, 1938), containing 10 μM sequestreen (Fe-ethylenediamide-di[*o*-hydroxyphenylacetic acid]; Novartis, Basel, Switzerland).

Induction treatments

For treatment with ISR-inducing rhizobacteria, 2-week-old seedlings were transplanted into soil containing WCS417r bacteria at 5 x 10^7 $CFU.g^{-1}$. Induction of ethylene-mediated resistance was performed 2 days before challenge by dipping the leaves of 5-week-old plants in a solution containing the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) with 0.015% (v/v) Silwet L-77. For RNA blot analysis, chemical treatments were performed by dipping the leaves of 5-week-old plants in a solution containing 0.015% (v/v) Silwet L77 and different concentrations of either methyl jasmonate (MeJA) or 1-aminocyclopropane-1-carboxylate (ACC).

Challenge inoculations and disease assessment

For assaying induced resistance, WCS417r- and control-treated plants were challenged when 5 weeks old by dipping the leaves in a suspension of virulent *Pst* bacteria at 2.5 x 10^7 $CFU.mL^{-1}$ in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77. One day before challenge inoculation, the plants had been placed at 100% relative humidity. Three or 4 days after challenge inoculation, the percentage of leaves

with symptoms was determined per plant (n = 20-25). Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased (Pieterse et al., 1996). For assaying basal resistance against *Pst*, leaves of 5-week-old, control-treated plants were inoculated by pressure-infiltration with a suspension of virulent *Pst* at 5 x 10^5 CFU.mL^{-1} in 10 mM $MgSO_4$. Immediately after pressure infiltration and 3 days later, replicate leaf samples from sixe plants per genotype were collected, weighed, and homogenized in 10 mM $MgSO_4$. Serial dilutions were plated on selective KB-agar plates supplemented with 100 mg.L^{-1} cycloheximide and 50 mg.L^{-1} rifampicin. After incubation at 28 °C for 2 days, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined and bacterial proliferation over the 3-day time interval was calculated.

Inhibition of primary root length by MeJA and ACC.

Seeds of Arabidopsis were surface sterilized for 5 minutes in 5% (v/v) sodium hypochlorite, washed in (v/v) 70% ethanol, and air-dried. Subsequently, seeds were distributed evenly on 1.0% (w/v) agar medium containing 0.5% (w/v) Murashige and Skoog salts (Duchefa BV, Haarlem, The Netherlands), 0.5% (w/v) sucrose, and different concentrations of either MeJA or ACC (pH 5.7). MeJA (Serva, Brunschwig Chemie BV, Amsterdam, The Netherlands) was added to the autoclaved medium from a filter-sterilized 1 mM stock (containing 0.96% (v/v) ethanol). ACC (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was added from a 10 mM stock in a similar manner. Seeds were germinated in the dark for 2 days at 4 °C. The effect of MeJA on primary root growth was determined essentially as described by Staswick et al. (1992). Plates were incubated in a climate chamber at 22 °C with an 8-hour day (≈200 μE.m^{-2}.s^{-1}) and a 16-hour night cycle. After 5 days, the primary root length was measured under a dissection microscope. In each case, 15-25 randomly selected seedlings were measured. The effect of ethylene on primary root length of etiolated seedlings was tested essentially according to Guzmán and Ecker (1990). After germination in the dark for 2 days at 4 °C, the seedlings were grown for an additional 3-7 days at 20 °C without light. The primary root length was measured as described above.

Ethylene measurements

Leaves of plants pretreated with 1 mM ACC were detached, weighed, and placed in 25-mL gas-tight serum flasks, and subsequently incubated at climate chamber conditions. At different time points over a 28-h interval, cumulative ethylene production was measured by gas chromatography as described by De Laat and Van Loon (1982).

RNA gel blot analysis

Total RNA was extracted by homogenizing frozen leaf tissue in extraction buffer (0.35 M glycine, 0.048 N NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 mL.g^{-1} of leaf tissue). The homogenates were extracted with phenol and chloroform and the RNA was precipitated using LiCl, as described by Sambrook et al. (1989). For RNA gel blot analysis, 15 μg of RNA was denatured using

glyoxal and DMSO (Sambrook et al., 1989). Subsequently, samples were electrophoretically separated on 1.5% agarose gels and blotted onto Hydobond-N+ membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. The electrophoresis buffer and blotting buffer consisted of 10 mM and 25 mM sodium phosphate (pH 7.0), respectively. RNA gel blots were hybridized and washed as described previously (Pieterse et al., 1994). DNA probes were labeled with α-^{32}P-dCTP by random primer labeling (Feinberg and Vogelstein, 1983). Probes for the detection of *Atvsp*, *Hel* and *Tub* transcripts were prepared by PCR with primers based on sequences obtained from GenBank accession numbers Z18377, U01880, and M21415, respectively. Probes to detect *Pdf1.2* transcripts were derived from an Arabidopsis *Pdf1.2* cDNA clone (Penninckx et al., 1996).

Genetic analysis

Previously, the F_1, F_2 and F_3 progenies of a cross between Col (*ISR1/ISR1*) and RLD (*isr1/isr1*) were tested for ISR inducibility and basal resistance against *Pst* (Chapter 2; Ton et al., 1999). From this cross, the F_2 progeny, five randomly selected F_3 families homozygous at the *ISR1* locus, and five randomly selected F_3 families homozygous at the *isr1* locus were tested for ethylene sensitivity and compared to that of the corresponding parents. Ethylene sensitivity was quantified by assaying triple response expression as described by Guzmán and Ecker (1990), using MS-agar plates containing different concentrations of ACC. Recombination between the *ISR1* and the *EIN3* locus was determined on 16 F_3 families homozygous at the *ISR1* locus and 16 F_3 families homozygous at the *isr1* locus, using a cleaved amplified polymorphism sequence (CAPS) marker (Konieczny and Ausubel, 1993) for the *EIN3* gene (accession no. AF004217; Chao et al., 1997). Amplification of the *EIN3* sequence was performed using the primers 5'-CTCCTTCTTTTTCCCATCAC CATA-3' (nucleotides 349 to 372) and 5'-TTCCCATCTCATTAAACATCATTG-3' (nucleotides 975 to 952). Subsequent digestion with *Bgl*II resulted in a polymorphism between accessions Col-0 (627 bp) and RLD1 (359 and 268 bp).

ACKNOWLEDGMENTS

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CHAPTER 4

Characterization of Arabidopsis enhanced disease susceptibility mutants that are affected in systemically induced resistance

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Submitted

Abstract

Selected nonpathogenic rhizosphere-colonizing *Pseudomonas fluorescens* bacteria trigger a jasmonate- and ethylene-dependent induced systemic resistance (ISR) that is effective against different pathogens. Arabidopsis genotypes that are unable to express rhizobacteria-mediated ISR against the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) show an enhanced disease susceptibility towards this pathogen (Pieterse et al., 1998; Ton et al., 1999). In order to identify novel components of the ISR signaling pathway, we screened a collection of Arabidopsis *eds* mutants with enhanced disease susceptibility to pathogenic *P. syringae* bacteria on their potential to express rhizobacteria-mediated ISR and pathogen-induced systemic acquired resistance (SAR) against *Pst*. Out of 11 *eds* mutants tested, *eds4-1*, *eds8-1*, and *eds10-1* were nonresponsive to induction of ISR by *Pseudomonas fluorescens* WCS417r, whereas mutants *eds5-1* and *eds12-1* were nonresponsive to induction of pathogen-induced SAR. Whereas *eds5-1* is known to be blocked in the synthesis of salicylic acid (SA), further analysis of *eds12-1* revealed that the SAR-impaired phenotype of this mutant is caused by a reduced sensitivity to SA. Analysis of the ISR-impaired *eds* mutants revealed that they are insensitive to induction of resistance by methyl jasmonate (MeJA) (*eds4-1*, *eds8-1*, and *eds10-1*), or the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (*eds4-1* and *eds10-1*). Moreover, *eds4-1* and *eds8-1* showed reduced expression of the plant defensin gene *Pdf1.2* after treatment with MeJA and ACC, which was associated with a reduced sensitivity to either ethylene (*eds4-1*), or MeJA (*eds8-1*). Although blocked in rhizobacteria-, MeJA-, and ACC-induced protection, mutant *eds10-1* showed normal responsiveness to both MeJA and ACC. Together, these results indicate that EDS12 is required for SAR and acts downstream of SA, whereas EDS4, EDS8, and EDS10 are required for ISR and act in either the jasmonate response (EDS8), the ethylene response (EDS4), or downstream of the jasmonate and ethylene response (EDS10) in the ISR signaling pathway.

Introduction

Plants possess multiple strategies to resist infection by virulent pathogens. The signaling molecules salicylic acid (SA), jasmonic acid (JA), and ethylene play important roles in defense signaling, because plant genotypes that are affected in the response to either of these signals show enhanced disease susceptibility to various virulent pathogens or insects (Delaney et al., 1994; Knoester et al., 1998; McConn et al., 1997; Pieterse et al., 1998; Staswick et al., 1998; Thomma et al., 1998; Ton et al., 2001; Vijayan et al., 1998). Over the past years, responses to virulent pathogens have been subjected to a series of mutant screens, using *Arabidopsis thaliana* as a model host plant. From a screen for enhanced disease susceptibility to *Pseudomonas syringae* pv. *maculicola*, 12 unique *eds* mutants have been characterized (*eds2* – *eds13*; Glazebrook et al., 1996; Volko et al., 1998). All these mutants allow at least 10-fold higher levels of growth of *P. syringae* pv. *maculicola* ES4326 upon infection of their leaves, but they vary in their susceptibility to other pathogens (Glazebrook et al., 1996; Rogers et al., 1997; Volko et al., 1998). The role of some of these *Eds* genes in basal disease resistance has been elucidated. For instance, mutant *eds5-1* was recently demonstrated to be allelic with the SA induction-deficient mutant *sid1-1* (Nawrath and Métraux, 1999). This mutation affects pathogen-induced accumulation of SA, and renders the plant more susceptible to a broad range of pathogens, including *Peronospora parasitica*, *Erisyphe orantii*, *P. syringae* pv. *tomato* DC3000 (*Pst*), and *Xanthomonas campestris* pv. *raphani* (Nawrath and Métraux, 1999; Volko et al., 1998). Furthermore, *eds4-1* was recently characterized as a mutant affected in SA-dependent defense responses (Gupta et al., 2000).

Besides basal resistance that protects the plant to some degree against primary attack by virulent pathogens, plants have the ability to develop an enhanced defensive capacity against a broad spectrum of pathogens after stimulation by specific biological or chemical agents. In Arabidopsis, two forms of biologically-induced disease resistance have been characterized relatively well: systemic acquired resistance (SAR) that is triggered upon infection by a necrotizing pathogen (Ryals et al., 1996; Sticher et al., 1997), and induced systemic resistance (ISR) that is triggered by selected strains of non-pathogenic rhizobacteria (Van Loon et al., 1998). Both SAR and rhizobacteria-mediated ISR are effective against different pathogens, but are regulated by distinct signaling pathways. Pathogen-induced SAR requires SA, whereas rhizobacteria-mediated ISR functions independently of SA (Gaffney et al., 1993; Pieterse et al., 1996; Pieterse and Van Loon, 1999). In the ISR signaling pathway, components from the JA and the ethylene response act in sequence in triggering a defense reaction that, like SAR, depends on the defense regulatory protein NPR1 (Cao et al., 1994; Pieterse et al., 1998; 2000). Simultaneous activation of both types of induced defense results in an enhanced level of induced protection against *Pst*, demonstrating that the defense responses activated via both pathways are compatible and additive (Van Wees et al., 2000).

Previously, we reported that two Arabidopsis ecotypes, RLD1 and Wassilewskija (Ws-0), are impaired in their ability to express ISR after treatment of the roots with ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria, whereas they express normal levels of pathogen-induced SAR (Chapter 2; Ton et al., 1999). This ISR-noninducible phenotype was associated with a remarkably low level of basal resistance against *Pst* in comparison to the ISR-inducible ecotype Columbia (Col-0). By using this naturally occurring variation among Arabidopsis ecotypes, a genetic approach was initiated. Analysis of the progeny from a RLD1 x Col-0 cross revealed that ISR inducibility and basal resistance against *Pst* are controlled by a single dominant locus (*ISR1*) that maps on chromosome III between cleaved amplified polymorphic sequence (CAPS) markers *Ein3* and *GL1* (Chapters 2 and 3; Ton et al., 1999; Ton et al., 2001). Genotypes carrying the recessive alleles of *ISR1* exhibit reduced sensitivity to ethylene, indicating that the *ISR1* locus encodes a component of the ethylene response that plays an important role in disease resistance signaling (Chapter 3; Ton et al., 2001).

The observed association between the inability to express WCS417r-mediated ISR, on the one hand, and enhanced susceptibility to *Pst*, on the other hand, prompted us to investigate whether a similar relationship might hold in mutants selected for enhanced disease susceptibility to *P. syringae* pathogens. To this end, mutants *eds3* - *eds13* were examined for their ability to develop *P. fluorescens* WCS417r-mediated ISR and SAR. Here, we show that out of the 11 mutants tested, three mutants are affected in their ability to express ISR, whereas two other mutants are SAR-deficient. The ISR- and SAR-deficient mutants were further characterized with regard to their responsiveness to SA, JA, and ethylene.

Results

Biologically-induced ISR and SAR in Arabidopsis mutants *eds3*- *eds13*

To investigate whether EDS3 to EDS13 play a role in the ISR signaling pathway, mutants *eds3-1* to *eds13-1* were tested for their ability to express *P. fluorescens* WCS417r-mediated ISR against *Pst*. In addition, the 11 mutants were tested for their capacity to express pathogen-induced SAR against this pathogen. ISR was induced by growing the plants in soil containing WCS417r bacteria for 3 weeks. SAR was induced 3 days before challenge inoculation by injecting two lower leaves with avirulent *Pst* carrying the avirulence gene *avrRpt2* (*Pst*(*avrRpt2*); Kunkel et al., 1993). ISR- and SAR-mediated protection was quantified by assessing the disease incidence at 3 days after challenge inoculation with virulent *Pst*. Except for *eds4-1*, *eds8-1* and *eds10-1*, all genotypes showed a statistically significant suppression of disease symptoms after treatment of the roots with WCS417r bacteria, indicating that the corresponding *Eds* genes do not influence WCS417r-mediated ISR (Figure 1A). The non-responsiveness to WCS417r of *eds4-1*, *eds8-1* and *eds10-1* could not be

attributed to poor root colonization by the ISR-inducing bacteria, because the extent of root colonization at the end of the bioassays was always above 10^6 $CFU.g^{-1}$ root fresh weight in all genotypes tested (data not shown).

With regard to pathogen-induced SAR, all genotypes, except *eds5-1* and *eds12-1*, showed a statistically significant reduction of disease incidence in *Pst*(*avrRpt2*)-pretreated plants (Figure 1B). From these results it can be concluded that *eds4-1*, *eds8-1*, and *eds10-1* are

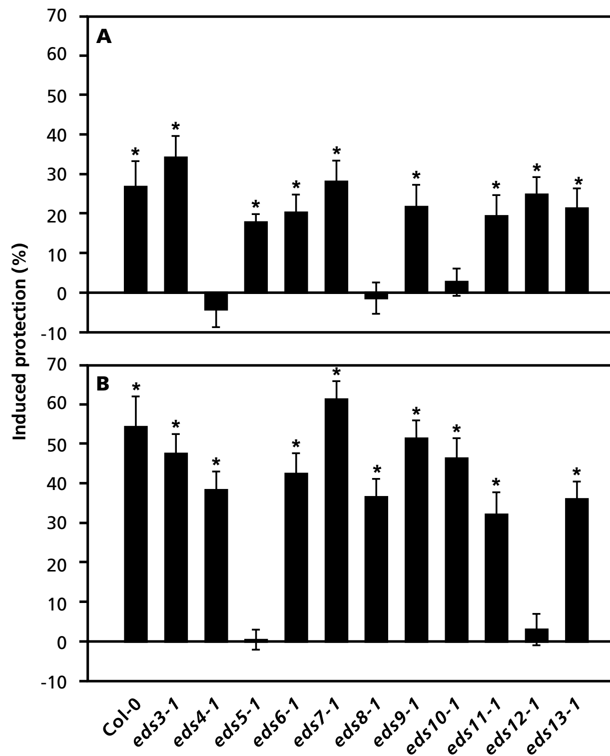

Figure 1: Levels of induced protection against *P. syringae* pv. *tomato* DC3000 (*Pst)* as a result of *P. fluorescens* WCS417r-mediated ISR (**A**) and pathogen-induced SAR (**B**) in wild-type Col-0 plants and *eds* mutants of Arabidopsis

(A) ISR was triggered by growing plants for three 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* at 2.5 x 10^7 $CFU.mL^{-1}$. 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 (Student's *t* test; $\alpha = 0.05$; n = 20-25). Data presented are means (± SD) from representative experiments that were performed at least twice with similar results.

(B) Induction of SAR was performed 3 days before challenge inoculation, either by pressure-infiltrating 2-3 lower leaves with a suspension of *Pst*(*avrRpt2*) bacteria at 10^7 $CFU.mL^{-1}$. Challenge inoculation and disease assessment were performed as described above.

blocked in the ISR pathway, whereas *eds5-1* and *eds12-1* are blocked in the SAR pathway. For *eds5-1*, this result confirms previous findings by Nawrath and Métraux (1999) that mutant *sid1-1*, which is allelic to mutant *eds5-1*, is impaired in its ability to express pathogen-induced SAR.

Chemically-induced resistance in ISR- and SAR-deficient *eds* mutants

In Arabidopsis, exogenous application of either MeJA or ACC has been shown to induce significant levels of protection against *Pst* (Pieterse et al., 1998; Van Wees et al., 1999). In the ISR signaling pathway leading to induced defense against *Pst*, components from the JA response act upstream of the ethylene response, because MeJA-induced protection against *Pst* is blocked in the ethylene response mutant *etr1-1*, while in the JA response mutant *jar1-1* ACC-induced protection is unaffected (Pieterse et al., 1998). To determine the position of EDS4, EDS8, and EDS10 in the ISR pathway, we tested whether *eds4-1*, *eds8-1*, and *eds10-1* are able to mount resistance against *Pst* in response to MeJA and ACC. Dipping the leaves of wild-type Col-0 plants in a solution containing 100 μM MeJA reduced the percentage of leaves with symptoms by almost 40% compared to non-treated control plants (Figure 2). Mutants *eds4-1*, *eds8-1* and *eds10-1* all three resembled the JA-response mutant *jar1-1* in that they failed to develop MeJA-induced protection against *Pst* (Figure 2). This indicates that EDS4, EDS8 and EDS10 all function downstream of JA perception in the ISR pathway.

Dipping the leaves in a solution containing 0.5 mM ACC resulted in a significant level of protection in Col-0 and *eds8-1* plants. However, *eds4-1* and *eds10-1* plants, like ethylene-insensitive *etr1-1* plants, failed to develop resistance after treatment with ACC

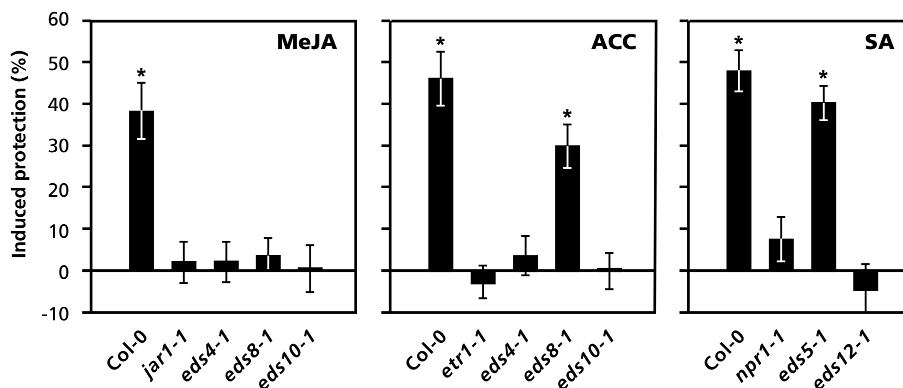

Figure 2: Levels of MeJA, ACC-, and SA-induced protection against *P. syringae* pv. *tomato* DC3000 in wild-type Col-0 plants, and in ISR- and SAR-impaired *eds* mutants. Five-week-old plants were induced by dipping the leaves in 0.015 (v/v) Silwet L-77 solutions containing either 100 μM MeJA, 0.5 mM ACC, or 1 mM SA. For challenge inoculation and disease assessment see caption to Figure 1.

(Figure 2). All genotypes converted ACC to ethylene with similar kinetics (data not shown), indicating that ACC uptake and ACC-converting capacity did not differ for all genotypes tested. It can thus be concluded that in the ISR pathway EDS8 functions downstream of the perception of JA, but upstream of ethylene signaling, whereas EDS4 and EDS10 both function downstream of the perception of ethylene.

To determine the locations of the mutations in the SAR signaling pathway, we dipped the leaves of Col-0, *eds5-1* and *eds12-1* plants in a solution containing 1 mM SA, quantified the level of protection against *Pst*, and analyzed the level of *PR-1* mRNA accumulation. As a control, mutant *npr1-1*, which is blocked downstream of SA in the SAR signaling pathway (Cao et al., 1994), was tested as well. Of the two SAR-impaired mutants, *eds5-1* was fully capable of expressing SAR upon treatment with SA (Figure 2). In response to SA, *eds5-1* also accumulated wild-type levels of *PR-1* transcripts (Figure 3). This intact responsiveness of *eds5-1* to SA is consistent with its inability to synthesize this signaling molecule in response to pathogen infection (Nawrath and Métraux, 1999). In contrast, *eds12-1* plants, like *npr1-1*, failed to develop resistance in response to SA treatment (Figure 2). Furthermore, SA-induced *PR-1* gene expression was significantly reduced in *eds12-1* plants, whereas it was nearly abolished in *npr1-1* plants (Figure 3). Thus, mutant *eds12-1* has reduced sensitivity to SA, suggesting that EDS12 functions downstream of SA in the SAR pathway. Treatment of *eds5-1* and *eds12-1* with either 100 μM MeJA or 0.5 mM ACC resulted in normal levels of expression of the JA- and ethylene-inducible, defense-related gene *Pdf1.2* (data not shown), indicating that they are not affected in JA or ethylene signaling.

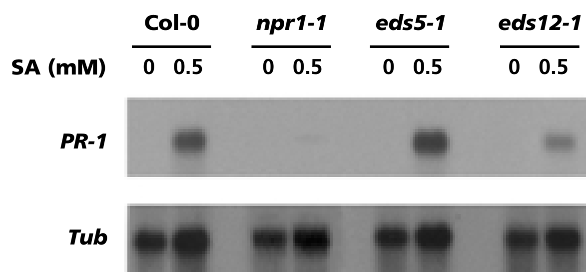

Figure 3: RNA blot analysis of the SA-inducible *PR-1* gene in wild-type Col-0 plants and in the SAR-impaired mutants *npr1-1*, *eds5-1* and *eds12-1*. Five-week-old plants were induced by dipping the leaves in a 0.015 % (v/v) Silwet L-77 solution containing 0.5 mM SA, 3 days before harvesting the leaves. Control-treated plants were dipped in a solution containing 0.015 % (v/v) Silwet L-77 only. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β-tubulin (*Tub*). The experiment was performed twice, yielding similar results.

***Pdf1.2* gene expression in ISR-impaired *eds* mutants.**

To further investigate the JA and ethylene responsiveness of the ISR-impaired mutants *eds4-1*, *eds8-1* and *eds10-1*, we examined the expression pattern of *Pdf1.2* after exogenous application of MeJA or ACC. Dipping the leaves of wild-type Col-0 plants in a solution containing either 50 μM MeJA or 0.5 mM ACC strongly activated *Pdf1.2* transcription (Figure 4). Mutant *eds4-1* showed strongly reduced levels of *Pdf1.2* transcript accumulation after treatment with either MeJA or ACC (Figure 4), consistent with its impaired expression of MeJA- and ACC-induced resistance against *Pst* (Figure 2). In mutant *eds8-1*, the level of *Pdf1.2* transcripts was also strongly reduced upon treatment with MeJA or ACC. However, *eds8-1* was unaffected in ACC-induced resistance (Figure 2). Since *Pdf1.2* gene expression is synergistically induced by JA and ethylene (Penninckx et al., 1998), the responsiveness of *eds8-1* to ACC with regard to the induction of resistance suggests that *eds8-1* is affected in JA signaling. Mutant *eds10-1* showed normal levels of *Pdf1.2* expression after treatment with either MeJA or ACC (Figure 4). Thus, the inability of *eds10-1* to develop MeJA- and ACC-induced resistance (Figure 2) cannot be explained by impaired sensitivity to JA or ethylene. Therefore, this mutant seems to be affected in a different trait. Since neither MeJA nor ACC induced resistance in *eds10-1*, this trait is likely to act downstream of JA and ethylene signaling in the ISR pathway. When treated with 1 mM SA, *eds4-1*, *eds8-1*, and *eds10-1* did not exhibit a reduced accumulation of *PR-1* transcripts compared to wild-

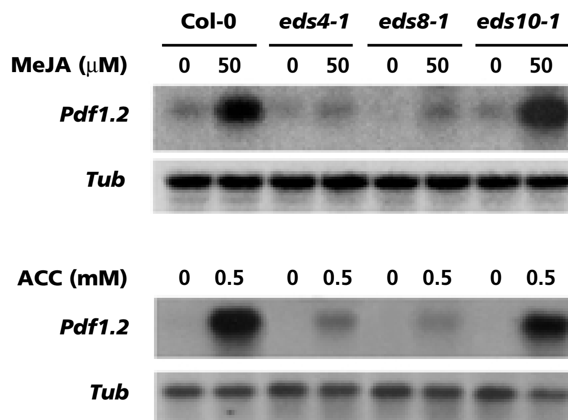

Figure 4: RNA blot analysis of the JA/ethylene-inducible *Pdf1.2* gene in wild-type Col-0 plants and in the ISR-impaired mutants *eds4-1*, *eds8-1* and *eds10-1*. Five-week-old plants were induced by dipping the leaves in a 0.015% (v/v) Silwet L-77 solution containing either 50 μM MeJA or 0.5 mM ACC, 3 days before harvest of the leaves. Control-treated plants were dipped in a solution containing 0.015 % (v/v) Silwet L-77 only. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β-tubulin (*Tub*). The experiment was performed twice, yielding similar results.

type Col-0 plants (data not shown), indicating that they are not impaired in SA responsiveness.

Mutant *eds4-1* is impaired in ethylene signaling

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). To investigate whether the ISR non-inducibility and impaired *Pdf1.2* gene expression of *eds4-1* and *eds8-1* are caused by a reduced sensitivity to ethylene, we examined ethylene-induced growth inhibition of the roots and hypocotyls, which are both characteristics of the triple response. In a comparative analysis, etiolated seedlings of Col-0, *eds4-1*, *eds8-1*, *eds10-1*, as well as the ethylene response mutant *etr1-1*, were grown on MS-agar plates containing different concentrations of ACC. At 5 days after germination, the lengths of the hypocotyls and roots were measured. At increasing concentrations of ACC, wild-type Col-0 and mutant *eds8-1*

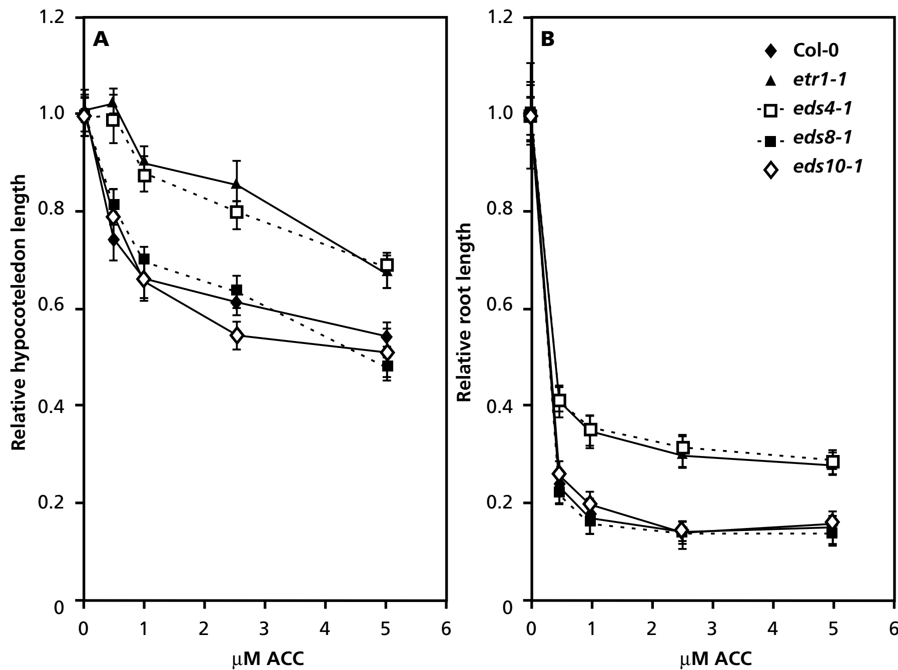

Figure 5: ACC-induced inhibition of growth of hypocotyl (**A**) and primary root (**B**) in wild-type Col-0, *etr1-1*, and the ISR-impaired *eds* mutants of Arabidopsis. Seeds were pregerminated for 2 days at 4 °C in darkness on MS-agar plates containing increasing concentrations of ACC. After an additional growth period of 5 days in darkness at 20 °C, the lengths of the etiolated seedlings were measured. Data presented are means (± SEM; n = 25) normalized with respect to the mean control value. The experiment was repeated several times, yielding similar results.

and *eds10-1* plants responded similarly to the ACC in a dose-dependent manner, resulting in a 45 – 50% reduction of hypocotyl length at 5 μM ACC. By contrast, mutant *eds4-1* behaved as *etr1-1* in failing to respond to 0.5 μM ACC, and showing significantly less responsiveness at increasing concentrations of ACC with no more than 25% reduction of hypocotyl length at 5 μM ACC. Similarly, the inhibition of root growth at increasing concentrations of ACC was significantly less extreme in *etr1-1* and *eds4-1* plants than that in Col-0, *eds8-1* and *eds10-1* plants (Figure 5B). These findings indicate that the recessive *eds4-1* mutant is as less responsive to ethylene as the dominant *etr1-1* mutant.

Mutant *eds8-1* is impaired in JA signaling

Previously, it was demonstrated that primary root growth of Arabidopsis seedlings is inhibited in response to treatment with MeJA (Staswick et al., 1992). To investigate whether the ISR non-inducibility and impaired *Pdf1.2* gene expression of *eds4-1* and *eds8-1* result from a reduced sensitivity to JA, we examined the level of MeJA-induced growth inhibition of the primary roots. Seedlings of Col-0, *eds4-1*, *eds8-1*, *eds10-1*, and the JA-response mutant *jar1-1* were grown on MS-agar plates containing different concentrations of MeJA. At five days after germination, Col-0, *eds4-1*, and *eds10-1* plants showed significant inhibition of root growth already at 0.1 μM MeJA (Figure 6A). In contrast, mutant *jar1-1* exhibited no significant root growth inhibition at 0.1 and 0.5 μM MeJA, and only a weak inhibition at 1.0 μM MeJA (Figure 6A). Mutant *eds8-1* exhibited an intermediate phenotype: at the relatively low concentration of 0.1 μM MeJA it resembled *jar1-1* in not showing a statistically significant response in root growth. However, at concentrations of 0.5 and 1.0 MeJA, *eds8-1* responded intermediately (Figure 6A), indicating that the roots of *eds8-1* are weakly insensitive to JA.

Application of MeJA has been reported to induce accumulation of anthocyanins in the leaves (Feys et al., 1994). Because this response was severely impaired in the JA-insensitive mutant *coi1-1*, we further quantified the extent of JA insensitivity in *eds8-1* by determining the level of anthocyanin accumulation in response to MeJA. At three days after treatment of the leaves with increasing concentrations of MeJA, wild-type Col-0 plants had accumulated anthocyanins in a dose-dependent manner, resulting in a 3-fold increase at 500 μM MeJA compared to water-treated control plants (Figure 6B). In contrast, mutant *eds8-1*, similar to *jar1-1*, failed to enhance anthocyanin accumulation at concentrations lower than 250 μM MeJA, and showed only weakly increased anthocyanin accumulation at concentrations of 250 and 500 μM MeJA (Figure 6B). These results confirm that *eds8-1* exhibits reduced sensitivity to JA.

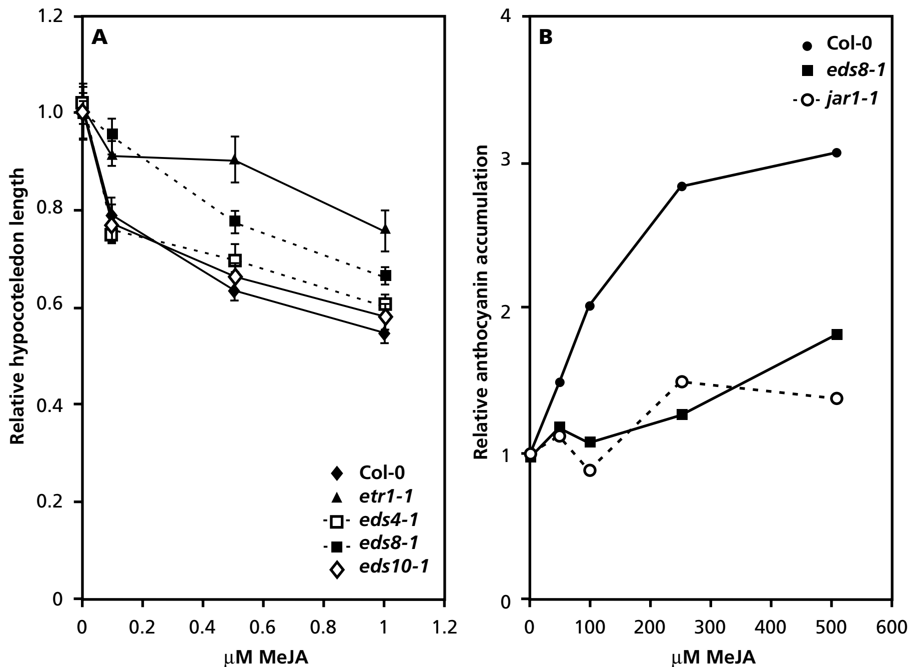

Figure 6: MeJA-dependent inhibition of primary root length (**A**) and accumulation of anthocyanins in the leaves (**B**). **(A)** Seeds of Col-0, *eds4-1*, *eds8-1*, *eds10-1* and *jar1-1* were pregerminated on MS-agar plates containing different concentrations of MeJA for 2 days at 4 °C in the dark. After an additional growth period of 5 days at 20 °C with an 8-h photoperiod, the length of the primary root was measured. Data presented are means (± SEM; n = 25), normalized with respect to the mean control value.

(B) Leaves of five-week-old Col-0, *eds8-1* and *jar1-1* were dipped in a solution of 0.015% (v/v) Silwet L-77 containing different concentrations of MeJA, 3 days before harvesting of the leaves. Data presented are the absorbencies (A_{615}) of the anthocyanin containing extracts, normalized with respect to the control value.

The *ISR1* locus is not allelic with *Eds4*

The phenotype of *eds4-1* resembles the phenotype of *isr1* genotypes in the inability to express WCS417r-mediated ISR, enhanced susceptibility to *Pst*, and reduced sensitivity to ethylene (Chapters 2 and 3; Ton et al., 1999; Ton et al., 2001). To investigate whether the *Eds4* gene is allelic with the *ISR1* locus, we performed a complementation cross between ecotype Ws-0 (*isr1*) and the *eds4-1* mutant. The F_1 progeny of this cross was fully capable of expressing WCS417r-mediated ISR (Figure 7A) and exhibited a similar level of basal resistance against *Pst* DC3000 as Col-0 wild-type plants, as indicated by similar symptom severity (data not shown) and similar rates of proliferation of the pathogen (Figure 7B). It can thus be concluded that the *ISR1* locus is not allelic with the *Eds4* gene.

Discussion

Previously, we demonstrated that Arabidopsis genotypes that are unable to express *P. fluorescens* WCS417r-mediated ISR against *Pst*, show an enhanced disease susceptibility towards this pathogen (Pieterse et al., 1998, Ton et al., 1999). Independently, two mutant screens for enhanced disease susceptibility to *P. syringae* pathogens yielded a large number of mutants with deficiencies in basal resistance against these pathogens (Glazebrook et al., 1996; Volko et al., 1998). To identify novel components of the ISR pathway, we made use of 11 of these *eds* mutants. Our results demonstrate that *eds4-1*, *eds8-1*, and *eds10-1* are impaired in *P. fluorescens* WCS417r-mediated ISR against *Pst* (Figure 1A), whereas *eds5-1* and *eds12-1* are affected in pathogen-induced SAR against this pathogen (Figure 1B). The ISR-impaired mutants showed normal levels of pathogen-induced SAR. Conversely, the SAR-impaired mutants showed normal levels of rhizobacteria-mediated ISR. These results corroborate our earlier demonstration that pathogen-induced SAR and WCS417r-mediated ISR are regulated by distinct defense pathways that are both effective against *Pst*.

More detailed analysis of the ISR-impaired *eds* mutants revealed that they are blocked in their ability to develop induced resistance against *Pst* in response to exogenous application of MeJA (*eds4-1*, *eds8-1*, and *eds10-1*) or ACC (*eds4-1* and *eds8-1*) (Figure 2). Moreover, *eds4-1* showed reduced sensitivity to ethylene (Figures 4 and 5), whereas mutant

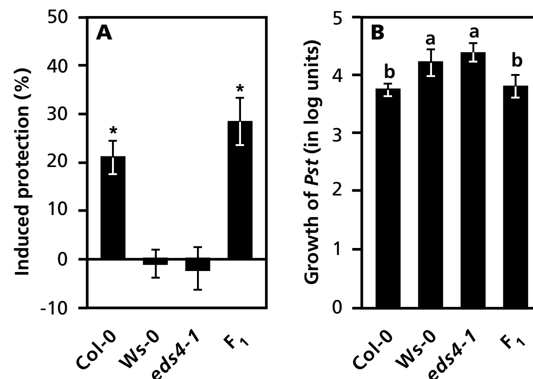

Figure 7: Levels of resistance against *P. syringae* pv. *tomato* DC3000 (*Pst*) as a result of *P. fluorescens* WCS417r-mediated ISR (**A**) and basal resistance (**B**) in Col-0, Ws-0, *eds4-1*, and F_1 plants of a cross between Ws-0 and *eds4-1*.
(A) For induction of ISR, challenge inoculation, and disease assessment, see caption to Figure 1A.
(B) Plants were infected by pressure-infiltrating a suspension of virulent *Pst* at 5 x 10^5 CFU.mL^{-1} into the leaves. Immediately afterwards and 3 days later, the number of *Pst* bacteria per gram fresh weight was determined and the proliferation over a 3-day time interval calculated. Values presented are means (± SD) of the log of the proliferation of *Pst*. Different letters indicate statistically significant different values between genotypes (LSD test; α = 0.05; n = 6).

eds8-1 showed reduced sensitivity to JA (Figures 4 and 6). Mutant *eds10-1* showed normal sensitivity to both JA and ethylene (Figures 2, 5 and 6A). These results indicate that the corresponding gene products act in either the JA response (EDS8), the ethylene response (EDS4), or downstream of the JA and ethylene response (EDS10) in the ISR signaling pathway. Figure 8 provides a model outlining the positions of the *eds* mutations in the ISR and the SAR signaling pathway.

Genotypes carrying the recessive alleles of the *ISR1* locus are non-responsive to ISR treatment, show enhanced susceptibility to *Pst* infection, and exhibit reduced sensitivity to ethylene (Chapters 2 and 3; Ton et al., 1999; Ton et al., 2001). These characteristics resemble the phenotype of *eds4-1*. However, F_1 plants of a cross between ecotype Ws-0 (*isr1/isr1*) and *eds4-1* showed full complementation of ISR inducibility and basal resistance against *Pst* (Figure 7), indicating that *ISR1* and *Eds4* are not allelic. Surprisingly, Gupta et al. (2000) reported that *eds4-1* failed to develop SAR upon induction treatment with avirulent *P. syringae* pv. *maculicola* carrying *avrRpt2*. Concurrently, *eds4-1* exhibited reduced *PR-1* gene expression after SA treatment, whereas it showed enhanced levels of *Pdf1.2* gene expression upon treatment with MeJA. These findings clearly differ from our results. However, Gupta et al. (2000) also reported that at low humidity the SA responsiveness in *eds4-1* was restored to wild-type levels. Therefore, the behavior of *eds4-1* plants appears to be strongly influenced by the environmental conditions. It may be significant that we cultivated *eds4-1* at a 9-h light and 15-h dark cycle at 65% relative humidity, whereas Gupta et al. (2000) used a 12/12-h light/dark cycle at 85% relative humidity. Depending on the growth conditions, *eds4-1* may act as a mutant affected in SA-dependent defenses (Gupta et al., 2000), or as a mutant affected in ethylene-dependent defenses (this study). Therefore, it may be postulated that EDS4 is involved in the control of the balance between SA-dependent and JA/ethylene-dependent defense pathways, as influenced by the prevailing environmental conditions (Figure 8).

Our results indicate that mutant *eds8-1* is impaired in the response to JA (Figures 2, 4 and 6). So far, two well-characterized JA-insensitive mutants have been described in Arabidopsis: the jasmonic acid-insensitive mutant *jar1-1* (Staswick et al., 1992), and the coronatin-insensitive mutant *coi1-1* (Feys et al., 1994). Based on the JA-insensitive phenotype of *eds8-1*, this mutant might be allelic with *jar1-1*. However, both mutants differed in other characteristics. Firstly, *eds8-1* showed an intermediate phenotype with regard to MeJA-induced inhibition of root growth (Figure 6A). Secondly, *jar1-1* and *eds8-1* showed striking differences in leaf morphology. Mutant *eds8-1* developed serrated leaves with relatively short petioles (Glazebrook et al., 1996; data not shown), whereas the leaf morphology of *jar1-1* resembles that of wild-type plants. Therefore, it seems unlikely that both mutants are allelic. Furthermore, it is highly unlikely that *eds8-1* is allelic with the JA-insensitive mutant *coi1-1*, since the latter mutant, unlike *eds8-1*, is male-sterile (Feys et al., 1994). Moreover, the *coi1-1* mutant exhibited enhanced resistance, rather than enhanced susceptibility to *P. syringae* pv.

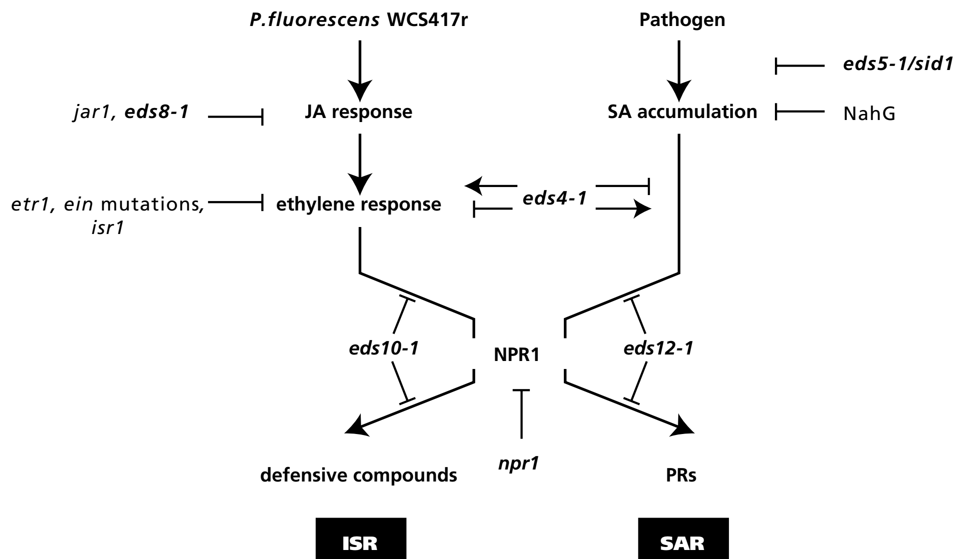

Figure 8: Putative positions of the ISR- and SAR-impaired *eds* mutants in the ISR and the SAR signaling pathway (adapted from Pieterse et al., 1998).

atropurpurea, probably as a result of its insensitivity to coronatin that functions as a virulence factor of *P. syringae* pathovars (Feys et al., 1994). Hence, we postulate that *Eds8* encodes a novel component in the JA-response pathway that plays an important role in disease resistance.

The only ISR-impaired *eds* mutant that was not affected in sensitivity to either JA or ethylene was *eds10-1*. However, it failed to develop induced resistance upon treatment with MeJA or ACC, indicating that *eds10-1*, like the defense regulatory mutant *npr1-1* (Pieterse et al., 1998), is blocked downstream of the ethylene response in the ISR pathway (Figure 8). Nevertheless, NPR1 and EDS10 clearly differ, because mutant *eds10-1* was fully capable of expressing pathogen-induced SAR (Figure 1B), whereas *npr1-1* is not (Cao et al., 1994; Figure 2). In this respect, it is tempting to speculate that EDS10 plays a role in the regulation of ISR-specific, NPR1-dependent defense responses.

Further characterization of the SAR-impaired mutants revealed that *eds12-1* is affected in SA-induced resistance and *PR-1* gene expression, whereas *eds5-1* showed full responsiveness to SA. For *eds5-1*, this confirms previous work by Nawrath and Métraux (1999), who showed that EDS5/SID1 regulates the accumulation of SA upon pathogen infection, and thus participates in the SAR signaling pathway upstream of SA (Figure 8). The reduced responsiveness of *eds12-1* to SA indicates that EDS12, like the defense regulator

NPR1, functions downstream of SA in the SAR signaling pathway (Figure 8). However, it is unlikely that *Eds12* is allelic with *Npr1*, because mutants *eds12-1* and *npr1-4* showed full complementation of basal resistance in their F_1 progeny (Volko et al., 1998). Moreover, mutant *npr1-1* is affected in the expression of WCS417r-mediated ISR (Pieterse et al., 1998), whereas mutant *eds12-1* is not (Figure 1A). Therefore, one may envisage that EDS12 plays a role in the regulation of SAR-specific, NPR1-dependent defense responses.

The Arabidopsis genotypes that are impaired in either ISR or SAR all exhibited enhanced susceptibility to *P. syringae* pathovars (Glazebrook et al., 1996; Volko et al., 1998). This association supports the earlier notion that induced resistance is an enhancement of extant basal defense mechanisms (Van Loon, 1997). However, six mutants with enhanced disease susceptibility to *P. syringae* were unaffected in their expression of SAR and ISR against *Pst* (Figure 1), indicating that they are affected in basal defense components that do not contribute to induced resistance. In view of the low frequency of allelic pairs in the *eds* mutant screens (Glazebrook et al., 1996; Volko et al., 1998), basal resistance must be controlled by a large number of genes regulating multiple defense mechanisms. Apparently, only subsets of these mechanisms are enhanced in plants expressing induced resistance, depending on the type of induced resistance that is activated.

Materials and Methods

Cultivation of rhizobacteria, pathogens and plants

Nonpathogenic, ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria (Pieterse et al., 1996) were grown on King's medium B (KB) agar plates (King et al., 1954) for 24 hours at 28 °C. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 colony-forming units (CFU) per mL. An avirulent strain of *Pseudomonas syringae* p*v. tomato* DC3000 carrying the avirulence gene *avrRpt2* (*Pst*(*avrRpt2*); Kunkel et al., 1993) was used for induction of SAR. *Pst*(*avrRpt2*) bacteria were grown overnight at 28 °C in liquid KB supplemented with 25 $mg.mL^{-1}$ kanamycin to select for the plasmid. The virulent strain of *P. syringae* p*v. tomato* DC3000 (*Pst)* (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 $MgSO_4$ with 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands) to a final density of 2.5 x 10^7 $CFU.mL^{-1}$.

Seedlings of wild-type *Arabidopsis thaliana* ecotypes Columbia (Col-0), Wassilewskija (Ws-0), F1 plants of the Ws-0 x *eds4-1* cross, and the Col-0 mutants *etr1-1* (Bleecker et al., 1988), *jar1-1* (Staswick et al., 1992), *eds3-1*, *eds4-1*, *eds5-1*, *eds6-1*, *eds7-1*, *eds8-1* (Glazebrook et al., 1996), *eds9-1* (Rogers et al., 1997), *eds10-1*, *eds11-1*, *eds12-1* and *eds13-1* (Volko et al., 1998) were grown in quartz sand for two weeks, and transferred to a sand/potting soil mixture as described previously (Pieterse et al., 1996). Plants were

cultivated in a growth chamber with a 9-hour day (≈200 $\mu E.m^{-2}.sec^{-1}$ at 24 °C) and 15-h night (20 °C) cycle at 65% relative humidity. For the duration of the experiments, all genotypes remained vegetative and developed at least 10-15 mature leaves. Plants were watered on alternate days, and once a week received modified half-strength Hoagland solution (Hoagland and Arnon, 1938; Pieterse et al., 1996).

Induction treatments

ISR was induced by transplanting 2-week-old Arabidopsis seedlings into the sand/potting soil mixture containing ISR-inducing WCS417r bacteria. Prior to transfer of the Arabidopsis seedlings to the pots, a suspension of ISR-inducing WCS417r bacteria (10^9 $CFU.mL^{-1}$) was mixed thoroughly through the soil to a final density of 5 x 10^7 $CFU.g^{-1}$. Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. Induction of SAR was performed 3 days before challenge inoculation by pressure-infiltrating 2-3 lower leaves with a suspension of *Pst*(*avrRpt2*) bacteria at 10^7 $CFU.mL^{-1}$. Induction treatments with salicylic acid (SA), methyl jasmonate (MeJA) and 1-aminocyclopropane-1-carboxylate (ACC) were performed three days before challenge inoculation by dipping the leaves in a solution containing either SA, MeJA, or ACC in 0.015% (v/v) Silwet L77. Control-treated plants were dipped in a solution containing 0.015% (v/v) Silwet L77 only.

Challenge inoculations and disease assessment

For assaying induced resistance, biologically and chemically induced plants were challenged when 5 weeks old by dipping the leaves in a suspension of virulent *Pst* bacteria at 2.5 x 10^7 $CFU.mL^{-1}$ 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. Three or 4 days after challenge inoculation, the percentage of leaves with symptoms was determined per plant (n = 20-25). Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased (Pieterse et al., 1996).

For assaying basal resistance against virulent *Pst*, leaves of 5-week-old, control-treated plants were inoculated by pressure-infiltration with a suspension of *Pst* at 5 x 10^5 $CFU.mL^{-1}$ in 10 mM $MgSO_4$. Immediately thereafter and 3 days later, replicate leaf samples from six plants per genotype were collected, weighed, and homogenized in 10 mM $MgSO_4$. Serial dilutions were plated on selective KB-agar plates supplemented with 100 $mg.L^{-1}$ cycloheximide and 50 $mg.L^{-1}$ rifampicin. After incubation at 28 °C for 2 days, the number of rifampicin-resistant CFU per g of infected leaf tissue was determined, and bacterial proliferation over the 3-day time interval was calculated.

Analysis of MeJA and ACC sensitivity

Seeds of Arabidopsis were surface-sterilized for 5 min in 5% sodium hypochlorite, washed in 70% ethanol, and air-dried. Subsequently, the seeds were distributed evenly on 1.0% (w/v) agar medium containing 0.5% (w/v) Murashige and Skoog salts (Duchefa BV, Haarlem, The Netherlands), 0.5% (w/v) sucrose, and different concentrations of either MeJA or ACC (pH 5.7). MeJA (Serva, Brunschwig Chemie BV, Amsterdam, The Netherlands) was added to the autoclaved medium from a

filter-sterilized 1 mM stock solution (containing 0.96% ethanol). ACC (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was added from a 10 mM stock solution in a similar manner.

Seeds were pre-germinated in the dark for 2 days at 4 °C. The effect of MeJA on primary root growth was determined essentially as described by Staswick et al. (1992). Plates were incubated in a climate chamber at 22 °C with an 8-h day (≈200 $\mu E.m^{-2}.s^{-1}$) and a 16-h night cycle. After 5 days, the primary root length was measured under a dissection microscope. In each case, 15-25 randomly selected seedlings were measured. The effect of ethylene on hypocotyl and primary root length in etiolated seedlings was determined essentially according to Guzmán and Ecker (1990). After pre-germination in the dark for 2 days at 4 °C, the seedlings were grown for an additional 5 days at 20 °C in darkness and the hypocotyl and primary root length were measured as described above.

Ethylene measurements

Leaves of plants pretreated with 1 mM ACC were detached, weighed, placed in 25-mL gas-tight serum flasks, and incubated under climate chamber conditions. At different intervals over a 28-h period, cumulative ethylene production was measured by gas chromatography as described by De Laat and Van Loon (1982).

Extraction and quantification of anthocyanins

Three days after treatment with MeJA, leaf material was collected and homogenized in extraction buffer (0.35 M glycine, 48 mM NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 $mL.g^{-1}$ of leaf tissue). After centrifugation for 2 min at 16.000 *g* to pellet non-soluble cell fragments, the supernatant was extracted twice with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1; v/v), and once with chloroform/isoamylalcohol (24:1; v/v). Subsequently, the relative amount of anthocyanins in the water phase (visible as a purple color) was assayed spectrophotometrically by determining the absorption spectrum between A_{400} and A_{800}. All samples containing anthocyanins showed an absorption maximum at A_{615}.

RNA gel blot analysis

Three days after induction treatments, total RNA was extracted by homogenizing frozen leaf tissue, as described previously (Ton et al., 2001). For RNA gel blot analysis, 15 µg of RNA was denatured using glyoxal and DMSO (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. The electrophoresis and blotting buffer consisted of 10 mM and 25 mM sodium phosphate (pH 7.0), respectively. RNA gel blots were hybridized with specific probes and washed, as described previously (Pieterse et al., 1994). To check for equal loading, the blots were stripped and hybridized with a probe for constitutively expressed β-tubulin (*Tub*) gene. DNA probes were labeled with α-^{32}P-dCTP by random primer labeling (Feinberg and Vogelstein, 1983). The probes to detect *Pdf1.2* and *PR-1* transcripts were derived from an

Arabidopsis *Pdf1.2* and a *PR-1* cDNA clone, respectively (Penninckx et al., 1996, Uknes et al., 1992). The probe for detection of *Tub* transcripts was prepared by PCR with primers based on the sequence obtained from Genbank accession number M21415.

CHAPTER 5

Differential effectiveness of salicylate-dependent, and jasmonate- and ethylene-dependent induced resistance in Arabidopsis

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Submitted

Abstract

The plant signal molecules salicylic acid (SA), jasmonic acid (JA), and ethylene are each involved in the regulation of basal resistance responses against different pathogens. These three signals play important roles in induced resistance as well: SA is a key regulator of pathogen-induced systemic acquired resistance (SAR), whereas JA and ethylene have been implicated in rhizobacteria-mediated induced systemic resistance (ISR) signaling. In this study, we compared the effectiveness of SAR and ISR against different Arabidopsis pathogens that, in non-induced plants, are primarily resisted through either SA-dependent defenses, e.g. *Peronospora parasitica* and turnip crinkle virus (TCV), JA/ethylene-dependent defenses, e.g. *Alternaria brassicicola*, or a combination of SA-, JA-, and ethylene defenses, e.g. *Xanthomonas campestris* pv. *armoraciae*. Induction of SAR and ISR was equally effective against *X. campestris* pv. *armoraciae*. Activation of ISR resulted in a significant level of protection against *A. brassicicola*, whereas SAR was ineffective against this pathogen. Conversely, activation of SAR resulted in a high level of protection against *P. parasitica* and TCV, whereas ISR conferred only weak and no protection against *P. parasitica* and TCV, respectively. These results indicate that SAR is effective against pathogens that in non-induced plants are resisted through SA-dependent basal resistance responses, whereas ISR is effective against pathogens that in non-induced plants are resisted through JA/ethylene-dependent basal resistance responses.

Introduction

Plants require a broad range of defense mechanisms in order to effectively combat invasions by micro-organisms. These mechanisms include preexisting physical and chemical barriers, as well as inducible defense responses that become activated after pathogen infection, such as synthesis of phytoalexins, enhanced strengthening of cell walls, and the production of antifungal proteins (Jackson and Taylor, 1996). The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene play key roles in the regulation of defense responses, because plant genotypes that are affected in their response to either of these signals are more susceptible to infection by certain virulent pathogens. Such enhanced susceptibility points to the presence of a certain level of basal resistance in wild-type plants that restrains colonization of these pathogens.

Evidence for the role of SA in basal resistance came from the analysis of transgenic plants expressing the bacterial salicylate hydroxylase (*NahG*) gene. Salicylate hydroxylase inactivates SA by converting it to catechol (Gaffney et al., 1993). NahG tobacco plants were found to be more susceptible to tobacco mosaic virus, the bacterium *Pseudomonas syringae* pv. *tabaci*, the oomycete *Phytophthora parasitica* and the fungus *Cercospora nicotianae* (Delaney et al., 1994). Reducing the biosynthesis of, or sensitivity to either JA or ethylene can also render plants more susceptible to pathogens and even insects. For instance, ethylene-insensitive tobacco plants transformed with the mutant *etr1-1* gene from Arabidopsis lost their ability to resist the soil-borne oomycete *Pythium sylvaticum* (Knoester et al., 1998). Similarly, Arabidopsis mutants affected in JA biosynthesis or signaling are more susceptible to *Pythium mastophorum* (Vijayan et al., 1998), *Pythium irregulare* (Staswick et al., 1998), as well as insect herbivory (McConn et al., 1997; Stout et al., 1999).

In Arabidopsis, SA, JA and ethylene are involved to different extents in basal resistance against specific pathogens. Basal resistance against the oomycetous pathogen *Peronospora parasitica* and to turnip crinkle virus (TCV) seems to be controlled predominantly by a SA-dependent pathway. Only SA-nonaccumulating *NahG* plants exhibited enhanced disease susceptibility to these pathogens (Delaney et al., 1994; Kachroo et al., 2000), whereas mutants affected in JA or ethylene signaling did not (Thomma et al., 1998; Kachroo et al., 2000). In contrast, basal resistance against the fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* was reduced only in JA- and ethylene-insensitive mutants, and not in NahG plants (Thomma et al., 1998; 1999a). Interestingly, basal resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was found to be affected in both *NahG* plants and in JA- and ethylene-response mutants (Pieterse et al., 1998), suggesting that basal resistance against this pathogen is controlled by a combined action of SA, JA and ethylene.

Besides basal resistance responses at the site of pathogen infection, plants are capable of developing a systemically induced resistance that is effective against further pathogen

infections. This induced disease resistance is generally manifest as a reduction of disease and a restriction of colonization of the challenging pathogen in comparison to non-stimulated control plants (Hammerschmidt, 1999). The classical way of inducing resistance is by predisposal infection with a necrotizing pathogen, which gives rise to an enhanced defensive capacity in distal plant parts. This type of systemically induced resistance is generally referred to as systemic acquired resistance (SAR; Ryals et al., 1996). The signaling pathway of pathogen-induced SAR depends on endogenous accumulation of SA, because transgenic NahG plants have lost their ability to express SAR (Gaffney et al., 1993). The expression of SAR, triggered by either pathogen infection or treatment with SA or its functional analogues 2,6-dichloroisonicotinic acid (INA) or benzothiodiazole (BTH), is tightly associated with the transcriptional activation of genes encoding pathogenesis-related proteins (PRs; Van Loon, 1997). Therefore, PRs are generally taken as markers for the induced state of SAR.

Another form of induced disease resistance is triggered by selected strains of non-pathogenic rhizobacteria. To facilitate distinguishing this type of induced resistance from pathogen-induced SAR, the term rhizobacteria-mediated induced systemic resistance (ISR) was introduced (Pieterse et al., 1996; Van Loon et al., 1998). In Arabidopsis, ISR triggered by the root-colonizing bacterial strain *Pseudomonas fluorescens* WCS417r was shown to be effective against the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani*, and the bacterial leaf pathogen *P. syringae* pv. *tomato* DC3000 (Pieterse et al., 1996; Van Wees et al., 1997). The signaling pathway controlling WCS417r-mediated ISR clearly differs from the SAR pathway, in that it is independent of SA but requires full responsiveness to JA and ethylene (Pieterse et al., 1996; 1998). Furthermore, the expression of WCS417r-mediated ISR is not accompanied by transcriptional activation of genes encoding PRs or other known defense-related genes (Pieterse et al., 1996; Van Wees et al., 1999). Interestingly, simultaneous activation of both the JA/ethylene-dependent ISR pathway and the SA-dependent SAR pathway results in an enhanced level of protection against *P. syringae* pv. *tomato* DC3000, demonstrating that the defense responses activated via both pathways are compatible and additive (Van Wees et al., 2000).

The differential effectiveness of SA-dependent basal resistance, on the one hand, and JA- and ethylene-dependent basal resistance, on the other hand, prompted us to reexamine the range of pathogens that are resisted by WCS417r-mediated ISR and SAR, respectively. To this end, we examined the effectiveness of WCS417r-mediated ISR and SAR against *P. parasitica* and TCV (primarily resisted through SA-dependent basal resistance; Delaney et al., 1994; Kachroo et al., 2000), and *A. brassicicola* (primarily resisted through JA-dependent basal resistance; Thomma et al., 1998). In addition, we analyzed the effectiveness WCS417r-mediated ISR and SAR against the bacterial leaf pathogen *X. campestris* pv. *armoraciae* that was resisted through a combination of SA-, JA-, and ethylene-dependent defenses in non-induced plants.

Results

Differential effectiveness of ISR and SAR against *Peronospora parasitica*

To compare the effectiveness of SA-dependent SAR and JA/ethylene-dependent ISR against the oomycetous leaf pathogen *P. parasitica*, the level of protection against race WACO9 was assessed in SAR- and ISR-expressing Arabidopsis ecotype Columbia (Col-0) plants. ISR was triggered by growing plants in soil containing ISR-inducing *P. fluorescens* WCS417r bacteria, whereas SAR was induced by spraying the leaves with INA. On leaves of control-treated plants, the first conidiophores started to appear 6 to 7 days after challenge inoculation. Figure 1 shows that by 11 days, 65% of the inoculated leaves showed sporulation.

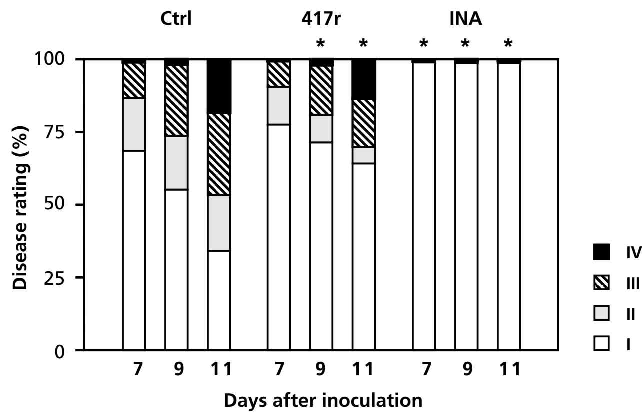

Figure 1. Quantification of ISR and SAR in Arabidopsis Col-0 plants against *Peronospora parasitica* WACO9. ISR was induced by growing plants in soil containing *Pseudomonas fluorescens* WCS417r bacteria at 5 x 10^7 CFU.g^{-1}. SAR was induced by spraying plants with a solution containing 0.1 mM INA, 3 days before challenge inoculation with *P. parasitica*. Plants (n = 40) were challenge inoculated when 3 weeks old by applying 3-µL droplets of 10 mM $MgSO_4$, containing 5 x 10^4 conidiospores per mL. At 7, 9 and 11 days after challenge, disease severity was determined. Disease rating is expressed as the percentages of leaves falling in classes I, no sporulation; II, <50 % of the leaf area covered by sporangia; III, >50 % of the leaf area covered by sporangia; IV, heavily covered with sporangia, with additional chlorosis and leaf collapse. Asterisks indicate statistically significant different frequency distributions of the disease-severity classes compared to the non-induced control treatments (Chi-square; n = 200; $\alpha < 0.05$). The data presented are from a representative experiment that was repeated with similar results.

In WCS417r-treated plants, disease development was suppressed, leading to a statistically significant reduction in the number of leaves with sporulation at 9 and 11 days after challenge. Furthermore, the number of conidiospores produced per gram of infected tissue was reduced 7-fold on the ISR-expressing leaves compared to the control plants (Table 1). As shown in Figures 2A and 2B, staining of the infected leaves with lactophenol trypan-blue revealed that the ISR-expressing leaves allowed considerably less colonization by the pathogen than leaves

Table 1. Production of conidiospores by *Peronospora parasitica* WACO9 on ISR- and SAR-expressing Arabidopsis leaves.

Treatmenta	No. of conidiospores per gram FW[b]
Control	10.8 x 10^5
ISR	1.5 x 10^5
SAR	0.8 x 10^5

[a] ISR was induced by transferring 2-week-old Col-0 seedlings to potting soil containing WCS417r bacteria at 5 x 10^7 CFU.g^{-1}. SAR was induced by spraying the plants with a solution containing 0.1 mM INA 3 days before challenge inoculation.

[b] Three-week-old plants were inoculated by applying 3-μL droplets of 10 mM M $MgSO_4$ containing 5 x 10^4 conidiospores per mL. Conidiospores were collected from entire plants at 11 days after challenge.

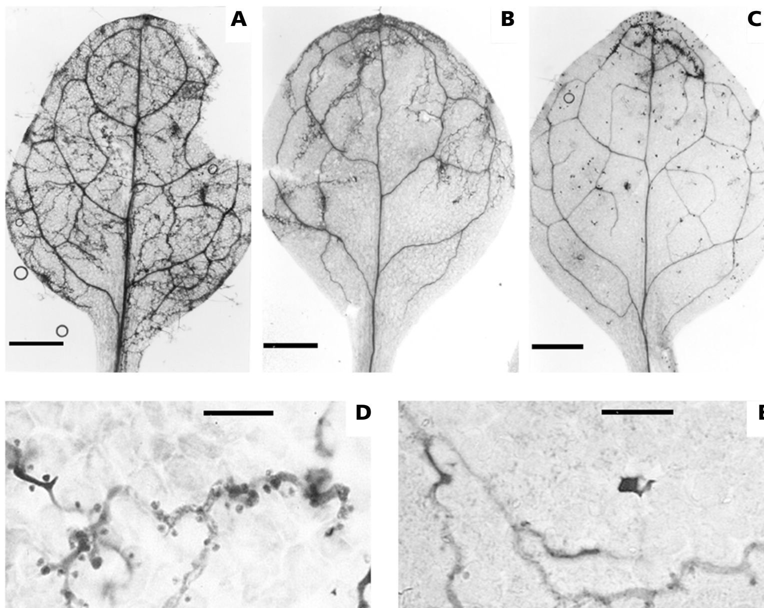

Figure 2. Tissue colonization of *Peronospora parasitica* WACO9 in ISR- and SAR-expressing leaves of Arabidopsis Col-0 plants. Leaves of water-treated control-plants **(A)**, *P. fluorescens* WCS417r-treated plants **(B)**, and INA-treated plants **(C)** were stained with lactophenol trypan-blue at 9 days after challenge inoculation with *P. parasitica* (scale bar = 0.5 mm). Fewer haustoria were present in ISR-expressing plants **(D)** than in control-treated plants **(E)** (scale bar = 50 μm).

of non-induced plants. Interestingly, the pathogen hyphae in ISR-expressing leaves had developed less haustoria compared to the hyphae in leaves of the control plants (Figure 2D and 2E).

Consistent with previous findings (Uknes et al., 1992), treatment with INA induced a high level of SAR against *P. parasitica*, as evidenced by a 98% decrease in the number of leaves showing sporulation, and a 13-fold lower number of conidiospores per gram of infected tissue at 11 days after inoculation. As shown in Figures 2A and 2C, lactophenol trypan-blue staining of the infected leaves revealed that colonization of SAR-expressing leaves by the pathogen was arrested predominantly at the stage of spore germination. These findings indicate that both WCS417r-mediated ISR and SAR are effective against *P. parasitica*, with INA-induced SAR being significantly more effective than WCS417r-mediated ISR.

Differential effectiveness of ISR and SAR against *Alternaria brassicicola*

The Arabidopsis ecotype Col-0 is resistant against the necrotrophic fungus *A. brassicicola* (Penninckx et al., 1996). However, the JA-insensitive mutant *coi1-1* and the phytoalexin-deficient mutant *pad3-1* are susceptible, indicating that the fungus is resisted through JA-dependent defense mechanisms, as well as phytoalexins (Thomma et al., 1998; 1999b). Both defenses seem unrelated, as *coi1-1* plants accumulate high levels of phytoalexins after infection by *A. brassicicola* (Thomma et al., 1999b), whereas *pad3-1* plants show restored levels of resistance against the fungus in response to MeJA treatment (Thomma et al., 1999b). Because expression of WCS417r-mediated ISR requires JA signaling, WCS417r-mediated ISR and SAR were determined in the *pad3-1* mutant, which is unaffected in its ability to express WCS417r-mediated ISR and SAR against *P. syringae* pv. *tomato* DC3000 (C.M.J. Pieterse; unpublished results).

In non-induced *pad3-1* plants, necrotic lesions appeared on the second leaf pair, within 3 days after inoculation. By 7 days after inoculation, the third and fourth leaf pairs developed spreading lesions surrounded by chlorosis, and the second leaf pair displayed extensive tissue damage and concurrent sporulation by the pathogen. Induction of ISR by WCS417r protected the plants substantially against fungal invasion, as was evident from reduced lesion expansion (Figure 3A and 3B) and a 2-fold reduction in fungal sporulation (Figure 3C). In contrast to WCS417r-mediated ISR, SAR induced by predisposal infection of the first pair of true leaves with the avirulent *P. syringae* pv. *tomato* DC3000(*avrRpt2*) failed to reduce the spread and sporulation of the pathogen (Figure 3). Besides biological induction of ISR and SAR, chemical induction was tested by exogenous application of the chemical agents MeJA and INA, which were previously demonstrated to activate the ISR and the SAR pathway, respectively (Pieterse et al., 1998; Vernooij et al., 1995). Consistent with previous findings of Thomma et al. (1998), treatment of the leaves with MeJA protected the

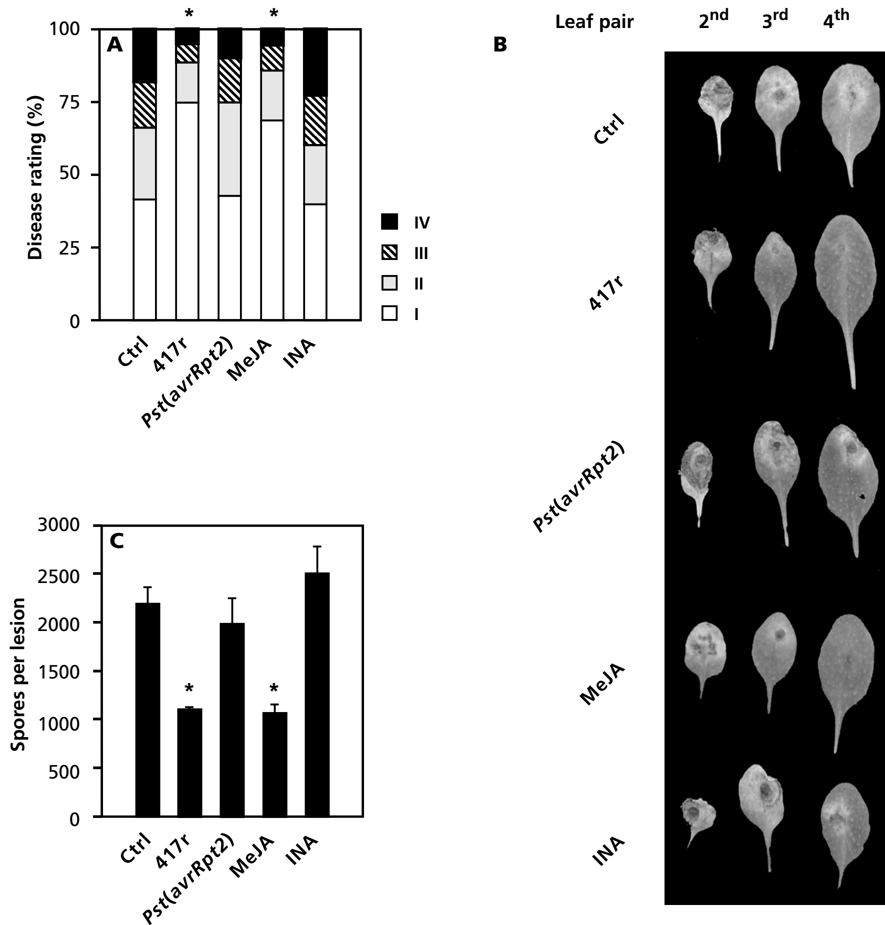

Figure 3. Quantification of ISR and SAR in Arabidopsis *pad3-1* plants against *Alternaria brassicicola* MUCL20297. ISR was induced by growing plants in soil containing *P. fluorescens* WCS417r bacteria at 5 x 10^7 CFU.g^{-1}, or by spraying with 100 µM MeJA, 3 days before challenge. SAR was induced by injecting the first pair of true leaves with a suspension of avirulent *P. syringae* pv. *tomato* DC3000(*avrRpt2*) at 10^7 CFU.mL^{-1}, or by spraying with 0.1 mM INA 3 days before challenge. Plants (n = 25) were challenge inoculated with *A. brassicicola* when four weeks old by applying 3-µL droplets containing 10^6 spores per mL on the second, third and fourth pair of true leaves. **(A)** Distribution of disease severity classes at 4 days after challenge. Disease severity is expressed as the percentage of leaves falling in disease severity classes: I, no visible disease symptoms; II, non-spreading lesion with a diameter <1.5 mm; III, spreading lesion with a diameter between 1.5 and 7 mm, surrounded by a chlorotic halo; IV, spreading lesion with a diameter >7 mm, with extensive tissue maceration and sporulation by the pathogen. Asterisks indicate statistically significant different frequency distributions of the disease-severity classes compared to the non-induced control treatment (Chi-square; n = 150; $\alpha < 0.05$). The data presented are from a representative experiment that was performed twice with similar results.
(B) Lesion development on the second, third, and fourth pair of true leaves at 7 days after challenge with *A. brassicicola*.
(C) Average number of newly formed spores per lesion (± SEM) at 7 days after challenge with *A. brassicicola*. Asterisks indicate statistically significant differences compared to the non-induced control treatment (Student's *t* test, n = 5; $\alpha < 0.05$).

plants significantly, whereas treatment of the leaves with INA did not (Figure 3). The level of MeJA-induced protection was similar to that observed in WCS417r-treated plants. These findings indicate that, in contrast to SAR, expression of ISR is effective against infection by *A. brassicicola*.

ISR and SAR are equally effective against *Xanthomonas campestris* pv. *armoraciae*

Little is known about the involvement of SA-, JA-, and ethylene-dependent defense responses in basal resistance against *X. campestris*. Rogers and Ausubel (1997) reported that mutant *eds5-1*, which is allelic with the SA-deficient mutant *sid1-1* (Nawrath and Métraux, 1999), displays enhanced susceptibility to *X. campestris* pv. *raphani*. This indicates that in non-induced plants, *X. campestris* is resisted, at least in part, through SA-dependent defenses. To investigate the role of SA, JA and ethylene in the basal resistance response against *X. campestris* pv. *armoraciae*, we determined growth of the pathogen in the leaves of non-induced Col-0 plants, SA-nonaccumulating NahG plants, JA-insensitive *jar1-1* plants, and ethylene-insensitive *ein2-1* plants. At three days after inoculation, NahG, *jar1-1* and *ein2-1* plants allowed 3- to 5-fold higher levels of growth of the pathogen in the leaves than wild-type plants (data not shown), indicating that basal resistance against *X. campestris* pv. *armoraciae* is resisted through a combination of SA-, JA-, and ethylene-dependent defenses.

To investigate the effectiveness of induced resistance against *X. campestris* pv. *armoraciae*, ecotype Col-0 was tested for WCS417r-mediated ISR and pathogen-induced SAR against the pathogen. Treatment of the roots with WCS417r bacteria resulted in a statistically significant reduction of disease symptoms (Figure 4). Furthermore, bacterial growth over a three-day time interval was suppressed 3.5-fold in the ISR-expressing plants

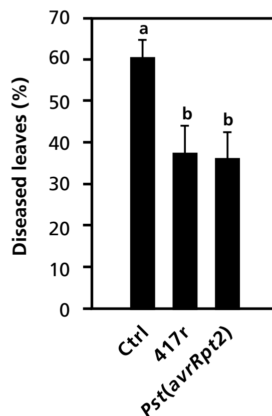

Figure 4. Quantification of ISR and SAR in Arabidopsis Col-0 plants against *Xanthomonas campestris* pv. *armoraciae*. ISR was induced by growing plants in soil containing *P. fluorescens* WCS417r bacteria at 5 x 10^7 CFU.g^{-1}. SAR was induced by pressure-infiltrating a suspension of avirulent *P. syringae* pv. *tomato* DC3000 (*Pst*(*avrRpt2*)) at 10^7 CFU.mL^{-1} into the first two leaves, 3 days prior to challenge inoculation of the upper leaves. Challenge inoculation was performed in five-week-old plants by dipping the leaves in a bacterial suspension containing 5 x 10^7 CFU.mL^{-1}. Disease symptoms were scored at 3 days after challenge and quantified as the proportion of leaves with symptoms. Different letters indicate statistically significant differences between treatments (Fisher's LSD test; $\alpha = 0.05$; n = 20-25). Data presented are means (± SD) from a representative experiment that was performed twice, yielding similar results.

(Table 2). These results demonstrate that WCS417r-mediated ISR effectively inhibits *X. campestris* pv. *armoraciae* infection. Induction of SAR by predisposal infection with avirulent *P. syringae* pv. tomato DC3000(*avrRpt2*) resulted in a suppression of both disease symptoms (Figure 4) and bacterial growth (Table 2) to the same level as found in the ISR-expressing plants. Thus, WCS417r-mediated ISR and pathogen-induced SAR are equally effective in protecting Arabidopsis against disease caused by *X. campestris* pv. *armoraciae*.

Table 2. Proliferation of *Xanthomonas campestris* pv. *armoraciae* in ISR- and SAR-expressing Arabidopsis leaves.

Treatment [a]	Proliferation (in log units)[b]
Control	3.35 ± 0.26 a
ISR	2.79 ± 0.10 b
SAR	2.80 ± 0.18 b

[a] ISR was induced by transferring 2-week-old Col-0 seedlings to potting soil containing WCS417r bacteria at 5 x 10^7 CFU.g^{-1}. SAR was induced by injecting two lower leaves with a suspension of avirulent *P. syringae* pv. *tomato* DC3000(*avrRpt2*) at 10^7 CFU.mL^{-1}, 3 days before challenge inoculation.

[b] Values presented are means with standard deviations of the log of the proliferation of *X. campestris pv. armoraciae* over a 3-day time interval. Five-week-old plants were infected by dipping the leaves in a bacterial suspension at 5 x 10^7 CFU.mL^{-1}. Immediately after inoculation and 3 days later, the number of bacterial cells per gram of leaf fresh weight was determined and proliferation over the 3-day time interval was calculated. Different letters indicate statistically significant differences between treatments (Fischer's LSD test; $\alpha = 0.05$; n = 5).

ISR is ineffective against turnip crinkle virus

Turnip crinkle virus (TCV) is virulent on most Arabidopsis ecotypes, including Col-0 (Simon et al., 1992), but avirulent on ecotype Dijon (Di-0 and Di-17), which develops a hypersensitive response (HR) and does not allow systemic spreading of the pathogen (Dempsey et al., 1997; Simon et al., 1992). To compare the effectiveness of SAR and ISR against this pathogen, the level of induced protection was examined in the compatible interaction with Col-0 and the incompatible interaction with Di-0.

In Col-0 plants, disease incidence was determined by scoring the number of non-inoculated leaves with symptoms, i.e. crinkled deformation and appearance of chlorotic spots around the vascular bundles. In addition, multiplication of TCV was assessed by gel blot analysis of RNA from systemically infected plants. In Col-0, INA treatment resulted in a 40% suppression of symptoms (Figure 5A), and a substantial reduction of TCV RNA accumulation (Figure 6A). In hypersensitively reacting Di-0 plants, the level of protection was determined

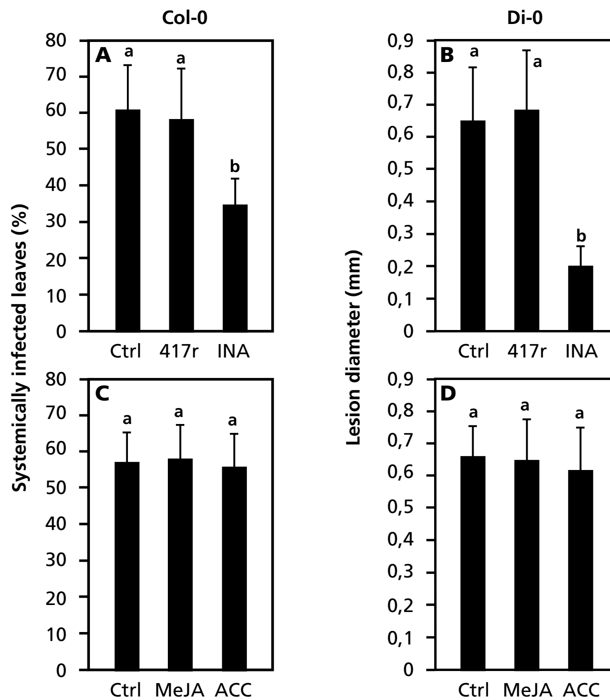

Figure 5. Quantification of induced resistance against turnip crinkle virus in Arabidopsis Col-0 and Di-0 plants.
(A) Rhizobacteria-mediated ISR and SAR in susceptible Col-0 plants.
(B) Rhizobacteria-mediated ISR and SAR in HR-developing Di-0 plants.
(C) ACC- and MeJA-induced protection in susceptible Col-0 plants.
(D) ACC- and MeJA-induced protection in HR-developing Di-0 plants.
ISR was induced by growing plants in soil containing *P. fluorescens* WCS417r bacteria at 5×10^7 CFU.g^{-1}. SAR was induced by spraying the plants with a solution containing 0.1 mM INA, 3 days before challenge inoculation. Treatments with MeJA and ACC were performed by dipping the leaves in a solution containing either 0.1 mM MeJA or 1 mM ACC, 3 days before challenge inoculation. Challenge inoculation was performed by rubbing 3-µL droplets of viral RNA suspension (0.1 µg.µL^{-1}) in bentonite buffer onto three lower leaves. For Di-0, average lesion diameters of challenged leaves (n = 45) were measured 4 days later. For Col-0, the percentage of non-inoculated leaves with symptoms was determined per plant (n = 20) 11 days later. Non-inoculated leaves showing crinkled deformation of the leaves and chlorotic spots around the vascular bundles were scored as diseased. For each ecotype, different letters indicate statistically significant differences between treatments (Fisher's LSD test; $\alpha = 0.05$). The data presented are from a representative experiment that was performed twice with similar results.

from the reduction in lesion size and the level of TCV RNA in locally infected leaves. Consistent with previous findings (Uknes et al., 1993), induction of SAR by INA resulted in a 3-fold reduction in lesion size (Figure 5B) and a strong suppression of TCV RNA accumulation in Di-0 plants (Figure 6A). These findings indicate that SAR effectively inhibits viral multiplication

In contrast to INA-induced SAR, treatment of the roots with ISR-inducing WCS417r bacteria failed to suppress disease development (Figure 5A and 5B) and viral RNA accumulation (Figure 6A) in both Col-0 and Di-0 plants. To relate this ineffectiveness of WCS417r-mediated ISR to JA/ethylene signaling, we tested the response to exogenous application of the chemical agents MeJA and ACC, which were previously demonstrated to activate the ISR pathway (Pieterse et al., 1998). Neither ACC treatment nor MeJA treatment of either Col-0 or Di-0 plants resulted in a reduction of disease symptoms (Figure 5C and 5D) or viral RNA accumulation (Figure 6B). Thus, in contrast to SA-dependent SAR, JA- and ethylene-dependent ISR is not effective against a virus.

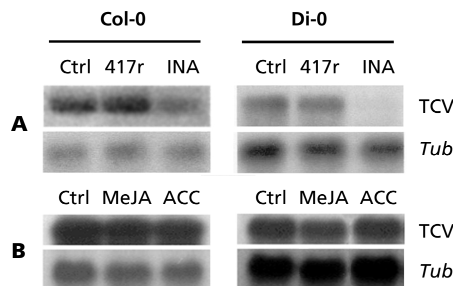

Figure 6. Accumulation of turnip crinkle virus (TCV) RNA in leaves of Arabidopsis ecotypes Col-0 and Di-0 after treatment with *P. fluorescens* WCS417r, INA, MeJA, or ACC. For the induction treatments and the challenge inoculation, see caption to Figure 5. For Di-0, the inoculated leaves were harvested at 4 days after challenge. For Col-0, the non-inoculated leaves were harvested at 11 days after challenge. Blots were hybridized with TCV-specific probes, derived from a TCV cDNA clone. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for ß-tubulin (*Tub*).

Discussion

P. fluorescens WCS417r-mediated ISR and pathogen-induced SAR are two inducible defense responses that are controlled by distinct signaling pathways (Pieterse et al., 1998). In this study, we tested both types of induced resistance in Arabidopsis for their effectiveness against four different pathogens, i.e. an oomycete, a fungus, a bacterium and a virus. ISR and SAR were equally effective against *X. campestris* pv. *armoraciae* (Figure 4). However, the effectiveness of ISR and SAR against *P. parasitica*, *A. brassicicola* and TCV diverged. Induction of SAR resulted in a high level of protection against *P. parasitica* and TCV (Figures 1, 2, 5 and 6), whereas WCS417r-mediated ISR yielded only moderate protection against *P. parasitica* (Figures 1 and 2) and no protection against TCV (Figures 5 and 6). Conversely, ISR was effective against *A. brassicicola*, whereas SAR was not (Figure 3).

By using Arabidopsis genotypes affected in their response to either SA, JA, or ethylene, it was previously demonstrated that basal resistance against *P. parasitica* and TCV is conferred by SA-dependent defense responses (Delaney et al., 1994; Kachroo et al., 2000), whereas basal resistance against *A. brassicicola* is mainly controlled by JA, and to a lower extent by ethylene (Thomma et al., 1998; Thomma et al., 1999a). Like *P. syringae* pv. *tomato* DC3000 (Pieterse et al., 1998), *X. campestris* pv. *armoraciae* proliferates faster in NahG, *jar1-1*, and *ein2-1* plants than in wild-type Col plants (data not shown), indicating that basal resistance against both bacterial pathogens is controlled by a combination of SA-, JA-, and ethylene-dependent pathways. When relating the effectiveness of ISR and SAR against these pathogens to the defense pathways that contribute to basal resistance, it is clear that SAR is effective predominantly against pathogens that are resisted through SA-dependent basal defenses, whereas WCS417r-mediated ISR is predominantly effective against pathogens that are resisted through JA/ethylene-dependent defense responses (Figure 7). In view of the earlier notion that induced disease resistance is an enhancement of genetically determined extant resistance (Van Loon et al., 1997), it is tempting to speculate that SAR constitutes an enhancement of SA-dependent basal resistance, whereas WCS417r-mediated ISR involves an enhancement of JA- and ethylene- dependent basal resistance.

If SAR is an enhancement of SA-dependent basal resistance, one may expect that SA-dependent responses are activated more strongly or more rapidly upon challenge of SAR-expressing plant parts. Similarly, JA- and ethylene-dependent responses should be boosted upon challenge of plants expressing WCS417r-mediated ISR. Indeed, SAR-expressing leaves of Arabidopsis showed a potentiated expression of the SA-inducible genes *PR-1*, *PR-2* and *PR-5* upon challenge inoculation with *P. syringae* pv. *tomato* DC3000 (Cameron et al., 1999; Van Wees et al., 1999), whereas this potentiation was absent for the JA-inducible genes *Pdf1.2*

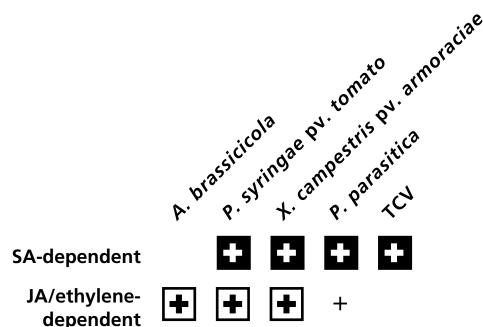

Figure 7. Model explaining the relationship between induced resistance and basal resistance for five different Arabidopsis pathogens. Pathogens sensitive to the expression of SAR (white plus signs), are resisted through SA-dependent basal resistance (black squares), whereas pathogens sensitive to *P. fluorescens* WCS417r-mediated ISR (black plus signs) are predominantly resisted through JA/ethylene-dependent basal resistance (white squares).

and *Atvsp* (Van Wees et al., 1999). Conversely, challenge-inoculated, ISR-expressing Arabidopsis leaves showed potentiated expression of *Atvsp*, while no potentation was detected for the SA-inducible *PR* genes (Van Wees et al., 1999). These observations support our conclusion that WCS417r-mediated ISR and SAR exploit different defense mechanisms that are controlled by an SA-dependent pathway in the case of SAR, and an JA/ethylene-dependent pathway in the case of WCS417r-mediated ISR. Recently, Van Wees et al. (2000) demonstrated that simultaneous activation of both pathways resulted in an enhanced level of protection against *P. syringae* pv. *tomato* DC3000, indicating that these defense mechanisms are complementary and act additively on the level of induced protection.

Consistent with earlier findings of Uknes et al. (1993), we showed that expression of INA-induced SAR limits TCV RNA accumulation and disease symptom development. However, no such effects were evident upon treatment with WCS417r, demonstrating that this type of induced resistance is not effective against TCV. Over the past years, there have been several reports showing that rhizobacteria-mediated ISR effectively inhibits infection by viral pathogens. In tobacco, Maurhofer et al. (1994) reported that *P. fluorescens* strain CHA0 induced resistance against tobacco necrosis virus (TNV), while De Meyer et al. (1998; 1999a) demonstrated that *P. aeruginosa* strain 7NSK2 triggered resistance against tobacco mosaic virus (TMV). However, in both cases, the mechanisms of induced resistance appeared to depend on SA. The resistance induced by strain CHA0 coincided with systemic accumulation of SA and PRs in the plant, and two SA-deficient mutants of strain 7NSK2 were no longer capable of inducing resistance against TMV. Furthermore, Maurhofer et al. (1998) demonstrated that introduction of SA biosynthetic gene cluster into *P. fluorescens* strain P3, which does not produce SA, significantly improved its ability to induce systemic resistance against TNV. These observations indicate that both these cases of rhizobacteria-mediated ISR against viral pathogens are phenotypically and mechanistically similar to pathogen-induced SAR, with SA implicated as the inducing determinant.

Recently, it was suggested that SA-dependent resistance against viruses is achieved through a signaling pathway that partially differs from SA-dependent resistance acting against pathogenic fungi and bacteria (Murphy et al., 1999). This difference may be related to the intimate intracellular relationship between viruses and their hosts, as opposed to the extracellularly attacking fungi and bacteria. In our experiments, TCV was the only pathogen tested that was entirely insensitive to WCS417r-mediated ISR. This suggests that TCV is either completely insensitive to the mechanisms of WCS417r-mediated ISR, or that the virus is located at sites where these mechanisms are not active.

Our results support and extend previous observations that SA-dependent and SA-independent pathways have different specificities (Pieterse and Van Loon, 1999). Clearly, different types of induced resistance are differentially active against different pathogens. Therefore, the prevailing notion that SAR and ISR are both active against all types of

pathogens, needs adjustment. On the other hand, the sensitivity of fungal and bacterial pathogens to components of both the SAR and the ISR pathway, offers potential for improved disease control, as was already demonstrated by the additive effect of SAR and rhizobacteria-mediated ISR on the level of induced protection against *P. syringae* pv. *tomato* DC3000 in Arabidopsis (Van Wees et al., 2000).

Materials and Methods

Cultivation of plants

Arabidopsis thaliana ecotypes Columbia (Col-0) and Dijon (Di-0), and the phytoalexin-deficient mutant *pad3-1* (ecotype Col-0; Glazebrook and Ausubel, 1994) were grown in quartz sand for two weeks. Subsequently, the seedlings were transferred to 60-mL pots, containing a sand/potting soil mixture that had been autoclaved twice for 20 min. Plants were further cultivated in a growth chamber with a 9-h day (≈200 $\mu.Em^{-2}.sec^{-1}$ at 24 °C) and 15-h night (20 °C) cycle at 65% relative humidity. Plants were watered on alternate days, and once a week supplied with modified half-strength Hoagland solution: 2 mM KNO_3, 5 mM $Ca[NO]_3$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, and trace elements, pH 7 (Hoagland and Arnon, 1938), containing 10 µM sequestreen (Fe-ethylenediamide-di[*o*-hydroxyphenylacetic acid]; Novartis, Basel, Switzerland).

Cultivation of rhizobacteria and pathogens

For treatment of the roots with ISR-inducing rhizobacteria, rifampicin-resistant *Pseudomonas fluorescens* strain WCS417r (Pieterse et al., 1996) was grown on King's medium B agar plates (King et al., 1954) for 24 h at 28 °C. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 colony-forming units (CFU) per mL. For induction of SAR, kanamycin-resistant *Pseudomonas syringae* pv. *tomato* strain DC3000 carrying the avirulence gene *avrRpt2*; Kunkel et al., 1993), was cultured overnight in liquid King's medium B at 28 °C, collected by centrifugation, and resuspended in 10 mM $MgSO_4$ to a final density of 10^7 $CFU.mL^{-1}$.

Peronospora parasitica race WACO9 was maintained on ecotype Col-0. Conidiospores were obtained by washing heavily sporulating leaves in 10 mM $MgSO_4$, collected by centrifugation, and resuspended in 10 mM $MgSO_4$ to a final density of 5 x 10^4 conidiospores per mL. *Alternaria brassicicola* strain MUCL20297 was grown on PDA agar plates for 2 weeks at 22 °C. Subsequently, the conidia were harvested as described previously (Broekaert et al., 1990). Rifampicin-resistant *Xanthomonas campestris* pv. *armoraciae* (Sahin and Miller, 1996) was cultured overnight in liquid 0.8 % Nutrient Broth medium (Difco, Detroit, USA) at 28 °C, collected by centrifugation, and resuspended in 10 mM $MgSO_4$ to a final density of 10^8 $CFU.mL^{-1}$. Turnip crinkle virus (TCV) inoculum was produced by in vitro transcription from plasmid pT7TCV66 (Oh et al., 1995), and adjusted to a concentration of 0.1 µg RNA per µL.

Induction treatments

ISR was induced by transplanting 2-week-old Arabidopsis seedlings into a sand/potting soil mixture containing ISR-inducing WCS417r bacteria. Prior to transfer of the Arabidopsis seedlings to the pots, a suspension of ISR-inducing WCS417r bacteria (10^9 CFU.mL^{-1}) was mixed thoroughly through the sand/potting soil mixture, to a final density of 5 x 10^7 CFU.g^{-1}. Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. Induction of SAR was performed 3 days before challenge inoculation, either by pressure-infiltrating the first pair of true leaves with a suspension of avirulent *P. syringae* pv. *tomato* DC3000(*avrRpt2*) at 10^7 CFU.mL^{-1}, or by spraying the plants with 0.1 mM 2,6-dichloroisonicotinic acid (INA; Novartis, Basel, Switzerland) formulated as 25 % active ingredient with 19 mg.L^{-1} wettable powder. Treatments with the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) and methyl jasmonate (MeJA) were performed at three days before challenge inoculation, by dipping the leaves in a solution of 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands), containing either 0.1 mM MeJA or 1 mM ACC. MeJA treatment prior to challenge with *A. brassicicola* was performed by spraying the leaves with 0.1 mM MeJA formulated with 19 mg.L^{-1} wettable powder. Control-treated plants were dipped either in a solution containing 0.015 % (v/v) Silwet L-77, or sprayed with 19 mg.L^{-1} wettable powder.

***Peronospora parasitica* bioassays**

Three-week-old Col-0 plants (n = 40) were challenge inoculated with *Peronospora parasitica* strain WACO9 by applying 3-µL drops of 10 mM $MgSO_4$, containing 5 x 10^4 conidiospores per mL. Inoculated plants were maintained at 17 °C and 100% relative humidity. Disease symptoms were scored for about 200 leaves per treatment at 7, 9 and 11 days after inoculation. Disease rating was expressed as intensity of disease symptoms and pathogen sporulation on each leaf: I, no sporulation; II, <50% of the leaf area covered by sporangia; III, >50% of the leaf area covered by sporangia; IV, heavily covered with sporangia, with additional chlorosis and leaf collapse. Leaves were washed in 10 mM $MgSO_4$, and conidiospores were collected by centrifugation at 1.500 *g* for 2 min, and resuspended in 10 mM $MgSO_4$. The number of conidiospores was determined microscopically, using a haemocytometer.

For determining leaf colonization, infected leaves were stained with lactophenol trypan-blue and examined microscopically at 9 days after inoculation, as described by Koch and Slusarenko (1990).

***Alternaria brassicicola* bioassays**

Four-week-old *pad3-1* mutant plants (n = 25) were challenge inoculated by applying 3-µL drops of 10 mM $MgSO_4$, containing 10^6 spores per mL, on the second, third and fourth true pair of leaves of each plant. Inoculated plants were kept at 100% relative humidity. At 3 and 4 days after challenge, the disease severity was determined. Disease rating was expressed as intensity of disease symptoms and lesion size: I, no visible disease symptoms; II, non-spreading lesion with a diameter < 1.5 mm; III, spreading lesion with a diameter ranging between 1.5 and 7 mm, surrounded by a chlorotic halo; IV, spreading lesion with a diameter > 7 mm with extensive tissue maceration and sporulation by

the pathogen. Isolation and determination of the number of newly formed spores was performed essentially as described by Thomma et al. (1999b). Batches of 15 leaves from 5 plants were placed in 9 mL of 0.1% Tween20 in a test tube. After vigorous shaking, the leaves were removed from the suspension. The spore suspension was centrifuged at 3.200 *g* for 15 min, and the spores were resuspended in 100 µL 0.1% Tween 20, after which they were counted in a haemocytometer.

Xanthomonas campestris bioassays

Five-week-old Col-0 plants (n = 20-25) were inoculated by dipping the leaves in a suspension of virulent *Xanthomonas campestris* pv. *armoraciae*, containing 5 x 10^7 CFU.mL^{-1} in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77. Three days after challenge inoculation, the percentage of leaves with symptoms was determined per plant. Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased. Growth of *X. campestris* pv. *armoraciae* in the leaves of wild-type Col-0, transgenic *NahG* (Delaney et al., 1994), and mutant *jar1-1* (Staswick et al., 1992) and *ein2-1* (Guzman and Ecker, 1990) plants was determined by collecting replicate samples from five plants per genotype. Approximately 30 min after challenge inoculation and three days later, leaf samples were collected, weighed, rinsed in water, and homogenized in 10 mM $MgSO_4$. Serial dilutions were plated on selective King's medium B agar, supplemented with 100 mg.L^{-1} cycloheximide and 50 mg.L^{-1} rifampicin. After incubation at 28 °C for 2 days, the number of rifampicin-resistant CFU per gram of infected leaf tissue was determined, and bacterial proliferation over the 3-day time interval was calculated.

Turnip crinkle virus bioassays

Four-week-old Arabidopsis plants (ecotypes Col-0 and Di-0) were challenge inoculated with TCV by applying 3 µL droplets of viral RNA suspension (0.1 µg.µL^{-1}) in bentonite buffer (0.05 M glycine, 0.03 M K_2HPO_4, 0.02 g bentonite per mL) on three lower leaves. The droplet was then rubbed across the leaf surface with a glass rod, and the treated leaves were marked. For ecotype Di-0, lesion diameters on the inoculated leaves (n = 45) were determined under a dissection microscope at 4 days after challenge inoculation. Subsequently, challenged leaves were collected for gel blot analysis of viral RNA accumulation. For ecotype Col-0, the percentage of non-inoculated leaves with symptoms was determined per plant (n = 20) at 11 days after challenge inoculation. Unchallenged leaves showing crinkled deformation of the leaves and chlorotic spots around the vascular bundles were scored as diseased. Subsequently, above-ground parts of five plants per treatment were collected for gel blot analysis of viral RNA accumulation.

RNA gel blot analysis

Total RNA was extracted from control- and WCS417r-treated Col-0 and Di-0 plants 14 days after challenge with TCV by homogenizing frozen leaf tissue in extraction buffer (0.35 M glycine, 0.048 N NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 mL.g^{-1} of leaf tissue). The homogenates

were extracted with phenol and chloroform, and the RNA was precipitated using LiCl, as described by Sambrook et al. (1989). For RNA gel blot analysis, 15 µg of RNA was denatured using glyoxal and DMSO (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. The electrophoresis buffer and blotting buffer consisted of 10 mM and 25 mM sodium phosphate (pH 7.0), respectively. RNA gel blots were hybridized with a TCV-specific probe as described previously (Pieterse et al., 1994). To check for equal loading, the blots were stripped and hybridized with a probe for the constitutively expressed β-tubulin (*Tub*) gene. DNA probes were labeled with α-^{32}P-dCTP by random primer labeling (Feinberg and Vogelstein, 1983). Probes for the detection of viral RNA were derived from the cDNA clone on plasmid pT7TCV66 (Oh et al., 1995). Probes for detection of *Tub* transcripts were prepared by PCR with primers based on the sequence of Arabidopsis obtained from GenBank accession no. M21415.

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CHAPTER 6

The Arabidopsis *ISR1* locus is required for rhizobacteria-mediated induced systemic resistance against different pathogens

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Submitted

Abstract

In Arabidopsis, non-pathogenic, root-colonizing *Pseudomonas fluorescens* WCS417r bacteria induce a systemic resistance (ISR) that is effective against different pathogens. In contrast to pathogen-induced systemic acquired resistance (SAR), WCS417r-mediated ISR is controlled by a salicylic acid-independent signaling pathway that requires an intact response to the plant hormones jasmonic acid and ethylene. Arabidopsis ecotypes RLD1 and Wassilewskija (Ws-0) fail to express WCS417r-mediated ISR against *Pseudomonas syringae* pv. *tomato* DC3000 and show enhanced disease susceptibility to this pathogen. Genetic analysis of progeny from crosses between WCS417r-responsive and non-responsive ecotypes demonstrated that ISR inducibility and basal resistance against *P. syringae* pv. *tomato* DC3000 are controlled by a single dominant locus (*ISR1*) on chromosome III (Ton et al., 1999). Here, we investigated the specificity of the *ISR1* locus in ISR, SAR, and basal resistance against *P. syringae* pv. *tomato* DC3000, *Xanthomonas campestris* pv. *armoraciae*, *Peronospora parasitica*, and turnip crinkle virus (TCV) in ecotypes Columbia (*ISR1*) and RLD1 (*isr1*) or Ws-0 (*isr1*). In contrast to basal resistance against *P. syringae* pv. *tomato* DC3000, basal resistance against *X. campestris* pv. *armoraciae*, *P. parasitica* and TCV was not reduced in the *isr1* genotypes, suggesting that the *ISR1* locus is not involved in basal resistance against these pathogens. Both *ISR1* and *isr1* genotypes were capable of expressing SAR against all pathogens tested, indicating that SAR against these pathogens functions independently of the *ISR1* locus. In contrast to the *ISR1* genotype, the *isr1* genotype did not express WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000, *X. campestris* pv. *armoraciae* and *P. parasitica*, indicating that the *ISR1* locus is required for WCS417r-mediated ISR against the various pathogens. Neither the *ISR1*, nor the *isr1* genotypes developed ISR against TCV, confirming previous findings that WCS417r-mediated ISR is ineffective against this virus.

Introduction

Plants are capable of developing an enhanced defensive capacity after appropriate stimulation. This induced disease resistance is generally manifest as a reduction of disease and a restriction of colonization of the challenging pathogen in comparison to non-stimulated control plants (Hammerschmidt, 1999). Rhizobacteria are present in large numbers on the root surface, where plant exudates and lysates provide nutrients (Lynch and Whipps, 1991). Selected strains of non-pathogenic, root-colonizing bacteria are capable of inducing systemic resistance against pathogen attack. This phenomenon is commonly referred to as rhizobacteria-mediated induced systemic resistance (ISR; Van Loon et al., 1998). Since the discovery of the resistance-inducing capacities of rhizobacteria in the early 1990's, ISR has been demonstrated in different plant species against various types of pathogens (Van Loon et al., 1998). Several rhizobacteria have been reported to also directly antagonize soil-borne pathogens and to stimulate plant growth (Bakker et al., 1991; Pieterse and Van Loon, 1999; Wei et al., 1996) and are, therefore, called plant growth-promoting rhizobacteria. The biological control activity of selected rhizobacterial strains is effective under field conditions (Tuzun and Kloepper, 1995; Wei et al., 1996) and in commercial greenhouses (Leeman et al., 1995c).

To study the molecular basis underlying rhizobacteria-mediated ISR, an *Arabidopsis thaliana*-based model system was developed (Pieterse et al., 1996), because this model has been proven to be excellently suited for molecular studies on biologically induced disease resistance (Cameron et al., 1994; Mauch-Mani and Slusarenko, 1994; Uknes et al., 1993). The non-pathogenic rhizobacterial strain *Pseudomonas fluorescens* WCS417r was used as the inducing agent, because this strain triggers ISR in several plant species, e.g. carnation, radish and tomato (Van Loon et al., 1998). Colonization of Arabidopsis roots by ISR-inducing WCS417r bacteria protects the plant against diseases caused by different types of pathogens, including the bacterial leaf pathogens *Pseudomonas syringae* pv. *tomato* DC3000 (Pieterse et al., 1996; Ton et al., 1999) and *Xanthomonas campestris* pv. *armoraciae* (Chapter 5), the oomycetous leaf pathogen *Peronospora parasitica* (Chapter 5), and the fungal pathogens *Fusarium oxysporum* f.sp. *raphani* (Pieterse et al., 1996) and *Alternaria brassicicola* (Chapter 5). Protection against these pathogens is typically manifested as a reduction in disease symptoms and an inhibition of pathogen growth.

Rhizobacteria-mediated ISR resembles pathogen-induced systemic acquired resistance (SAR; Ryals et al., 1996; Sticher et al., 1997), in that it is effective against a range of different pathogens. SAR is regulated by a SA-dependent signaling pathway and is associated with the coordinate expression of genes encoding pathogenesis-related (PR) proteins. In some cases, rhizobacteria have been shown to trigger the SAR pathway by producing SA at the root surface (De Meyer et al., 1997; 1999b; Maurhofer et al., 1998). However, in Arabidopsis the signaling pathways controlling WCS417r-mediated ISR and

SAR clearly differ. WCS417r-mediated ISR is controlled by a jasmonic acid (JA)- and ethylene-dependent pathway and is not associated with the activation of *PR* genes (Pieterse and Van Loon, 1999; Pieterse et al., 1996; 1998; 1999; 2000a). Also the spectrum of effectiveness partly diverges. In contrast to SAR, ISR is effective against pathogens that, in non-induced plants, are resisted through JA/ethylene-dependent basal defenses, e.g. *A. brassicicola*. Conversely, SAR is effective against pathogens that in non-induced plants are resisted through SA-dependent defenses, e.g. *P. parasitica* and turnip crinkle virus (TCV), whereas ISR is only weakly effective (*P. parasitica*) or not effective at all (TCV) against these pathogens. (Chapter 5). Both ISR and SAR are effective against the bacterial pathogens *P. syringae* pv. *tomato* DC3000 and *X. campestris* pv. *armoraciae* that in non-induced plants are resisted through a combination of SA-, JA-, and ethylene-dependent basal defenses (Pieterse et al., 1996; 1998; Chapter 5). Interestingly, simultaneous activation of both types of induced resistance results in an enhanced level of protection against *P. syringae* pv. *tomato* DC3000, indicating that ISR and SAR can have an additive effect on the level of induced protection (Van Wees et al., 2000).

Elicitation of ISR is specific with regard to both the host species and the rhizobacterial strain. For instance, the ISR-inducing rhizobacterial strains *P. putida* WCS358 and *P. fluorescens* WCS374 perform differently on different plant species (Van Loon, 1997). Arabidopsis is responsive to WCS358, whereas radish and carnation are not. Conversely, radish is responsive to WCS374r, whereas Arabidopsis is not. Strain WCS417r has the ability to elicit an ISR response in both plant species. However, differential induction of WCS417r-mediated ISR occurs in Arabidopsis ecotypes. Most ecotypes, e.g. Columbia (Col-0) and Landsberg *erecta*, are responsive to treatment with WCS417r, whereas ecotypes RLD1 and Wassilewskija (Ws-0) are not (Van Wees et al., 1997; Ton et al., 1999). These findings indicate that induction of ISR requires a specific interaction between the plant and the plant-beneficial rhizobacterium.

Further characterization of several ISR-inducible ecotypes and the non-inducible ecotypes RLD and Ws revealed that the inability to express WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000 is associated with an enhanced susceptibility to this pathogen (Chapter 2; Ton et al., 1999). Genetic analysis of progeny of crosses between inducible and non-inducible Arabidopsis ecotypes showed that inducibility of ISR and basal resistance against *P. syringae* pv. *tomato* DC3000 are controlled by a single dominant locus (*ISR1*), that maps to chromosome III between cleaved amplified polymorphic sequence (CAPS) markers *Ein3* and *GL1*. Interestingly, both the non-inducible ecotypes RLD1 and Ws-0 showed a reduced sensitivity to ethylene. This reduced sensitivity to ethylene co-segregated with the recessive alleles at the *ISR1* locus in the F_2 progeny of a cross between Col-0 and RLD1 (Chapter 3; Ton et al., 2001). Therefore, it was proposed that the Arabidopsis *ISR1* locus encodes a novel component of the ethylene response pathway that plays an important role in

disease resistance.

The dual involvement of the *ISR1* locus in WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000, on the one hand, and basal resistance against *P. syringae* pv. *tomato* DC3000, on the other hand, prompted us to investigate whether the *ISR1* locus plays a similar role in both ISR and basal resistance against other pathogens. In a comparative study between ecotype Col-0, carrying the dominant alleles at the *ISR1* locus (*ISR1*), and ecotypes RLD1 and Ws-0, carrying the recessive alleles (*isr1*), we investigated the role of the *ISR1* locus in WCS417r-mediated ISR against the Arabidopsis pathogens *P. syringae* pv. *tomato* DC3000, *X. campestris* pv. *armoraciae*, and *P. parasitica*. In addition, we examined the role of the *ISR1* locus in SAR and basal resistance against *P. syringae* pv. *tomato* DC3000, *X. campestris* pv. *armoraciae*, *P. parasitica*, and TCV.

Results

Involvement of the *ISR1* locus in basal resistance against *X. campestris* pv. *armoraciae*, *P. parasitica* and TCV

Previously, we demonstrated that the recessive alleles at the *ISR1* locus confer a reduced level of basal resistance against *P. syringae* pv. *tomato* DC3000, indicating that the dominant alleles of the *ISR1* locus contribute to basal resistance against this pathogen (Chapter 2; Ton et al., 1999). To investigate whether the locus is also involved in basal resistance against other pathogens, *ISR1* plants were compared with *isr1* plants for their level of basal resistance against another bacterium, an oomycete, and a virus. Consistent with our previous findings (Chapter 2; Ton et al., 1999), the *isr1* genotype Ws-0 showed enhanced disease severity after inoculation with *P. syringae* pv. *tomato* DC3000 (Figure 1A), and allowed significantly more growth of the pathogen in the non-induced leaves compared to the *ISR1* genotype Col-0 (Figure 1B). However, a decrease rather than an increase in basal resistance was apparent for the bacterial pathogen *X. campestris* pv. *armoraciae* and the oomycetous pathogen *P. parasitica*. Three days after primary inoculation with *X. campestris* pv. *armoraciae*, *ISR1* genotype Col-0 developed more bacterial spot disease (Figure 1C), and allowed higher levels of growth of the pathogen than *isr1* genotype RLD1 (Figure 1D). Similarly, *ISR1* genotype Col-0 was more susceptible to *P. parasitica* than *isr1* genotype Ws-0, as was evident from enhanced disease incidence (Figure 1E) and a two-fold higher production of spores by the pathogen (Figure 1F). For TCV, the disease severity in non-inoculated leaves of infected RLD1 plants (*isr1*) was somewhat enhanced compared to that of similarly treated Col-0 plants (*ISR1*) (Figure 1G). Nevertheless, the extent of viral multiplication in these plants was similar for both genotypes (Figure 1H), indicating that both the *ISR1* genotype Col-0 and the *isr1* genotype RLD1 exhibit equal levels of susceptibility to TCV. Together, these results indicate

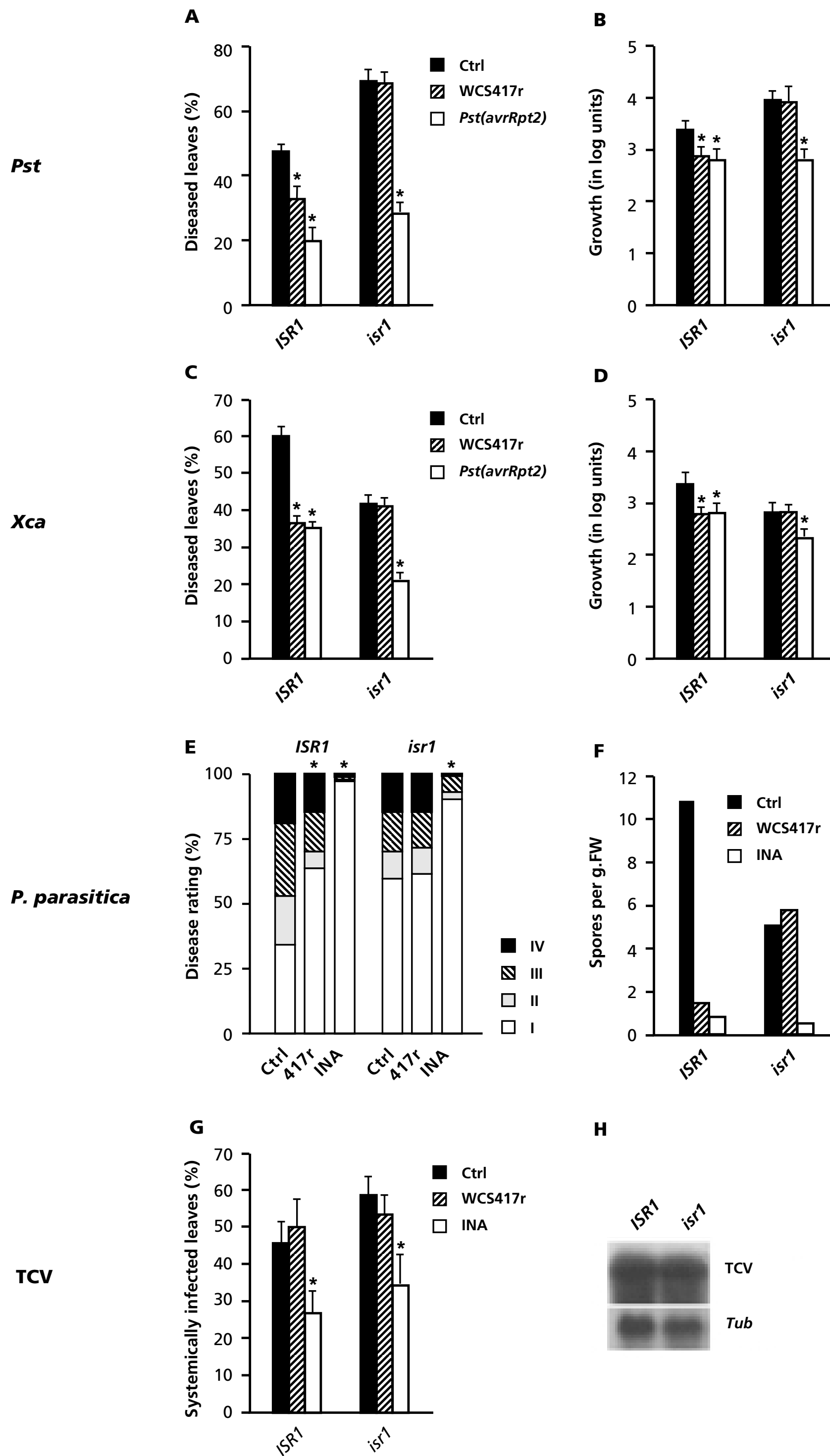
A
B
C
D
E
F
G
H
Pst
Xca
P. parasitica
TCV
Diseased leaves (%)
Growth (in log units)
Disease rating (%)
Spores per g.FW
Systemically infected leaves (%)
Ctrl
WCS417r
Pst(avrRpt2)
INA
IV
III
II
I
ISR1
isr1
417r
TCV
Tub

Figure 1. Quantification of basal and induced resistance against four different Arabidopsis pathogens in *ISR1* and *isr1* plants.
(A) and **(B)** Basal and induced resistance against *P. syringae* pv. *tomato* DC3000 *(Pst)*.
(C) and **(D)** Basal and induced resistance against *X. campestris* pv. *armoraciae (Xca)*.
(E) and **(F)** Basal and induced resistance against *P. parasitica*.
(G) and **(H)** Basal and induced resistance against TCV
Differences in basal resistance are reflected by the differences in disease severity and pathogen proliferation in non-induced control plants (Ctrl). ISR was induced by treatment of the roots with ISR-inducing *P. fluorescens* WCS417r bacteria. SAR was induced three days prior to challenge inoculation, either by infection of two lower leaves with *P. syringae* pv. *tomato* DC3000(*avrRpt2*), or by spraying the leaves with INA. For *P. syringae* pv. *tomato* DC3000 and *X. campestris* pv. *armoraciae* bioassays, 5-week-old plants were challenged by dipping the leaves in a bacterial suspension containing 2.5 x 10^7 CFU.mL^{-1} or 5 x 10^7 CFU.mL^{-1}, respectively. Data presented are means (± SD) of the percentage of leaves with symptoms (n = 20) (**A, C**) and bacterial proliferation (n = 5) (**B, D**) at 3 days after challenge. Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased. For *P. parasitica* bioassays, 3-week-old plants were challenged by applying 3-µL droplets containing 5 x 10^4 conidiospores per mL. Disease symptoms (**E**), and spore production (**F**) were determined at 11 days after challenge. Disease rating was expressed as percentage of leaves (n = 200) in disease-severity classes: I, no sporulation; II, <50 % of the leaf area covered by sporangia; III, >50 % of the leaf area covered by sporangia; IV, leaves heavily covered by sporangia, with additional chlorosis and leaf collapse. For TCV bioassays, 4-week-old plants were challenged by rubbing 3 µL droplets of viral RNA suspension (0.1 µg.µL^{-1}) in bentonite buffer onto three lower leaves**.** At 14 days after challenge, the percentage of systemically infected leaves with symptoms was determined per treatment (n = 20) (**G**), and 5 representative control-treated plants were harvested for RNA-blot analysis (**H**). Systemically infected leaves showing crinkled deformation of the leaves and chlorotic spots around the vascular bundles were scored as diseased. Error bars indicate standard errors of the mean. Asterisks indicate statistically significant differences between induction and control treatments according to the Student's *t* test (**A, B, C, D, G**), or the Chi-square test (**E**) (α = 0.05). All experiments were repeated with similar results. *ISR1*: ecotype Col-0; *isr1*: ecotype RLD1 (**C, D, G, H)** or Ws-0 (**A, B, E, F**).

that there is no consistent effect of the *ISR1* locus on the level of basal resistance against *X. campestris* pv. *armoraciae*, *P. parasitica* and TCV.

The *ISR1* locus is required for rhizobacteria-mediated ISR against different pathogens

Besides for basal resistance against *P. syringae* pv. *tomato* DC3000, the *ISR1* locus is also required for WCS417r-mediated ISR against this pathogen (Chapter 2; Ton et al., 1999). Indeed, *ISR1* genotype Col-0, unlike *isr1* genotype Ws-0, developed significantly less disease symptoms (Figure 1A) and allowed lower levels of growth of *P. syringae* pv. *tomato* DC3000 (Figure 1B) after treatment of the roots with WCS417r. Similarly, disease symptoms caused by *X. campestris* pv. *armoraciae*, as well as growth of this bacterium in the leaves, were significantly reduced in *ISR1* genotype Col-0 after treatment with WCS417r, whereas the *isr1* genotype RLD1 failed to develop WCS417r-mediated ISR against this pathogen (Figure 1C and 1D). Upon challenge with *P. parasitica*, the same situation applied. Only the *ISR1* genotype Col-0 expressed WCS417r-mediated ISR, as was evident by a reduction in disease

severity (Figure 1E) and a 7.5-fold reduction of pathogen sporulation in WCS417r-treated plants (Figure 1F). The *isr1* genotype Ws-0 failed to develop resistance against *P. parasitica* after treatment with WCS417r (Figure 1E and 1F). These results demonstrate that the *ISR1* locus is required for the expression of WCS417r-mediated ISR against these three pathogens. In contrast, neither the *ISR1* genotype Col-0, nor the *isr1* genotype RLD1 showed a reduction in viral disease symptoms caused by TCV after treatment of the roots with WCS417r (Figure 1G). These latter results support our previous finding that WCS417r-mediated ISR is ineffective against TCV (Chapter 5).

SAR functions independently of the *ISR1* locus

To elucidate whether the *ISR1* locus also influences the expression of SAR, *ISR1* plants (Col-0) were compared with *isr1* plants (RLD1 or Ws-0) for their ability to express SAR against the four pathogens. SAR was triggered either by predisposal infection of two lower leaves with avirulent *P. syringae* pv. *tomato* DC3000 carrying the avirulence gene *avrRpt2*, or by spraying the plants with the chemical SAR inducer 2,6-dichloroisonicotinic acid (INA). After induction of SAR, both the *ISR1* genotype Col-0 and the *isr1* genotypes Ws-0 and RLD1 showed a significant reduction in disease symptoms and bacterial proliferation after challenge inoculation with *P. syringae* pv. *tomato* DC3000 and *X. campestris* pv. *armoraciae*, respectively (Figure 1A-1D). Similarly, induction of SAR against *P. parasitica* and TCV was equally effective in *ISR1* genotype Col-0 and *isr1* genotypes Ws-0 and RLD1, respectively (Figure 1E-1G). It can thus be concluded that the *ISR1* locus is not involved in the expression of SAR against these different pathogens.

Discussion

Compared to several other Arabidopsis ecotypes, RLD1 and Ws-0 have reduced levels of basal resistance against *P. syringae* pv. *tomato* DC3000. This naturally occurring variation is due to a difference at the *ISR1* locus on chromosome III (Chapter 2; Ton et al., 1999). Accordingly, primary-inoculated *isr1* plants allowed significantly higher levels of growth of *P. syringae* pv. *tomato* DC3000 than *ISR1* genotype Col-0 (Figure 1B). In contrast, *isr1* genotypes RLD1 and Ws-0 did not exhibit reduced levels of basal resistance against *X. campestris* pv. *armoraciae*, *P. parasitica* and TCV (Figures 1C – 1H). Thus, the *ISR1* locus does not appear to contribute to basal resistance against these pathogens. Recently, we demonstrated that the *isr1* genotypes RLD1 and Ws-0 exhibit reduced responsiveness to ethylene, indicating that the *ISR1* locus is involved in ethylene signaling (Chapter 3; Ton et al., 2001). This suggests that ethylene signaling is of less relevance to basal resistance against *X. campestris* pv. *armoraciae*, *P. parasitica*, and TCV. Accordingly, Thomma et al. (1998) reported that basal resistance against

P. parasitica is unaffected in the ethylene-insensitive mutant *ein2-1*, whereas Kachroo et al. (2000) demonstrated that the *ein2-1* mutation does not confer enhanced susceptibility to TCV. However, *ein2-1* plants did allow higher levels of growth of *X. campestris* pv. *armoraciae* in the leaves compared to wild-type Col plants (Chapter 5). These observations suggest that additional genetic differences between *isr1* genotype RLD1 and *ISR1* genotype Col-0 influence the level of basal resistance against *X. campestris* pv. *armoraciae*. Arabidopsis ecotypes have been reported to exhibit considerable genetic variation for many different traits, including responses to pathogens (Alonso-Blanco and Koornneef, 2000; Erschadi et al., 2000; Kunkel et al., 1996). In order to more precisely define the role of the *ISR1* locus in basal resistance, a comparative study should be performed, using two near-isogenic inbred lines carrying either the dominant alleles of *ISR1* or the recessive alleles of *ISR1*.

In addition to basal resistance against *P. syringae* pv. *tomato* DC3000, the *ISR1* locus is also required for the expression of WCS417r-mediated ISR against this pathogen (Chapter 2; Ton et al., 1999). Indeed, *isr1* genotype Ws-0 failed to suppress disease and growth by *P. syringae* pv. *tomato* DC3000 upon treatment with WCS417r (Figure 1A and 1B). The *isr1* genotypes RLD1 and Ws-0 also failed to develop ISR against *X. campestris* pv. *armoraciae* (Figure 1C and 1D) and *P. parasitica* (Figure 1E and 1F), respectively, whereas *ISR1* genotype Col-0 expressed significant levels of ISR against these pathogens. Previously, Van Wees et al. (1997) reported that RLD1 failed to express WCS417r-mediated ISR against the fungal pathogen *F. oxysporum* f.sp. *raphani*. In contrast to these pathogens, disease caused by TCV

Table 1. WCS417r-mediated ISR and SAR against five different pathogens in *ISR1* and *isr1* plants[a].

Pathogen	ISR		SAR	
	ISR1	*isr1*	*ISR1*	*isr1*
P. syringae pv. *tomato* DC3000[b]	+	-	+	+
X. campestris pv. *armoraciae*	+	-	+	+
P. parasitica	+	-	+	+
F. oxysporum f.sp. *raphani*[c]	+	-	+	+
TCV	-	-	+	+

[a] ISR was induced by growing the plants for three weeks in soil containing ISR-inducing *P. fluorescens* WCS417r bacteria; SAR was induced either biologically by prediposal infection with *P. syringae* pv. *tomato* DC3000(*avrRpt2*), or chemically by exogenous application of SA or INA. *ISR1*: ecotype Col-0; *isr1*: ecotype RLD1 or Ws-0; **+**: expression of induced resistance; -: no expression of induced resistance.

[b] Chapter 2; Ton et al., 1999.

[c] Van Wees et al., 1997.

was not reduced in either *ISR1* genotype Col-0 or *isr1* genotype RLD1 upon treatment with WCS417r (Figure 1G). This supports our previous conclusion that WCS417r-mediated ISR, and JA/ethylene-dependent defense responses in general, are ineffective against this pathogen (Chapter 5). As summarized in Table 1, our results clearly demonstrate that the *ISR1* locus plays a key role in WCS417r-mediated ISR against various plant pathogens, including oomycetes, fungi, and bacteria. Because the *ISR1* locus is involved in ethylene signaling (Chapter 3; Ton et al., 2001), this indicates that intact responsiveness to ethylene is an important prerequisite for the broad-spectrum resistance conferred by WCS417r-mediated ISR.

Expression of SAR against all pathogens tested was unaffected in both the *isr1* and the *ISR1* genotypes (Figure 1A-1G). Additionally, Van Wees et al. (1997) reported that SAR induced by exogenous application of SA was fully effective against *F. oxysporum* f.sp. *raphani* in RLD1 plants. It can thus be concluded that the *ISR1* locus plays no significant role in the signaling pathway and expression of SAR against the various pathogens (Table 1), and that responsiveness to ethylene is not essential for the establishment of SAR. The latter agrees with previous findings by Lawton et al. (1994; 1995), who demonstrated that the ethylene-insensitive Arabidopsis mutants *etr1-1* and *ein2-1* display normal SAR gene expression and develop normal levels of SAR against *P. parasitica* after treatment with the SAR-inducing chemicals SA and INA.

Materials and Methods

Cultivation of plants

Arabidopsis thaliana ecotype Columbia (Col-0), carrying the dominant alleles at the *ISR1* locus (*ISR1* genotype; Ton et al., 1999), and ecotypes RLD1 and Wassilewskija (Ws-0), carrying the recessive alleles (*isr1* genotypes), were used. Seeds were sown in quartz sand. After two weeks, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that had been autoclaved twice for 20 min. Plants were cultivated in a growth chamber with a 9-h day (≈200 $\mu E.m^{-2}.sec^{-1}$ at 24 °C) and 15-h night (20 °C) cycle and 65% RH, as described previously (Chapter 2; Ton et al., 1999).

Cultivation of rhizobacteria and pathogens

For treatment of the roots with ISR-inducing rhizobacteria, rifampicin-resistant *Pseudomonas fluorescens* strain WCS417r (Pieterse et al., 1996) was grown on King's medium B agar plates (King et al., 1954) for 24 h at 28 °C. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 colony-forming units (CFU) per mL. For induction of SAR, *Pseudomonas syringae* pv. *tomato* strain DC3000, carrying the avirulence gene *avrRpt2* (Kunkel et al., 1993), was cultured overnight at 28 °C in liquid King's medium B with 25 $mg.L^{-1}$ kanamycin to select

for the plasmid. Subsequently, bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a final density of 10^7 CFU.mL^{-1}. For challenge inoculations, virulent *P. syringae* pv. *tomato* strain DC3000 (Whalen et al., 1991) was cultured overnight in liquid King's medium B at 28 °C. After centrifugation, bacterial cells were resuspended to a final density of 10^7 CFU.mL^{-1} in 10 mM $MgSO_4$ containing 0.015% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). A rifampicin-resistant derivative of *Xanthomonas campestris* pv. *armoraciae* (Sahin and Miller, 1996) was cultured overnight in liquid 0.8% Nutrient Broth medium (Difco, Detroit, USA) at 28 °C, collected by centrifugation, and resuspended in 10 mM $MgSO_4$ with 0.015% (v/v) Silwet L-77 to a final density of 5 x 10^7 CFU.mL^{-1}. *Peronospora parasitica* strain WACO9 was obtained from the Plant Research Institute, Wageningen, The Netherlands. The pathogen was maintained on susceptible Col-0 or Ws-0 plants as described by Koch and Slusarenko (1990). Conidiospores were collected by immersion of heavy-sporulating leaves in 10 mM $MgSO_4$. After centrifugation at 1400 *g* for 2 min, the spores were resuspended in 10 mM $MgSO_4$ to a final density of 5x10^4 conidiospores per mL. Turnip crinkle virus (TCV) RNA was produced by in vitro transcription from plasmid pT7TCV66 as described by Oh et al. (1995), and adjusted to a final concentration of 0.1 μg.μL^{-1}.

Induction treatments

ISR was induced by transplanting 2-week-old Arabidopsis seedlings into the autoclaved sand/potting soil mixture through which WCS417r bacteria were thoroughly mixed to a final density of 5 x 10^7 CFU.g^{-1}. Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. Induction of SAR was performed 3 days before challenge inoculation, either biologically by pressure-infiltrating two lower leaves with a suspension of avirulent *P. syringae* pv. *tomato* DC3000(*avrRpt2*) at 10^7 CFU.mL^{-1}, or chemically by spraying the plants with 0.1 mM 2,6-dichloroisonicotinic acid (INA; Novartis, Basel, Switzerland).

***P. syringae* pv. *tomato* DC3000 and *X. campestris* pv. *armoraciae* bioassays**

Five-week-old plants were challenge inoculated by dipping the leaves into a suspension of either virulent *P. syringae* pv. *tomato* DC3000 at 10^7 CFU.mL^{-1} (Arabidopsis ecotypes Col-0 and Ws-0), or *X. campestris* pv. *armoraciae* at 5 x 10^7 CFU.mL^{-1} (ecotypes Col-0 and RLD1) in 10 mM $MgSO_4$ with 0.015% (v/v) Silwet L-77. Three days after challenge inoculation, the percentage of leaves with symptoms was determined per plant (n = 20). Leaves showing necrotic or water-soaked lesions surrounded by chlorosis, were scored as diseased. Bacterial growth was determined in replicate leaf samples from five plants per treatment. Immediately after challenge inoculation and three days later, the leaf samples were collected, weighed, and homogenized in 10 mM $MgSO_4$. Serial dilutions were plated on selective King's medium B agar plates (*P. syringae* pv. *tomato* DC3000) or Nutrient Broth agar plates (*X. campestris* pv. *armoraciae*), supplemented with 100 mg.L^{-1} cycloheximide and 50 mg.L^{-1} rifampicin. After incubation at 28 °C for 2 days, the number of rifampicin-resistant CFU per g of infected leaf tissue was determined, and bacterial proliferation over the 3-day time interval was calculated.

P. parasitica bioassays

Three-week-old Arabidopsis Col-0 and Ws-0 plants were challenge inoculated with *P. parasitica* WACO9 by applying 3-µL droplets of a conidiospore suspension containing 5 x 10^4 conidiospores per mL to individual leaves. Inoculated plants were maintained at 17 °C and 100% RH. Disease incidence was quantified either by determining the disease severity or by counting the number of conidiospores per g of infected leaf tissue at 11 days after challenge. Disease severity ratings were expressed on the basis of symptom severity and pathogen sporulation on each leaf: I, no sporulation; II, <50% of the leaf area covered by sporangia; III, >50% of the leaf area covered by sporangia; IV, leaves heavily covered with sporangia, with additional chlorosis and leaf collapse. Conidiospores were collected by immersing the leaves in 10 mM $MgSO_4$ and centrifugation at 1400 *g* for 2 min. Subsequently, the conidiospores were resuspended in 10 mM $MgSO_4$ and the number of conidiospores was determined in an haemocytometer.

TCV bioassays

Four-week-old Col-0 and RLD1 plants were challenge inoculated with TCV by applying 3 µL- droplets of viral RNA suspension (0.1 $\mu g.\mu L^{-1}$) in bentonite buffer (0.02 g of bentonite per mL of 0.05 M glycine, 0.03 M K_2HPO_4) on three lower leaves. The droplets were then rubbed across the leaf surface with a glass rod and the treated leaves were marked. At 14 days after challenge inoculation, the percentage of non-inoculated leaves with symptoms was determined per plant (n = 20). Newly developed leaves showing crinkled deformation and chlorotic sites around the vascular bundles were scored as diseased. Subsequently, systemically infected leaves of five representative plants per treatment were collected for RNA gel blot analysis of viral RNA accumulation.

RNA gel blot analysis

Total RNA was extracted from control and WCS417r-treated Col-0 and RLD1 plants 14 days after challenge with TCV, as described by Linthorst et al. (1993). Subsequently, RNA gel blots were prepared and hybridized with random primer-labeled DNA probes for detection of TCV RNA and β-tubulin, as described previously (Chapter 3; Ton et al., 2001). Probes for the detection of viral RNA were derived from the plasmid pT7TCV66, containing the full-length cDNA clone of TCV (Oh et al., 1995). Probes for detection of β-tubulin transcripts (*Tub*) were prepared by PCR with primers based on the sequence of GenBank accession no. M21415.

CHAPTER 7

General Discussion

Rhizobacteria-mediated ISR in Arabidopsis

Plants have the ability to develop an enhanced defensive capacity upon stimulation by pathogenic or non-pathogenic microorganisms. This induced disease resistance is generally expressed as a restriction of pathogen growth and a reduction of symptom development (Hammerschmidt, 1999). The signaling pathways controlling pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR) are relatively well studied. Pathogen-induced SAR is controlled by a signaling pathway that depends on endogenous accumulation of salicylic acid (SA), and is associated with the accumulation of pathogenesis-related proteins (PRs; Ryals et al., 1996; Sticher et al., 1997; Van Loon, 1997). In some cases, rhizobacteria have been shown to activate the SAR pathway by producing SA at the root surface (Maurhofer et al., 1994; 1998; De Meyer et al., 1997; 1999b). However, in Arabidopsis, ISR triggered by *Pseudomonas fluorescens* WCS417r functions independently of SA and, instead, requires intact responsiveness to the plant hormones jasmonic acid (JA) and ethylene (Pieterse et al., 1996; 1998). Despite these differences, the SAR and the ISR pathway are both controlled by the regulatory protein NPR1 (Cao et al., 1994; Delaney et al., 1995; Pieterse et al., 1998). Because ISR is not accompanied by transcriptional activation of *PR* genes (Pieterse et al., 1996; Van Wees et al., 1997), both pathways must diverge downstream of NPR1. This indicates that NPR1 differentially regulates defense responses depending on the pathway that is activated upstream of it (Pieterse et al., 1998). Based on the apparent differences in signal transduction between SAR and ISR, the research described in this thesis was mainly focussed on elucidating the following questions:

- What is the genetic and physiological basis of non-inducibility of ISR in specific ecotypes of Arabidopsis?
- Do pathogen-induced SAR and WCS417r-mediated ISR act differently against different pathogens?

Approaches to identify novel genes contributing to rhizobacteria-mediated ISR

Over the past years, several approaches have been initiated to identify ISR-related gene expression. The expression pattern of a large set of known, well-characterized defense-related genes of Arabidopsis was analyzed upon induction of WCS417r-mediated ISR. None of these defense-related genes were upregulated in roots or leaves of ISR-expressing plants (Van Wees et al., 1999). Furthermore, a differential screening from a cDNA library representing

mRNAs of ISR-expressing leaves did not result in the identification of genes that were upregulated consistently upon induction of ISR (Van Wees, 1999; C.M.J. Pieterse, unpublished data). Thus, unlike SAR, WCS417r-mediated ISR in Arabidopsis is not associated with major changes in defense-related gene expression. In an alternative approach, a collection of Arabidopsis *Ds*-transposon enhancer trap lines containing the β-glucuronidase (GUS) reporter gene was screened for promotors that are differentially activated in induced and non-induced seedlings. Out of the 700 lines screened, only one line showed enhanced GUS activity in the roots upon colonization by WCS417r bacteria. Interestingly, this enhancer trap line also showed increased GUS activity in the roots in response to treatment with the ethylene precursor 1-aminopropane-1-carboxylate (ACC), indicating that the *Ds*-transposon is in the vicinity of an ethylene-responsive promotor (Leon-Kloosterziel et al., 2000). cDNA microarray analysis offers further potential for identifying changes in gene expression upon elicitation of WCS417r-mediated ISR.

In this thesis, we took a genetic approach in order to identify novel traits involved in WCS417r-mediated ISR in Arabidopsis. This genetic analysis was based on the naturally occurring variation among Arabidopsis ecotypes in the ability to express rhizobacteria-mediated ISR, as discovered previously by Van Wees et al. (1997). In an alternative approach, we tested a collection of enhanced disease susceptibility mutants (Glazebrook et al., 1996; Volko et al., 1998) for their ability to express WCS417r-mediated ISR and pathogen-induced SAR.

Identification of an ISR-related locus using naturally occurring variation of ISR inducibility.

Van Wees et al. (1997) demonstrated that the capacity to express WCS417r-mediated ISR in *Arabidopsis* is ecotype-dependent. Ecotypes Columbia (Col-0) and Landsberg *erecta (Ler*) were responsive to induction of ISR by WCS417r, whereas ecotype RLD1 was not. Such dependency on the plant genotype is not unique. In carnation, WCS417r-mediated ISR against Fusarium wilt was considerably more effective in the moderately resistant cultivar Pallas than in the susceptible cultivar Lena. (Van Peer et al., 1991). Similarly, in cucumber it was found that out of three cultivars tested, two cultivars expressed rhizobacteria-mediated ISR, whereas a third did not (Liu et al., 1995).

In Chapter 2, seven more Arabidopsis ecotypes were tested for their ability to express both WCS417r-mediated ISR and pathogen-induced SAR. This screen revealed that not only ecotype RLD1, but also ecotype Wassilewskija (Ws-0) was unable to express ISR, whereas none of the ecotypes were impaired in pathogen-induced SAR. Ecotypes RLD1 and Ws-0 also stood out by showing a remarkably high level of susceptibility to the challenging pathogen *P. syringae* pv. *tomato* DC3000. A genetic analysis of crosses between ISR-inducible and ISR-noninducible ecotypes revealed that RLD1 and Ws-0 are both affected in a single

dominant locus, designated *ISR1*, that was mapped to chromosome III (Chapter 2; Ton et al., 1999). Thus, the naturally occurring variation in both ISR-inducibility and basal resistance was based on differences at the *ISR1* locus.

Although ecotypes RLD1 and Ws-0 also failed to express WCS417r-mediated ISR against *Xanthomonas campestris* pv. *armoraciae* and *Peronospora parasitica*, they did not show reduced levels of basal resistance against these pathogens. Instead, they exhibited elevated levels of basal resistance against these two pathogens compared to the ISR-inducible ecotype Col-0 (Chapter 6). Furthermore, ecotype RLD1 harbors a remarkably high number of race-specific resistance genes against different races of *Peronospora* pathogens (Bittnereddy et al., 1999; 2000; Joos et al., 1996; Kunkel, 1996). Our seemingly contradicting finding that ecotype RLD1 with its reduced sensitivity to ethylene (Chapter 3) is relatively resistant against *X. campestris* pv. *armoracia* (Chapter 6), whereas the ethylene-insensitive mutant *ein2-1* exhibits enhanced susceptibility to this pathogen (Chapter 5), may be explained by (a) gene(s) conferring partial resistance in an ethylene-independent manner. Thus, the impaired ISR response against different pathogens associated with the reduced basal resistance against *P. syringae* pv. *tomato* DC3000 could be compensated by genes conferring either horizontal or vertical resistance against other pathogens. The situation is more complex, however, because RLD1 and Ws-0 can still enhance their defensive capacity through the expression of SAR, even though they have lost their ability to express ISR (Chapter 6). These results indicate that generalizations as to the effectiveness of plant defenses against different pathogens cannot be made.

Specific genes contributing to basal and induced resistance are involved in SA, JA, or ethylene signaling.

Previous analyses of mutant and transgenic plants revealed that the capacity of a plant to develop induced resistance is associated with a certain level of basal resistance. For instance, SA-nonaccumulating NahG plants are affected in the expression of pathogen-induced SAR (Gaffney et al., 1993; Lawton et al., 1995), and at the same time exhibit enhanced disease susceptibility to a variety of pathogens (Delaney et al., 1994). Similarly, Arabidopsis plants mutated in the *Npr1* gene are not only blocked in the expression of pathogen-induced SAR (Cao et al., 1994; Delaney et al., 1995) and WCS417r-mediated ISR (Pieterse et al., 1998), but are also affected in basal resistance against *P. syringae* and *P. parasitica* (Delaney et al., 1995; Glazebrook et al., 1996). In addition, the JA-insensitive *jar1-1* mutant and the ethylene-insensitive *etr1-1* mutant were affected in the expression of WCS417r-mediated ISR (Pieterse et al., 1998), and concurrently allowed 10-fold higher levels of growth of *P. syringae* pv. *tomato* DC3000 than wild-type plants upon primary infection.

Phenotypically, mutants *jar1-1* and *etr1-1* strongly resemble the *isr1* phenotype of ecotypes RLD1 and Ws-0. Therefore, we investigated the possibility that ecotypes RLD1

and Ws-0 are impaired in either JA or ethylene signaling. As described in Chapter 3, both ecotypes were less responsive to ethylene: not only the triple response and ethylene-inducible gene expression were reduced in RLD1 and Ws-0, but also ethylene-induced resistance against *P. syringae* pv. *tomato* DC3000 was abolished in both ecotypes. Moreover, analysis of the F_2 and F_3 progeny of a cross between Col-0 (*ISR1/ISR1*) and RLD1 (*isr1/isr1*) revealed that this reduced sensitivity to ethylene co-segregated with the recessive alleles of the *ISR1* locus, indicating that the *ISR1* locus is involved in ethylene signaling. Thus, the observed association between ISR-noninducibility and reduced basal resistance against *P. syringae* can be attributed to a reduced sensitivity to ethylene.

Because of the association between induced resistance and basal resistance among the Arabidopsis ecotypes, a collection of enhanced disease susceptibility mutants with reduced basal resistance against *P. syringae* was tested for WCS417r-mediated ISR and pathogen-induced SAR (Chapter 4). Out of 11 *eds* mutants tested, three mutants (*eds4*, *eds8* and *eds10*) were impaired in the ability to express ISR, whereas two others (*eds5* and *eds12*) did not display pathogen-induced SAR. Further characterization of the ISR-impaired *eds* mutants showed that the *Eds8* gene is involved in JA signaling, whereas the *Eds4* gene is involved in ethylene signaling. The involvement of the *Eds10* gene was located downstream of JA and ethylene signaling. The SAR-impaired mutants were found to be blocked in either SA signaling (*eds12*), or SA biosynthesis (*eds5/sid1*; Nawrath and Métraux, 1999). Together, these results not only confirm the dual involvement of JA, ethylene and SA in induced resistance and basal resistance, but they also demonstrate that *P. syringae* is resisted through a combined action of JA-, ethylene-, and SA-dependent basal resistance.

Induced resistance: an enhancement of SA-dependent or JA/ethylene-dependent basal resistance.

Over the past years, plant genotypes affected in SA, JA, or ethylene signaling have been linked repeatedly to enhanced disease susceptibility to specific pathogens and even insects (Delaney et al., 1994; Knoester et al., 1998; McConn et al., 1997; Staswick et al., 1998; Vijayan et al., 1998). Evidence is accumulating that SA, JA, and ethylene-dependent defenses contribute to basal resistance against different pathogens. For instance, JA- and ethylene-insensitive Arabidopsis genotypes exhibit enhanced susceptibility to necrotrophic pathogens, i.e. *Alternaria brassicola* and *Botrytis cinerea*, indicating that basal resistance against these pathogens is, at least in part, conferred by JA- and ethylene-dependent defenses. Conversely, genotypes impaired in SA accumulation exhibit enhanced susceptibility to predominantly biotrophic pathogens, i.e. *Peronospora parasitica* and TCV (Kachroo et al., 2000; Nawrath and Métraux, 1999; Thomma et al., 1998), indicating that these pathogens are predominantly

resisted through SA-dependent defenses.

As demonstrated in chapter 5, the fungal pathogen *A. brassicicola*, which is resisted through JA/ethylene-dependent basal defenses, was inhibited considerably in plants expressing WCS417r-mediated ISR, whereas expression of SAR was ineffective against this pathogen. Conversely, *P. parasitica* and TCV, which are both resisted through predominantly SA-dependent basal defenses, were strongly inhibited by the expression of SAR, while ISR yielded only weak and no protection, respectively. Thus, ISR is predominantly effective against pathogens that are resisted through JA/ethylene-dependent basal defenses, and SAR is more effective against pathogens that are resisted through SA-dependent basal defenses. In chapters 3 and 4, we showed that Arabidopsis genotypes affected in JA/ethylene-dependent basal resistance against *P. syringae* are impaired in WCS417r-mediated ISR, whereas genotypes affected in SA-dependent basal resistance against *P. syringae* are impaired in pathogen-induced SAR. In accordance with the earlier notion that induced disease resistance is an enhancement of genetically determined basal resistance (Van Loon et al., 1997; Chapter 1), these results strongly suggest that WCS417r-mediated ISR involves an enhancement of JA- and ethylene dependent basal resistance, whereas SAR constitutes an enhancement of SA-dependent basal resistance. Consequently, pathogens such as *P. syringae* and *X. campestris*, which are resisted through a combined action of SA-dependent and JA/ethylene-dependent basal defenses, are sensitive to both SAR and ISR (Pieterse et al., 1998; Chapters 4 and 5).

Induced resistance: a potentiated expression of SA-dependent or JA/ethylene-dependent basal resistance?

The enhanced defensive capacity of plants expressing induced resistance is either based on biochemical changes in response to the resistance-inducing treatment, or on mechanisms that are expressed only after pathogen challenge of the induced tissues. In the case of SAR, accumulation of PRs is an example of a mechanism that is triggered upon the inducing treatment. However, the contribution of PRs to induced resistance remains uncertain (Van Loon, 1997). PRs may contribute to resistance against oomycetes, fungi, or bacteria by their hydrolytic action on pathogen cell walls, but it is difficult to envisage a function in viral resistance. Despite several attempts in the case of WCS417r-mediated ISR, metabolic changes before challenge inoculation with a pathogen have not been identified. This suggests that the enhanced defensive capacity of plants expressing induced resistance is to a large extent based on increased post-challenge defense responses. Indeed, examples of potentiated expression of defense mechanisms have been reported for both SAR and ISR. For instance, upon infection with *Colletotrichum lagenarium* cucumber plants expressing pathogen-induced SAR developed significantly more papillae at the sites of appressorium formation than non-induced plants (Kovats et al., 1991). Similarly, challenge-inoculated carnation plants expressing rhizobacteria-mediated ISR against *Fusarium oxysporum* f.sp.

dianthi, accumulated phytoalexins earlier and to a greater extent than non-induced plants (Van Peer et al., 1991). In both examples, the induced resistance appeared as a faster and stronger expression of defense mechanisms that also contributed to the basal resistance of non-induced plants.

Interestingly, SA, JA, and ethylene have all been implicated in the regulation of potentiation of defense responses. For instance, parsley cells pretreated with either JA, SA, or its functional analogues, showed potentiated accumulation of active oxygen species, secretion of cell wall phenolics, accumulation of coumarin phytoalexins, and *Pal* gene expression upon treatment with the *Pmg* elicitor of *Phytophthora megasperma* f.sp. *glycinea* (Katz et al., 1998; Kauss et al., 1992; 1993; 1994; Thulke and Conrath, 1998). Notably, in intact plants these defense responses all contribute to local resistance responses after primary pathogen attack. In tobacco, Mur et al. (1996) demonstrated that SAR-expressing plants showed potentiated *PR-10* and *Pal* gene expression upon infection with different pathogenic pseudomonads. In Arabidopsis, Lawton et al. (1994) showed that plants pre-exposed to ethylene were sensitized to SA-induced *PR-1* gene expression, suggesting that ethylene potentiates defense mechanisms that contribute to SAR. Indeed, ethylene-insensitive tobacco plants expressing the mutant *etr1-1* gene of Arabidopsis showed a reduced SAR response (Knoester, 1998). Nevertheless, various ethylene-insensitive mutants of Arabidopsis were unaffected in their SAR response (Lawton et al., 1994; 1995; Knoester et al., 1999). Interestingly, upon challenge inoculation with *P. syringae* pv. *tomato*, SAR-expressing Arabidopsis plants showed a potentiated expression of SA-inducible *PR* genes (Cameron et al., 1999; Van Wees et al., 1999), whereas ISR-expressing Arabidopsis plants displayed a potentiated expression of the JA-inducible *Atvsp* gene (Van Wees et al., 1999). These results clearly indicate that both types of induced resistance are associated with potentiation of different defense responses. Therefore, it is tempting to speculate that SAR is achieved through a potentiated expression of SA-dependent basal defenses, whereas WCS417r-mediated ISR is achieved through a potentiated expression of JA/ethylene-dependent basal resistance. A model is schematically represented in Figure 1.

Combining SAR and ISR as a method to improve biocontrol of plant diseases

Van Wees et al. (2000) demonstrated that simultaneous activation of the ISR and the SAR pathway results in an enhanced level of induced protection against *P. syringae* pv. *tomato*. This indicates that the JA/ethylene-dependent ISR pathway and the SA-dependent SAR pathway act independently and additively on the level of protection against this pathogen. In this thesis we demonstrated that besides *P. syringae* pv. *tomato* DC3000, also *X. campestris* pv. *armoraciae*

is resisted through a combined action of JA/ethylene-dependent and SA-dependent defense pathways (Chapters 4 and 5). Therefore, one can predict that simultaneous activation of SAR and ISR will result in an enhanced level of protection against *X. campestris* pv. *armoraciae* as well. We provided evidence that SAR and ISR confer differential protection against different types of pathogens (Chapter 5). Thus, combining SAR and ISR can protect the plant against a complementary spectrum of pathogens, and can even result in an additive level of induced protection against pathogens that are resisted through both the JA/ethylene- and the SA-dependent pathways.

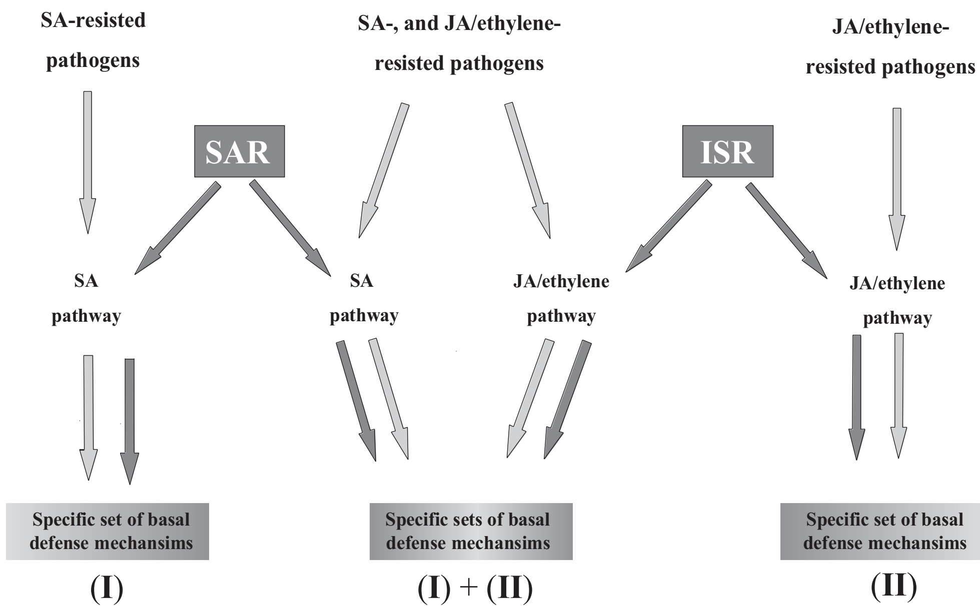

Figure 1: Model explaining SAR and ISR as a potentiated expression of basal defense mechanisms.
SA-dependent basal defense mechanisms (**I**) are potentiated in SAR-induced plants. Consequently, infection of SAR-expressing tissue triggers a faster and stronger activation of SA-dependent defense mechanisms, resulting in an effective protection against pathogens that are resisted through SA-dependent basal resistance, i.e. *P. parasitica* and TCV. Conversely, pathogen infection of plants pretreated with ISR-inducing WCS417r bacteria results in potentiation of JA/ethylene-dependent basal defense mechanisms (**II**). Accordingly, ISR-expressing tissues show a faster and stronger expression of JA/ethylene-dependent defense mechanisms upon infection, resulting in an effective protection against pathogens that are resisted through JA/ethylene-dependent basal resistance, i.e. *A. brassicicola*. Pathogens that are resisted through a combination of SA- and JA/ethylene-dependent basal resistance, i.e. *P. syringae* and *X. campestris*, are sensitive to both SAR and ISR.

Biological control of plant diseases is still in its infancy, because the level of protection and its consistency are generally not sufficient to compete with conventional methods of disease control. One approach to improve the efficacy and consistency of biological control against soil-borne pathogens is to apply combinations of antagonistic micro-organisms with different mechanisms of action (De Boer, 2000). Alternatively, micro-organisms can be engineered to express disease suppressive traits constitutively at high levels. Manipulation of the plants by introducing race-specific *R* genes into plants is an another attractive approach, because it renders the plant completely resistant to a pathogen. However, resistance based on gene-for gene resistance offers protection against only a single pathogen, and the pathogen can overcome the resistance by mutation. Transgenic approaches to engineer durable and broad-spectrum resistance are promising, but are still under development. Our findings that the combination of SAR and ISR confers protection against a complementary spectrum of pathogens and results in enhanced levels of protection against specific bacterial pathogens (Van Wees et al., 1999), offers great potential for integrating both forms of induced resistance in future agricultural practices.

Recently, the chemical plant activator Bion has been introduced, which suppresses plant diseases through BTH-mediated activation of the SAR response (Friedrich et al., 1996; Lawton et al., 1996). Nevertheless, SAR does not protect the plant against necrotrophic pathogens such as *A. brassicicola* and *B. cinerea* (Thomma et al., 1998; Chapter 5). Furthermore, Bion has been reported to reduce plant growth and seed set under field conditions (Heil et al., 2000). In contrast, resistance-inducing rhizobacteria generally improve plant growth under field conditions. This rhizobacteria-mediated growth promotion results mainly from their antagonizing action against soil-borne pathogens and other deleterious micro-organisms (Kloepper et al., 1980; Schippers et al., 1987). Furthermore, resistance-inducing rhizobacteria in general do not solely induce resistance through JA/ethylene-dependent ISR. Some rhizobacteria have also been demonstrated to activate the SAR response by producing SA at the root surface (De Meyer et al., 1997; 1999b; Maurhofer et al., 1994; 1998). Combining ISR-inducing rhizobacteria with SA-producing rhizobacteria would have the advantage that three disease suppressive mechanisms, i.e. antagonism, ISR and SAR, are combined. Therefore, dual application of ISR- and SAR-inducing rhizobacteria offers not only great potential to improve the efficacy and consistency of biological control, but it would also broaden the effectiveness of biological control.

References

Agrios, G.N. (1997). Plant Pathology, 4th Ed., Academic Press Inc., San Diego, USA.

Alonso-Blanco, C., and Koornneef, M. (2000). Naturally occurring variation in Arabidopsis: an underexploited resource for plant genetics. Trends Plant Sci. **5,** 22-29.

Bakker, P.A.H.M., Van Peer, R., and Schippers, B. (1991). Suppression of soil-borne plant pathogens by fluorescent pseudomonads: mechanisms and prospects. *In:* Biotic interactions and soil-borne diseases (Beemster A.B.R. et al. eds.), Elsevier Scientific Publishers, Amsterdam, The Netherlands, Vol. **23,** pp. 217-230.

Bell, E., Creelman, R.A., and Mullet, J.E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc. Natl. Acad. Sci. USA **92,** 8675-8679.

Bent, A.F., Innes, R.W., Ecker, J.R., and Staskawicz, B.J. (1992). Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. Mol. Plant-Microbe Interact. **5,** 372-378.

Bittnereddy, P., Can, C., Gunn, N., Pinel, M., Tor, M., Crute, I.R., Holub, E.B., and Beyon, J.L. (1999). Genetic and physical mapping of the *RPP13* locus in Arabidopsis, responsible for specific recognition of several *Peronospora parasitica* (downy mildew) isolates. Mol. Plant-Microbe Interact. **9,** 792-802.

Bittnereddy, P., Crute, I.R., Holub, E.B., and Beyon, J.L. (2000). *RPP13* is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. Plant J. **21,** 177-188.

Bleecker, A.B., Estelle, M.A., Sommerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science **241**, 1086-1089.

Boller, T. (1991). Ethylene in pathogenesis and disease resistance. *In:* The plant hormone ethylene (Mattoo, A.K., and Suttle, J.D. eds.), CRS press, Boca Raton, USA, pp. 293-314.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Vanderleyden, J. (1990). An automated quantitative assay for fungal growth. FEMS Microb. Lett. **69,** 55-90.

Cameron, R.K., Dixon, R.A., and Lamb, C.J. (1994). Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. Plant J. **5,** 715-725.

Cameron, R.K., Paiva, N.L., Lamb, C.J., and Dixon, R.A. (1999). Accumulation of salicylic acid and *PR-1* gene expression in relation to systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. *tomato* in Arabidopsis. Physiol. Mol. Plant Pathol. **55,** 121-130.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583-1592.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. (1997). The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88,** 57-63.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997).

Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein Ethylene-insensitive3 and related proteins. Cell **89,** 1133-1144.

Ciardi, J.A., Tieman, D.M., Lund, S.T., Jones, J.B., Stall, R.E, and Klee, H.J. (2000). Response to *Xanthomonas campestris* pv. *vesicatoria* in tomato involves regulation of ethylene receptor gene expression. Plant Physiol. **123,** 81-92.

Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. (1998). Uncoupling *PR* gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis *cpr6-1* mutant. Plant Cell **10,** 557-569.

Cohen, Y., and Gisi U. (1994). Systemic translocation of ^{14}C-DL-3-aminobutyric acid in tomato plants in relation to induced resistance against *Phytophthora infestans*. Phys. Mol. Plant Pathol. **45,** 441-456.

De Boer, M. (2000). Combining *Pseudomonas* strains to improve biological control of fusarium wilt in radish. PhD thesis , Utrecht University, The Netherlands.

De Laat, A.M.M., and Van Loon, L.C. (1982). Regulation of ethylene biosynthesis in virus-infected tobacco leaves. II. Time course of levels of intermediates and *in vivo* conversion rates. Plant Physiol. **69**, 240-245.

Delaney, T.P., Friedrich, and Ryals, J. (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced systemic disease resistance. Proc. Natl. Acad. Sci. USA **92,** 6602-6606.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negretto, D., Gaffney, T., Gur-Rella, M., Kesmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247-1250.

De Meyer, G., Audenaert, K., and Höfte, M. (1999a). *Pseudomonas Aeruginosa* 7NSK2-induced systemic resistance in tobacco depends on *in planta* salicylic acid accumulation, but is not associated with *PR-1a* expression. Eur. J. Plant Pathol. **105,** 513-517.

De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Métraux, J.-P., and Höfte, M. (1999b). Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. Mol. Plant-Microbe Interact. **12,** 450-458.

De Meyer, G., and Höfte, M. (1997). Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by *Botrytis cinerea* on bean. Phytopathology **87,** 588-593.

De Meyer, G., and Höfte, M. (1998). Induction of systemic resistance by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 is a salicylic acid dependent phenomenon in tobacco. *In:* Molecular Approaches to Biological Control (Duffy, B.K., Rosenberger, U., and Défago, G., eds.), IOBC/WPRS, Vol. **21(9),** 117-120.

Dempsey, D.A., Pathirana, M.S., Wobbe, K.K., and Klessig, D.F. (1997). Identification of an Arabidopsis locus required for resistance to turnip crinkle virus. Plant J. **11,** 301-311.

Dempsey, D.A., Shah, J., and Klessig, D.F. (1999). Salicylic acid and disease resistance in plants. Crit. Rev. Plant Sci. **18,** 547-575.

Després, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P.R. (2000). The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell **12,** 279-290.

Emery, R.J.N., Reid, D.M., and Chinnappa, C.C. (1996). Phenotypic plasticity of stem elongation in two ecotypes of *Stellaria longipes*: the role of ethylene and response to wind. Plant Cell Environm. **19,** 423-1430.

Erschadi, S., Haberer, G., Schöniger, M., and Torres-Ruiz, R.A. (2000). Estimating genetic diversity of *Arabidopsis thaliana* ecotypes with amplified fragment length polymorphisms (AFLP). Theor. Appl. Genet. **100,** 633-640.

Feinberg, A.P., and Vogelstein, G. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6-13.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. **18,** 265-276.

Feys, B.J.F., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6,** 751-759.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Gut-Rella, M.G., Meier, B., Dincher, S., Staub, T., Métraux, J.-P., Kessmann, H., and Ryals, J. (1996). A benzothiadiazole derivate induces systemic resistance in tobacco. Plant J. **10,** 61-70.

Gaffney, T.P., Friedrich, L., Vernooij, B., Negrotto, D., Neye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261,** 754-756.

Glazebrook J., and Ausubel, F.M. (1994). Isolation of phytoalexin deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc. Natl. Acad. Sci. USA **91,** 8955-8959.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics **143,** 973-982.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol. Cell: **5,** 1003-1012.

Gupta, V., Willits, M.G., and Glazebrook, J. (2000). *Arabidopsis thaliana* EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: evidence for inhibition of jasmonic acid signaling by SA. Mol. Plant-Microbe Interact. **13,** 503-511.

Guzmán, P., and Ecker, J.R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2,** 513-523.

Hammerschmidt, R. (1999). Induced disease resistance: how do induced plants stop pathogens? Physiol. Mol. Plant Pathol. **55,** 77-84.

Hammerschmidt, R., and Kuć, J. (1982). Lignification as a mechanism for induced systemic resistance in cucumber. Physiol. Plant Pathol. **17,** 61-71.

Hammerschmidt, R., and Kuć, J. (1995). Induced resistance to diseases in plants. Kluwer Academic Press, Dordrecht, The Netherlands.

Hammond-Kosack, K.E., and Jones, J.D.G. (1996). Resistance gene-dependent plant defense responses. Plant Cell **8,** 1773-1791.

Heil, M., Hilper, A., Kaiser, W., and Linsenmair, K.E. (2000). Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? J. Ecol. **88,** 645-654.

Hoagland, D.R., and Arnon, D.I. (1938). The water culture method for growing plants without soil. Calif. Agric. Exp. Stn. Bull. **347,** 36-39.

Jackson, A.O., and Taylor, C.B. (1996). Plant-microbe interactions: life and death at the interface. Plant Cell **8,** 1651-1668.

Joos, H.J., Mauch-Mani, B., and Slusarenko, A.J. (1996). Molecular mapping of the Arabidopsis locus *RPP11*, which conditions isolate-specific hypersensitive resistance against downy mildew in ecotype RLD. Theor. Appl. Genet. **92,** 281-284.

Kachroo, P., Yoshioka, K., Shah, J., Dooner, H.K., and Klessig, D.F. (2000). Resistance to turnip crinkle virus in Arabidopsis is regulated by two host genes and is salicylic acid dependent but *NPR1*, ethylene and jasmonate independent. Plant Cell **12,** 677-690.

Katz, V.A., Thulke, O.U., and Conrath, U. (1998). A benzothiadiazole primes parsley cells for augmented elicitation of defense responses. Plant Physiol. **117,** 1333-1339.

Kauss, H., Franke., R, Krause, K., Conrath, U., Jeblick, W., Grimmig, B., and Matern, U. (1993). Conditioning of parsley (*Petroselium crispum*) suspension cells increases elicitor-induced incorporation of cell wall phenolics. Plant Physiol. **102,** 459-466.

Kauss, H, Jeblick, W., Ziegler, J., and Krabler, W. (1994). Pretreatment of parsley (*Petroselinum crispum* L.) suspension cultures with methyl jasmonate enhances elicitation of activated oxygen species. Plant Physiol. **105,** 89-104.

Kauss, H., Theisinger-Hinkel, E., Mindermann, R., and Conrath, U. (1992). Dichloroisonicotinic and salicylic acid, inducers of systemic acquired resistance, enhance fungal elicitor responses in parsley cells. Plant J. **2,** 655-660.

King, E.O., Ward, M.K., and Raney, D.E. (1954). Two simple media for the demonstration of phycocyanin and fluorescin. J. Lab. Clin. Med. **44,** 301-307.

Kloepper, J.W., Leong, J., Teintze, M., and Schroth, M.N. (1980). Enhanced plant growth by siderophores produced by plant-growth-promoting rhizobacteria. Nature **286,** 885-886.

Knoester, M. (1998). The involvement of ethylene in plant disease resistance. PhD thesis, Utrecht University, The Netherlands.

Knoester, M., Pieterse, C.M.J., Bol, J.F., and Van Loon, L.C. (1999). Systemic resistance in *Arabidopsis* induced by rhizobacteria requires ethylene-dependent signaling at the site of application.

Mol. Plant-Microbe Interact. **12,** 720-727.

Knoester, M., Van Loon, L.C., Van den Heuvel, J., Hennig, J., Bol, J.F., and Linthorst, H.J.M. (1998). Ethylene-insensitive tobacco lacks non-host resistance against soil-borne fungi. Proc. Natl. Acad. Sci. USA **95,** 1933-1937.

Koch, E., and Slusarenko, A. (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. Plant Cell **2,** 437-445.

Konieczny, A., and Ausubel, F.M. (1993). A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J. **4,** 403-410.

Kovats, K., Binder, A., and Hohl, H,L. (1991). Cytology of induced systemic resistance of tomato to *Colletotrichum lagenarium*. Planta **183,** 484-490.

Kuć, J. (1982). Induced immunity to plant disease. Bioscience **32,** 854-860.

Kunkel, B.N. (1996). A useful weed put to work: genetic analysis of disease resistance in *Arabidopsis thaliana*. Trends Genet. **12,** 62-69.

Kunkel, B.N., Bent, A., Dahlbeck, D., Innes, R.W., and Staskawicz, B.J. (1993). *RPS2*, an Arabidopsis disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene *avrRpt2*. Plant Cell **5,** 865-875.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., and Ryals, J. (1996). Benzatiodiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. Plant J. **10,** 71-82.

Lawton, K.A., Potter, S.L., Uknes, S., and Ryals, J. (1994). Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell **6,** 581-588.

Lawton, K., Weimann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals J. (1995). Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. Mol. Plant-Microbe Interact. **8,** 863-870.

Leeman, M., Van Pelt, J.A., Den Ouden, F.M., Heinsbroek, M., Bakker, P.A.H.M., and Schippers, B. (1995b). Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. Eur. J. Plant Pathol. **101,** 655-664.

Leeman, M., Van Pelt, J.A., Hendrickx, M.J., Scheffer, R.J., Bakker, P.A.H.M., and Schippers, B. (1995a). Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. Phytopathology **85,** 1021-1027.

Leeman, M., Van Pelt, J.A., Hendrickx, M.J., Scheffer, R.J., Bakker, P.A.H.M., and Schippers, B. (1995c). Biocontrol of fusarium wilt of radish in commercial greenhouse trials by seed treatment with *Pseudomonas fluorescens* WCS374r. Phytopathology **85,** 1301-1305.

Léon-Kloosterziel, K.M., Keurentjes, J.J.B., Verhagen, B.W.M., Pieterse, C.M.J., and Van Loon, L.C. (2000). Identification of genes involved in rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis. *In:* Abstract book of the 11th International Conference on Arabidopsis research, Madison, WI, USA, p. 79.

Li, X., Zhang, Y.L., Clarke J.D., and Dong, X. (1999). Identification and cloning of a negative

regulator of systemic acquired resistance. Cell **98,** 329-339.

Linthorst, H.J.M., Brederode, F.T., Van der Does, C., and Bol, J.F. (1993). Tobacco proteinase inhibitor-I genes are locally, but not systemically induced by stress. Plant Mol. Biol. **21,** 985-992.

Liu, L., Kloepper, J. W., and Tuzun, S. (1995). Induction of systemic resistance in cucumber against angular leaf spot by plant growth-promoting rhizobacteria. Phytopathology **85,** 1064-1068.

Lund, S.T., Stall, R.E., and Klee, J.J. (1998). Ethylene regulates the susceptible response to pathogen infection in tomato. Plant Cell **10,** 371-382.

Lynch, J.M., and Whipps, J.M. (1991). Substrate flow in the rhizosphere. *In*: The rhizosphere and plant growth (Keister D.L., and Cregan, P.B., eds.), Kluwer Academic Press, Dordrecht, The Netherlands, pp. 15-24.

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science **250,** 1002-1004.

Mauch-Mani, B., and Slusarenko, A.J. (1994). Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. Mol. Plant-Microbe Interact. **7,** 378-383.

Maurhofer, M., Hase, C., Meuwly, P., Métraux, J.-P., and Défago, G. (1994). Induction of systemic resistance to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: Influence of the *gacA* gene and pyoverdine production. Phytopathology **84,** 139-146.

Maurhofer, M., Reimmann, C., Schmidli-Sacherer, P., Heeb, S.D., and Défago, G. (1998). Salicylic acid biosynthesis genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. Phytopathology **88,** 678-684.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., and Browse, J. (1997). Jasmonate is essential for insect defense in Arabidopsis. Proc. Natl. Acad. Sci. USA **94,** 5473-5477.

Métraux, J.-P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M. M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990). Increase of salicylic acid at the onset of systemic acquired resistance in cucumber. Science **250,** 1004-1006.

Meyer, J.-M., Azelvandre, P., and Georges, C. (1992). Iron metabolism in *Pseudomonas*: Salicylic acid, a siderophore of *Pseudomonas fluorescens* CHA0. Biofactors **4,** 23-27.

Mur, L.A.J., Naylor, G., Warner, S.A.J., Sugars, F.M., White, R.F., and Draper, J. (1996). Salicylic acid potentiates defence gene expression in tissue exhibiting acquired resistance to pathogen attack. Plant J. **9,** 559-571.

Murphy, A.M., Chivasa, S., Singh, D.P., and Carr, J.P. (1999). Salicylic acid-induced resistance to viruses and other pathogens: a parting of the ways? Trends Plant Sci. **4,** 155-160.

Nawrath, C., and Métraux, J.-P. (1999). Salicylic acid induction-deficient mutants of Arabidopsis express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11,** 1393-1404.

Norman-Setterblad, C., Vidal, S., and Palva, T.E. (2000). Interacting signal pathways control defense gene expression in Arabidopsis in response to cell wall-degrading enzymes from *Erwinia*

carotovora. Mol. Plant-Microbe Interact. **13,** 430-438.

Oh, J.-W., Kong, Q., Song, C., Carpenter, C., and Simon, A.E. (1995). Open reading frames of turnip crinkle virus involved in satellite symptom expression and incompatibility with *Arabidopsis thaliana* ecotype Dijon. Mol. Plant-microbe Interact. **6,** 979-987.

Pallas, J.A., Paiva, N.L., Lamb, C., and Dixon, R.S. (1996). Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. Plant J. **10,** 281-293.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, J.-P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. Plant Cell **8,** 2309-2323.

Penninckx, I.A.M.A., Thomma, B.P.H.J., Buchala, A., Métreaux, J.-P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10,** 2103-2113.

Pieterse, C.M.J., Derksen, A.-M.C.E., Folders, J., and Govers, F. (1994). Expression of the *Phytophthora infestans ipiB* and *ipiO* genes *in planta* and *in vitro*. Mol. Gen. Genet. **244,** 269-277.

Pieterse, C.M.J., and Van Loon, L.C. (1999). Salicylic acid independent plant defence pathways. Trends Plant Sci. **4,** 52-58.

Pieterse, C.M.J., Van Pelt, J.A., Ton, J., Parchmann, S., Mueller, M.J., Buchala, A.J., Métraux, J.-P., and Van Loon, L.C. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol. Mol. Plant Pathol. **57**, 123-134.

Pieterse, C.M.J., Van Wees, S.C.M., Hoffland, E., Van Pelt, J.A., and Van Loon, L.C. (1996). Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell **8,** 1225-1237.

Pieterse, C.M.J., Van Wees S.C.M., Ton J., Léon-Kloosterziel, K.M., Van Pelt, J.A., Keurentjes, J.J.B., Knoester M., and Van Loon L.C. (1999). Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis: involvement of jasmonate and ethylene. *In*: Biology of Plant-Microbe Interactions (De Wit, P.J.G.M., Bisseling, T., Stiekema, W.J., eds.), International society for molecular plant-microbe interactions, St. Paul, MN, USA, Vol. **2,** pp. 291-296.

Pieterse, C.M.J., Van Wees, S.C.M., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., and Van Loon, L.C. (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell **10,** 1571-1580.

Potter, S., Uknes, S., Lawton, K., Winter, A.M., Chandler, D., DiMaio, J., Novitzky, R., Ward, E., and Ryals, J. (1993). Regulation of a hevein-like gene in Arabidopsis. Mol. Plant-Microbe Interact. **6,** 680-685.

Press, C.M., Wilson, M., Tuzun, S., and Kloepper, J.W. (1997). Salicylic acid produced by *Serratia marcerescens* 91-166 is not the primary determinant of induced systemic resistance in cucumber or

tobacco. Mol. Plant-Microbe Interact. **10,** 761-768.

Raaijmakers, J.M., Leeman, M., Van Oorschot, M.M.P., Van de Sluis, I., Schippers, B., and Bakker, P.A.H.M. (1995). Dose-response relationships in biological control of fusarium wilt of radish by *Pseudomonas* spp. Phytopathology **85,** 1075-1081.

Rogers, E.E., and Ausubel, F.M. (1997). Arabidopsis enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. Plant Cell **9,** 305-316.

Roman, G., Lubarsky, B., Kieber, J.J., Rothenberg, M., and Ecker, J.R. (1995). Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway. Genetics **139,** 1393-1409

Ross, A.F. (1961). Systemic acquired resistance induced by localized virus infections in plants. Virology **14,** 340-358.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H., and Hunt, M.D. (1996). Systemic acquired resistance. Plant Cell **8,** 1809-1819.

Ryals, J.A., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.-Y., Johnson, J., Delaney, T.P., Jesse, T., Vos, P., and Uknes, S. (1997). The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor IκB. Plant Cell **9,** 425-439.

Sahin F., and Miller, S.A. (1996). Occurrence and identification of a bacterial spot disease of radish in Ohio. *In*: Abstract SAPS/MSA Joint Annual Meeting, July 27-31, 1996, Indianapolis, Indiana, USA, p. 79.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schippers, B., Bakker, A.W., and Bakker, P.A.H.M. (1987). Interactions of deleterious and beneficial rhizosphere micoorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. **115,** 339-358.

Shah, J., Tsui, F., and Klessig, D.F. (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Mol. Plant-Microbe Interact. **10,** 69-78.

Shulaev, V., Leon, J., and Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell **7,** 1691-1701.

Simon, A.E., Li, X.H., Lew, J.E., Stange, R., Zhang, C., Polacco, M., and Carpenter, C.D. (1992). Susceptibility and resistance of *Arabidopsis thaliana* to turnip crinkle virus. Mol. Plant-Microbe Interact. **5,** 496-503.

Smith-Becker, J., Marois, E., Huguet, E.J., Midland, S.L., Sims, J., and Keen, N.T. (1998). Accumulation of salicylic acid and 4-hydroxybenzoic acid in phloem fluids of cucumber during systemic acquired resistance is preceded by a transient increase in phenylalanine ammonia-lyase activity in petioles and stems. Plant Physiol. **116,** 231-238.

Staswick, P.E., Yuen, G.Y., and Lehman, C.C. (1992). Methyl jasmonate inhibition of root

growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc. Natl. Acad. Sci. USA **89,** 6837-6840.

Staswick, P.E., Yuen, G.Y., and Lehman, C.C. (1998). Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus *Pythium irregulare*. Plant J. **15,** 747-754.

Sticher, L., Mauch-Mani, B., and Métraux, J.-P. (1997). Systemic acquired resistance. Annu. Rev. Phytopathol. **35,** 235-270.

Stout, M.J., Fidantsef, A.L., Duffey, S.S., and Bostock R.M. (1999). Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. Physiol. Mol. Plant Pathol. **54,** 115-130.

Thomma, B.P.H.J., Eggermont, K., Broekaert, W.F., and Cammue, B.P.A. (2000). Disease development of several fungi on Arabidopsis can be reduced by treatment with methyl jasmonate. Plant. Phys. Biochem. **38,** 421-427.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA **95,** 15107-15111.

Thomma, B.P.H.J., Eggermont, K., Tierens, F.M.-J., and Broekaert, W.F. (1999a). Requirement of functional Ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol. **121,** 1093-1101.

Thomma, B.P.H.J., Nelissen, I., Eggermont, K., and Broekaert, W.F. (1999b). Defiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J. **19,** 163-171.

Thulke O.U., and Conrath U. (1998). Salicylic acid has a dual role in the activation of defense-related genes in parsley Plant J. **14,** 35-42.

Ton, J., Davison, S., Van Pelt, J.A., Van Loon L.C., and Pieterse C.M.J. (2001). The *ISR1* locus of Arabidopsis controlling induced systemic resistance is involved in ethylene signaling. Plant Physiol. **125**, 652-661.

Ton, J., Pieterse, C.M.J., and Van Loon, L.C. (1999). Identification of a locus in Arabidopsis controlling both the expression of rhizobacteria-mediated induced systemic resistance (ISR) and basal resistance against *Pseudomonas syringae* pv. *tomato*. Mol. Plant-Microbe Interact. **12**, 911-918.

Tuzun, S., and Kloepper, J. (1995). Practical application and implementation of induced resistance. *In:* Induced resistance to diseases in plants (Hammerschmidt, R., and Kuć, J. eds.), Kluwer Academic Press, Dordrecht, The Netherlands, pp. 152-168.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S.C., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals J. (1992). Acquired resistance in Arabidopsis. Plant Cell **4,** 645-656.

Uknes, S., Winter, A.M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter S., Ward, E., and Ryals, J. (1993). Biological induction of systemic acquired resistance in

Arabidopsis. Mol. Plant-Microbe Interact. **6,** 692-698.

Van Loon, L.C. (1985). Pathogenesis-related proteins. Plant Mol. Biol. **4,** 111-116.

Van Loon, L.C. (1997). Induced resistance in plants and the role of pathogenesis-related proteins. Eur. J. Plant Pathol. **103,** 753-765.

Van Loon, L.C., Bakker, P.A.H.M., and Pieterse, C.M.J. (1998). Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. **36,** 453-485.

Van Peer, R. (1990). Microbial interactions and plant responses in soilless cultures – Root colonization by pseudomonads: mechanisms, plant responses and effects on fusarium wilt. PhD thesis, Utrecht University, The Netherlands.

Van Peer, R., Niemann, G. J., and Schippers, B. (1991). Induced resistance and phytoalexin accumulation in biological control of fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. Phytopathology **91,** 728-734.

Van Peer, R., and Schippers, B. (1992). Lipopolysaccharides of plant growth-promoting *Pseudomonas* sp. strain WCS417r induce resistance in carnation to fusarium wilt. Neth. J. Plant Pathol. **98,** 129-139.

Van Wees, S.C.M. (1999). Rhizobacteria-mediated induced systemic resistance in Arabidopsis – signal transduction and expression. PhD thesis, Utrecht University, The Netherlands.

Van Wees, S.C.M., De Swart, E.A.M., Van Pelt, J.A., Van Loon, L.C., and Pieterse, C.M.J. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **95,** 1933-1937.

Van Wees, S.C.M., Luijendijk, M., Smoorenburg, I., Van Loon, L.C. and Pieterse, C.M.J. (1999). Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on known defense-genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. Plant Mol. Biol. **41,** 537-549.

Van Wees, S.C.M., Pieterse, C.M.J., Trijssenaar, A., Van 't Westende, Y.A.M., Hartog, F., and Van Loon, L.C. (1997). Differential induction of systemic resistance in Arabidopsis by biocontrol bacteria. Mol. Plant-Microbe Interact. **10,** 716-724.

Vernooij, B., Friedrich, L., Ahl Goy, P., Staub, T., Kessmann, H., and Ryals, J. (1995). 2,6-Dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. Mol. Plant-Microbe Interact. **8,** 228-234.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6,** 959-965.

Visca, P., Ciervo, A., Sanfilippo, V., and Orsi, N. (1993). Iron-regulated salicylate synthesis by *Pseudomonas* spp. J. Gen. Microbiol. **139,** 1995-2001.

Vijayan, P., Shockey, J., Lévesque, C.A., Cook, R.J., and Browse, J. (1998). A role for jasmonate in pathogen defense of Arabidopsis. Proc. Natl. Acad. Sci. USA **95,** 7209-7214.

Voesenek, L.A.C.J., Banga, M., Rijnders, J.G.H.M., Visser, E.J.W., and Blom, C.W.P.M. (1996). Hormone sensitivity and plant adaptations to flooding. Folia Geobot. Phytotax. **31,** 47-56.

Volko, S.M., Boller, T., and Ausubel, F.M. (1998). Isolation of new Arabidopsis mutants with enhanced disease susceptibility to *Pseudomonas syringae* by direct screening. Genetics **149,** 537-548.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3,** 1085-1094.

Wei, G., Kloepper, J.W., and Tuzun, S. (1991). Induction of systemic resistance of cucumber to *Colletrotichum orbiculare* by select strains of plant-growth promoting rhizobacteria. Phytopathology **81,** 1508-1512.

Wei, G., Kloepper, J.W., and Tuzun, S. (1996). Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. Phytopathology **86,** 221-224.

Whalen, M.C., Innes, R.W., Bent, A.F., and Staskawicz, B.J. (1991). Identification of *Pseudomonas syringae* pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. Plant Cell **3**, 49-59.

White, R.F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology **99,** 410-412.

Zhang, Y., Fan, W., Kinkema, M., and Dong, X. (1999). Interaction of NPR1 with basic leucin zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. Proc. Natl. Acad. Sci. USA **96,** 6523-6528.

Zhou, J-M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., and Klessig, D.F. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the *PR-1* gene required for induction by salicylic acid. Mol. Plant-Microbe Interact. **12,** 279-290.

Zimmerli, L., Jakab, G., Métraux, J.-P., and Mauch-Mani, B. (2000). Potentiation of pathogen-specific defense mechanisms in Arabidopsis by β-aminobutyric acid. Proc. Natl. Acad. Sci. USA **97,** 12920-12925.

Summary

During primary pathogen attack, plants activate a diverse array of defense mechanisms at the site of infection. Besides this so-called basal resistance, plants can also enhance their defensive capacity against future pathogen attack. This phenomenon is referred to as induced resistance. There are at least two types of biologically induced resistance. Classic induced resistance results from localized infection by a necrotizing pathogen, leading to a systemic acquired resistance (SAR) in plant parts distant from the site of infection. Alternatively, root colonization by non-pathogenic rhizobacteria can trigger an induced systemic resistance (ISR) response as well. Most ISR-eliciting rhizobacteria belong to the group of the fluorescent *Pseudomonas* spp. Both pathogen-induced SAR and rhizobacteria-mediated ISR are effective against different types of pathogens, and are typically characterized by a restriction of pathogen growth and a suppression of disease development compared to primary infected, non-induced plants. However, the signaling pathways controlling pathogen-induced SAR and rhizobacteria-mediated ISR differ. Whereas SAR requires endogenous accumulation of salicylic acid (SA), the signaling pathway controlling ISR functions independently of SA, and requires intact responsiveness to the plant hormones jasmonic acid (JA) and ethylene. Apart from these differences, SAR and ISR are both dependent on the defense-regulatory protein NPR1. Downstream NPR1 both signaling pathways diverge, because, unlike ISR, SAR is accompanied by a transcriptional activation of genes encoding pathogenesis-related proteins (PRs). Interestingly, tobacco and Arabidopsis genotypes that are impaired in the expression of SAR and/or ISR are often characterized by a reduced level of basal resistance against primary infection. This suggests that there are components that contribute to both induced resistance and basal resistance. In this thesis, the molecular and genetic relationship between induced resistance and basal resistance was investigated.

Previous findings revealed that the Arabidopsis ecotype RLD1 failed to express ISR upon treatment of the roots with *Pseudomonas fluorescens* WCS417r, in contrast to other ecotypes. To further investigate this naturally occurring variation in ISR-inducibility, a collection of ten Arabidopsis ecotypes was tested for their ability to express WCS417r-mediated ISR and pathogen-induced SAR. This screen revealed that, besides ecotype RLD1, also ecotype Ws-0 is impaired in the expression of WCS417r-mediated ISR. The remaining eight ecotypes were normally responsive to ISR induction by WCS417r bacteria. The potential to express SAR after infection with an avirulent strain of the bacterial leaf pathogen *P. syringae* pv. *tomato* DC3000 (*Pst*) was unaffected in all ecotypes tested, providing additional evidence that SAR and ISR constitute different induced resistance responses. Interestingly, ecotypes RLD1 and Ws-0 also displayed a remarkably low level of basal resistance against the virulent strain of *Pst*. Based on this association between ISR-inducibility and basal resistance

against *Pst*, a genetic approach was initiated to identify (a) genetic determinant(s) involved in the regulation of ISR and basal resistance against *Pst*. Analysis of the progeny from crosses between ISR-inducible and ISR-noninducible Arabidopsis ecotypes revealed that both the potential to express ISR and the relatively high basal resistance against *Pst* of the ISR-inducible ecotypes are controlled by a single locus on chromosome III, designated *ISR1*.

The *isr1* phenotype of RLD1 and Ws-0, i.e. non-inducibility of ISR and reduced basal resistance against *Pst*, strikingly resembles the phenotype of the JA- and ethylene insensitive Arabidopsis mutants *jar1-1* and *etr1-1*, respectively. Analysis of the responsiveness of RLD1 and Ws-0 to JA and ethylene revealed that they react normally to JA, but have a reduced sensitivity to ethylene. Compared to the ISR-inducible ecotype Columbia (Col-0), ecotypes RLD1 and Ws-0 exhibited a reduced triple response, a decrease in the expression of ethylene-inducible genes, and no induced resistance against *Pst* after treatment with various concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). Moreover, in the F_3 progeny of a cross between the ISR-inducible ecotype Col-0 and the ISR-noninducible ecotype RLD1, the reduced ethylene sensitivity of the RLD1 parent co-segregated with the recessive alleles of the *ISR1* locus, whereas the unaffected ethylene sensitivity of the Col-0 parent co-segregated with the dominant alleles of the *ISR1* locus. Furthermore, the *ISR1* locus is not allelic with the previously characterized ethylene response gene *Ein3*, which maps to the same region on chromosome III. These results strongly suggest that the *ISR1* locus encodes a novel component of the ethylene response pathway that plays an important role in disease resistance. Further studies on the contribution of the *ISR1* locus to resistance against different pathogens, revealed that it is required not only for the expression of ISR against *Pst*, but also for the expression of ISR against the bacterial leaf pathogen *Xanthomonas campestris* pv. *armoraciae* and the oomycetous pathogen *Peronospora parasitica*. Together with results obtained previously for known ethylene signal-transduction mutants, these observations indicate that a fully functional ethylene response pathway is essential for the expression of ISR against different pathogens.

Based on other observations that plant genotypes impaired in ISR and/or SAR commonly show a reduced level of basal resistance against different pathogens, a collection of Arabidopsis mutants with enhanced disease susceptibility to pathogenic *P. syringae* was screened for the ability to express pathogen-induced SAR and WCS417r-mediated ISR. Out of 11 *eds* mutants tested, three mutants (*eds4*, *eds8* and *eds10*) were affected in the expression of WCS417r-mediated ISR, whereas two mutants (*eds5* and *eds12*) were blocked in the expression of pathogen-induced SAR. Further analysis of the ISR-impaired mutants revealed that *eds8* is disturbed in JA signaling, whereas *eds4-1* is affected in ethylene signaling. Although blocked in rhizobacteria-, MeJA-, and ACC-induced resistance against *Pst*, mutant *eds10* showed normal responsiveness to both methyl jasmonate (MeJA) and ACC, indicating that it harbors a mutation downstream of the perception of ethylene in the ISR signaling pathway.

Whereas *eds5* is known to be blocked in pathogen-inducible accumulation of SA, further analysis of *eds12* revealed that the SAR-impaired phenotype of this mutant is caused by a reduced sensitivity to SA. Thus, the characterization of the collection of *eds* mutants resulted in the identification of three novel components involved in the ISR response (EDS4, EDS8, and EDS10), and one novel component of the SAR response (EDS12). Additionally, these results demonstrate that components contributing to SA-dependent basal resistance against *P. syringae* are required for the expression of SAR, whereas components contributing to JA/ethylene-dependent basal resistance against *P. syringae* are required for WCS417r-mediated ISR.

To investigate whether a similar association between basal resistance and induced resistance also holds for other pathogens, we compared the effectiveness of SAR and ISR against different pathogen of Arabidopsis that are primarily resisted through either SA-dependent, JA/ethylene-dependent, or a combination of SA- and JA/ethylene-dependent basal resistance. Activation of ISR resulted in a significant level of protection against the fungal pathogen *Alternaria brassicicola*, which is resisted through JA/ethylene-dependent basal defenses. Conversely, SAR was ineffective against this pathogen. Diseases caused by the oomycete *Peronospora parasitica* or by turnip crinkle virus, which are both predominantly resisted through SA-dependent basal resistance, were considerably reduced in plants expressing SAR, whereas activation of ISR yielded only weak, and no protection against these pathogens, respectively. Induction of SAR or ISR was equally effective against *X. campestris* pv. *armoraciae* that, like *Pst*, is resisted through a combined action of SA- and JA/ethylene-dependent basal resistance. Apparently, SAR is effective against pathogens that are resisted predominantly through SA-dependent basal resistance, whereas ISR is effective against pathogens that are resisted through JA/ethylene-dependent basal resistance.

Collectively, the results described in this thesis indicate that induced resistance constitutes an enhancement of SA-dependent basal defenses in the case of pathogen-induced SAR, and of JA/ethylene-dependent basal resistance in the case of WCS417r-mediated ISR. Thus, defense responses that are active locally upon primary pathogen attack are intensified by induction of ISR or SAR. Such association between induced resistance and basal resistance fits perfectly with the phenomenon variously referred to as "priming", "potentiation" or "sensitization". Potentiation is manifested upon challenge inoculation of plants expressing induced resistance as a stronger and faster activation of specific defenses compared to non-induced plants. Therefore, it is tempting to conclude that ISR is achieved by a potentiated expression of JA- and ethylene-dependent basal defenses, whereas SAR is achieved by a potentiated expression of SA-dependent defenses. Another important conclusion is that the spectrum of effectiveness of SAR and ISR differs. Previous findings demonstrated that simultaneous activation of SAR and ISR results in an additive level of protection against *Pst*. In addition, plants expressing both SAR and ISR are better protected

against a broader range of pathogens. Therefore, integration of SAR and ISR in agricultural practice offers not only potential to improve the efficacy and consistency of biological crop protection, but it could also increase its spectrum of effectiveness.

Samenvatting

Planten zijn voortdurend blootgesteld aan potentiële ziekteverwekkers. Als een plant aangevallen wordt door een virulent pathogeen worden op de plek van infectie diverse afweermechanismen geactiveerd. Als gevolg hiervan ondervindt het pathogeen weerstand om de plant verder te koloniseren, waardoor de ziekte-ontwikkeling vertraagd wordt. Een dergelijke resistentie na primaire infectie wordt basisresistentie genoemd. Naast basisresistentie bezitten planten ook het vermogen hun weerstand tegen verdere infecties te verhogen. Dit verschijnsel wordt geïnduceerde resistentie genoemd. Er zijn tenminste twee manieren waarop micro-organismen een verhoogde resistentie in planten kunnen induceren. Als een plant lokaal reageert op een necrotiserend pathogeen, verwerft hij een verhoogde resistentie die systemisch (in alle plantendelen) tot expressie komt en effectief is tegen verschillende typen pathogenen. Deze vorm van geïnduceerde resistentie wordt systemisch verworven resistentie genoemd (afgekort SAR). Naast SAR zijn er ook bepaalde niet-pathogene, wortelkoloniserende bacteriën die systemische resistentie kunnen induceren in planten. Deze vorm van induceerbare resistentie wordt geïnduceerde systemische resistentie genoemd (afgekort ISR). De meeste wortelkoloniserende bacteriën die ISR kunnen opwekken behoren tot het geslacht *Pseudomonas*.

SAR and ISR uiten zich beiden in een verminderde kolonisatie van de plant na infectie met een pathogeen, waardoor zich minder ziektesymptomen ontwikkelen dan in een niet geïnduceerde plant. Desalniettemin verschillen de signaal-transductiewegen van beide vormen van geïnduceerde resistentie. Zo is de signaal-transductie van SAR afhankelijk van het hormoon salicylzuur (SA), terwijl de signaal-transductie van ISR onafhankelijk van SA verloopt, maar gevoeligheid voor de hormonen jasmonzuur (JA) en ethyleen vereist. Behalve deze verschillen in signaal-transductie zijn er ook aantoonbare verschillen in de expressie van SAR en ISR. Zo wordt SAR gekenmerkt door een systemische accumulatie van zgn. "pathogenesis-related proteins" (PR-eiwitten), terwijl een dergelijke respons afwezig is na inductie van ISR. In de afgelopen jaren zijn er verscheidene mutanten en transgenen van *Arabidopsis thaliana* (de zandraket) geïsoleerd die verstoord zijn in de signaal-transductiewegen van SAR en/of ISR. Opmerkelijk is dat deze planten niet alleen verstoord zijn in de expressie van SAR of ISR, maar dat zij tegelijkertijd een verlaagde basisresistentie tegen diverse pathogenen vertonen. Dit suggereert dat basisresistentie en geïnduceerde resistentie gebruik maken van dezelfde signaal-transductiecomponenten. De relatie tussen geïnduceerde resistentie en basisresistentie staat centraal in het onderzoek dat beschreven is in dit proefschrift.

Voorafgaand onderzoek wees uit dat het ecotype RLD1 van Arabidopsis, in tegenstelling tot twee andere Arabidopsis ecotypen, geblokkeerd is in de expressie van ISR na behandeling van de wortels met de bacteriestam *Pseudomonas fluorescens* WCS417r. Deze

natuurlijke variatie in geïnduceerde resistentie werd onderworpen aan verder onderzoek door tien Arabidopsis ecotypen te testen op hun vermogen ISR en SAR tot expressie te brengen. Behalve ecotype RLD1 bleek ook ecotype Ws-0 geen ISR te vertonen na behandeling met WCS417r, terwijl de overige acht ecotypen wel in staat waren ISR tot expressie te brengen. De expressie van SAR was niet verminderd in alle tien ecotypen. Behalve dat RLD1 and Ws-0 niet in staat waren ISR tot expressie te brengen, bleken deze beide ecotypen ook een opmerkelijk laag niveau van basisresistentie te bezitten tegen het bacteriële bladpathogeen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). Deze natuurlijke variatie in ISR en basisresistentie diende als basis voor een genetische analyse om nieuwe componenten te identificeren die betrokken zijn bij de regulatie van ISR en basisresistentie. Experimenten met nakomelingen van diverse kruisingen tussen wel- en niet-induceerbare Arabidopsis ecotypen wezen uit dat zowel het vermogen om ISR tot expressie te brengen, als een relatief hoge basisresistentie tegen *Pst* gereguleerd worden door een enkel dominant gen op chromosoom III. Het desbetreffende locus werd *ISR1* genoemd.

Net als de *isr1* ecotypen RLD1 en Ws-0, zijn de JA-ongevoelige mutant *jar1-1* en de ethyleen-ongevoelige mutant *etr1-1* niet in staat ISR tot expressie te brengen en vertonen zij ook een verlaagd niveau van basisresistentie tegen *Pst*. Deze overeenkomst was aanleiding om te onderzoeken of RLD1 en Ws-0 wellicht verstoord zijn in de gevoeligheid voor JA of ethyleen. Vergeleken met het ISR-induceerbare ecotype Col-0 reageerden RLD1 en Ws-0 normaal op JA, maar in verminderde mate op ethyleen. Tevens bleek in de F_3 nakomelingen van een kruising tussen Col-0 en RLD1 de verminderde ethyleengevoeligheid van RLD1 gekoppeld te zijn aan de recessieve allelen van het *ISR1* locus, terwijl de ethyleengevoeligheid van Col-0 gekoppeld was aan de dominante allelen van het *ISR1* locus. Deze resultaten wijzen op een betrokkenheid van het *ISR1* locus bij de ethyleensignaal-transductie. Blijkbaar codeert het *ISR1* gen voor een produkt dat, als onderdeel van de ethyleensignaal-transductieroute, zowel de door WCS417r geïnduceerde ISR als de basisresistentie tegen *Pst* reguleert. Verdere studies naar de functie van het *ISR1* locus bij de regulatie van ISR tegen andere pathogenen van Arabidopsis, wezen uit dat de *isr1* ecotypen RLD1 en Ws-0 geblokkeerd zijn in de expressie van ISR tegen verschillende bladpathogenen. Dit suggereert dat een intacte ethyleensignaal-transductieroute nodig is voor de expressie van ISR tegen verschillende pathogenen.

Op basis van eerdere waarnemingen dat Arabidopsis genotypen die verstoord zijn in de signaaltransductie van ISR en/of SAR een verlaagd niveau van basisresistentie bezitten, d.w.z. gevoeliger zijn voor infectie, werd een collectie van 11 zgn. *eds* mutanten met een verminderde basisresistentie tegen pathogene *P. syringae* bacteriën getest op hun vermogen ISR en SAR tot expressie te brengen. Drie mutanten (*eds4*, *eds8*, en *eds10*) bleken verstoord te zijn in de expressie van ISR, en twee (*eds5* en *eds12*) in de expressie van SAR. Vervolgexperimenten wezen uit dat het niet optreden van ISR in mutant *eds8* geassocieerd

is met een verminderde gevoeligheid voor JA. Hetzelfde fenotype van *eds4* lijkt te berusten op een verminderde gevoeligheid voor ethyleen. Mutant *eds10* bleek normaal gevoelig voor zowel JA als ethyleen. Desalniettemin was deze mutant niet in staat resistentie te ontwikkelen na behandeling met JA of een directe precursor van ethyleen. Dit wijst erop dat het *Eds10* gen codeert voor een component die de expressie van ISR reguleert na de perceptie van JA en ethyleen. Van de mutanten die geen SAR tot expressie brachten, bleek mutant *eds12* verminderd gevoelig te zijn voor SA, terwijl voorgaand onderzoek reeds had uitgewezen dat *eds5* geblokkeerd is in de induceerbare biosynthese van SA. Daarmee leidden deze resultaten tot de identificatie van drie nieuwe genen die betrokken zijn bij de signaal-transductie van ISR: *Eds4*, *Eds8*, en *Eds10*, en een nieuwe component die betrokken is bij de signaaltransductie van SAR: *Eds12*. Bovendien bleek dat componenten die een rol spelen bij de JA- of ethyleen-afhankelijke basisresistentie tegen *P. syringae* tevens betrokken zijn bij de signaal-transductie van ISR, terwijl componenten die een rol spelen bij de SA-afhankelijke basisresistentie tegen *P. syringae* betrokken zijn bij de signaal-transductie van SAR.

Onderzoek naar het spectrum van pathogenen dat geremd wordt door de expressie van ISR en SAR, wees uit dat beide vormen van geïnduceerde resistentie bescherming bieden tegen verschillende groepen van pathogenen. Zo bleek de door WCS417r geïnduceerde ISR zeer effectief te zijn tegen de schimmel *Alternaria brassicicola*, waartegen de plant een voornamelijk JA- en ethyleen-afhankelijke basisresistentie bezit. SAR bleek geen bescherming te bieden tegen deze schimmel, maar juist effectief te zijn tegen andere pathogenen, zoals de oomyceet *Peronopora parasitica* en het “turnip crinkle virus”, die beide geremd worden door een SA-afhankelijke basisresistentie. ISR bleek slechts zwakke bescherming te bieden tegen *P. parasitica* en geen enkele bescherming tegen het virus. Tegen het bacteriële pathogeen *Xanthomonas campestris*, dat door een combinatie van JA/ethyleen-afhankelijke en SA-afhankelijke basisresistentie wordt geremd, bleken beide vormen van geïnduceerde resistentie in gelijke mate bescherming te bieden. Blijkbaar is ISR vooral effectief tegen pathogenen die door een JA/ethyleen-afhankelijke basisresistentie geremd worden, terwijl SAR vooral effectief is tegen pathogenen die geremd worden door een SA-afhankelijke basisresistentie.

Tezamen wijzen de resultaten erop dat SAR het resultaat is van een verhoogde SA-afhankelijke basisresistentie en ISR van een verhoogde JA/ethyleen-afhankelijke basisresistentie. Een dergelijke associatie tussen geïnduceerde resistentie en basisresistentie zou goed uitgelegd kunnen worden als een vorm van “potentiëring” of “sensitisering”. Potentiëring treedt op als geïnduceerde planten tijdens infectie met een pathogeen een specifiek basisresistentiemechanisme sneller of in sterkere mate tot expressie brengen dan niet-geïnduceerde planten. Het is daarom aannemelijk dat SAR gerealiseerd wordt via een gepotentieerde expressie van SA-afhankelijke basisresistentie en ISR via een gepotentieerde expressie van JA/ethyleen-afhankelijke basisresistentie. Een andere belangrijke conclusie is

dat SAR en ISR verschillen in het spectrum van pathogenen waartegen zij bescherming bieden. Voorafgaand onderzoek heeft uitgewezen dat gelijktijdige activering van ISR en SAR een verhoogd niveau van bescherming tegen *Pst* oplevert, hetgeen aantoont dat beide vormen van geïnduceerde resistentie niet antagonistisch zijn. Daarom zou een gelijktijdige activering van SAR en ISR de plant effectiever kunnen beschermen tegen een breder spectrum van pathogenen. Integratie van beide vormen van geïnduceerde resistentie biedt nieuwe mogelijkheden voor biologische gewasbescherming: niet alleen zou de effectiviteit en betrouwbaarheid verhoogd worden, maar ook zou het spectrum van bescherming uitgebreid worden tot meer pathogenen.

Nawoord

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List of publications

Ton, J., Pieterse, C.M.J., and Van Loon, L.C. (1998). Genetic analysis of induced systemic resistance in *Arabidopsis thaliana*: association between induced and basal resistance. *In:* Molecular Approaches to Biological Control (Duffy, B.K., Rosenberger, U., and Défago, G., eds), IOBC/WPRS, Vol. **21(9),** 111-116.

Ton, J., Pieterse, C.M.J., and Van Loon, L.C. (1999). Identification of a locus in Arabidopsis controlling both the expression of rhizobacteria-mediated induced systemic resistance (ISR) and basal resistance against *Pseudomonas syringae* pv. *tomato*. Mol. Plant-Microbe Interact. **12**, 911-918.

Pieterse, C.M.J., Van Wees, S.C.M., **Ton J.**, Léon-Kloosterziel, K.M., Van Pelt, J.A., Keurentjes, J.J.B., Knoester, M., and Van Loon, L.C. (1999). Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis: involvement of jasmonate and ethylene. *In*: Biology of Plant-Microbe Interactions, (De Wit, P.J.G.M., Bisseling,T., Stiekema W.J., eds), International society for molecular plant-microbe interactions, St. Paul, MN, USA, Vol. **2,** pp. 291-296.

Pieterse, C.M.J., Van Pelt, J.A., **Ton, J.**, Parchmann, S., Mueller, M.J., Buchala, A.J., Métraux, J.-P., and Van Loon, L.C. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol. Mol. Plant Pathol. **57**, 123-134.

Ton, J., Davison, S., Van Pelt, J.A., Van Loon L.C., and Pieterse C.M.J. (2001). The *ISR1* locus of Arabidopsis controlling induced systemic resistance is involved in ethylene signaling. Plant Physiol. **125**, 652-661.

Ton, J., Davison, S., Van Loon, L.C., and Pieterse, C.M.J. (2001). Heritability of rhizobacteria-mediated induced systemic resistance and basal resistance in Arabidopsis. Eur. J. Plant Pathol. **106,** *in press*.

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. Eur. J. Plant Pathol. **106**, *in press*.

Ton, J., Van Pelt, J.A., Van Loon, L.C., and Pieterse, C.M.J. Differential effectiveness of SA-dependent and jasmonate- and ethylene-dependent induced resistance in Arabidopsis. *Submitted*.

Ton, J., De Vos, M., Robben, C, Van Loon, L.C., and Pieterse, C.M.J. Characterization of Arabidopsis enhanced disease susceptibility mutants that are affected in systemically induced resistance. *Submitted*.

Ton, J., Van Pelt, J.A., Van Loon, L.C., and Pieterse, C.M.J. The Arabidopsis *ISR1* locus is required for rhizobacteria-mediated induced systemic resistance against different pathogens. *Submitted*.

Curriculum vitae

Jurriaan Ton werd geboren op 19 maart 1973 te Klaaswaal. In 1991 behaalde hij het Gymnasium-*ß* diploma aan Het Rotterdamsch Lyceum te Rotterdam. In datzelfde jaar werd aangevangen met de studie Biologie aan de Universiteit Utrecht. Tijdens zijn derde studiejaar nam hij deel aan een Erasmus uitwisselingsproject in Italië voor een cursus en onderzoeksstage in de Paleobiologie. In de doctoraalfase werden twee onderzoeksstages vervuld: Fytopathologie, onder begeleiding van Drs. H. Steyl (onderzoek naar de signaaltransductie en expressie van systemische resistentie in radijs opgewekt door rhizobacteriën) en Plantenfysiologie, onder begeleiding van Dr. A.C. Borstlap (onderzoek naar de transcriptie van het *Aap3* gen in de *raz1* mutant van Arabidopsis). In september 1996 studeerde hij af. Vanaf oktober 1996 tot november 2000 was hij werkzaam als AIO bij de projectgroep 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 beschreven is.