

Rhizobacteria-mediated induced systemic resistance in *Arabidopsis*

signal transduction and expression

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**Door rhizobacteriën geïnduceerde systemische resistentie
in *Arabidopsis***

signaaltransductie en expressie

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Believe

*Bravely I look further than I see
knowing things I know I can not be, not now
I'm so aware of what I am
but I don't know where that is
And there is something right in front of me and I
touch the fingers of my hand
and I wonder if it's me
Holding on and on to
theories of prosperity
someone who can promise me
I believe in me
Tomorrow I was nothing, yesterday I'll be
time has fooled me into thinking it's a part of me
Nothing in this room but empty space
no me, no world, no mind, no face
Touch the fingers of my hand
and tell me if it's me
Holding on and on to
love, what else is real
a religion that appeals to me
I believe in me
Wait for me, I'm nothing on my own
I'm willing to go on but not alone, not now
I'm so aware of everything
but nothing seems for real and
as long as you're in front me then I'll
watch the fingers of our hands
and I'm grateful that it's me
Holding on and on...
I believe in me*

K's choice, Cocoon crash
(1998)

*Aan mijn ouders
en Mark*

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General introduction

Biological disease control by rhizosphere-colonizing fluorescent *Pseudomonas* spp

Rhizobacteria are present in large numbers on the root surface, where plant exudates and lysates provide nutrients (Lynch and Whipps, 1991; Rovira and Davey, 1974). Certain strains of rhizobacteria are referred to as plant growth-promoting rhizobacteria (PGPR), because they can stimulate growth of the plant (Kloepper *et al.*, 1980; Lynch, 1976). Growth promotion results mainly from suppressing soil-borne pathogens and other deleterious microorganisms (Schippers *et al.*, 1987), but also direct effects on plant growth have been reported (Lynch, 1976; Pieterse and Van Loon, 1999; Van Peer and Schippers, 1989). Fluorescent *Pseudomonas* spp are among the most effective PGPR and reduce soil-borne diseases in natural disease-suppressive soils (Kloepper *et al.*, 1980; Scher and Baker, 1980).

Research on the mechanisms of biological control by fluorescent *Pseudomonas* spp revealed that antagonism, leading to a direct inhibitory effect on pathogens, plays an important role. Antagonism can result from the production of toxic metabolites, e.g. hydrogen cyanide and antibiotics, such as phenazine-1-carboxylic acid, and 2,4-diacetylphloroglucinol (Thomashow and Weller, 1996), the synthesis of lytic enzymes like chitinase (Chet *et al.*, 1993), or competition for nutrients, in particular iron (Bakker *et al.*, 1991).

In 1991, two research groups independently demonstrated that besides a direct effect on soil-borne pathogens, fluorescent *Pseudomonas* spp can have an indirect effect on different types of pathogens as well. Selected *Pseudomonas* strains were capable of triggering a plant-mediated resistance response (Van Peer *et al.*, 1991; Wei *et al.*, 1991). Phenotypically, this resistance response resembles classic pathogen-induced resistance, in which noninfected parts of previously pathogen-infected plants become more resistant to further infection. The first systematic study of this phenomenon was performed with *N* gene-containing tobacco plants that were inoculated with tobacco mosaic virus (TMV). Upon development of necrotic lesions caused by TMV infection, the remainder of the plant developed resistance against subsequent infection by different viral pathogens (Ross, 1961a,b). This form of induced resistance was designated local acquired resistance (LAR) when expressed locally around the primary lesions, and systemic acquired resistance (SAR) when expressed distant from the primary site of infection (Ross, 1961a,b). In the past decade, the molecular mechanisms underlying pathogen-induced SAR have been studied extensively. In contrast, the molecular basis of rhizobacteria-mediated resistance is to a large extent unknown. This thesis focusses in particular on the mechanisms involved in induction, signalling, and expression of rhizobacteria-mediated induced disease resistance, using *Arabidopsis thaliana* as a model plant.

Induced disease resistance

R gene-mediated resistance

Resistance of plants to pathogenic microorganisms is based on the combined effects of preformed defensive barriers and inducible resistance mechanisms. Preformed barriers, such as a thick cuticle or inhibitory secondary metabolites in the outer cell layers of plant organs (Osbourn, 1996) form the first line of defense against pathogen infection. Upon recognition of the pathogen, the plant activates resistance mechanisms to inhibit growth and spread of the pathogen. In interactions that follow a ‘gene-for-gene’ relationship, the host plant carries a resistance gene (*R*) whose product directly or indirectly recognizes the product of a matching avirulence gene (*avr*) expressed by the pathogen. This genetic incompatibility often leads to the elicitation of the characteristic hypersensitive response (HR), which involves rapid death of infected cells and is manifested as a small necrotic lesion. Concomitantly, the pathogen is prevented from spreading throughout the rest of the plant (Dangl *et al.*, 1996). In cells adjacent to the lesion, a diverse group of defense-related genes is activated, the products of which play a role in limiting pathogen growth, either indirectly, by helping to reinforce host cell walls, or directly, by providing antimicrobial compounds like phytoalexins and pathogenesis-related proteins (PRs). These defense responses may also be induced in compatible interactions that involve a pathogen and host plant that lack matching *avr* and *R* genes. However, in a compatible interaction, the susceptible plant does not specifically recognize the virulent pathogen, and defense responses appear to be

activated too slowly or too weakly to restrict pathogen growth and/or spread, resulting in severe damage or even death of the plant (Van Loon, 1997).

Systemically induced disease resistance

If, for whatever reason, the plant's defense mechanisms are already stimulated, subsequent infection by a virulent pathogen can be diminished. This state of induced resistance depends on either defensive products that accumulate in the induced plants before challenge, or a quicker and stronger activation of extant defense mechanisms after challenge (Van Loon, 1997). Thus, a first triggering factor can predispose the plant to resist further pathogenic attacks. The enhanced resistance is expressed not only locally at the site in direct contact with the inducer, but extends systemically, conferring disease resistance to the entire plant. This phenomenon of induced resistance was designated induced systemic resistance (ISR) and is defined as the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (Kloepper *et al.*, 1992).

ISR has been taken to encompass at least two types of induced disease resistance:

- 1) Systemic acquired resistance (SAR) that is triggered by pathogens causing restricted lesions, either as part of the HR (the pathogen is avirulent), as has been described by Ross (1961a,b), or as a symptom of disease caused by a mildly virulent pathogen (reviewed by Ryals *et al.*, 1996).
- 2) Rhizobacteria-mediated induced systemic resistance (ISR) that is triggered by nonpathogenic rhizobacteria that do not cause any harmful symptoms on the plant, and can even have a growth-promoting effect (reviewed by Van Loon *et al.*, 1998).

The term 'ISR' is commonly used for both the general phenomenon of systemically induced resistance (Kloepper *et al.*, 1992), and the specific type that is triggered by nonpathogenic (rhizo)bacteria. In this thesis, the term 'ISR' will be used in the latter sense to distinguish it from pathogen-induced SAR. Both pathogen-induced SAR and rhizobacteria-mediated ISR have been demonstrated in several plant species and are effective against a broad spectrum of root and foliar pathogens including fungi, bacteria, and viruses (Sticher *et al.*, 1997; Van Loon *et al.*, 1998). For the establishment of systemically induced resistance, (a) signal(s) must be transported from the site of induction, which is the primary infection site in the case of SAR, and the root colonization site in the case of ISR. The signal(s) are systemically translocated to trigger the induced state in distant plant parts (Figure 1).

Induced resistance as a mechanism to control plant diseases

The phenomenon of induced resistance offers great opportunities to control plant diseases in a biological way as part of integrated pest management strategies. It may serve as an alternative for conventional chemical control and *R* gene breeding. Many chemicals have been developed to protect plants against disease. Most of them are inexpensive and easy to obtain, but both their production and their persistence in the soil after use are potentially harmful to the environment. This is particularly true for

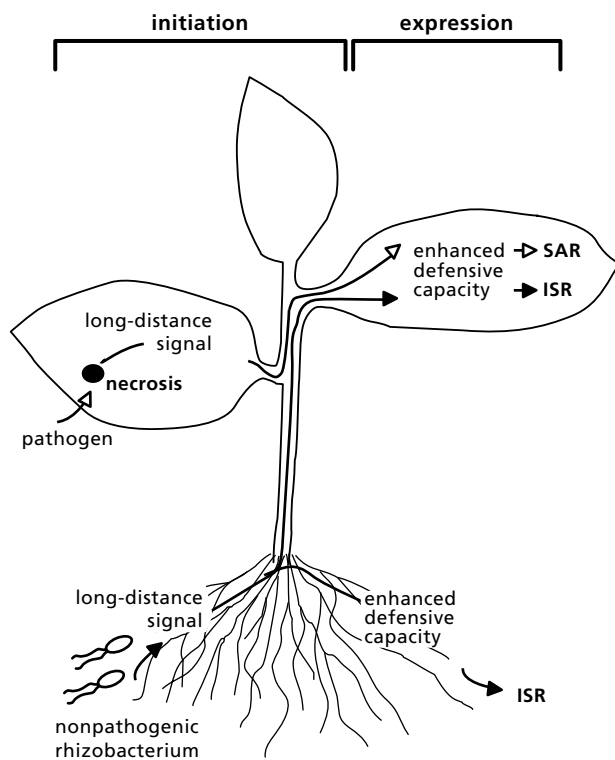


Figure 1. Schematic representation of pathogen-induced SAR and rhizobacteria-mediated ISR.

Either necrotic resistance responses triggered by pathogen infection, or colonization of the rhizosphere by selected nonpathogenic rhizobacteria elicit the production of a long-distance signal. The signal is perceived by distant tissues, leading to the expression of SAR or ISR, respectively, which protects plants against several types of pathogens. The biochemical changes during SAR and ISR can be divided into two phases, initiation and expression. The initiation phase may be transient and includes the generation of the systemically transported signal. The expression phase reflects the systemic resistant state resulting from initiation, and involves (1) a direct activation of defense-related gene expression, (2) potentiation of defense-related gene expression, and/or (3) posttranslational modification of existing proteins. These three possibilities are contained by the term 'enhanced defensive capacity'. Partly according to Ryals (1994).

chemicals employed in the control of soil-borne fungal pathogens, because often they must be applied repeatedly in large, toxic amounts (Buchenauer, 1998). The introduction of *R* genes into plants is attractive because it can render plants completely resistant to a pathogen. However, resistance breeding is dependent on the availability of an *R* gene, which is not always the case. Moreover, resistance based on a gene-for-gene relationship offers protection against only a single pathogen, which can overcome resistance by mutation, leading to a limited durability of disease resistance in the fields.

Induced resistance is an attractive alternative form of plant protection as it is based on the activation of extant resistance mechanisms of the plant. It may, therefore, be considered natural and safe, and offers resistance against a wide range of pathogens (Sticher *et al.*, 1997; Van Loon, 1997; Van Loon *et al.*, 1998). Both SAR and ISR confer resistance for the lifetime of the plant (Hammerschmidt and Kuć, 1995; Liu *et al.*, 1995; Raaijmakers *et al.*, 1995). Both SAR and ISR have been shown to be effective under field conditions (Tuzun and Kloepper, 1995; Wei *et al.*, 1996) and in commercial greenhouses (Leeman *et al.*, 1995c). Recently, the commercial product Bion, which is based on the active compound benzothiadiazole (BTH) that activates the plant to express induced resistance (Friedrich *et al.*, 1996; Görlach *et al.*, 1996), has

been released on the market. In this age, where the importance of environmentally friendly and sustainable agriculture is highly recognized, the contribution of induced resistance to control plant diseases can be of great value. Understanding of the molecular mechanisms underlying rhizobacteria-mediated ISR may contribute to exploitation of the potential of ISR in the control of plant diseases.

Signals associated with disease resistance

Systemically induced resistance requires activation of the plant, leading to an enhanced level of protection against pathogen attack. Salicylic acid (SA), jasmonate, and ethylene have emerged as important signalling molecules in plant defense. Pathogen infection provokes the accumulation of these three signalling compounds (Boller, 1991; Malamy *et al.*, 1990; Métraux *et al.*, 1990; Penninckx *et al.*, 1996). Moreover, when applied exogenously they induce various levels of resistance and activate specific sets of defense-related genes (Boller, 1991; Cohen *et al.*, 1993; Reymond and Farmer, 1998; Ryals *et al.*, 1996).

Analyses of plant genotypes, particularly mutants and transgenics of *Arabidopsis* and tobacco that either do not accumulate or do not respond to SA, jasmonate, or ethylene revealed that all three signalling molecules are essential for basal resistance to varying pathogens. The 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 inactive catechol (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). As a result, NahG plants are severely affected in SA accumulation (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). Tobacco and *Arabidopsis* NahG plants are more susceptible to a variety of fungal, bacterial, and viral pathogens, indicating that SA is required for full expression of basal resistance against these pathogens (Delaney *et al.*, 1994). The significance of jasmonate in disease resistance was demonstrated in the jasmonate response mutant *coi1* (coronatine insensitive) of *Arabidopsis*, that shows enhanced susceptibility to several necrotrophic fungi compared to the wild-type plant (Thomma *et al.*, 1998). Moreover, the *coi1* mutant as well as the *Arabidopsis* mutant *jar1* (jasmonate response mutant), and the triple mutant *fad3, fad7, fad8* (defective in the biosynthesis of jasmonate) are all susceptible to normally nonpathogenic soil-borne fungi from the genus *Pythium* (Staswick *et al.*, 1998; Vijayan *et al.*, 1998). The same phenomenon was observed in transgenic tobacco (Tetr) plants that are ethylene insensitive through the expression of a mutant *Arabidopsis etr1* gene, encoding a defective ethylene receptor (Knoester *et al.*, 1998). However, the role of ethylene in plant disease resistance is ambiguous, because in other cases ethylene insensitivity has been shown to be associated with increased tolerance to pathogen infection. For instance, ethylene-insensitive mutants of *Arabidopsis* (*ein2*) and tomato (*nr*, never ripe) displayed less severe symptoms than wild-type plants after infection with virulent chlorosis-inducing pathogens such as *Xanthomonas campestris* and *Pseudomonas syringae*, while *in planta* growth of the pathogens was unaffected (Bent *et al.*, 1992; Lund *et al.*, 1998). This could be explained by the fact that ethylene is a hormone involved in senescence. Pathogen infection induces ethylene production in the plant,

which contributes to chlorosis near the infection site. If the plant can not sense ethylene, this will automatically lead to a reduction in chlorosis development.

SAR signalling pathway

Salicylic acid and pathogenesis-related proteins

In the past decade, major efforts have been made to unravel the steps in the signalling pathway controlling classic pathogen-induced SAR, mainly using tobacco, cucumber, and *Arabidopsis* as models. The resistant state is characterized by an increase in endogenously synthesized SA prior to the onset of SAR, both in primary infected leaves, and to a lesser extent, in noninfected leaves (Malamy *et al.*, 1990; Métraux *et al.*, 1990). Furthermore, SAR is characterized by the systemic activation of so-called SAR genes (Ryals *et al.*, 1996; Ward *et al.*, 1991). These SAR genes include genes encoding pathogenesis-related proteins (PRs; Van Loon and Antoniw, 1982). Some of these PRs possess antifungal activity and are thought to contribute to SAR (Kombrink and Somssich, 1997). As SAR is invariably linked to the accumulation of these PRs, they are commonly used as markers for SAR.

Exogenous application of SA can mimic pathogen-induced SAR, because it induces resistance to the same spectrum of pathogens and concurrently activates the expression of SAR genes. The synthetic plant-activating compounds 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) are functional analogs of SA (Görlach *et al.*, 1996; Métraux *et al.*, 1991). Exogenous application of INA and BTH has been shown to trigger the SAR signalling pathway in both tobacco and *Arabidopsis* (Friedrich *et al.*, 1996; Lawton *et al.*, 1996; Uknes *et al.*, 1992; Ward *et al.*, 1991). Conclusive evidence for the requirement of SA in the expression of SAR came from the analysis of the SA-nonaccumulating tobacco and *Arabidopsis* NahG transformants. Gaffney *et al.* (1993), Delaney *et al.* (1994) and Lawton *et al.* (1995) showed that NahG plants exhibit poor expression of PR genes and fail to develop SAR in response to either pathogen infection or exogenous application of SA, indicating that SA plays a crucial role in the SAR signalling pathway (Figure 2).

SA is not the long-distance signal in SAR

Before the expression of pathogen-induced SAR, SA levels in the phloem of infected cucumber plants increase (Métraux *et al.*, 1990). Moreover, SA accumulating in noninfected leaves of pathogen-infected plants was partially imported from primary infected leaves (Shulaev *et al.*, 1995), indicating SA as a potential candidate for the long-distance signal inducing SAR. However, grafting experiments between tobacco NahG and wild-type plants demonstrated that NahG rootstocks were capable of releasing the SAR-inducing signal that was translocated to wild-type scions, whereas no SAR could be induced in the reciprocal graft (Vernooij *et al.*, 1994). Similar results were found with grafting experiments between tobacco wild types and transformants that show epigenetic cosuppression of the *Pal* gene (encoding phenylalanine-ammonia lyase), which results in a defective SA production (Pallas *et al.*, 1996). Moreover, leaf

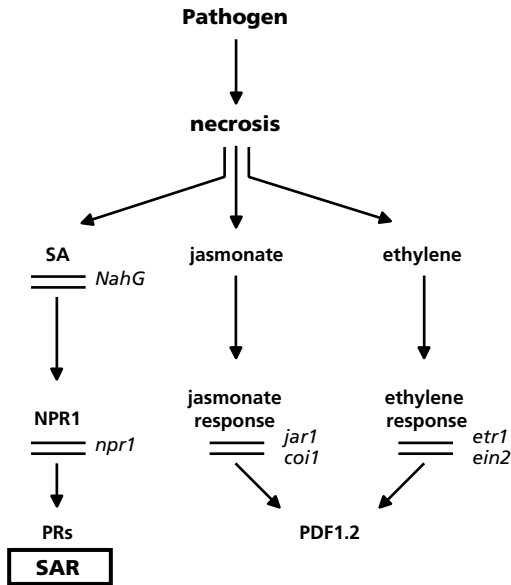


Figure 2. SA-, jasmonate-, and ethylene-dependent signalling pathways induced upon pathogen infection.

Pathogen-induced necrosis triggers the synthesis of SA, jasmonate and ethylene. *PR* gene expression and SAR are controlled by the SA- and NPR1-dependent pathway. *Pdf1.2* expression requires a concerted action of jasmonate and ethylene. For simplicity, not all mutants mentioned in the text are presented in the scheme. The SAR pathway is according to Ryals *et al.* (1996), and the pathway leading to *Pdf1.2* gene expression according to Penninckx *et al.* (1996, 1998).

excision experiments in cucumber showed that a SAR-inducing signal is translocated from a *Pseudomonas syringae*-infected leaf before a rise in SA levels in both infected and noninfected leaves could be detected (Rasmussen *et al.*, 1991). Both types of observations rule out the possibility that SA is the (only) primary long-distance signal responsible for the elicitation of SAR. Instead, SA seems to be required in the noninfected tissue for SAR to be expressed (Vernooij *et al.*, 1994; Willits and Ryals, 1998). It was recently proposed that SA is produced *de novo* in stems and petioles in response to a mobile signal from the leaf lamina (Smith-Becker *et al.*, 1998).

SAR mutants

To better understand the mechanism of SAR, mutant screens of *Arabidopsis* designed to identify components of the SAR signalling pathway have been conducted by various research groups. These screens yielded both gain-of-function and loss-of-function mutants. Gain-of-function mutants such as those from the *lsd* (lesions simulating disease; Dietrich *et al.*, 1994), the *acd* (accelerated cell death; Greenberg *et al.*, 1994), and the *cpr* (constitutive expressor of *PR* genes; Bowling *et al.*, 1994, 1997; Clarke *et al.*, 1998) series contain elevated levels of SA, leading to constitutive expression of *PR* genes and SAR. Loss-of-function mutants, such as the allelic mutants *npr1* (non-expressor of *PR* genes), *nim1* (noninducible immunity; Delaney *et al.*, 1995) and *sai1* (SA insensitive; Shah *et al.*, 1997) are affected in a gene that acts downstream of SA in the SAR pathway, leading to the inability to express *PR* genes and SAR after treatment with SAR inducers (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997) (Figure 2).

Analysis of double mutants with a mutation in a gene from the gain-of-function class and a mutation in a gene from the loss-of-function class revealed that *cpr* genes act

upstream of SA and NPR1 in the SAR pathway (Bowling *et al.*, 1997; Clarke *et al.*, 1998; Dong, 1998). However, *cpr5* and *cpr6* seem to activate an NPR1-independent defense pathway as well (Bowling *et al.*, 1997; Clarke *et al.*, 1998), indicating that the SAR pathway is part of a more complex signalling network.

The regulatory factor NPR1

The key regulatory gene *Npr1/Nim1/Sai1* (from now on called *Npr1*) was recently cloned and demonstrated to show some homology to a gene encoding the mammalian signal transduction factor I κ B α , which is involved in the transcriptional activation of immune and inflammatory responses (Ryals *et al.*, 1997). *Npr1* encodes a protein containing functionally important ankyrin repeats which have been implicated in protein-protein interactions (Cao *et al.*, 1997; Ryals *et al.*, 1997). The *Npr1* gene is constitutively expressed in *Arabidopsis* (Ryals *et al.*, 1997). In the absence of a pathogen, the basal expression level of *Npr1* does not lead to expression of *PR* genes or resistance, suggesting that the NPR1 protein is active only upon stimulation. The carboxyl end of NPR1 contains a nuclear localization signal which is essential for protein function (Dong, 1998). Using a fusion of NPR1 with the green fluorescent protein (GFP), it was demonstrated that NPR1 is translocated to the nucleus in response to both biological and chemical inducers of SAR (Kinkema *et al.*, 1998), suggesting that NPR1 functions as a transcription activator in conjunction with other factors.

Jasmonate- and ethylene-dependent defense responses

Besides SA, also jasmonate and ethylene are rapidly produced when the plant is attacked by a pathogen, particularly during necrotizing infections when the rise in jasmonate levels extends to systemic tissue (Penninckx *et al.*, 1996). Lawton *et al.* (1994, 1996) demonstrated that the jasmonate response mutant *jar1* and the ethylene response mutant *etr1* of *Arabidopsis* express normal levels of SAR after induction, indicating that in *Arabidopsis*, SAR functions independently of jasmonate and ethylene. Nevertheless, pathogen-induced production of jasmonate and ethylene, or exogenous application of these two signalling molecules, induces a particular set of defense-related genes that might be involved in a parallel pathway leading to systemically induced resistance. Among those are basic *PR* genes in tobacco (Linthorst, 1991) that are inducible by ethylene, and in *Arabidopsis* the genes *Pdf1.2* (plant defensin; Penninckx *et al.*, 1996), *Hel* (hevein-like protein; Potter *et al.*, 1993) and *ChiB* (chitinase; Samac *et al.*, 1990), which are responsive to both jasmonate and ethylene, and *Thi2.1* (thionin; Epple *et al.*, 1995), *Pal1* (phenylalanine-ammonia lyase; McConn *et al.*, 1997), *Lox1*, and *Lox2* (lipoxygenase; Bell and Mullet, 1993; Melan *et al.*, 1993), which are responsive to jasmonate.

Recently, it was demonstrated that some of these genes are activated through an SA-independent pathway. Vidal *et al.* (1997) demonstrated that in tobacco the systemic induction of ethylene-responsive basic *PR-2* and *PR-3* genes by *Erwinia*

carotovora was unaffected in transgenic NahG plants. Moreover, NahG plants showed a normal level of enhanced resistance after treatment with *Erwinia* elicitors, indicating that the activated pathway differs from the SA-dependent SAR pathway (Vidal *et al.*, 1998). In *Arabidopsis*, Penninckx *et al.* (1996) showed that after infection with *Alternaria brassicicola* local and systemic activation of the antifungal *Pdf1.2* gene occurred equally well in wild-type and NahG plants. Moreover, pathogen-induced expression of *Pdf1.2* was unaffected in the SAR mutant *npr1*, indicating that this defense reaction functions independently of the SA/NPR1-dependent SAR pathway. In contrast, the jasmonate response mutant *coi1* and the ethylene response mutants *etr1* and *ein2* did not express *Pdf1.2* upon infection with *A. brassicicola*, demonstrating that this particular defense reaction requires components of both the jasmonate and ethylene response (Penninckx *et al.*, 1996, 1998) (Figure 2).

Cross-talk between defense signalling pathways

Apparently, defense responses are controlled by multiple signal transduction pathways in which either SA, or jasmonate and ethylene are implicated. Cross-talk between the different signalling pathways might help the plant to either prioritize the action of a particular pathway over another, or activate multiple pathways if necessary (Pieterse and Van Loon, 1999; Reymond and Farmer, 1998).

In this respect, jasmonate and ethylene often act in concert in activating defense responses. Penninckx *et al.* (1998) demonstrated that treatment of either *coi1* or *ein2* mutants with ethylene or methyl jasmonate (MeJA) respectively, did not induce *Pdf1.2* gene expression as it did in wild-type plants. This indicates that the jasmonate and ethylene signalling pathways need to be triggered concomitantly, and not sequentially, to activate *Pdf1.2*. The concerted action of jasmonate and ethylene was further illustrated by a synergistic effect of both signals on the expression level of *Pdf1.2* (Penninckx *et al.*, 1998). Cooperation between the jasmonate and ethylene responses was also demonstrated in the induction of genes encoding PR-1 and PR-5 type proteins in tobacco (Xu *et al.*, 1994), and the activation of the *Pin2* gene of tomato, encoding proteinase inhibitor, that is implicated in defense against insects (O'Donnell *et al.*, 1996).

Evidence is emerging that SA-responsive pathways and jasmonate- and/or ethylene-responsive pathways influence each other's activity. For example, in tomato, both the wound-induced accumulation of jasmonate, as well as the jasmonate-induced expression of the *Pin2* gene could be prevented by treating plants with SA (Doares *et al.*, 1995; Doherty *et al.*, 1988; Peña-Cortés *et al.*, 1993). Also, pathogen-induced expression of ethylene-responsive basic *PR-2* and *PR-3* genes in tobacco was antagonized by exogenous application of SA (Vidal *et al.*, 1997). In *Arabidopsis*, expression of the jasmonate- and ethylene-responsive gene *Pdf1.2* was enhanced in SA-nonaccumulating NahG transformants (Penninckx *et al.*, 1996), suggesting that its expression is repressed in SA-accumulating wild-type plants.

Conversely, jasmonate and ethylene have varying effects on the level of SA-induced gene transcripts. The *Arabidopsis* ethylene response mutant *etr1* showed a decreased level of expression of the SA-responsive *PR-1* gene after pathogen infection, suggesting that the ethylene response modulates SA action (Lawton *et al.*, 1995). This was confirmed by the observation that pretreatment of wild-type *Arabidopsis* plants with ethylene results in an enhanced *PR-1* gene expression upon induction by SA (Lawton *et al.*, 1995). MeJA has been reported to enhance the level of SA-induced *PR-1* gene expression in tobacco seedlings (Xu *et al.*, 1994). However, also inhibitory effects of MeJA on SA-induced *PR-1* gene expression in tobacco leaf discs, and no effects of MeJA on SA-induced expression of the *PR-3* gene in tomato have been demonstrated (Doares *et al.*, 1995; Niki *et al.*, 1998).

Potentialiation of defense responses

Obviously, pathogen-induced SAR is associated with a direct activation of specific defense responses. However, some defense responses are not directly activated but are manifested to a greater extent after challenge inoculation of induced tissue, a phenomenon referred to as potentiation. Potentiation is taken to represent an increase in sensitivity of induced tissue to defensive signals, which upon challenge infection leads to a more rapid and more effective defense response. For example, SAR-expressing leaves of cucumber showed a more rapid and increased lignification of host cell walls in response to challenge inoculation with *Colletotrichum lagenarium* (Hammerschmidt and Kuć, 1982). Likewise, tobacco plants exhibiting pathogen-induced SAR showed an enhanced expression of *PR-10* and *Pal* genes upon challenge with TMV (Mur *et al.*, 1996).

SA, jasmonate, and ethylene have all been shown to act as potentiating signals of defense-related gene expression. The enhanced levels of SA in noninfected leaves of pathogen-inoculated plants have been demonstrated to affect the timing and the level of expression of various responses induced by challenge inoculation, including *PR* gene activation (Mur *et al.*, 1996), *Pal* gene activation (Katz *et al.*, 1998; Mur *et al.*, 1996), and phytoalexin accumulation (Kauss *et al.*, 1992, 1993). Jasmonate potentiates elicitor-induced accumulation of active oxygen species in cultured parsley cells (Kauss *et al.*, 1994). As described earlier, ethylene potentiates *PR-1* gene expression in SAR-expressing *Arabidopsis* plants (Lawton *et al.*, 1994, 1995). This demonstrates that induction of systemic resistance extends beyond a direct effect on the production of defensive compounds and also involves sensitization of the tissue to react more effectively to invading pathogens.

Rhizobacteria-mediated ISR

Protection against plant diseases by nonpathogenic rhizosphere-colonizing bacteria has been proven in many cases to be mediated by the activation of ISR in the plant (reviewed by Van Loon *et al.*, 1998). In all these cases, the resistance-inducing

rhizobacterial strain was spatially separated from the challenging pathogen, preventing direct antagonism between the two microorganisms. Rhizobacteria-mediated ISR has been demonstrated in cucumber (Wei *et al.*, 1991), tobacco (Maurhofer *et al.*, 1994), radish (Leeman *et al.*, 1995a), tomato (Duijff *et al.*, 1998), and bean (De Meyer and Höfte, 1997), and is effective against a broad spectrum of pathogens, including fungi, bacteria, and viruses (reviewed by Van Loon *et al.*, 1998).

The mechanisms by which rhizobacteria elicit ISR are to a large extent unknown. For the induction of ISR against the pathogen *Fusarium oxysporum* f sp *raphani* in radish, a threshold level of the initial population density of inducing *P. fluorescens* WCS374r bacteria of 10^5 colony-forming units per gram of roots was required (Leeman *et al.*, 1995a; Raaijmakers *et al.*, 1995). Higher bacterial densities did not result in a higher level of protection. A time span of one day between application of the inducing rhizobacteria and pathogen challenge was shown to be required for ISR to be manifested (Leeman *et al.*, 1995a). Liu *et al.* (1995) demonstrated that for the establishment of a long-lasting ISR by *P. putida* 89B-27 against *Colletotrichum orbiculare* in cucumber plants, a similar threshold level of the *Pseudomonas* bacteria on the roots needs to be reached for only a limited period (less than 1 week) and may dwindle later.

Bacterial determinants involved in ISR

Several determinants of rhizobacteria have been implicated in the elicitation of ISR. The strain-specific O-antigenic side chain of the outer membrane lipopolysaccharide (LPS) from some fluorescent *Pseudomonas* spp has been demonstrated to be an important factor in triggering ISR. In carnation and radish, heat-killed *P. fluorescens* WCS417r bacteria (Van Peer and Schippers, 1992), and purified LPS of WCS417r and WCS374r (Leeman *et al.*, 1995b) were shown to trigger ISR to the same extent as live cells. Mutants of WCS417r and WCS374r that lack the O-antigenic side chain of the LPS had lost their ISR-inducing capacity, indicating that the O-antigenic side chain is the part of the LPS responsible for induction of ISR (Leeman *et al.*, 1995b). Both live cells of *P. putida* WCS358r and its purified LPS were incapable of inducing ISR in radish (Leeman *et al.*, 1995b), showing that induction of ISR is strain specific. Plants appear to specifically recognize the outer membrane LPS of different types of bacteria. The LPS of the plant pathogenic bacterium *X. campestris*, for instance, has been shown to be essential for the elicitation of host resistance responses, such as PR-2 accumulation in tobacco (Newman *et al.*, 1995). Moreover, LPS preparations of pathogenic *Pseudomonas* strains prevent the HR induced by avirulent *Pseudomonas* strains, and reduce symptoms caused by virulent *Pseudomonas* bacteria in tobacco (Mazzucchi *et al.*, 1979). Also in symbiotic interactions, LPS plays an important role. For example, the LPS of *Rhizobium* is required for the formation of an infection thread in white clover root hair cells in preparation for the formation of nitrogen-fixing root nodules (Dazzo *et al.*, 1991).

Another group of bacterial determinants shown to have ISR-inducing activity are siderophores. Siderophores are iron chelators that are secreted by microorganisms in

iron-limited environments. Subsequent uptake of these strain-specific siderophores provides the microorganisms with iron (reviewed by Höfte, 1993). The fluorescent siderophores produced by *Pseudomonas* rhizobacteria function in antagonizing soil-borne pathogens by depriving them of iron. But, siderophores can protect plants through the activation of ISR as well. Leeman *et al.* (1996) showed that apart from the LPS, the pseudobactin siderophore of WCS374r can induce ISR in radish. Maurhofer *et al.* (1994) reported that, compared to wild-type *P. fluorescens* CHAO, a siderophore-minus mutant was less effective in inducing resistance against tobacco necrosis virus (TNV) in tobacco, pointing to a role for siderophores in the activation of resistance.

Several resistance-inducing bacteria produce SA as a siderophore at low-iron conditions *in vitro* (Buysens *et al.*, 1996; De Meyer and Höfte, 1997; Leeman *et al.*, 1996; Maurhofer *et al.*, 1994; Press *et al.*, 1997), suggesting that SA produced by the bacteria themselves could serve as a signal triggering the SAR pathway in plants. For the rhizobacterial strain *P. aeruginosa* 7NSK2 this was reported to be the case, because SA-deficient mutants had lost the ability to induce resistance against *Botrytis cinerea* in bean (De Meyer and Höfte, 1997). The resistance against TNV in tobacco leaves induced by CHAO was associated with bacterially produced SA as well (Maurhofer *et al.*, 1994, 1998). Introduction of SA biosynthetic genes from CHAO in the noninducing strain *P. fluorescens* P3 provided this strain with resistance-inducing activity against TNV in tobacco, indicating that SA functions as an inducing bacterial determinant in this system (Maurhofer *et al.*, 1998). However, the involvement of other inducing factors can not be ruled out.

Rhizobacteria-induced plant defense responses

Rhizobacteria-mediated ISR is phenotypically similar to pathogen-induced SAR, in that both result in an enhanced level of broad-spectrum disease resistance. Some reports suggest that both forms of induced resistance are based on the activation of similar defense mechanisms, but others indicate significant differences. The rhizobacterium 7NSK2 was suggested to activate the SAR pathway in bean against *B. cinerea* by producing nanogram amounts of SA, because treatment with either 7NSK2 or 1 nM of SA resulted in comparable increases in both PAL activity in the roots and SA levels in the leaves, two characteristics of enhanced resistance in this system (De Meyer *et al.*, 1999). The rise in SA appeared to be essential for induction of systemic resistance by the 7NSK2 strain, because 7NSK2-mediated resistance against TMV in tobacco plants was blocked in SA-nonaccumulating NahG transformants (De Meyer *et al.*, 1997). Resistance induced by the rhizobacterium CHAO in tobacco against TNV was associated with increased levels of SA and accumulation of acidic PRs in the leaves (Maurhofer *et al.*, 1994), indicating that CHAO triggered the SAR signalling pathway. In contrast, WCS417r-mediated ISR in radish, which is effective against different types of pathogens, was not associated with elevated levels of PRs (Hoffland *et al.*, 1995, 1996). Similar results were found for WCS417r-mediated ISR in carnation against *F.*

oxysporum f sp *dianthi* (E. Hoffland and S.C.M. Van Wees, unpublished result). This shows that some rhizobacteria can elicit ISR without triggering the SAR pathway.

Analogous to SAR, potentiation of defense responses has also been reported for rhizobacteria-mediated ISR. Van Peer and Schippers (1992) reported that in carnation, plants expressing WCS417r-induced ISR showed higher levels of phytoalexins following challenge inoculation with *F. oxysporum* compared to challenged control plants. Similarly, pea roots that were colonized by ISR-inducing *P. fluorescens* 63-28R showed an enhanced level of cell wall rigidifications upon infection with *F. oxysporum* f sp *pisi*, leading to restricted growth of the pathogen (Benhamou *et al.*, 1996).

Apart from these reports, little is known about rhizobacteria-induced metabolic changes in the plant. One may conclude that some rhizobacteria appear to elicit resistance by triggering the SAR signalling pathway, while others do not. This thesis aims at gaining more insight into the molecular mechanisms underlying rhizobacteria-mediated ISR.

Outline of this thesis

In the past decade, the molecular aspects of classic pathogen-induced SAR have been the subject of many studies. In 1994, when the work described in this thesis was started, research on rhizobacteria-mediated ISR was still in its infancy. In this thesis, the first steps in unraveling the molecular basis of ISR are described.

As a start, a model system using *Arabidopsis thaliana* was developed. *Arabidopsis* is a model plant for molecular studies and is frequently used in research on plant-microbe interactions, including SAR. Most conveniently, there is a growing collection of well-characterized *Arabidopsis* mutants, thus making the examination of particular genetic requirements for the expression of ISR directly accessible. Fluorescent *Pseudomonas* spp strains *P. fluorescens* WCS417r, *P. fluorescens* WCS374r, and *P. putida* WCS358r, which in previous studies had been shown to acts as biocontrol agents against fusarium wilt in carnation and radish (Duijff, 1994; Leeman, 1995; Raaijmakers, 1994; Van Peer, 1990) were tested for ISR-inducing activities. Besides *Fusarium oxysporum* f sp *raphani*, the pathogen *P. syringae* pv *tomato* (*Pst*) was chosen as challenging pathogen, because it is a well-established pathogen of *Arabidopsis*.

In Chapters 2 and 3 the *Arabidopsis*-based model system developed to study ISR is described. This research revealed that rhizobacteria-mediated ISR is differentially expressed depending on the host-rhizobacterium combination. Moreover, ISR was found to function independently of SA accumulation and SAR gene expression, indicating that the ISR signalling pathway differs from the one controlling SAR.

The ISR pathway was further investigated in Chapter 4. In this chapter it is demonstrated that in contrast to pathogen-induced SAR, WCS417r-mediated ISR requires responsiveness to jasmonate and ethylene. Evidence is provided that the jasmonate and the ethylene response act sequentially in the ISR signalling pathway. Like SAR, rhizobacteria-mediated ISR is regulated through NPR1. Both upstream and

downstream of NPR1, the signalling pathways diverge, indicating that NPR1 differentially regulates ISR- and SAR-related defense responses.

Chapter 5 shows that simultaneous activation of the ISR and the SAR pathway results in an additive effect on the level of induced protection against *Pst*. Evidence is provided that there is no significant cross-talk between the ISR and the SAR pathway, and that the enhanced level of induced resistance is caused by the parallel activation of complementary defense responses that must both be active against *Pst*.

Chapter 6 describes the expression of a large set of well-characterized defense-related genes in WCS417r-induced plants. None of the genes investigated was significantly induced locally in the roots or systemically in the leaves, at different times after treatment of the plants with WCS417r. Only after challenge inoculation with *Pst*, an enhanced expression of the jasmonate-responsive gene *Atvsp* was observed in ISR-expressing plants, indicating that WCS417r systemically potentiates the expression of selected jasmonate-responsive genes.

In Chapter 7, the attempt to identify ISR-specific molecular markers is described. Two-dimensional gel electrophoresis of proteins from control plants and WCS417r-treated plants was performed to screen for changes in protein accumulation. No differences in the protein patterns were apparent. In addition, a cDNA library representing mRNAs from ISR-expressing leaves was screened to identify mRNAs that accumulate specifically during ISR. Thirteen cDNA clones were isolated and found to be 2- to 9-fold upregulated in ISR-expressing tissue, suggesting that for plants to develop ISR no major changes in gene expression are required.

In Chapter 8, the results are discussed with reference to current ideas about the signalling network regulating inducible plant defenses.

Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression

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Abstract

Systemic acquired resistance is a pathogen-inducible defense mechanism in plants. The resistant state is dependent on endogenous accumulation of salicylic acid (SA) and is characterized by the activation of genes encoding pathogenesis-related proteins (PRs). Recently, selected nonpathogenic, root-colonizing biocontrol bacteria have been shown to trigger a systemic resistance response as well. To study the molecular basis underlying this type of systemic resistance, we developed an *Arabidopsis*-based model system using *Fusarium oxysporum* f.sp. *raphani* and *Pseudomonas syringae* pv. *tomato* (*Pst*) as challenging pathogens. Colonization of the rhizosphere by the biological control strain WCS417r of *P. fluorescens* resulted in a plant-mediated resistance response that significantly reduced symptoms elicited by both challenging pathogens. Moreover, growth of *Pst* in infected leaves was strongly inhibited in WCS417r-treated plants. Transgenic *Arabidopsis* NahG plants, unable to accumulate SA, and wild-type plants were equally responsive to WCS417r-mediated induction of resistance. Furthermore, WCS417r-mediated systemic resistance did not coincide with the accumulation of PR mRNAs before challenge inoculation. These results indicate that WCS417r induces a pathway different from that controlling classical systemic acquired resistance, leading to a form of systemic resistance that is independent of SA accumulation and PR gene expression.

Introduction

Induced disease resistance is the phenomenon by which a plant exhibits an increased level of resistance to infection by a pathogen after appropriate stimulation. This resistance response, first characterized by Ross (1961a,b), is expressed systemically throughout the plant and is effective against a broad spectrum of viral, bacterial, and fungal pathogens (reviewed by Hammerschmidt and Kuć, 1995).

In general, induced resistance can be triggered in three ways: (1) by a predisposing infection with a necrotizing pathogen (Kuć, 1982; Ross, 1961a,b); (2) by treatment with certain chemicals, such as salicylic acid (SA; Malamy and Klessig, 1992; White, 1979) or 2,6-dichloroisonicotinic acid (INA; Métraux *et al.*, 1991); or (3) by colonization of the rhizosphere with selected plant growth-promoting rhizobacteria (PGPR; Van Peer *et al.*, 1991; Wei *et al.*, 1991). Systemic resistance induced by the first two types of inducers is termed systemic acquired resistance (SAR; Ross, 1961b), whereas PGPR-mediated protection is generally referred to as induced systemic resistance (ISR; Kloepper *et al.*, 1992).

Pathogen-induced SAR has been studied most extensively in tobacco and cucumber (Kuć, 1982; Ward *et al.*, 1991) and was recently demonstrated in *Arabidopsis* as well (Cameron *et al.*, 1994; Mauch-Mani and Slusarenko, 1994; Uknes *et al.*, 1993). The resistant state is characterized by an increase in endogenously synthesized SA at the onset of SAR (Malamy *et al.*, 1990; Métraux *et al.*, 1990). Accumulation of SA appears to be critical for the induction of the SAR signaling pathway because transgenic plants unable to accumulate SA are incapable of developing SAR (Gaffney *et al.*, 1993). Furthermore, SAR is associated with the coordinate expression of a set of so-called SAR genes (Ward *et al.*, 1991). These SAR genes include genes encoding pathogenesis-related proteins (PRs; Van Loon, 1985), of which some exhibit limited antifungal activity *in vitro* (Mauch *et al.*, 1988; Roberts and Selitrennikoff, 1988; Vigers *et al.*, 1991; Woloshuk *et al.*, 1991) as well as *in vivo* (Alexander *et al.*, 1993; Broglie *et al.*, 1991; Liu *et al.*, 1994). Exogenous application of the chemicals SA or INA has been reported to mimic pathogen-induced SAR because they induce resistance to the same spectrum of pathogens and concurrently activate the expression of SAR genes (Uknes *et al.*, 1992, 1993; Ward *et al.*, 1991). Although these observations are highly suggestive of a causal relationship between accumulation of PRs and SAR, definitive evidence that they are responsible for broad-spectrum resistance is still lacking.

Selected PGPR, mainly fluorescent *Pseudomonas* spp, have been demonstrated to control plant diseases effectively by suppressing pathogens and deleterious microorganisms through siderophore-mediated competition for iron, or antibiosis (reviewed by Schippers, 1992; Thomashow and Weller, 1996). Recently, research on mechanisms of biological control by PGPR revealed that some PGPR strains protect plants against pathogen infection through induction of systemic resistance, without provoking any symptoms themselves. *P. fluorescens*-mediated ISR was demonstrated in carnation against fusarium wilt by Van Peer *et al.* (1991), and in cucumber against *Colletotrichum orbiculare* infection by Wei *et al.* (1991). More recently PGPR-

mediated induction of ISR has been reported for several other plant-pathogen systems (Leeman *et al.*, 1995a; Liu *et al.*, 1995; Maurhofer *et al.*, 1994; Zhou and Paulitz, 1994).

Maurhofer *et al.* (1994) showed that ISR induced by strain CHA0 of *P. fluorescens* in tobacco against tobacco necrosis virus was accompanied by an increase in the accumulation of PRs, suggesting that PGPR-mediated ISR and pathogen-induced SAR are manifestations of a similar defense mechanism. However, Hoffland *et al.* (1995) were unable to establish an accumulation of PRs in radish displaying substantial ISR against *Fusarium oxysporum* when plants were treated with strain WCS417r of *P. fluorescens*. Therefore, it is unclear whether PGPR-mediated ISR and pathogen-induced SAR share a common signal transduction pathway. With the goal of addressing whether a common pathway is shared, two bioassays for PGPR-mediated ISR were developed by using *Arabidopsis* as the host plant and a rifampicin-resistant mutant of the nonpathogenic, root-colonizing PGPR strain WCS417 of *P. fluorescens* (WCS417r; Van Peer *et al.*, 1991) as an inducer. WCS417 is an effective biocontrol agent of the take-all disease in wheat caused by *Gaeumannomyces graminis* pv *tritici* (Lamers *et al.*, 1988) and has been demonstrated to be a strong inducer of ISR against vascular wilt caused by *F. oxysporum* in carnation and radish (Leeman *et al.*, 1995a; Van Peer *et al.*, 1991). Previously, Van Peer and Schippers (1992) and Leeman *et al.* (1995b) demonstrated that the extracellular lipopolysaccharide (LPS) of WCS417r is sufficient to elicit ISR in carnation and radish, respectively, indicating that this type of resistance is not based on microbial antagonism but is plant mediated. In this study, we describe WCS417r-mediated ISR in *Arabidopsis* against the fungal root pathogen *F. oxysporum* f sp *raphani* (*For*) and the bacterial leaf pathogen *P. syringae* pv *tomato* (*Pst*). In addition, we provide evidence that, in contrast to classical SAR, induction of WCS417r-mediated ISR is independent of both endogenous SA accumulation and *PR* gene activation.

Results

PGPR-mediated ISR against For

The soil-borne fungus *F. oxysporum* is the causal agent of vascular wilt in a wide range of host plants, including economically important crops. The fungus invades the roots and colonizes the vascular tissue. *Arabidopsis* is susceptible to infection by *For*. To study PGPR-mediated ISR against this pathogen in *Arabidopsis*, a system ensuring spatial separation of the inducing agent and the challenging root pathogen was used as described by Leeman *et al.* (1995a). For induction of ISR, a suspension of WCS417r in 10 mM MgSO₄, mixed with talcum powder as a carrier, was applied to the lower part of the root system. As a control, 10 mM MgSO₄ was applied in a similar manner. Three days later, the upper parts of the roots were inoculated with *For* mixed with sterilized peat, or mock-inoculated with sterilized peat. After inoculation, a relatively long latent period (2 weeks) during which no fusarium wilt symptoms were visible was followed by a phase of rapid symptom development. Thereafter, leaves turned yellow, beginning

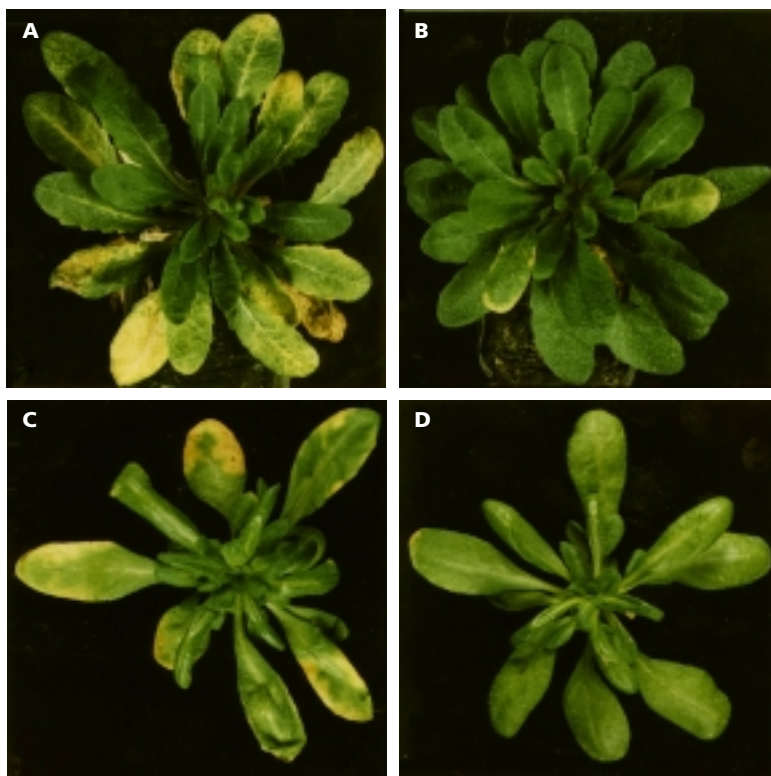


Figure 1. Response of *Arabidopsis* to *For* and *Pst* infection following WCS417r-mediated ISR.

A and **B** show fusarium wilt symptoms on Col-0 plants pretreated with either 10 mM MgSO_4 (**A**) or WCS417r (**B**) 19 days after challenge inoculation with *For*. Inoculations were performed by applying 1×10^6 conidia of *For* in 0.25 g of peat to the upper parts of the roots that were spatially separated from the treated lower parts of the roots. **C** and **D** show symptoms caused by *Pst* infection on *Ler* plants grown in soil supplemented with either 10 mM MgSO_4 (**C**) or WCS417r (**D**) 4 days after challenge inoculation. Leaves were inoculated with *Pst* by dipping them into a 1×10^8 cfu/ml bacterial suspension in 10 mM MgSO_4 , 0.01% (v/v) Silwet L-77.

with the veins, then wilted, and finally died. Occasionally, wilting of the leaves started before yellowing became apparent. Progressive development of disease eventually led to death of the plant.

Figures 1A and B show typical differences in symptom expression of fusarium wilt between nontreated *A. thaliana* ecotype Columbia (Col-0) plants and WCS417r-treated Col-0 plants. As shown in Figure 2A, in control plants the percentage of leaves per plant showing yellowing and wilting increased markedly between 17 and 24 days after inoculation. In WCS417r-treated plants, disease progressed substantially more slowly, and the number of plants with 76-100% diseased leaves was reduced by 80% at 24 days after inoculation. Thus, colonization of the rhizosphere by WCS417r resulted in a delay in symptom development and reduction of disease severity. Similar results were obtained with ecotype Landsberg *erecta* (*Ler*) (data not shown).

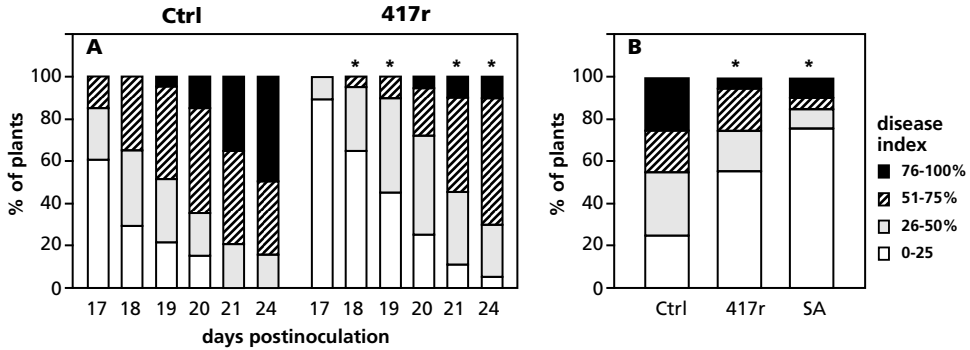


Figure 2. Classification of diseased plants in the *Arabidopsis-For* bioassay.

A shows a time course of fusarium wilt symptom development in control and WCS417r-treated *Arabidopsis* plants 17 to 24 days after inoculation. In **B**, disease severity in SA-, WCS417r-, and noninduced *Arabidopsis* plants 19 days after inoculation with *For* is depicted. The lower parts of the roots, which were spatially separated from the inoculated upper parts of the roots, were treated with 10 mM MgSO_4 (Ctrl), WCS417r (417r), or 1 mM SA (SA). The disease index is based on the percentage of leaves per plant showing fusarium wilt symptoms. Asterisks indicate statistically significant differences in frequency distribution compared to the noninduced control plants (In **A**, Wilcoxon's two sample test, $n=20$, $\alpha=0.05$, was applied; In **B**, Kruskal-Wallis multiple comparison test, $n=20$, $\alpha=0.05$, was applied).

To examine the inducing potential of WCS417r in comparison to SA, which is known to be an effective inducer of SAR (Malamy and Klessig, 1992; Uknes *et al.*, 1993), induction treatments with 1 mM SA and WCS417r were performed. Both agents caused a statistically significant reduction of symptoms compared to the control treatment (Figure 2B). There were no statistically significant differences in disease severity between the SA- and WCS417r-treated plants. In all experiments, mock-inoculated control plants remained healthy, and no apparent differences in the growth and development of control, WCS417r-, and SA-treated plants were observed.

PGPR-mediated ISR against *Pst*

Pst causes bacterial speck disease of tomato and has been demonstrated to be virulent on *Arabidopsis* as well (Dong *et al.*, 1991; Whalen *et al.*, 1991). Previously, *Arabidopsis* was shown to develop SAR against infection with *Pst* when induced by necrotizing pathogens (Uknes *et al.*, 1993) or chemical agents (Uknes *et al.*, 1992). To test for PGPR-mediated ISR in this pathosystem, *Arabidopsis* seedlings were planted in soil with or without WCS417r. Five-week-old plants were challenge inoculated with *Pst*. As a positive control, a solution of 1 mM SA was applied to the plants as a soil drench 7 and 4 days before challenge inoculation.

Four days after inoculation with *Pst*, leaves of control *Ler* plants displayed necrotic lesions surrounded by extensive spreading chlorosis (Figure 1C), whereas *Ler* plants pretreated with WCS417r (Figure 1D) or SA showed significantly fewer symptoms. Mock-inoculated control plants remained symptomless. Plants grown in sand/potting soil mixture supplemented with WCS417r bacteria were slightly larger than control

and SA-treated plants, indicating that this PGPR strain can stimulate plant growth in *Arabidopsis* as observed in other species (Van Peer and Schippers, 1989).

Protection against *Pst* was quantified by assessing the proportion of leaves per plant showing symptoms 4 or 5 days after challenge inoculation. Figure 3A shows that treatment of *Ler* plants with WCS417r or SA resulted in a statistically significant reduction in the percentage of leaves with symptoms. The level of protection against *Pst* infection induced by WCS417r was similar to that induced by SA (disease severity 60 and 65%, respectively, of that observed in the control).

To examine whether the observed reduction of symptoms was associated with diminished pathogen growth in the leaves, the number of *Pst* cells was monitored in inoculated leaves of control, WCS417r- and, SA-treated plants. In mock-inoculated plants, no rifampicin-resistant bacteria were detected (data not shown). The number of rifampicin-resistant cells per gram of infected leaf tissue was assessed at 15 min after inoculation to determine the number of *Pst* bacteria that entered the leaves (typically about 10^6 per gram of leaf tissue). Subsequently, at two time points after inoculation, the number of colony-forming units (cfu) of *Pst* in the leaves was determined. Figure 3B illustrates that treatment with WCS417r or with SA resulted in inhibition of growth of *Pst* in the leaves compared to control plants. Growth of *Pst* in WCS417r- and SA-treated plants was inhibited up to 10-fold by day 1 after inoculation. Three days later, differences in bacterial proliferation between control and protected plants increased up to 22-fold.

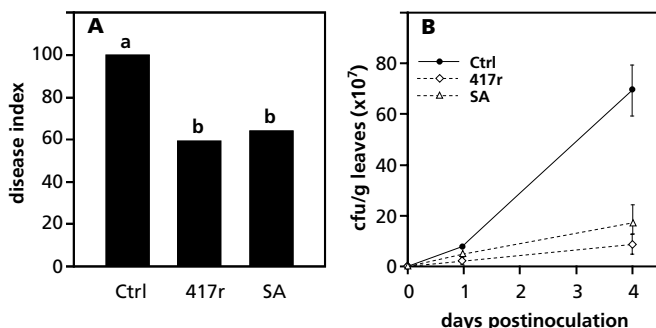


Figure 3. Quantification of induced resistance in the *Arabidopsis-Pst* bioassay.

ISR was induced by growing plants in soil supplemented with WCS417r bacteria (417r). SAR was induced by supplying the plants with 1 mM SA (SA) as a soil drench 4 and 7 days before challenge inoculation. Control plants were cultured in soil supplemented with 10 mM MgSO_4 (Ctrl). The disease index depicted in **A** is the mean ($n=25$ plants) of the proportion of leaves with symptoms per plant compared to that of control plants (set at 100%), 4 days after challenge inoculation with *Pst*. The absolute proportion of diseased leaves of control plants was 74.3%. Different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha=0.05$). Time course of growth of *Pst* in the leaves is shown in **B**. Data points are means (cfu/g) with standard errors from two sets of 20 randomly selected leaves from plants of the bioassay shown in **A**. The values presented are the averages of two independent experiments.

WCS417r-induced protection is plant mediated

In the bioassays performed, WCS417r and the challenging pathogens were spatially separated to prevent direct interaction. To verify whether WCS417r and *For* remained spatially separated for the duration of the assay, the presence of WCS417r in both root zones was evaluated at the end of each experiment. Table 1 shows that WCS417r bacteria were recovered from the induction treatment zone at mean population densities of 5.4×10^5 cfu/g of root fresh weight. No rifampicin-resistant bacteria were recovered from the *For*-inoculated zone. This observation shows that WCS417r remained spatially separated from the inoculated zone throughout the experiment. In addition, possible spreading of WCS417r from roots to leaves was assessed by plating leaf extracts from root-induced plants onto selective King's medium B agar plates (King *et al.*, 1954). Table 1 shows that in all bioassays performed, WCS417r bacteria were absent from the leaves of root-induced plants, indicating that WCS417r remained localized at the roots. These data clearly demonstrate that WCS417r-mediated protection is not due to microbial antagonism but results from increased resistance in the plant.

In spite of the demonstrated spatial separation of the inducing WCS417r bacteria and the challenging pathogens, indirect interaction between both microorganisms would be possible if PGPR-mediated production of antibiotics antagonized the

Table 1. Colonization of *Arabidopsis* by WCS417r.

Bioassay	cfu/g fresh weight ($\times 10^5$)	
	Control	WCS417r
<i>For</i> - Col-0		
Induction treatment root zone ¹	b.d. ³	5.4 ± 0.5
Inoculated root zone ¹	b.d.	b.d.
Leaves ²	n.d. ⁴	b.d.
<i>Pst</i> - Ler		
Roots ¹	b.d.	2.6 ± 0.4
Leaves ²	n.d.	b.d.
<i>Pst</i> - Col-0		
Roots ¹	b.d.	2.8 ± 0.6
Leaves ²	n.d.	b.d.
<i>Pst</i> - NahG		
Roots ¹	b.d.	3.3 ± 0.6
Leaves ²	n.d.	b.d.

¹ Roots were harvested at the end of the bioassays.

² Leaves were harvested at different time points to check for systemic colonization by WCS417r.

³ B.d. = below detection (detection limit 10^3 cfu/g).

⁴ N.d. = not determined.

pathogen at distant sites. However, such antagonism did not play a role. First, WCS417r did not inhibit growth of *For* and *Pst* *in vitro*. Second, treatment of *Arabidopsis* roots with cell wall extracts of WCS417r resulted in induced protection, whereas cell wall extracts from WCS417rOA⁻, a mutant lacking the O-antigenic side chain of the LPS, showed no inducing activity. This indicates that bacterial LPS is involved in eliciting the ISR response in *Arabidopsis* (Chapter 3).

PGPR-mediated ISR is expressed in NahG plants

Previously, Gaffney *et al.* (1993) demonstrated that transgenic tobacco plants expressing the bacterial salicylate hydroxylase (*NahG*) gene are unable to accumulate SA after pathogen infection. Consequently, NahG plants do not develop SAR when induced by a pathogen. To investigate whether PGPR-mediated ISR is also dependent on SA production, *Arabidopsis*-*Pst* bioassays were performed using transgenic Col-0 plants expressing the *NahG* gene (NahG plants; Delaney *et al.*, 1994) and wild-type Col-0 plants. Both were treated with WCS417r, INA, or SA. WCS417r was applied to the soil before planting, whereas INA and SA were sprayed onto the leaves 4 days before challenge inoculation. Protection against *Pst* infection was assessed by determining the percentage of leaves with symptoms 5 days after challenge inoculation and by monitoring growth of the pathogen in the leaves.

Figures 4A and B show that in Col-0 plants, WCS417r induced resistance against *Pst* to an extent similar to that in *Ler* plants (disease severity was 48% of that observed in the control). Spraying Col-0 plants with INA and SA resulted in a significantly higher level of protection (disease severity was 13 and 3%, respectively, of that observed in the control). Control NahG plants were more sensitive to *Pst* infection than were wild-type Col-0 plants in that symptoms elicited by *Pst* were more severe and the proliferation rate of this pathogen was considerably higher (166-fold) (Figure 4). This result agrees with those of others (Delaney *et al.*, 1994; Gaffney *et al.*, 1993; Vernooij *et al.*, 1994, 1995) who found that the disease symptoms in tobacco and *Arabidopsis* NahG plants were significantly more severe than in wild-type plants. Based on these results, the authors postulated that SA has a role not only in SAR but also in the primary resistance response to pathogen infection.

INA-treated NahG plants showed reduced disease severity and inhibition of growth of the pathogen to an extent similar to that found in INA-treated wild-type plants (Figure 4). This observation is consistent with previous findings (Delaney *et al.*, 1994; Vernooij *et al.*, 1995) demonstrating that INA induction is not affected by the *NahG* gene. In contrast, SA-treated NahG plants showed no inhibition of growth of *Pst*. Moreover, a considerably lower level of protection was observed in SA-treated NahG plants compared to similarly treated wild-type plants (disease severity was 60 and 3%, respectively, of that observed in the controls). However, SA-treated NahG plants were clearly less diseased than control NahG plants, possibly because NahG plants need time to convert the excess of exogenously applied SA (5 mM) and as a result show a delay in symptom development.

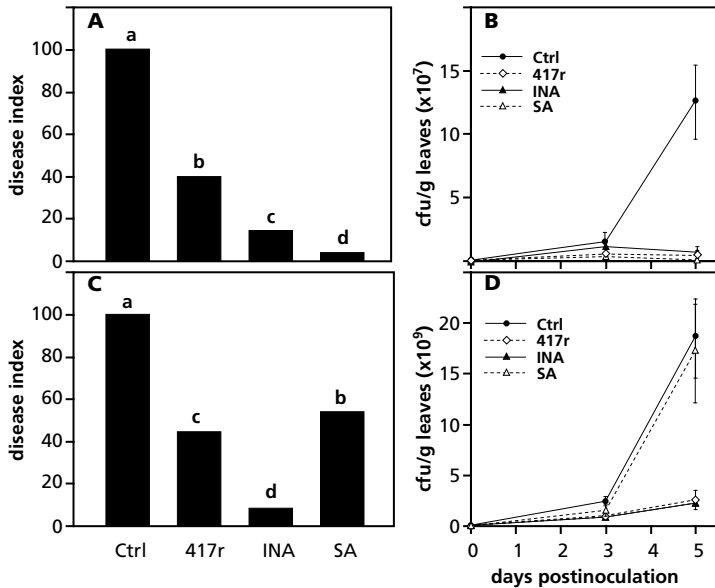


Figure 4. Quantification of protection against *Pst* in *Arabidopsis* Col-0 and NahG plants treated with WCS417r, INA, or SA.

Induction treatments were performed by culturing the plants in soil supplemented with 10 mM MgSO_4 (Ctrl) or WCS417r bacteria (417r), or by spraying the leaves until imminent runoff with a solution of 325 μM INA (INA) or 5 mM SA (SA) 4 and 7 days before challenge inoculation of Col-0 (**A** and **B**) or NahG (**C** and **D**) plants. The disease index depicted in **A** and **C** is the mean ($n=25$ plants) of the proportion of leaves with symptoms per plant compared to that of control plants (set at 100%), 5 days after challenge inoculation with *Pst*. The absolute proportions of diseased leaves of control plants depicted in **A** and **C** were 57.3% and 94.8%, respectively. Different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha=0.05$). Time course of growth of *Pst* in the leaves is shown in **B** and **D**. Data points are means (cfu/g) with standard errors from two sets of 20 randomly selected leaves from plants of the bioassay shown in **A** and **C**, respectively. The data presented are from a representative experiment that was repeated twice with similar results.

Notably, in WCS417r-treated Col-0 and NahG plants, the level of induced protection was similar (disease severity was 48 and 43%, respectively, of that observed in the controls). Table 1 shows that the roots of Col-0 and NahG plants harbored similar levels of WCS417r bacteria and that the inducing bacteria remained confined to the rhizosphere. Thus, in contrast to SA but like INA, WCS417r induced resistance to similar levels in wild-type and NahG plants, indicating that WCS417r-mediated induction of ISR is independent of SA accumulation.

Expression of PR genes

Induction of SAR by necrotizing pathogens or selected abiotic agents in *Arabidopsis* is associated with the coordinated accumulation of *PR-1*, *PR-2*, and *PR-5* mRNAs (Uknes *et al.*, 1992, 1993). Analysis of *PR-1* mRNA levels in treated Col-0 and NahG plants using the competitive reverse transcriptase-polymerase chain reaction (RT-PCR)

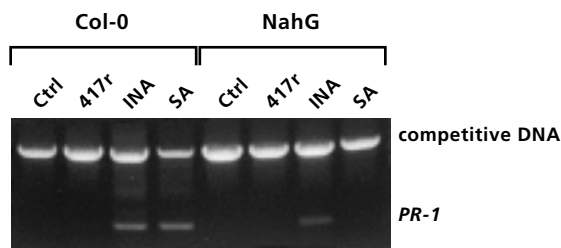


Figure 5. Competitive RT-PCR analysis of *PR-1* gene expression in *Arabidopsis* Col-0 and NahG plants treated with WCS417r, INA, or SA. Shown is an ethidium bromide-stained agarose gel with competitive RT-PCR products obtained after amplification of equal portions of first-strand cDNA and 500 pg of heterologous competitor DNA using *PR-1* specific primers. First-strand cDNA was synthesized on mRNA isolated from leaves of the

indicated plant and treatment combinations. Leaves were harvested just before challenge inoculation. For treatments see legend to Figure 4. Competitive DNA indicates the 900-bp heterologous competitor DNA amplified from 500 pg; *PR-1* indicates the 422-bp DNA fragment amplified from *PR-1* cDNA.

(Figure 5) shows that INA induced *PR-1* mRNA accumulation in both wild-type Col-0 plants and NahG plants, whereas SA induced *PR-1* gene expression in Col-0 plants but not in NahG plants. This result agrees with the expression of SAR in these plants. In contrast, WCS417r-treated Col-0 and NahG plants do not show increased accumulation of *PR-1* mRNA, whereas systemic resistance was clearly induced (Figures 4A and B).

To compare induction of *PR* gene expression by different inducers of systemic resistance, the expression of the *PR-1*, *PR-2*, and *PR-5* genes was studied in

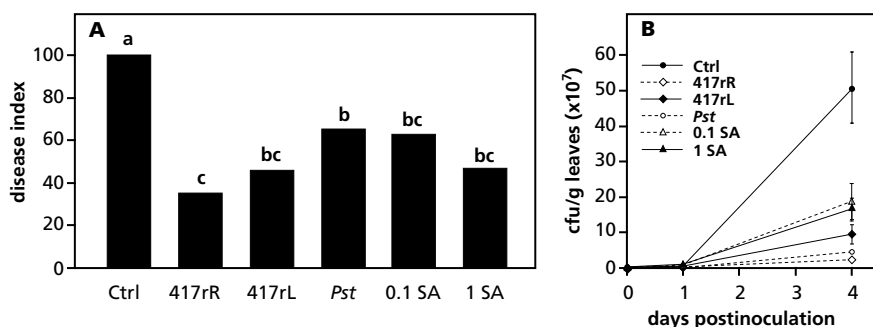


Figure 6. Quantification of resistance against *Pst* in *Arabidopsis* induced by pressure infiltrating leaves with WCS417r, *Pst*, or SA, or through colonization of the rhizosphere by WCS417r.

Induction treatment of the roots was performed by culturing the plants in soil supplemented with WCS417r bacteria (417rR). Treatment of the leaves was carried out by pressure infiltrating three leaves either with a solution of 10 mM MgSO_4 (Ctrl), 0.1 mM SA (0.1 SA) or 1 mM SA (1 SA), or with a bacterial suspension of 10^7 cfu/ml of WCS417r (417rL) or *Pst* (*Pst*). Leaves were treated 8 days before challenge inoculation. A shows the disease index, which is the mean ($n=25$ plants) of the proportion of leaves with symptoms per plant compared to that of control plants (set at 100%), 4 days after challenge inoculation with *Pst*. The absolute proportion of diseased leaves of control plants was 54.4%. Different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha=0.05$). Time course of growth of *Pst* in the leaves is shown in B. Data points are means (cfu/g) with standard errors from two sets of 20 randomly selected leaves from plants of the bioassay shown in A. The data presented are from a representative experiment that was repeated twice with similar results.

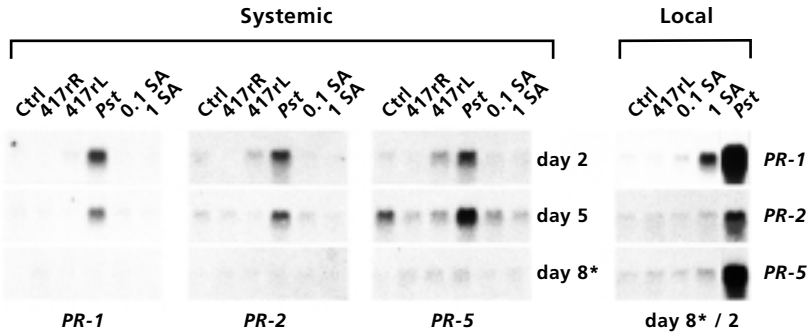


Figure 7. RNA blot analyses of *PR* gene expression in *Arabidopsis* in response to induction of resistance by WCS417r, *Pst*, and SA.

A subset of plants from the *Arabidopsis*-*Pst* bioassay (the results of which are presented in Figure 6) was used for RNA extractions. See for treatments the legend to Figure 6. RNA was isolated from pressure-infiltrated leaves (Local) and noninfiltrated leaves (Systemic) that were harvested at indicated time points after infiltration. Leaves infiltrated with *Pst* were sampled at day 2, whereas the other infiltrated leaves were harvested at day 8. The asterisks indicate that leaves were harvested just prior to challenge inoculation. Plants induced by colonization of the roots by WCS417r (417rR) were harvested at the same time points as plants induced by pressure infiltration. *Arabidopsis* *PR-1*, *PR-2*, and *PR-5* gene-specific probes were used for RNA blot hybridizations.

WCS417r-, *Pst*-, and SA-induced plants. Induction of ISR in *Ler* plants through colonization of the rhizosphere by WCS417r was performed as described above for the *Pst* bioassay. At the same time, induction treatments were performed by pressure infiltration of 10 mM MgSO₄ (control), WCS417r (10⁷ cfu/ml), *Pst* (10⁷ cfu/ml), or SA (0.1 and 1 mM) into three lower leaves of 4-week-old *Ler* plants. *Pst*-infiltrated leaves showed heavy necrosis after 4 days, whereas leaves infiltrated with 10 mM MgSO₄, WCS417r, or SA remained symptomless. Figures 6A and B show that infiltration of the leaves with WCS417r, *Pst*, or SA and colonization of the rhizosphere by WCS417r resulted in a significant level of systemic protection after challenge inoculation of upper leaves with *Pst*.

PR gene expression was studied in infiltrated as well as in nontreated leaves of induced and noninduced plants harvested at different time points after induction treatment. RNA blot analyses (Figure 7) show that *Pst* infection caused a high, local accumulation of *PR-1*, *PR-2*, and *PR-5* transcripts 2 days after infiltration. Later time points could not be studied because of the formation of heavy necrosis. Systemic accumulation of *PR* mRNAs was observed up to 5 days after induction by *Pst* infiltration. At 8 days, the day of challenge inoculation, *PR* mRNA levels had dropped to undetectable levels. In contrast, no local accumulation of *PR* mRNA was observed in leaves infiltrated with WCS417r or 0.1 mM SA, whereas the increase in *PR* transcript levels after infiltration with 1 mM was moderate. Moreover, neither colonization of the rhizosphere nor infiltration of leaves with WCS417r bacteria or SA resulted in a systemic induction of *PR-1*, *PR-2*, and *PR-5* gene expression. Nonetheless, these treatments clearly induced systemic resistance (Figure 6). To rule out the possibility

that in protected plants PRs accumulated from low mRNA levels, proteins were extracted from leaves and roots of control plants and from plants protected by colonization of the rhizosphere with WCS417r. Protein blot analyses showed no specific accumulation of the proteins PR-1, PR-2, or PR-5 in leaves or roots from protected plants compared to control plants (data not shown). These results clearly demonstrate that WCS417r-mediated induction of systemic resistance is not associated with the activation of *PR* genes and subsequent accumulation of PRs before challenge inoculation.

Discussion

Systemic resistance in Arabidopsis induced by biocontrol bacteria

With the goal of developing *Arabidopsis* as a model system for studying PGPR-mediated ISR, we demonstrated that WCS417r effectively protects *Arabidopsis* against infection by *For* as well as *Pst*. Root colonization by WCS417r resulted in a marked delay in symptom development and reduction in disease severity after challenge inoculation with *For* (Figure 2). Likewise, WCS417r reduced both the visible symptoms caused by *Pst* infection and growth of this pathogen in the leaves (Figures 3, 4, and 6). Because inducing bacteria and challenging pathogens remained spatially separated throughout the experiment, antagonism by direct interactions could be ruled out, demonstrating that WCS417r-induced protection is plant mediated. Among the bacterial determinants implicated in eliciting metabolic events in plants is the outer membrane LPS (Dazzo *et al.*, 1991; Graham *et al.*, 1977; Mazzucchi *et al.*, 1979; Newman *et al.*, 1995). Previously, it was demonstrated that the LPS of WCS417r is involved in eliciting systemically enhanced resistance in carnation (Van Peer and Schippers, 1992), radish (Leeman *et al.*, 1995b), and *Arabidopsis* (Chapter 3), indicating that PGPR-mediated protection is accomplished by induction of ISR in the plant. In *Arabidopsis*, this resistance response is effective against a fungal root pathogen as well as a bacterial leaf pathogen. This demonstrates that, like pathogen-induced SAR, WCS417r-mediated ISR is effective against different types of pathogens.

WCS417r-mediated ISR is independent of SA accumulation and PR gene expression

Endogenous accumulation of SA is one of the characteristics of pathogen-induced SAR (Malamy *et al.*, 1990; Métraux *et al.*, 1990). Transgenic NahG plants that are unable to accumulate SA do not develop a SAR response when treated with a pathogen, indicating that SA accumulation is a crucial step in the signal transduction pathway leading to SAR (Gaffney *et al.*, 1993). WCS417r is equally capable of inducing ISR in *Arabidopsis* NahG and wild-type plants (Figure 4). This demonstrates that, in contrast to pathogen-induced SAR, endogenous accumulation of SA is not required for induction of WCS417r-mediated ISR. It can thus be concluded that an SA-independent signalling pathway is involved in the elicitation of WCS417r-mediated ISR.

Another important feature of pathogen-induced SAR is the activation of *PR* gene expression in nontreated leaves (Uknes *et al.*, 1993; Ward *et al.*, 1991). Accordingly, we observed systemic accumulation of *PR-1*, *PR-2*, and *PR-5* mRNAs when using *Pst* as a pathogenic inducer of SAR (Figure 7). Exogenous application of SA and INA is reported to induce SAR and *PR* gene expression as well (Malamy and Klessig, 1992; Uknes *et al.*, 1992; Ward *et al.*, 1991). In *Arabidopsis* leaves sprayed with SA and INA, we indeed observed an induced accumulation of *PR-1* mRNA (Figure 5). However, we never observed a detectable systemic activation of *PR* gene expression after application of SA as a soil drench (data not shown) or upon pressure infiltration of leaves with SA (Figure 7), whereas systemic protection was clearly induced in these plants (Figures 2, 3, and 6, respectively). Hence, SA is acting only locally on the expression of *PR* genes in *Arabidopsis*, as has also been observed in tobacco (Van Loon and Antoniw, 1982; Vernooij *et al.*, 1994).

In contrast to inducers of SAR in *Arabidopsis*, WCS417r induces systemic resistance without activating *PR* gene expression (Figures 5 and 7) and subsequent accumulation of PRs (data not shown). This demonstrates that activation of *PR* genes is not a prerequisite for the induction of ISR in *Arabidopsis*. This result supports and extends recent findings of Hoffland *et al.* (1995), who demonstrated that WCS417r-mediated ISR against *For* is not associated with accumulation of PRs in roots and leaves of radish. WCS417r thus appears to be a definitive biological inducer of systemic resistance that does not simultaneously activate *PR* gene expression. These results differ from those of Maurhofer *et al.* (1994), who showed that systemic resistance induced by PGPR strain CHA0 of *P. fluorescens* in tobacco against tobacco necrosis virus coincides with the accumulation of proteins of the PR-2 and PR-3 family. However, those authors suggested that induction of resistance may be due to enhanced stress caused by strain CHA0. On the contrary, we observed stimulation of growth rather than stress effects in WCS417r-treated *Arabidopsis* plants, suggesting that different mechanisms are involved.

PRs and systemic resistance

The ability of *Arabidopsis* to develop a systemic resistance response without concomitant *PR* gene activation sheds new light on the role of PRs in systemic resistance. Although not required for WCS417r-mediated ISR, accumulation of PRs seems to be essential for the expression of pathogen-induced SAR. In their attempt to genetically dissect the signal transduction pathway regulating SAR in *Arabidopsis*, Cao *et al.* (1994) showed that mutant *npr1*, which is a nonexpresser of *PR* genes, is nonresponsive to induction of SAR by SA, INA, or avirulent pathogens. Similarly, Delaney *et al.* (1995) showed that the noninducible immunity mutant *nim1*, which is insensitive to chemical and biological induction of SAR, is also unable to activate *PR* genes upon treatment with inducers of SAR. Moreover, mutant *cpr1*, which is a constitutive expresser of *PR* genes, also expresses SAR constitutively (Bowling *et al.*, 1994). Although these findings are suggestive of a critical role of PRs in SAR, the evidence is circumstantial as other factors in the signalling pathway downstream of

npr1, *nim1*, and *cpr1* may contribute to the induced resistant state as well. Furthermore, in other plant species, a causal relationship between accumulation of PRs and SAR has never been convincingly demonstrated. Nevertheless, the fact that transgenic tobacco plants, constitutively expressing one or more *PR* genes, show enhanced resistance to a limited number of fungal pathogens (Alexander *et al.*, 1993; Broglie *et al.*, 1991; Liu *et al.*, 1994) demonstrates that PRs can contribute to resistance. However, it is unlikely that they are responsible for the broad-spectrum induced resistance characteristic of SAR.

WCS417r-mediated ISR versus pathogen-induced SAR

Disease reduction in plants expressing WCS417r-mediated ISR typically ranged from 40 to 60%. Significantly higher levels of protection (up to 97%) were achieved when using chemical inducers of SAR as a spray (Figure 4). Nevertheless, our results clearly demonstrate that, when using WCS417r as inducing agent, systemic resistance manifested effectively in the absence of both endogenous accumulation of SA and expression of the *PR* genes. This indicates that WCS417r induces an SA-independent signalling pathway that is different from that controlling SAR. Whether the signalling pathways regulating WCS417r-mediated ISR and pathogen-induced SAR converge to elicit a similar or partially similar phenotypic effect remains to be determined. It is possible that certain defense-related activities induced in both pathways are responsible for enhancing resistance to an intermediate level. Pathogenic and chemical inducers of *PR* gene expression might further enhance resistance through activity of PR proteins. In this scenario, SAR and the observed PGPR-mediated ISR may share a part of the signal transduction pathway(s) controlling systemic resistance. Alternatively, WCS417r could trigger a completely different, parallel pathway unconnected with SA accumulation and activation of *PR* genes. Whatever the case may be, further study of the *Arabidopsis*-WCS417r system will provide important information on the regulation of ISR.

Materials and methods

Fungal cultures

Fusarium oxysporum f. sp. *raphani* WCS600 (*For*) was initially isolated from the roots of a naturally infected radish plant (Leeman *et al.*, 1995a). The fungus was maintained on potato-dextrose agar. A suspension of 10^8 conidia/ml in 10 mM MgSO_4 was prepared from a culture grown for 7 days at 22°C on aerated 2% malt extract. This conidial suspension (0.1 ml/g) was added to sterile peat (Agrifutur s.r.l., Alfianello, Italy) and allowed to germinate and grow for 2 days at 24°C. The final density of colony-forming units (cfu) in the peat was determined by plating dilutions of a peat suspension on potato-dextrose agar. An inoculum density of 4×10^6 cfu/g of peat was used in the *Arabidopsis*-*For* bioassays.

Bacterial cultures

A rifampicin-resistant mutant of the biocontrol strain *Pseudomonas fluorescens* WCS417 (WCS417r), which was originally isolated from the rhizosphere of wheat, grown in a field suppressive to take-all disease caused by *Gaeumannomyces graminis* pv. *tritici* (Lamers *et al.*, 1988), was used throughout this study. WCS417r was grown on King's medium B (KB) agar plates (King *et al.*, 1954) for 24 h at 28°C. The bacterial cells were collected and resuspended in 10 mM MgSO_4 .

The necrotizing leaf pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*; Whalen *et al.*, 1991) was cultured overnight in liquid KB at 28°C. After we collected the cells by centrifugation, bacterial cells were resuspended in 10 mM MgSO₄.

Cultivation of plants

For the *Arabidopsis thaliana*-*For* bioassay, ecotype Columbia (Col-0) or ecotype Landsberg *erecta* (*Ler*) seeds were sown singly in 1-ml pipette tips filled with sterile quartz sand to stimulate root elongation. The tips were drenched daily in water for 30 min and once a week in modified half-strength Hoagland's nutrient solution (2 mM KNO₃, 5 mM Ca(NO₃)₂, 1 mM KH₂PO₄, 1 mM MgSO₄, and trace elements, pH 7; Hoagland and Arnon, 1938) containing 10 µM Sequestreen (CIBA-Geigy, Basel, Switzerland). After 2 weeks, seedlings were gently washed out of the pipette tips and transferred to a rock wool system containing two spatially separated compartments, allowing an induction treatment and a challenge inoculation of the same root system (Leeman *et al.*, 1995a). Briefly, rock wool cubes (Rock-wool/Grodan B.V., Roermond, The Netherlands) were drenched in nutrient solution (see above) and placed in two polyethylene bags. Three-week-old seedlings with roots 2-3 cm in length were positioned horizontally on two separated rock wool cubes through a vertical incision in the bags; the lower part of the roots on one cube and the upper part, along with the rosette, on the other. Both parts of the roots were covered with small rock wool cubes to prevent desiccation and exposure to light.

For the *Arabidopsis*-*Pst* bioassay, seeds of *Ler*, Col-0, and Col-0 plants harboring the bacterial *NahG* gene (Delaney *et al.*, 1994) were sown in sterile quartz sand. Two weeks later, seedlings were transferred to pots (60 ml) containing a sand/potting soil mixture that had been autoclaved twice before application of WCS417r bacteria or 10 mM MgSO₄.

Plants were cultivated in a growth chamber with a 9-h day (200 µE/m²/sec at 24°C) and 15-h night (20°C) cycle at 65% relative humidity. Plants were watered or supplied with modified Hoagland's nutrient solution twice a week.

Arabidopsis-For ISR bioassay

After transfer of the *Arabidopsis* seedlings to the rock wool separate inoculation system, the lower parts of the roots were covered with either 1 ml of a 1:1 (v/w) WCS417r/talcum suspension (final concentration of 5×10^8 cfu/g), a 1:1 (w/v) mixture of talcum with 1 mM salicylic acid (SA), pH 6, or a 1:1 (w/v) mixture of talcum with 10 mM MgSO₄. Three days after induction treatment, the plants were inoculated with *For* by applying about 0.25 g of peat inoculum (4×10^6 cfu/g) to the upper parts of the roots. Control plants were mock inoculated with about 0.25 g of sterile peat. For each treatment, a set of 20 individual plants was used. Protection against *For* was analyzed at different time points after inoculation by determining the frequency distribution of plants over four distinct disease severity classes, i.e. 0-25, 26-50, 51-75, and 76-100% of the leaves showing fusarium wilt symptoms. Data were statistically analyzed using Wilcoxon's two-sample test at $\alpha=0.05$ or the Kruskal-Wallis multiple comparison test at $\alpha=0.05$.

Arabidopsis-Pst ISR bioassay

Before transfer of the *Arabidopsis* seedlings to the sand/potting soil mixture, a WCS417r suspension (10^9 cfu/ml) was mixed thoroughly through the sterile sand/potting soil mixture to a final density of 5×10^7 cfu/g. Nontreated soil was supplemented with an equal volume of sterile 10 mM MgSO₄. SA induction treatment was performed either by applying 20 ml of a 1 mM SA solution (pH 6) as soil drench to the plants 7 and 4 days before challenge inoculation, or by spraying a 5 mM SA solution on the leaves to the point of imminent runoff 4 days before inoculation. 2,6-Dichloroisonicotinic acid (INA; 325 µM), formulated as a 25% active ingredient in a wettable powder carrier (Ciba-Geigy AG), was suspended in water and sprayed on the leaves in a similar manner.

Five-week-old plants were challenge inoculated with *Pst* by dipping the leaves in a bacterial suspension containing 10^8 cfu/ml (*Ler*) or 2.5×10^7 cfu/ml (Col-0 and *NahG*) in 10 mM MgSO₄, supplemented with 0.01% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). Mock inoculations were performed with a solution of 10 mM MgSO₄, 0.01% (v/v) Silwet L-77 without bacteria. One day before challenge inoculation, the plants were placed at 100% relative humidity. Growth of *Pst* was assessed in inoculated leaves of control and treated plants at different time points after inoculation. Two sets of 20 randomly selected leaves per treatment were weighed, rinsed thoroughly in sterile water, and homogenized in 10 mM MgSO₄. Subsequently, dilutions were plated onto KB agar supplemented with rifampicin (50 mg/l) and cycloheximide (100 mg/l). After an incubation time of 48 h at 28°C, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined.

Four or 5 days after inoculation, the proportion of leaves per plant with symptoms was determined (20-25 plants per treatment). Data were statistically analyzed using analysis of variance followed by Fisher's test for least significant differences (LSD) at $\alpha=0.05$.

Pressure infiltration of leaves

Three fully expanded leaves of 4-week-old *Ler* plants were pressure infiltrated at the lower side of the leaf with *Pst* (10^7 cfu/ml), WCS417r (10^7 cfu/ml), SA (0.1 or 1 mM), or 10 mM MgSO_4 by using a 1-ml syringe without a needle as described by Swanson *et al.* (1988). Two, 5, and 8 days later, pressure-infiltrated as well as noninfiltrated leaves were harvested from a subset of plants of each treatment. Subsequently, the leaves were frozen in liquid nitrogen and stored at -80°C until RNA extractions were performed. Eight days after induction, another subset of plants from each treatment was challenge inoculated with *Pst* and induced resistance was quantified as described above.

Rhizosphere colonization

Roots (0.5 g fresh weight) were harvested in duplicate and shaken vigorously for 1 min in 5 ml of 10 mM MgSO_4 containing 0.5 g of glass beads (0.17 mm). Subsequently, dilutions were plated onto KB agar supplemented with cycloheximide (100 mg/l), ampicillin (50 mg/l), chloramphenicol (13 mg/l), and rifampicin (150 mg/l), which is selective for rifampicin-resistant, fluorescent *Pseudomonas* spp (Geels and Schippers, 1983). After overnight incubation at 28°C , the number of rifampicin resistant colony-forming units per gram of root fresh weight was determined.

RNA extraction and RNA blot analysis

Total RNA was isolated from frozen leaf samples using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). For RNA blot analysis, 15 μg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond- N^+ membranes (Amersham) by capillary transfer as described by Sambrook *et al.* (1989). RNA gel blots were hybridized and washed as described previously (Pieterse *et al.*, 1994) and exposed to a Kodak X-OMAT AR film. DNA probes were labelled with α - ^{32}P -dCTP by random primer labelling (Feinberg and Vogelstein, 1983), using Pharmacia Biotech's Oligolabeling Kit. The *PR*-2 probe was derived from plasmid A-2237 carrying the *Arabidopsis* β -1,3-glucanase gene (Dong *et al.*, 1991). Probes for *PR*-1 and *PR*-5 were derived from an *Arabidopsis* *PR*-1 and a *PR*-5 cDNA clone, respectively (Uknes *et al.*, 1992).

Competitive reverse transcriptase-polymerase chain reaction

Analysis of *PR*-1 gene expression was performed using the competitive reverse transcriptase-polymerase chain reaction (RT-PCR) as described by Siebert and Larrick (1992). A *PR*-1-specific primer pair (5'-GTAGGTGCTCTTGTCTTCC-3' and 5'-TTCACATAATCCCCACGAGG-3'), yielding RT-PCR products of 422 bp, was prepared based on the *Arabidopsis* *PR*-1 cDNA sequence described by Uknes *et al.* (1992). A 900-bp heterologous competitor DNA fragment, competing for the same set of primers, was obtained as described by Siebert and Larrick (1992). Fifty nanograms of poly(A)⁺ RNA, isolated from frozen leaves using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech), was converted into first-strand cDNA using a T-Primed First-Strand Kit (Pharmacia Biotech). Subsequently, equal portions of cDNA were amplified in the presence of 500 pg of competitive DNA using the *PR*-1-specific primer pair. The products were then resolved on an agarose gel stained with ethidium bromide.

Protein extraction and immunoblot analyses

Proteins were extracted 3, 10, and 20 days (leaves) or 7 and 14 days (roots) after induction treatment. Protein extraction and immunoblot analyses, using specific antisera against tobacco proteins PR-1, PR-2, and PR-5, were performed as described previously (Hoffland *et al.*, 1995).

Acknowledgements

We thank Dr. Maarten Koornneef for providing Col-0 and *Ler* seeds; Dr. Brian Staskawicz for the gift of *Pst*; Dr. John Ryals for kindly providing the *PR*-1 and *PR*-5 cDNA clones and the *Arabidopsis* NahG seeds; Dr. Fred Ausubel for the gift of plasmid A-2237; and Corine Van der Weele for performing immunoblot analyses.

Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria

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Abstract

Selected nonpathogenic, root-colonizing bacteria are able to elicit induced systemic resistance (ISR) in plants. To elucidate the molecular mechanisms underlying this type of systemic resistance, an *Arabidopsis*-based model system was developed in which *Pseudomonas syringae* pv *tomato* and *Fusarium oxysporum* f.sp. *raphani* were used as challenging pathogens. In *Arabidopsis thaliana* ecotypes Columbia and Landsberg *erecta*, colonization of the rhizosphere by *P. fluorescens* strain WCS417r induced systemic resistance against both pathogens. In contrast, ecotype RLD did not respond to WCS417r treatment, whereas all three ecotypes expressed systemic acquired resistance upon treatment with salicylic acid (SA). *P. fluorescens* strain WCS374r, previously shown to induce ISR in radish, did not elicit ISR in *Arabidopsis*. The opposite was found for *P. putida* strain WCS358r, that induced ISR in *Arabidopsis* but not in radish. These results demonstrate that rhizosphere pseudomonads are differentially active in eliciting ISR in related plant species. The outer membrane lipopolysaccharide (LPS) of WCS417r is the main ISR-inducing determinant in radish and carnation, and LPS-containing cell walls also elicit ISR in *Arabidopsis*. However, mutant WCS417rOA⁻, lacking the O-antigenic side chain of the LPS, induced levels of protection similar to wild-type WCS417r. This indicates that ISR-inducing bacteria produce more than a single factor triggering ISR in *Arabidopsis*. Furthermore, WCS417r and WCS358r induced protection in both wild-type *Arabidopsis* and SA-nonaccumulating NahG plants without activating pathogenesis-related gene expression. This suggests that elicitation of an SA-independent signalling pathway is a characteristic feature of ISR-inducing biocontrol bacteria.

Introduction

Induced resistance is defined as an enhancement of the plant's defensive capacity against a broad spectrum of pathogens that is acquired after appropriate stimulation (reviewed by Hammerschmidt and Kuć, 1995). The classic way of eliciting induced resistance is by a predispositional infection with a pathogen causing a hypersensitive reaction. The resulting elevated resistance response upon challenge inoculation of plant parts distant from the site of primary infection is known as systemic acquired resistance (SAR). SAR was first characterized in tobacco plants that expressed increased resistance systemically after infection by tobacco mosaic virus (Ross, 1961a,b). Pathogen-induced SAR is associated with an early increase in endogenously synthesized salicylic acid (SA; Malamy *et al.*, 1990; Métraux *et al.*, 1990). Accumulation of SA is critical in the signalling pathway controlling SAR, since SA-nonaccumulating plants are incapable of expressing induced resistance (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). Furthermore, SAR is characterized by the activation of so-called SAR genes (Ward *et al.*, 1991), including genes encoding pathogenesis-related proteins (PRs; Linthorst, 1991; Van Loon, 1985), which are often used as markers for the state of induced resistance. Both *PR* genes and induced resistance are expressed in plants treated with SA (Ward *et al.*, 1991; White, 1979). In addition, chemical agents such as 2,6-dichloroisonicotinic acid (INA; Métraux *et al.*, 1991), and benzothiadiazole (BTH; Lawton *et al.*, 1996) have been shown to induce resistance to the same spectrum of pathogens and to concurrently activate expression of SAR genes.

In 1991, an alternative approach to induce systemic resistance was reported by Van Peer *et al.* (1991) and Wei *et al.* (1991). These authors independently demonstrated that selected strains of nonpathogenic plant growth-promoting rhizobacteria (PGPR), that colonize the rhizosphere of the plant, are able to elevate plant resistance. Until then, these bacteria, mainly fluorescent *Pseudomonas* spp, had been studied for their ability to control soilborne pathogens through competition for nutrients, siderophore-mediated competition for iron, or antibiosis (Bakker *et al.*, 1991; Schippers, 1992; Thomashaw and Weller, 1996). Induction of systemic resistance in the plant thus appeared to be an additional mechanism by which these bacteria could protect the plant against disease. To date, induced systemic resistance (ISR; Kloepper *et al.*, 1992) mediated by nonpathogenic rhizobacteria has been demonstrated in several plant species (Van Loon *et al.*, 1998), and shown to be effective against bacterial, viral and fungal diseases. So far, little is known about the molecular basis underlying this type of ISR. Maurhofer *et al.* (1994) showed that ISR induced by *P. fluorescens* strain CHAO in tobacco was associated with the accumulation of PRs, suggesting that nonpathogen-induced ISR and pathogen-induced SAR share similar mechanisms. However, PRs did not accumulate in radish plants expressing ISR elicited by *P. fluorescens* strain WCS417r (Hoffland *et al.*, 1995; 1996). Moreover, Pieterse *et al.* (1996) demonstrated that, in *Arabidopsis*, ISR induced by WCS417r was not associated with *PR* gene activation, and was elicited in transgenic *Arabidopsis* plants

unable to accumulate SA. This indicates that in contrast to pathogen-induced SAR, WCS417r-mediated ISR is controlled by an SA-independent signalling pathway.

Previously, Van Peer and Schippers (1992) and Leeman *et al.* (1995b) showed that the O-antigenic side chain of the outer membrane lipopolysaccharide (LPS) of strain WCS417r is the main determinant for the induction of ISR against fusarium wilt disease in both carnation and radish. A bacterial mutant lacking the O-antigenic side chain did not induce resistance, whereas LPS-containing cell walls and purified LPS of WCS417r induced ISR to the same extent as living bacteria. Other bacterial determinants suggested to contribute to ISR are siderophores and SA (Leeman *et al.*, 1996; Maurhofer *et al.*, 1994).

The main objective of this study was to elucidate the basic mechanisms underlying nonpathogenic *Pseudomonas* spp-mediated ISR in the *Arabidopsis* model system. Here, we demonstrate that ISR-inducing fluorescent *Pseudomonas* spp are differentially active in eliciting ISR in *Arabidopsis*. Furthermore, we provide evidence that in contrast to what is observed in carnation and radish, the LPS of WCS417r plays only a minor role in the elicitation of ISR in *Arabidopsis*, indicating that WCS417r possesses more than a single ISR-inducing determinant.

Results

Differential expression of WCS417r-mediated ISR in Arabidopsis

Recently, Pieterse *et al.* (1996) demonstrated that colonization of the rhizosphere by strain WCS417r induces ISR in *Arabidopsis* against diseases caused by the bacterial leaf pathogen *P. syringae* pv *tomato* (*Pst*; Whalen *et al.*, 1991) and the fungal root pathogen *Fusarium oxysporum* f sp *raphani* (*For*; Leeman *et al.*, 1995a). To investigate whether different ecotypes of *A. thaliana* are equally able to express WCS417r-mediated ISR, ecotypes Columbia (Col-0), Landsberg *erecta* (*Ler*), and RLD were tested in bioassays using *Pst* and *For* as challenging pathogens. In these bioassays, the resistance-inducing potential of WCS417r was compared with that of SA, an established inducer of SAR (Malamy and Klessig, 1992). Leaves of noninduced control plants challenged with *Pst* developed necrotic lesions, surrounded by extensive, spreading chlorosis. Upon challenge inoculation with *For*, control plants showed wilting and yellowing of the leaves after 3 to 4 weeks. Induced protection against either pathogen was quantified by determining the percentage of leaves with symptoms. In plants challenge inoculated with *Pst*, proliferation of the pathogen in the leaves was assessed also. Figures 1A and C show that root treatment of ecotype Col-0 with WCS417r resulted in a reduction of about 50% in the symptoms caused by either of the pathogens. The level of protection induced by WCS417r was similar to or only slightly less than that induced by SA applied to the roots as a soil drench. As shown in Figure 1B, growth of *Pst* was significantly inhibited in WCS417r- and SA-treated Col-0 plants, indicating that the reduction of symptoms is associated with inhibition of bacterial multiplication. Ecotype *Ler* responded similarly to WCS417r and SA treatments. Both inducers

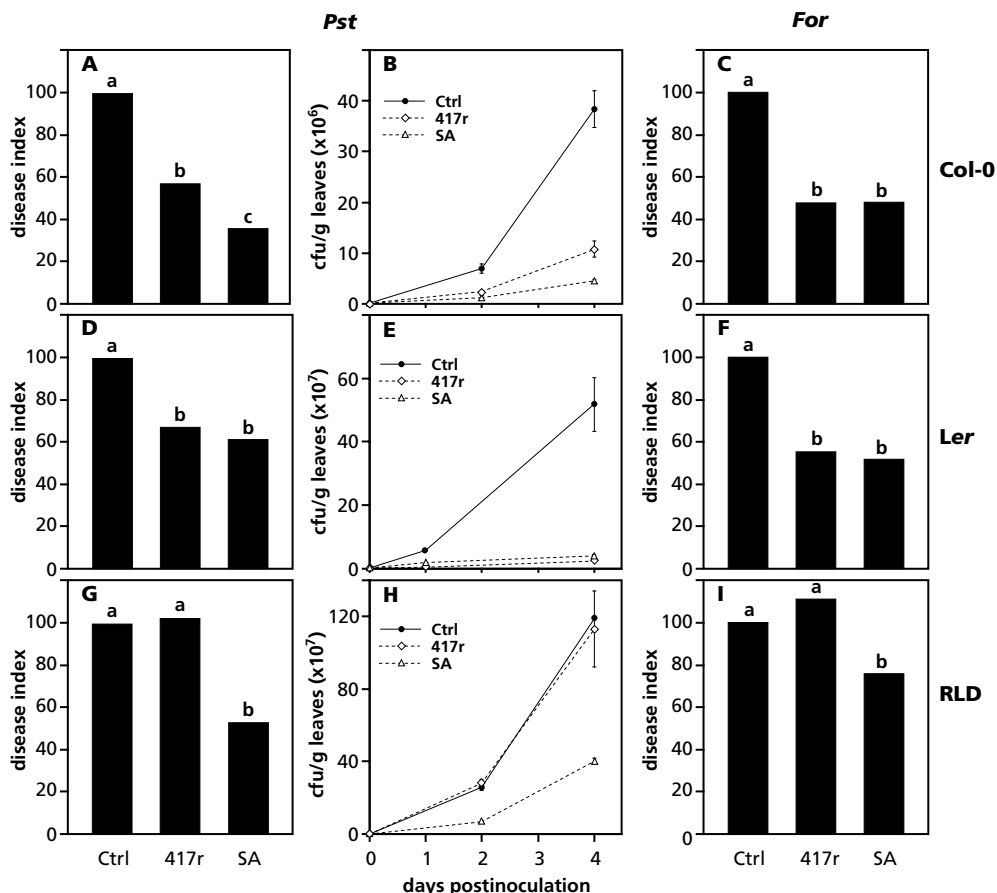


Figure 1. Quantification of protection against *Pst* or *For* infection in *A. thaliana* ecotypes Col-0 (A-C), *Ler* (D-F), and RLD (G-I) induced by WCS417r or SA.

Plants were grown in soil that was either mixed with WCS417r (417r) or 10 mM MgSO_4 (Ctrl), or supplied with a soil drench of 1 mM SA (SA). The disease index is the mean ($n=30$) of the proportion of leaves with symptoms per plant compared to that of control plants (set at 100%), 4 days after challenge inoculation with *Pst* (A, D, and G), or 3 to 4 weeks after challenge inoculation with *For* (C, F, and I). The absolute proportions of diseased leaves of the controls depicted in A, C, D, F, G and I were 58.9%, 53.4%, 80.9%, 61.2%, 54.4%, and 35.8 %, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fischer's LSD test; $\alpha=0.05$). Growth of *Pst* in challenged leaves (B, E, and H) was assessed at the indicated days after inoculation. Data points are means (cfu/g) with standard errors from two sets of 20 leaves randomly selected from plants of the bioassays shown in A, D and G, respectively. The data presented are from representative experiments that were repeated at least twice with similar results.

decreased disease symptoms to the same extent (Figures 1D and F) and caused a 20-fold reduction in bacterial multiplication in leaves challenged with *Pst* (Figure 1E). In ecotype RLD, however, WCS417r did not reduce symptoms provoked by either pathogen (Figures 1G and I), nor did it inhibit growth of *Pst* in challenged leaves (Figure 1H). In contrast, treatment with SA resulted in a significant reduction in

Table 1. Colonization of the rhizosphere of *A. thaliana* ecotypes Col-0, *Ler*, and RLD by WCS417r.

Ecotype	cfu/g fresh weight ($\times 10^5$) ¹	
	<i>Pst</i> bioassay	<i>For</i> bioassay
Col-0	2.2 $\pm$ 0.2	6.4 $\pm$ 0.4
<i>Ler</i>	3.1 $\pm$ 0.3	3.9 $\pm$ 0.2
RLD	6.0 $\pm$ 1.0	8.3 $\pm$ 0.7

¹ Values presented are the average population densities $\pm$ SE of multiple bioassays. Roots were harvested at the end of the bioassays. On nontreated roots or root parts, no rifampicin-resistant bacteria were detected (detection limit 10^3 cfu/g).

symptoms caused by either pathogen, as in ecotypes Col-0 and *Ler*. Moreover, proliferation of *Pst* in challenged leaves was clearly decreased. These results demonstrate that WCS417r induces ISR in ecotypes Col-0 and *Ler* but fails to do so in ecotype RLD, whereas in all three ecotypes SAR can be induced by SA.

To determine whether the inability of RLD to exhibit WCS417r-mediated ISR might be attributed to a less effective colonization of the roots, the population density of WCS417r in the rhizosphere of treated Col-0, *Ler* and RLD plants was examined. Table 1 shows that the number of rifampicin-resistant bacteria present in the rhizosphere of the three ecotypes was of the same order of magnitude ($2.2\text{--}8.3 \times 10^5$ cfu/g of root fresh weight). No rifampicin-resistant bacteria were detected on nontreated roots. Therefore, it can be concluded that WCS417r colonized the rhizosphere of the three ecotypes to comparable levels.

Differential ability of fluorescent Pseudomonas spp strains to elicit ISR

Previously, Leeman *et al.* (1995a) showed that in radish plants, strains WCS417r and WCS374r induce ISR against fusarium wilt, whereas strain WCS358r does not. To investigate whether *Arabidopsis* responds similarly, the ability of these strains to induce ISR against *Pst* or *For* infection was tested. In contrast to WCS417r, WCS374r did not reduce disease symptoms provoked by either *Pst* or *For* (Figures 2A and C), nor did it inhibit proliferation of *Pst* in challenged leaves (Figure 2B). WCS358r induced significant ISR against both pathogens. However, the extent of symptom reduction was less than that induced by WCS417r. On the other hand, multiplication of *Pst* upon challenge was reduced almost to the same level. Whereas WCS417r induced resistance in both radish and *Arabidopsis*, the resistance-inducing capacities of WCS374r and WCS358r clearly differed in these two species.

To exclude that the observed protection was caused by a direct effect of the inducing *Pseudomonas* strains on the pathogen, their spatial separation on the plant was verified. To this end, the population densities of the rhizobacterial strains on treated and nontreated plant parts were determined at the end of each bioassay by plating root washes or leaf extracts on selective King's medium B agar plates (King *et al.*, 1954).

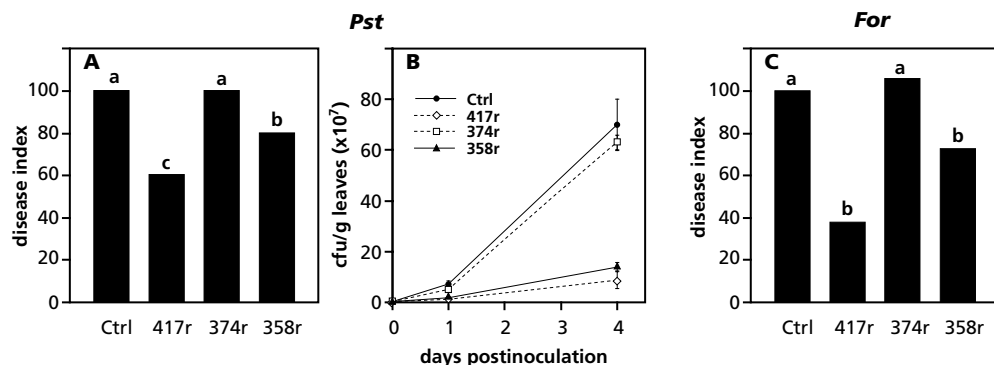


Figure 2. Quantification of protection against *Pst* or *For* infection in *Arabidopsis* plants treated with different fluorescent *Pseudomonas* spp strains.

Plants were grown in soil that was mixed with either 10 mM MgSO₄ (Ctrl), WCS417r (417r), WCS374r (374r), or WCS358r (358r). The disease index is the mean ($n=30$) of the proportion of leaves with symptoms per plant compared to that of control plants (set at 100%), 4 days after challenge inoculation of *Ler* plants with *Pst* (A), or 3 to 4 weeks after challenge inoculation of Col-0 plants with *For* (C). The absolute proportions of diseased leaves of the controls depicted in A and C were 75.9% and 39.0%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fischer's LSD test; $\alpha=0.05$). Growth of *Pst* in challenged leaves (B) was assessed at the indicated days after inoculation. Data points are means (cfu/g) with standard errors from two sets of 20 leaves randomly selected from plants of the bioassay shown in A. The data presented are from representative experiments that were repeated at least twice with similar results.

Table 2 shows that from WCS417r- and WCS358r-treated roots similar amounts of rifampicin-resistant *Pseudomonas* bacteria were recovered, whereas approximately 10-fold lower numbers were detected on WCS374r-treated roots. In the leaves used for challenge inoculation with *Pst* or on the root parts inoculated with *For*, rifampicin-resistant bacteria were never detected, demonstrating that for the duration of the bioassays, the inducing *Pseudomonas* strains remained spatially separated from the challenging pathogens. Moreover, *in vitro* antagonism assays showed no significant inhibition of growth of *Pst* or *For* by either of the three bacterial strains (data not shown), indicating that the induced protection is unlikely to be caused by accumulation of *Pseudomonas*-produced antibiotics in the plant.

Both WCS417r- and WCS358r-mediated ISR are independent of SA

Using transgenic *Arabidopsis* NahG plants that did not accumulate SA (Delaney *et al.*, 1994), Pieterse *et al.* (1996) demonstrated that, in contrast to pathogen-induced SAR, WCS417r-mediated ISR is independent of endogenous SA accumulation and PR gene activation. To investigate whether WCS358r-mediated ISR is independent of SA as well, bioassays were performed with NahG plants and wild-type Col-0 plants. In accordance with previous results, treatment of the roots with WCS417r resulted in a significant reduction of symptoms caused by *Pst* infection in both Col-0 and NahG plants (Figure 3). WCS358r similarly induced protection in both wild-type and NahG

Table 2. Colonization of *Arabidopsis* by WCS417r, WCS374r, and WCS358r.

Treatment ²	cfu/g fresh weight ($\times 10^5$) ¹		
	<i>Pst</i> bioassay treated roots ³	<i>For</i> bioassay treated roots ³	challenged plant parts ⁴
Control	b.d.	b.d.	b.d.
WCS417r	3.1 ± 0.2	5.8 ± 0.4	b.d.
WCS374r	0.3 ± 0.0	0.7 ± 0.1	b.d.
WCS358r	3.0 ± 0.3	6.7 ± 0.6	b.d.

¹ Values presented are the average population densities $\pm$ SE of multiple bioassays. b.d. = below detection (detection limit 10^3 cfu/g).

² In the *Pst* bioassays a solution of 10 mM MgSO_4 (control) or a suspension of *Pseudomonas* spp strains in 10 mM MgSO_4 was mixed through the soil (5×10^7 cfu/g) prior to planting of *Ler*. In the *For* bioassays talcum powder mixed with a solution of 10 mM MgSO_4 (control) or a suspension of *Pseudomonas* spp strains in 10 mM MgSO_4 (5×10^8 cfu/g) was applied to the lower part of the roots of 2-week-old Col-0 seedlings.

³ Roots were harvested at the end of the bioassays.

⁴ In the *Pst* bioassays leaves were harvested prior to challenge inoculation. In the *For* bioassays, inoculated upper root parts were harvested at the end of the bioassays.

plants, whereas plants treated with WCS374r did not show increased resistance. The level of protection induced by WCS417r and WCS358r is somewhat lower in NahG plants compared to that in wild-type plants, suggesting a modulating role for SA in the level of expression of ISR. RNA blot analyses demonstrated that none of the

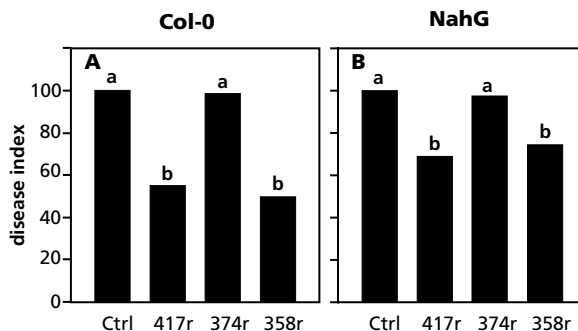


Figure 3. Quantification of protection against *Pst* infection in *Arabidopsis* Col-0 (A) or NahG (B) plants treated with different fluorescent *Pseudomonas* spp strains.

Plants were grown in soil that was mixed with either 10 mM MgSO_4 (Ctrl), WCS417r (417r), WCS374r (374r), or WCS358r (358r). For experimental details see the text and legend to Figure 2. The absolute proportions of diseased leaves of the controls depicted in A and B were 58.5% and 80.1%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fischer's LSD test; $n=30$, $\alpha=0.05$). The data presented are from representative experiments that were repeated at least twice with similar results.

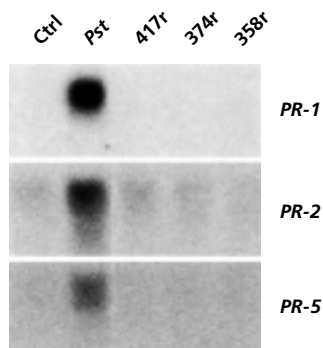


Figure 4. RNA blot analyses of the expression of the SAR marker genes *PR-1*, *PR-2* and *PR-5* in *Arabidopsis* plants treated with different fluorescent *Pseudomonas* spp strains.

Ler plants were treated with either 10 mM MgSO_4 (Ctrl), WCS417r (417r), WCS374r (374r), or WCS358r (358r) by growing them in soil mixed with these agents for 3 weeks. Inoculation with *Pst* was performed by pressure infiltrating three lower leaves with *Pst* 2 days before harvest of the noninoculated leaves. *PR-1*, *PR-2* and *PR-5* gene-specific probes of *Arabidopsis* were used.

rhizosphere pseudomonads induced *PR-1*, *PR-2*, or *PR-5* mRNAs (Figure 4). In contrast, *PR* mRNA accumulated in noninoculated leaves of plants expressing SAR induced by a predisposal infection of primary leaves with pathogenic *Pst*. These results demonstrate that like WCS417r, WCS358r elicits an SA-independent signalling pathway leading to ISR without concomitant activation of *PR* genes.

Involvement of bacterial LPS in the elicitation of ISR in Arabidopsis

In radish, purified LPS and LPS-containing cell wall preparations of WCS417r are as effective as living WCS417r bacteria in inducing ISR (Leeman *et al.*, 1995b). To investigate whether the LPS of WCS417r also elicits ISR in *Arabidopsis*, cell wall preparations of WCS417r and its mutant WCS417rOA⁻ (Leeman *et al.*, 1995b), which lacks the O-antigenic side chain of the LPS, were tested in *Pst* bioassays. Cell walls of the noninducing strain WCS374r were used as a control. Figure 5 shows that treatment of the roots with cell walls of WCS417r reduced symptoms by 20%, whereas the cell walls of WCS417rOA⁻ or WCS374r were ineffective. The reduction was significantly lower than the level of protection obtained with living bacteria, suggesting that the O-antigenic side chain of the LPS of WCS417r contributes to elicitation of ISR but is probably not sufficient for full induction.

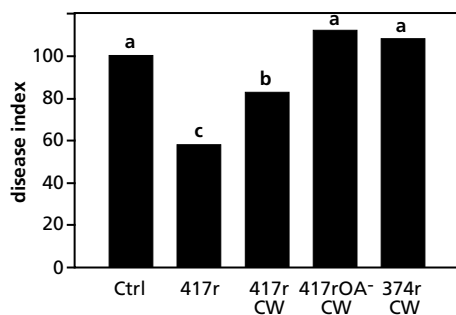


Figure 5. Quantification of protection against *Pst* infection in *Arabidopsis* plants treated with cell wall preparations of fluorescent *Pseudomonas* spp strains.

Ler plants were grown in soil that was either mixed with 10 mM MgSO_4 (Ctrl) or WCS417r (417r), or supplied with a soil drench of cell wall preparations of WCS417r (417r cw), WCS417rOA⁻ (417rOA⁻ cw), or WCS374r (374r cw). For experimental details see the text and legend to Figure 1. The absolute proportion of diseased leaves of the control was 65.5%. Different letters indicate statistically significant differences between treatments (Fischer's LSD test; $\alpha=0.05$, $n=30$). The data presented are from a representative experiment that was repeated twice with similar results.

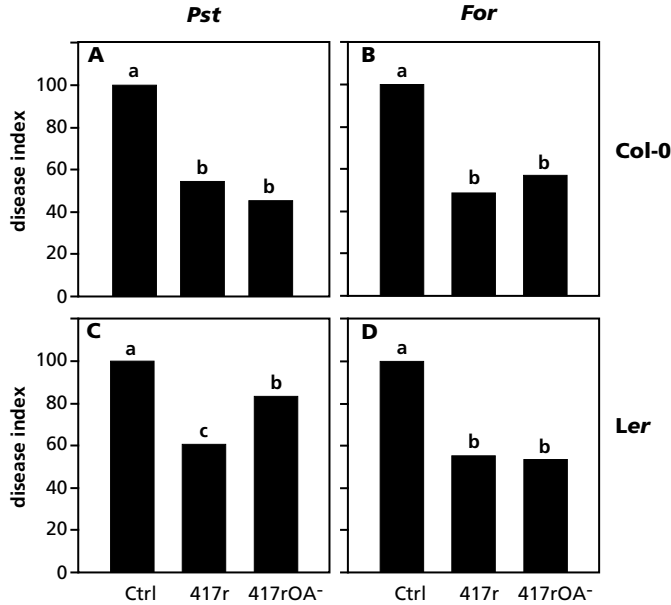


Figure 6. Quantification of ISR against *Pst* (A and C) or *For* (B and D) infection in *Arabidopsis* plants induced by the WCS417r wild-type and an LPS O-antigen mutant. *Col-0* (A and B) and *Ler* (C and D) plants were grown in soil mixed with either 10 mM MgSO₄ (Ctrl), WCS417r (417r), or an LPS O-antigen mutant WCS417rOA⁻ (417rOA⁻). For experimental details see the text and legend to Figure 1. The absolute proportions of diseased leaves of the controls depicted in A–D were 58.9%, 57.6%, 73.3%, and 61.2%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fischer's LSD test; $\alpha=0.05$, $n=30$). The data presented are from representative experiments that were repeated at least twice with similar results.

Comparison of the resistance-inducing ability of living cells of WCS417r and its OA⁻ mutant in *Col-0* and *Ler* plants revealed that in most experiments, wild-type and mutant bacteria induced similar levels of protection against both *Pst* and *For* infection (Figures 6A, B and D). However, in some bioassays, the mutant was significantly less effective (Figure 6C). WCS417r and WCS417rOA⁻ colonized the rhizosphere of *Arabidopsis* to similar levels (average of 3.6×10^5 and 3.4×10^5 cfu/g of root fresh weight, respectively). These results demonstrate that, in *Arabidopsis*, elicitation of ISR by WCS417r is not dependent on the O-antigenic side chain of the LPS, although cell wall components can induce resistance and may contribute to the level of protection attained.

Discussion

Induction of systemic resistance is one of the mechanisms by which selected strains of nonpathogenic *Pseudomonas* spp can reduce diseases. WCS417r has been demonstrated to induce resistance in several plant species (Duijff *et al.*, 1998; Leeman

et al., 1995a; Van Peer *et al.*, 1991). With the aim to study the molecular and mechanistic basis underlying this type of systemic resistance, we recently developed *Arabidopsis* as a model host using WCS417r as the inducing agent and *Pst* and *For* as challenging pathogens (Pieterse *et al.*, 1996). ISR against *Pst* is manifested by both a reduction in the number of leaves showing symptoms and a decrease in multiplication of the pathogen in the leaves. ISR against *For* was measured as a reduction in the percentage of leaves showing symptoms only.

Using three *A. thaliana* ecotypes and three rhizobacterial strains, we now demonstrate that specific interactions between the bacterial strains and the plant ecotypes determine induction of systemic resistance. On the one hand, the tested ecotypes are differentially responsive to WCS417r treatment. In contrast to ecotypes Col-0 and *Ler*, ecotype RLD did not develop ISR upon treatment of the roots with WCS417r (Figure 1). Nevertheless, all three ecotypes readily expressed SAR upon SA treatment. Colonization of the rhizosphere by WCS417r was similar in the three ecotypes (Table 1), suggesting that ecotype RLD either does not recognize elicitors of WCS417r or is impaired in the ISR signalling pathway. On the other hand, bacterial strains WCS417r, WCS374r, and WCS358r were differentially active in the induction of ISR. WCS417r and WCS358r triggered an ISR response in *Arabidopsis*, whereas WCS374r did not (Figure 2). In contrast, in radish, Leeman *et al.* (1995a) demonstrated induction by WCS417r and WCS374r but not by WCS358r. Apparently, all three strains have the potential to induce ISR but do so only in selected plant species.

Compared to the ISR-inducing strains WCS417r and WCS358r, the noninducing strain WCS374r was present at a 10-fold lower level in the rhizosphere of *Arabidopsis* by the end of the bioassays (Table 2). Therefore, it can not be ruled out that the inability of WCS374r to trigger ISR in *Arabidopsis* is caused by insufficient root colonization. However, in the *For* bioassay, in which plants were challenged as soon as 3 days after application of the rhizobacterial strains, only the initial density of bacteria applied to the roots appeared critical for the induction of ISR, and bacterial numbers often dropped to noninducing levels by the end of the bioassays (Leeman *et al.*, 1995a; Raaijmakers *et al.*, 1995). Since treatments constituted equal amounts of the different bacteria at a concentration 500-fold higher than the threshold for ISR in radish (Raaijmakers *et al.*, 1995), the inability of WCS374r to induce ISR in this bioassay is more likely caused by a lack of response by the plant. This explanation is supported by our observation that cell wall preparations of WCS374r were ineffective in inducing ISR in *Arabidopsis*, in contrast to those of WCS417r, whereas cell wall preparations from both strains were active in eliciting ISR in radish (Leeman *et al.*, 1995b).

The ecotype-specific induction of resistance in *Arabidopsis* by WCS417r further indicates that protection against *Pst* and *For* is dependent on specific interactions between the bacteria and the plant. Direct suppression of the pathogen by bacterial antagonism can be ruled out since the inducing pseudomonads and the challenging pathogens remained spatially separated (Table 2). Moreover, none of the bacterial

strains significantly inhibited the pathogens *in vitro*, making it highly unlikely that accumulation of antibiotics produced by the rhizobacterial pseudomonads contributed to the increased protection.

A major bacterial trait implicated in the elicitation of resistance responses in plants by pathogens is the outer membrane LPS (Sequeira, 1983). LPS-containing cell walls of WCS417r, which were able to elicit a full resistance response in radish and carnation (Leeman *et al.*, 1995b; Van Peer and Schippers, 1992), also induced protection in *Arabidopsis* (Figure 5). However, the level of protection was significantly lower than that elicited by living bacteria. Moreover, the OA⁻ mutant of WCS417r, which no longer induced ISR in radish (Leeman *et al.*, 1995b), did reduce the disease symptoms in *Arabidopsis* in most experiments to the same extent as the wild-type (Figure 6). This indicates that the LPS of WCS417r plays only a minor role in the elicitation of ISR in *Arabidopsis*, and that other bacterial component(s) constitute the primary determinant.

SA produced by rhizosphere pseudomonads has been implicated in the activation of systemic resistance in radish (Leeman *et al.*, 1996). However, bacterially produced SA is unlikely to be a determinant for eliciting ISR in *Arabidopsis*. First, both WCS417r and WCS358r induced ISR in *Arabidopsis*, but only WCS417r has the capacity to produce SA (Leeman *et al.*, 1996). Moreover, WCS374r can produce the largest amount of SA but does not induce resistance in *Arabidopsis*. Second, inducing strains were equally effective in wild-type and in NahG plants, that readily inactivate SA. In addition, the OA⁻ mutants of these strains had the same resistance-inducing capacity in NahG plants as the wild-type strains (data not shown), indicating that SA does not contribute to the ISR response elicited by the non-LPS determinant. Third, WCS417r did not trigger ISR in ecotype RLD, although this ecotype is responsive to induction by SA (Figure 1).

Another metabolite implicated in ISR induction is the iron-regulated pyoverdine siderophore (Maurhofer *et al.*, 1994). Leeman *et al.* (1996) demonstrated that the siderophore of WCS374r can act as an elicitor of ISR in radish, even though its effect is overridden by that of the LPS during the induction by living bacteria. We are currently investigating the involvement of siderophores in the elicitation of ISR in *Arabidopsis*.

As previously demonstrated for strain WCS417r (Pieterse *et al.*, 1996), WCS358r induces a plant-mediated resistance response in both wild-type and NahG plants without concomitant activation of *PR* genes (Figures 3 and 4). These results indicate that both biocontrol strains induce a signalling pathway different from the one that controls classic SAR. Press *et al.* (1997) found that biocontrol strain *Serratia marcescens* 90-166 is able to induce protection in both wild-type and NahG tobacco plants against *P. syringae* pv *tabaci* as well. Hence, it seems that the ability to trigger an SA-independent pathway controlling systemic resistance is a common trait of ISR-inducing biocontrol bacteria.

Materials and methods

Microbial cultures

Pseudomonas fluorescens strain WCS417 was initially isolated from the rhizosphere of wheat grown in a field suppressive to take-all disease caused by *Gaeumannomyces graminis* pv *tritici* (Lamers *et al.*, 1988), and *P. fluorescens* strain WCS374 and *P. putida* strain WCS358 were collected from the rhizosphere of potato (Geels and Schippers, 1983). Rifampicin-resistant mutants of these strains (WCS417r, WCS374r, WCS358r) were used throughout this study (Geels and Schippers, 1983; Glandorf *et al.*, 1992; Leeman *et al.*, 1991). WCS417rOA⁻ is a spontaneous phage-resistant mutant of WCS417r lacking the O-antigenic side chain of the outer membrane LPS (Leeman *et al.*, 1995b). The bacteria were cultured for 24 h on King's medium B (KB) agar plates (King *et al.*, 1954) at 28°C. Subsequently, the cells were collected and resuspended in 10 mM MgSO₄.

The virulent bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*; Whalen *et al.*, 1991) was cultured in liquid KB at 28°C. After overnight incubation, the cells were collected by centrifugation and resuspended in 10 mM MgSO₄.

The fungal pathogen *Fusarium oxysporum* f.sp. *raphani* WCS600 (*For*) was initially isolated from tubers of a naturally infected radish plant (Leeman *et al.*, 1995a), and a culture was maintained on potato-dextrose agar. The inoculum was prepared by incubating mycelial patches in aerated 2% malt extract at 22°C for 7 days. Subsequently, cultures were filtered and conidia were collected by centrifugation. Conidia were mixed with sterile peat (Agrifutur s.r.l., Alfianello, Italy) to a density of 10⁷ conidia/g and allowed to germinate and grow at 24°C for 2 days. The final density of colony-forming units (cfu) in the peat was determined by dilution plating on potato-dextrose agar.

Preparation of bacterial cell walls

Cell walls of WCS417r, WCS417rOA⁻, and WCS374r were isolated from overnight cultures grown in liquid KB at 28°C, essentially as described by Leeman *et al.* (1995b). The bacteria were collected by centrifugation and resuspended in 50 mM Tris-HCl, 2 mM EDTA (pH 8.5). The cells were then sonicated eight times for 15 s on ice at resonance amplitude. Intact cells were removed from the sonicated suspension by centrifugation at 600 × *g* for 20 min. After centrifugation of the supernatant at 8000 × *g* for 60 min, the pellet of LPS-containing cell walls was resuspended in 10 mM phosphate buffered saline (pH 7.2), 0.01% sodium azide, and stored at -80°C until further use. The absence of living bacteria was verified by plating on KB agar plates.

Pst bioassay

Seeds of *Arabidopsis thaliana* ecotypes Columbia (Col-0), Landsberg *erecta* (*Ler*), RLD, and transgenic NahG plants harboring the bacterial *NahG* gene encoding salicylate hydroxylase (Delaney *et al.*, 1994) were sown in sterile quartz sand. Once a day, the seedlings were supplied with modified half-strength Hoagland nutrient solution (2 mM KNO₃, 5 mM Ca(NO₃)₂, 1 mM KH₂PO₄, 1 mM MgSO₄, and trace elements, pH 7; Hoagland and Arnon, 1938) containing 10 µM Sequestreen (Fe-ethylenediamine-di[*o*-hydroxyphenylacetic acid]; CIBA-Geigy, Switzerland, Basel). Two-week-old seedlings were transferred to 60-ml pots containing a sand/potting soil mixture that had been autoclaved twice for 1 h before it was mixed with either a suspension of pseudomonads to a final density of 5 × 10⁷ cfu/g, or an equal volume of a solution of 10 mM MgSO₄ (50 ml/kg). Treatment of the roots with bacterial cell walls was performed by applying 20 ml of a cell wall preparation as a soil drench 7 and 4 days before challenge inoculation (cell walls from 2.5 × 10⁶ cfu/ml, resulting in an equal amount of cell walls as present in soil containing 5 × 10⁷ cfu/ml at the beginning of the bioassay). SA treatment was performed by applying 20 ml of a solution of 1 mM SA (pH 6) as a soil drench 7 and 4 days before challenge inoculation. Plants were cultivated in a growth chamber with a 9-h day (200 µE/m²/s at 24°C) and 15-h night (20°C) cycle at 70% relative humidity. The plants were watered on alternate days and once a week supplied with nutrient solution.

Plants were challenge inoculated when 5 weeks old. One day before challenge the plants were placed at 100% relative humidity. Inoculation was carried out by dipping the leaves in a suspension of *Pst* in 10 mM MgSO₄ supplemented with 0.01% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). Inoculation densities were chosen such that 4 days after challenge, approximately 70% of the leaves of control plants showed symptoms (2.5 × 10⁷ cfu/ml for Col-0 and NahG, 1 × 10⁸ cfu/ml for *Ler*, and 1 × 10⁷ cfu/ml for RLD). At that time, the proportion of leaves with disease symptoms per plant was determined for 30 plants per treatment. Data were statistically analyzed using one-way analysis of variance

(ANOVA) for a single experiment and two-way ANOVA for combined experiments, followed by Fisher's test for least significant differences at $\alpha=0.05$.

Multiplication of *Pst* was assessed in challenged leaves at different time points after inoculation. Two pools of 1 g of randomly selected leaves (15 to 20) per treatment were rinsed thoroughly in sterile water and homogenized in a sterile solution of 10 mM MgSO_4 . Dilutions were plated onto KB agar supplemented with rifampicin (50 mg/l) and cycloheximide (100 mg/l). After incubation at 28°C for 2 days, the number of colony-forming units per g of infected leaf tissue was determined.

For bioassay

Seeds of ecotypes Col-0, *Ler*, and RLD were sown singly in 1-ml pipette tips filled with sterile quartz sand to stimulate root elongation. The tips were drenched in water daily and in modified half-strength Hoagland nutrient solution once a week. After 2 weeks, seedlings were rinsed out of the pipette tips and placed horizontally on a system of rock wool cubes (Rock-wool/Grodan B.V., Roermond, The Netherlands), consisting of two spatially separated compartments. This system allows an induction treatment and a challenge inoculation of the same root system at different sites (Leeman *et al.*, 1995a; Pieterse *et al.*, 1996). The lower part of the root system was covered with 1 ml of a 1:1 (w/v) mixture of talcum powder and either *Pseudomonas* bacteria in 10 mM MgSO_4 (final density 5×10^8 cfu/g), a solution of 1 mM SA (pH 6), or a solution of 10 mM MgSO_4 as a control. Three days after the induction treatment, the plants were challenge inoculated by applying approximately 0.25 g of the *For* inoculum (4×10^6 cfu/g of peat) to the upper part of the roots. Subsequently, plants were cultivated as described above.

Thirty plants per treatment were analyzed for induced protection against *For* by determining the percentage of fully expanded leaves per plant with symptoms of fusarium wilt at 3 to 4 weeks after challenge inoculation. The data were statistically analyzed as described above.

Rhizosphere colonization

Bacterial colonization of the root (parts) was determined by the time the bioassays were discontinued. The roots of six plants of each treatment were harvested, weighed, rinsed briefly in water, and shaken vigorously for 1 min in glass tubes containing 5 ml of 10 mM MgSO_4 and 0.5 g of glass beads (0.17 mm). Appropriate dilutions were plated on KB agar supplemented with cycloheximide (100 mg/l), ampicillin (50 mg/l), chloramphenicol (13 mg/l), and rifampicin (150 mg/l), which is selective for rifampicin-resistant *Pseudomonas* spp (Geels and Schippers, 1983). After overnight incubation at 28°C the number of cfu/g of root fresh weight was determined.

RNA analysis

For RNA extraction, leaves were harvested from 5-week-old plants that were either nontreated, treated with *Pseudomonas* rhizobacteria, or inoculated with *Pst*. Inoculation with *Pst* was performed by pressure infiltrating three lower leaves with a suspension of 1×10^7 cfu/ml of 10 mM MgSO_4 by using a syringe without a needle, as described by Swanson *et al.* (1988). Leaves were frozen in liquid nitrogen and stored at -80°C. RNA was extracted using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). Total RNA (15 μg) was electrophoretically separated on denaturing formaldehyde-agarose gels and blotted onto Hybond-N⁺ membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer as described by Sambrook *et al.* (1989). RNA blots were hybridized and washed as described previously (Pieterse *et al.*, 1994), and exposed to a Kodak X-OMAT AR film. The DNA probes were labelled with α -³²P-dCTP by random primer labeling (Feinberg and Vogelstein, 1983) using a Ready-To-Go DNA Labeling Kit (Pharmacia Biotech, Roosendaal, The Netherlands). *PR-1*, *PR-2* and *PR-5* probes were originated from *Arabidopsis PR-1*, *PR-2* and *PR-5* cDNA clones, respectively (Uknes *et al.*, 1992).

In vitro antagonism assay

To test antibiotic activity by WCS417r, WCS374r and WCS358r, the bacterial strains were spotted at three positions on KB and rhizosphere medium (RSM; Buyer *et al.*, 1989) agar plates supplemented with 200 μM FeCl_3 (Duijff *et al.*, 1993). After incubation at 28°C for 2 days, a suspension of *Pst* (1×10^7 cfu/ml) or *For* (5×10^6 conidia/ml) in 10 mM MgSO_4 was sprayed evenly onto the plates. After an additional incubation for 2 days at 28°C for *Pst* or at 24°C for *For*, plates were inspected for the occurrence of zones of inhibited growth of *Pst* or *For* around the colonies of the biocontrol bacteria.

Acknowledgements

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A novel signalling pathway controlling induced systemic resistance in *Arabidopsis*

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Abstract

Plants have the ability to acquire an enhanced level of resistance to pathogen attack after being exposed to specific biotic stimuli. In *Arabidopsis*, nonpathogenic, root-colonizing *Pseudomonas fluorescens* bacteria trigger an induced systemic resistance (ISR) response against infection by the bacterial leaf pathogen *P. syringae* pv *tomato* (*Pst*). In contrast to classic, pathogen-induced systemic acquired resistance (SAR), this rhizobacteria-mediated ISR response is independent of salicylic acid (SA) accumulation and pathogenesis-related gene activation. Using the jasmonate response mutant *jar1*, the ethylene response mutant *etr1*, and the SAR regulatory mutant *npr1*, we demonstrate that signal transduction leading to *P. fluorescens* WCS417r-mediated ISR requires responsiveness to jasmonate and ethylene, and is dependent on NPR1. Similar to WCS417r, methyl jasmonate (MeJA) and the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) were effective in inducing resistance against *Pst* in SA-nonaccumulating NahG plants. Moreover, MeJA-induced protection was blocked in *jar1*, *etr1*, and *npr1* plants, whereas ACC-induced protection was affected in *etr1* and *npr1* plants but not in *jar1* plants. Hence, we postulate that rhizobacteria-mediated ISR follows a novel signalling pathway in which components from the jasmonate and ethylene response are engaged successively to trigger a defense reaction that, like SAR, is regulated by NPR1. We provide evidence that the processes downstream of NPR1 in the ISR pathway are divergent from those in the SAR pathway, indicating that NPR1 differentially regulates defense responses depending on the signals that are elicited during induction of resistance.

Introduction

Plants of which the roots have been colonized by selected strains of nonpathogenic fluorescent *Pseudomonas* spp develop an enhanced level of protection against pathogen attack (reviewed by Van Loon *et al.*, 1998). Strain WCS417r of *P. fluorescens* is a biological control strain that has been shown to trigger an induced systemic resistance (ISR) response in several plant species, including carnation (Van Peer *et al.*, 1991), radish (Leeman *et al.*, 1995), tomato (Duijff *et al.*, 1998), and *Arabidopsis* (Pieterse *et al.*, 1996). In *Arabidopsis*, WCS417r-mediated ISR has been demonstrated against the bacterial leaf pathogen *P. syringae* pv *tomato* (*Pst*), the fungal root pathogen *Fusarium oxysporum* f sp *raphani* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997), and the oomycetous leaf pathogen *Peronospora parasitica* (J. Ton and C.M.J. Pieterse, unpublished result), indicating that this type of biologically induced resistance is effective against different types of pathogens.

ISR-inducing rhizobacteria show host specificity in regard to elicitation of resistance (Van Wees *et al.*, 1997), which indicates that specific recognition between protective bacteria and the plant is a prerequisite for the activation of the signalling cascade leading to ISR. The downstream signalling events in the rhizobacteria-mediated ISR pathway clearly differ from those in the pathway leading from pathogen infection to classic systemic acquired resistance (SAR). SAR is a form of systemically induced disease resistance that is triggered upon infection by a necrotizing pathogen (reviewed by Ryals *et al.*, 1996). The state of SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA; Malamy *et al.*, 1990; Métraux *et al.*, 1990) and the concomitant activation of genes encoding pathogenesis-related proteins (PRs; Ward *et al.*, 1991). SA-nonaccumulating NahG plants expressing the bacterial salicylate hydroxylase (*NahG*) gene are incapable of developing SAR and do not show PR gene activation upon pathogen infection, indicating that SA is a necessary intermediate in the SAR signalling pathway (Gaffney *et al.*, 1993; Delaney *et al.*, 1994). In contrast to pathogen-induced SAR, rhizobacteria-mediated ISR is not associated with the activation of PR genes (Hoffland *et al.*, 1995; Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Moreover, NahG plants that are unable to express SAR develop normal levels of ISR after treatment of the roots with ISR-inducing rhizobacteria (Pieterse *et al.*, 1996; Press *et al.*, 1997; Van Wees *et al.*, 1997). This demonstrates that biologically induced disease resistance can be controlled by at least two pathways that diverge in their requirement for SA accumulation.

Besides SA, the plant growth regulators jasmonic acid and ethylene have been implicated in plant defense responses (Boller, 1991; Wasternack and Parthier, 1997). Jasmonic acid and derivatives, collectively referred to as jasmonates, induce the expression of genes encoding defense-related proteins such as thionins (Epple *et al.*, 1995) and proteinase inhibitors (Farmer *et al.*, 1992), whereas ethylene activates several members of the PR gene superfamily (Brederode *et al.*, 1991; Potter *et al.*, 1993). Jasmonate and ethylene also have been shown to act synergistically in stimulating elicitor-induced PR gene expression (Xu *et al.*, 1994). Moreover, both signalling

molecules are implicated in the activation of genes encoding plant defensins (Penninckx *et al.*, 1996) and enzymes involved in phytoalexin biosynthesis (Ecker and Davis, 1987; Gundlach *et al.*, 1992). Both jasmonate and ethylene have been reported to be involved in systemically induced defense responses (Farmer and Ryan, 1992; Penninckx *et al.*, 1996; Van Loon, 1977), although their role is in many cases still unclear.

Several *Arabidopsis* mutants affected in their response to the signalling molecules jasmonate, ethylene, or SA have been characterized in the past years. To gain more insight into the signalling pathway controlling nonpathogenic rhizobacteria-mediated ISR, we examined whether the jasmonate response mutant *jar1* (Staswick *et al.*, 1992), the ethylene response mutant *etr1* (Bleecker *et al.*, 1988), and the SAR regulatory mutant *npr1* (Cao *et al.*, 1994) are able to express ISR after colonization of the roots by strain WCS417r. Mutant *jar1* exhibits reduced sensitivity to methyl jasmonate (MeJA), leading to a decrease in MeJA-inducible inhibition of primary root growth and MeJA-inducible accumulation of a vegetative storage protein (Staswick *et al.*, 1992). Mutant *etr1* (Bleecker *et al.*, 1988) is altered in its ability to perceive and react to ethylene due to a mutation in the *etr1* gene, encoding an ethylene receptor (Chang *et al.*, 1993; Schaller and Bleecker, 1995). *Arabidopsis jar1* plants, as well as ethylene-insensitive tobacco plants expressing the mutant *Arabidopsis etr1* gene, are susceptible to opportunistic microorganisms (Knoester *et al.*, 1998; Staswick *et al.*, 1997), whereas wild-type plants show a resistant phenotype, indicating that both mutations affect signalling events leading to disease resistance. *Arabidopsis* mutants *npr1*, *nim1*, and *sai1* are affected downstream of SA in the SAR signalling pathway and as a result are blocked in the SAR response (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). The genes involved are allelic and code for an ankyrin repeat-containing protein with homology to the mammalian signal transduction factor I κ B α which is implicated in disease resistance responses in a wide range of higher organisms (Cao *et al.*, 1997; Ryals *et al.*, 1997).

Using the *Arabidopsis* mutants *jar1*, *etr1*, and *npr1*, we demonstrate that WCS417r-mediated ISR against *Pst* in *Arabidopsis* follows a novel signalling pathway that is dependent on responsiveness to both jasmonate and ethylene. Moreover, we show that similar to SAR, the regulatory protein NPR1 plays a crucial role in the expression of ISR.

Results

Rhizobacteria-mediated ISR requires components of the jasmonate and ethylene response

To investigate whether jasmonate and/or ethylene play a role in rhizobacteria-mediated ISR, the jasmonate response mutant *jar1* and the ethylene response mutant *etr1* were tested for their ability to develop biologically induced resistance against infection by *Pst*. Wild-type Col-0 plants, transgenic SA-nonaccumulating NahG plants, and mutant *jar1* and *etr1* plants were grown in soil containing ISR-inducing WCS417r

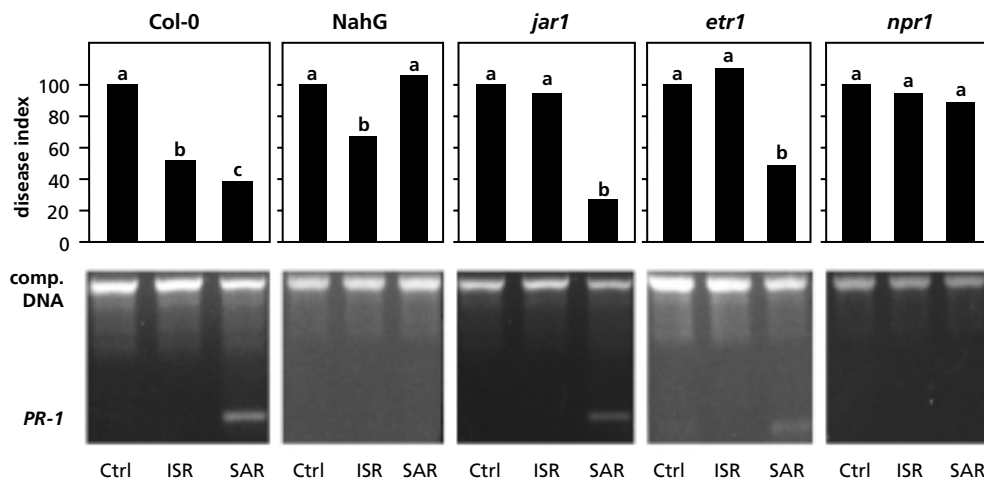


Figure 1. Quantification of ISR and SAR against *Pst* infection in *Arabidopsis* Col-0, NahG, *jar1*, *etr1*, and *npr1* plants and analysis of *PR-1* gene expression.

Shown at top is the disease index of control plants (Ctrl) and plants that received an ISR or SAR treatment. Plants treated with ISR-inducing bacteria were grown in soil containing WCS417r. SAR was induced 3 days before challenge inoculation by infiltrating three leaves per plant with the avirulent pathogen *Pst*(*avrRpt2*). The disease index is the mean ($n=20$ plants) of the percentage of leaves with symptoms relative to control plants (set at 100%), 4 days after challenge inoculation with the virulent pathogen *Pst*. The absolute proportions of diseased leaves of control-treated Col-0, NahG, *jar1*, *etr1*, and *npr1* plants were 60.1, 82.9, 80.6, 60.2, and 68.0%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha=0.05$). Shown below are ethidium bromide-stained agarose gels with competitive RT-PCR products obtained after amplification of equal portions of first-strand cDNA and 500 pg of heterologous competitor DNA (comp. DNA) by using *PR-1*-specific primers. First-strand cDNA was synthesized on mRNA that was isolated from leaves of the indicated plant and treatment combinations that were harvested just before challenge inoculation. The data shown are taken from representative experiments that were repeated at least twice with similar results.

bacteria. Another subset of plants received SAR treatment by inoculating three lower leaves with the avirulent pathogen *Pst*(*avrRpt2*) (Kunkel *et al.*, 1993) 3 days before challenge inoculation with *Pst*. Control plants received no treatment before challenge. Figure 1 shows that in wild-type Col-0 plants, colonization of the roots by WCS417r and predisposing infection with *Pst*(*avrRpt2*) resulted in a significant reduction of symptoms 4 days after challenge inoculation with *Pst*. Moreover, in Col-0 plants pretreated with WCS417r or *Pst*(*avrRpt2*) growth of the challenging pathogen was inhibited (Table 1), indicating that WCS417r-mediated ISR and pathogen-induced SAR were triggered in these plants.

Figure 1 and Table 1 show that SA-nonaccumulating NahG plants mounted resistance against *Pst* infection after WCS417r treatment but not after preinfection with the avirulent pathogen. Furthermore, only plants expressing SAR concomitantly showed accumulation of *PR-1* transcripts (Figure 1), whereas plants expressing ISR did not, confirming that ISR and SAR are controlled by distinct signalling pathways that diverge in their requirement for SA. Both *jar1* and *etr1* plants developed SAR after

Table 1. Number of *Pst* bacteria in challenged leaves of control plants and WCS417r-, and *Pst(avrRpt2)*-treated *Arabidopsis* plants.

Plant type	cfu/g fresh weight ($\times 10^7$) ¹		
	Control	WCS417r	<i>Pst(avrRpt2)</i>
Col-0	2.5 $\pm$ 0.2 ^a	0.6 $\pm$ 0.2 ^b	0.5 $\pm$ 0.2 ^b
NahG	143.0 $\pm$ 21.6 ^a	91.7 $\pm$ 6.5 ^b	161.7 $\pm$ 27.3 ^a
<i>jar1</i>	31.1 $\pm$ 2.9 ^a	27.6 $\pm$ 1.3 ^a	1.9 $\pm$ 0.7 ^b
<i>etr1</i>	28.1 $\pm$ 1.2 ^b	43.0 $\pm$ 1.7 ^a	9.6 $\pm$ 0.8 ^c
<i>npr1</i>	138.8 $\pm$ 17.3 ^a	110.5 $\pm$ 26.5 ^a	147.3 $\pm$ 39.6 ^a

¹ Values presented are average numbers of cfu/g ($\pm$ SE) of leaves, harvested 4 days after challenge inoculation with virulent *Pst*. Within each row, different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha=0.05$).

preinoculation with *Pst(avrRpt2)* and showed activation of *PR-1* gene expression (Figure 1), supporting previous findings (Lawton *et al.*, 1995, 1996) that SAR signal transduction in *Arabidopsis* does not require components of the jasmonate or ethylene response. However, neither *jar1* nor *etr1* plants developed ISR when the roots were colonized by WCS417r, indicating that an intact response to both jasmonate and ethylene is required for the development of rhizobacteria-mediated ISR. Evidently, both of the phytohormones jasmonate and ethylene play a crucial role in the ISR signalling pathway but SA does not.

Rhizobacteria-mediated ISR is dependent on NPR1

NPR1 has been shown to be an important regulatory factor in the SA-dependent SAR response (Cao *et al.*, 1994). To investigate whether NPR1 is involved in the SA-independent ISR response as well, *Arabidopsis* mutant *npr1* was tested. Figure 1 and Table 1 show that *npr1* plants failed to develop SAR and did not show *PR-1* gene activation after predisposing infection with *Pst(avrRpt2)*, confirming that the SAR response was effectively blocked in these plants. Surprisingly, *npr1* plants were also affected in the expression of WCS417r-mediated ISR, indicating that both types of biologically induced disease resistance are dependent on NPR1.

Colonization of the rhizosphere by WCS417r

To investigate whether the inability to express ISR in the mutants was caused by insufficient colonization of the rhizosphere by WCS417r, we determined the number of WCS417r bacteria per gram of root fresh weight at the end of each bioassay. Table 2 shows that WCS417r colonizes the rhizosphere of Col-0, NahG, *jar1*, *etr1*, and *npr1* plants with equal efficiency. Thus, the loss of the capacity to express WCS417r-mediated ISR in *jar1*, *etr1*, and *npr1* plants is not caused by changes in bacterial root colonization but must be the result of alterations in properties of the mutants.

Table 2. Colonization of the rhizosphere of *Arabidopsis* Col-0, NahG, *jar1*, *etr1*, and *npr1* plants by WCS417r.

Plant type	cfu/g fresh weight ($\times 10^6$) ¹
Col-0	8.4 $\pm$ 0.4
NahG	7.5 $\pm$ 1.1
<i>jar1</i>	9.7 $\pm$ 0.8
<i>etr1</i>	6.6 $\pm$ 0.6
<i>npr1</i>	7.3 $\pm$ 1.1

¹ Values presented are average population densities ($\pm$ SE). Roots were harvested at the end of the bioassays. On nontreated roots, no rifampicin-resistant bacteria were detected (detection limit 10^3 cfu/g).

Sequence of signalling events

To elucidate the sequence of the signalling events involved in the jasmonate-, ethylene-, and NPR1-dependent ISR response, we tested the resistance-inducing ability of MeJA and 1-aminocyclopropane-1-carboxylate (ACC), the natural precursor of ethylene, in Col-0, NahG, *jar1*, *etr1*, and *npr1* plants. Figure 2A shows that applied ACC was readily converted to ethylene by endogenous ACC oxidase activity. H₂O- and MeJA-treated plants showed basal levels of ethylene production, whereas in ACC-treated plants a 10- to 25-fold increase in ethylene production was observed (Figure 2B). As shown in Figure 2C, pretreatment of Col-0 plants with MeJA or ACC resulted in a 50% reduction of the symptoms caused by *Pst* infection. Table 3 shows that growth of *Pst* was inhibited as well, indicating that the observed reduction of symptoms is caused by the activation of a resistance response.

Application of MeJA or ACC to NahG plants also resulted in a reduction of the symptoms, although the level of protection was somewhat lower than that observed in wild-type Col-0 plants. In *jar1* plants, application of MeJA did not elicit a resistance response, whereas application of ACC resulted in wild-type levels of protection. Mutant *etr1* plants were nonresponsive to ACC treatment but also failed to respond to MeJA treatment, indicating that components of the ethylene response act downstream of jasmonate in the signalling pathway leading to protection against *Pst*. In *npr1* plants, responsiveness to MeJA or ACC was blocked and strongly reduced, respectively, indicating that components of the jasmonate and ethylene response act upstream of NPR1 in regulating the expression of induced resistance against *Pst*.

ISR is not associated with jasmonate- and ethylene-responsive gene activation

The involvement of components from the jasmonate and ethylene response in rhizobacteria-mediated ISR suggests that ISR might be associated with jasmonate- and ethylene-induced processes. To investigate whether treatment with WCS417r stimulates known jasmonate- or ethylene-inducible responses, we studied the expression of the jasmonate-inducible gene *Atvsp*, encoding a vegetative storage protein (Berger *et al.*, 1995), the ethylene-inducible *Hel* gene, encoding a hevein-like protein with antifungal activity (Potter *et al.*, 1993), and the jasmonate- and ethylene-inducible plant defensin gene *Pdfl.2*, encoding a small protein with antifungal activity

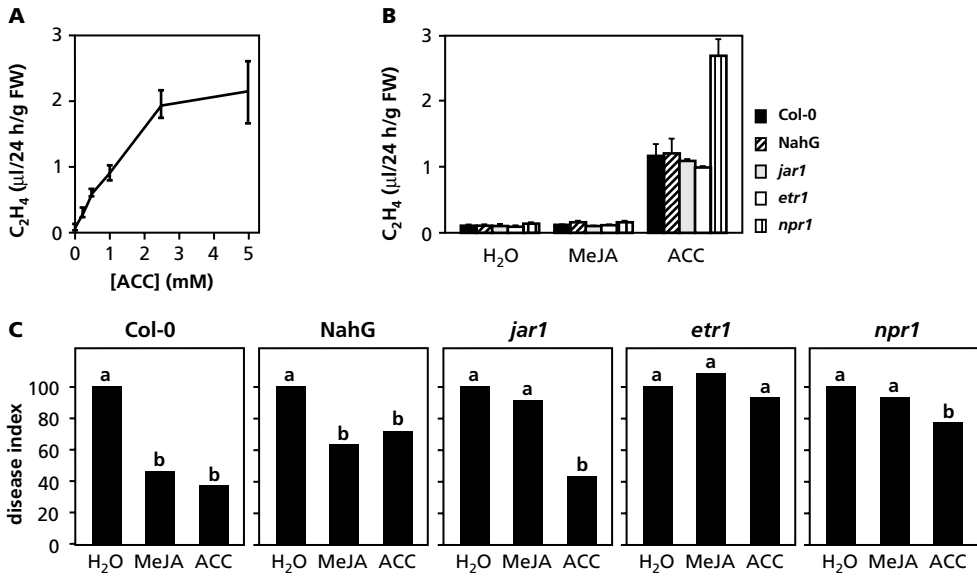


Figure 2. Ethylene production and quantification of induced protection in MeJA-, and ACC-treated *Arabidopsis* plants.

A shows the ethylene production in leaves of *Arabidopsis* ecotype Col-0 after treatment of the leaves with different concentrations of ACC. Data points are means (μl of ethylene produced per gram fresh weight of leaf tissue in the first 24 h after treatment) with standard errors from three independent samples that received the same treatment. In **B**, the ethylene production in leaves of Col-0, NahG, *jar1*, *etr1*, and *npr1* plants in the first 24 h after treatment with H₂O, 100 μM MeJA, or 1 mM ACC is depicted. Bars represent standard errors from six independent samples that received the same treatment. **C** shows the protection against *Pst* induced by MeJA and ACC in Col-0, NahG, *jar1*, *etr1* and *npr1* plants. Plants were pretreated with H₂O, 100 μM MeJA, or 1 mM ACC 3 days before challenge inoculation with *Pst*. Four days after challenge inoculation, the disease index was determined (see legend to Figure 1). The absolute proportions of diseased leaves of control-treated Col-0, NahG, *jar1*, *etr1*, and *npr1* plants were 49.9, 78.2, 74.8, 61.0, and 70.0%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fisher's LSD test, $n=20$, $\alpha=0.05$).

(Penninckx *et al.*, 1996). Figure 3 shows that application of MeJA or ACC to the leaves activated the expression of the *Atvsp* or *Hel* gene, respectively, demonstrating that both MeJA and ACC triggered their corresponding response pathway specifically. As expected, both MeJA and ACC induced *Pdfl.2* transcript accumulation in the leaves.

Table 3. Number of *Pst* bacteria in challenged leaves of control plants and MeJA- and ACC-treated *Arabidopsis* Col-0 plants.

Treatment	cfu/g fresh weight ($\times 10^6$) ¹
H ₂ O	6.0 $\pm$ 0.4 ^a
MeJA	2.4 $\pm$ 0.3 ^b
ACC	2.6 $\pm$ 0.2 ^b

¹ Values presented are average numbers of cfu/g ($\pm$ SE) per gram of leaves, harvested 4 days after challenge inoculation with virulent *Pst*. Different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha=0.05$).

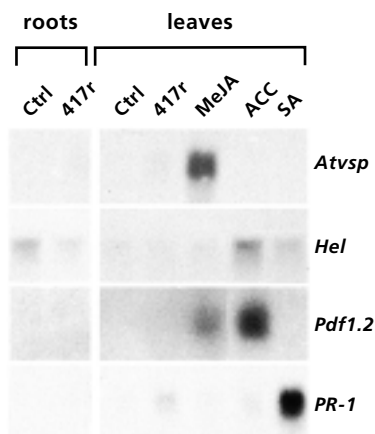


Figure 3. Expression of jasmonate-, ethylene-, and SA-inducible genes in response to WCS417r, MeJA, ACC, and SA treatment.

The results of RNA blot analysis of *Atvsp*, *Hel*, *Pdf1.2* and *PR-1* gene expression are shown. Roots and leaves of plants that were grown in soil supplemented with 10 mM MgSO_4 (Ctrl) or WCS417r bacteria (417r) were harvested when the plants were 5 weeks old. Chemical treatments were performed by dipping the leaves of 5-week-old plants in a solution containing 0.01% (v/v) Silwet L-77, and either MeJA (100 μM), ACC (1 mM), or SA (5 mM). Control leaves were treated with 0.01% (v/v) Silwet L-77 only. Chemically treated leaves were harvested 2 days after application of the chemicals. *Arabidopsis Atvsp*, *Hel*, *Pdf1.2* and *PR-1* gene-specific probes were used for RNA blot hybridizations.

However, in roots and leaves of WCS417r-induced plants, no increase in *Atvsp*, *Hel*, or *Pdf1.2* transcript levels was detected, indicating that the expression of WCS417r-mediated ISR does not coincide with a strong stimulation of the jasmonate and the ethylene response.

Discussion

We demonstrated previously that plants expressing pathogen-induced SAR or rhizobacteria-mediated ISR against *Pst* infection develop significantly fewer symptoms compared to noninduced plants, and show a strong inhibition of pathogen growth in the leaves (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Despite these phenotypical similarities, the signalling pathways leading to both biologically induced resistance responses diverge in their requirement for SA. Moreover, the expression of SAR is accompanied by the activation of *PR* genes, whereas this response is lacking during expression of ISR (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). In this study, we used well-characterized *Arabidopsis* mutants in our attempt to elucidate the steps involved in the SA-independent signalling pathway controlling rhizobacteria-mediated ISR. Systemic resistance induced by nonpathogenic rhizobacteria was blocked in the *Arabidopsis* mutants *jar1*, *etr1*, and *npr1* (Figure 1 and Table 1), indicating that components of the jasmonate and ethylene response, as well as NPR1 play a crucial role in the ISR signalling pathway. Consistent with our observations, Lawton *et al.* (1995, 1996) previously demonstrated that both *jar1* and *etr1* are not impaired in their ability to develop SAR. Thus, the rhizobacteria-mediated ISR and pathogen-induced SAR signalling pathways diverge in their requirement for SA, on the one hand, and for jasmonate and ethylene, on the other hand.

Several lines of evidence indicate that MeJA- and ACC-induced protection against *Pst* follow the same signalling pathway as WCS417r-mediated ISR. First, WCS417r, MeJA, and ACC induce resistance against *Pst* in NahG plants (Figures 1 and 2C), indicating that these agents activate an SA-independent resistance mechanism. This is

supported by the fact that WCS417r-, MeJA-, and ACC-treated plants do not show an increase in SA-inducible *PR-1* gene expression (Figure 3). The level of protection in NahG plants after induction by these agents was lower compared to that observed in wild-type Col-0 plants. This may be due to the fact that NahG plants are more susceptible to pathogen infection (Delaney *et al.*, 1994; Figures 1 and 2, and Table 1), resulting in a lower efficacy of the ISR-inducing agents. Nevertheless, a modulating role of SA in the ISR response cannot be ruled out. The second line of evidence indicating that WCS417r, MeJA, and ACC trigger the same signalling pathway controlling induced resistance against *Pst* is the observation that resistance induced by these three agents requires responsiveness to ethylene and is dependent on NPR1 to be fully expressed. All together, this strongly suggests that resistance induced by WCS417r, MeJA, or ACC is reached by activating the same defense pathway.

Using MeJA and ACC as inducing agents, we determined the sequence of signalling events involved in the pathway leading to resistance against *Pst*. Figure 2C clearly shows that MeJA-mediated protection against *Pst* requires an intact response to ethylene, whereas ACC is fully active in *jar1* plants. Hence, components of the ethylene response act downstream of jasmonate. Moreover, MeJA- and ACC-induced protection are blocked or highly diminished in *npr1* plants, indicating that NPR1 acts downstream of jasmonate and ethylene in the signalling pathway leading to resistance against *Pst*. Therefore, we postulate that during signal transduction leading to WCS417r-mediated ISR, the jasmonate and ethylene responses are engaged successively to trigger a defense response that is regulated by NPR1 (Figure 4).

The observation that ACC-mediated protection was not completely blocked in *npr1* plants (Figure 2C) suggests the existence of a parallel ethylene-inducible defensive pathway that does not require NPR1. A candidate pathway might be the ethylene-inducible pathway leading to *Pdf1.2* gene expression that has been shown to be NPR1 independent (Penninckx *et al.*, 1996). Alternatively, this low level of protection in *npr1* plants may be caused by the twofold higher production of ethylene after ACC treatment (Figure 2B). However, the latter possibility seems unlikely because a twofold increase in ethylene production in wild-type Col-0 plants, by applying 2.5 mM of ACC to the leaves instead of 1 mM, does not result in a higher level of protection against *Pst* infection (Chapter 6). In itself, the enhanced level of ethylene production in ACC-treated *npr1* plants is intriguing as it demonstrates that *npr1* plants show twofold higher ACC oxidase activity than do wild-type plants. Interestingly, pathogen infection also causes a significantly higher increase in ethylene production in *npr1* plants (C.M.J. Pieterse, unpublished result), suggesting that not only SA responsiveness but also ethylene metabolism is altered by the *npr1* mutation.

Elicitation of a similar SA-independent defense pathway against *Pst* infection by WCS417r, MeJA, and ACC implies that ISR is associated with an increase in the production of jasmonate or ethylene. However, WCS417r-mediated ISR does not coincide with jasmonate- and ethylene-responsive gene expression (Figure 3), suggesting that the production of jasmonate and ethylene is not strongly stimulated. When plants were treated with lower concentrations of MeJA or ACC (5 μ M and 0.1

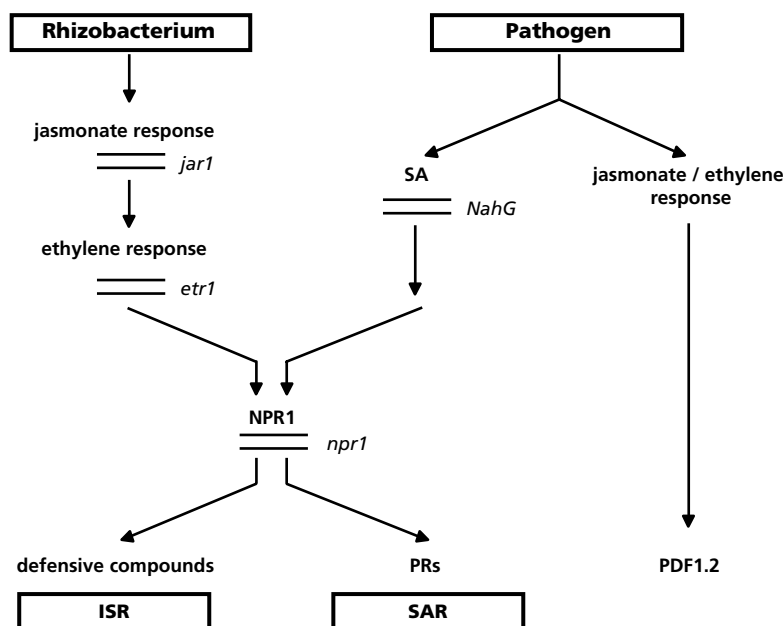


Figure 4. Proposed model for the nonpathogenic rhizobacteria-mediated ISR signalling pathway as part of the network of pathways controlling biologically induced systemic resistance.

WCS417r bacteria trigger an SA-independent pathway in which components from the jasmonate and ethylene response act in sequence to activate a systemic resistance response that is dependent on the regulatory protein NPR1. The ISR pathway shares signalling events that are initiated upon pathogen infection but is not associated with *PR* or *Pdf1.2* gene expression. This indicates that WCS417r bacteria trigger a novel signalling pathway and that resistance induced by these nonpathogenic rhizobacteria involves the production of so far unidentified defensive compounds that are active against *Pst*. The NPR1-dependent pathway controlling *PR* gene expression and the NPR1-independent pathway leading to *Pdf1.2* gene expression are according to Ryals *et al.* (1996) and Penninckx *et al.* (1996), respectively.

mM rather than 100 μ M and 1 mM, respectively), they clearly developed enhanced protection against *Pst*, without activating *Atvsp*, *Hel*, or *Pdf1.2* gene expression (Chapter 6). Hence, WCS417r-mediated ISR may involve a moderate or localized stimulation of the jasmonate and ethylene response that is below the threshold level needed for *Atvsp*, *Hel*, and *Pdf1.2* gene activation. Nevertheless, it cannot be ruled out that simply the availability of jasmonate and ethylene signalling intermediates is sufficient to facilitate induction of ISR. Recently, Schweizer *et al.* (1997b) demonstrated that during infection of rice with the fungal pathogen *Magnaporthe grisea*, jasmonate-inducible genes are activated without an increase in endogenous jasmonate levels. Moreover, Tsai *et al.* (1996) provided evidence that an increase in ethylene sensitivity rather than ethylene production is the initial event to trigger jasmonate-enhanced senescence in detached rice leaves. Thus, ethylene- and jasmonate-dependent plant responses can be triggered without a concomitant increase in the levels of these phytohormones. Whether enhanced sensitivity to either jasmonate or ethylene plays a role in rhizobacteria-mediated ISR needs to be elucidated.

Pathogen-induced systemic activation of the *Arabidopsis* plant defensin gene *Pdf1.2* is independent of SA and requires components from both the jasmonate and the ethylene response pathway (Penninckx *et al.*, 1996). Therefore, this defense reaction appears to share specific signalling events with WCS417r-mediated ISR. However, the latter is not associated with an increase in *Pdf1.2* transcript levels (Figure 3). Moreover, signal transduction leading to *Pdf1.2* gene activation was reported to be independent of NPR1 (Penninckx *et al.*, 1996), whereas WCS417r-mediated ISR requires NPR1 (Figure 1). Thus, the corresponding signalling pathways must be dissimilar (Figure 4). Recently, analysis of the SAR signal transduction mutant *cpr5* revealed that the signalling pathways controlling NPR1-dependent SAR and NPR1-independent *Pdf1.2* gene expression are connected in early signal transduction steps and branch upstream of SA (Bowling *et al.*, 1997). Here, we show that the ISR pathway is connected with that of SAR as well in that they both require NPR1. Apparently, biologically induced systemic resistance responses in plants are connected via a complex network of signalling pathways that involve not only SA but also the concerted action of jasmonate and ethylene (Figure 4).

Mutant *npr1* was originally isolated in a screen for SAR mutants that are blocked in the response pathway leading from SA to *PR* gene activation (Cao *et al.*, 1994). Although ISR is independent of SA accumulation and is not associated with *PR* gene activation, this resistance response is blocked in mutant *npr1* as well. Hence, NPR1 is not only required for the SA-dependent expression of *PR* genes that are activated during SAR but also for the jasmonate- and ethylene-dependent activation of thus so far unidentified defense responses that are involved in rhizobacteria-mediated ISR. Hence, NPR1 differentially regulates defense gene expression, depending on the signalling pathway that is activated upstream of it. Future research should reveal the molecular basis underlying this phenomenon.

Materials and methods

Bacterial cultures

ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria (Van Peer *et al.*, 1991) were grown on King's medium B (KB) agar plates (King *et al.*, 1954) for 24 h at 28°C. The bacterial cells were collected, resuspended in 10 mM MgSO₄, and adjusted to a concentration of 10⁹ cfu/ml before mixing throughout the soil.

The avirulent *P. syringae* pv *tomato* strain DC3000 carrying a plasmid with avirulence gene *avrRpt2* (*Pst*(*avrRpt2*); Kunkel *et al.*, 1993) was used for induction of SAR. Bacteria were cultured overnight at 28°C in liquid KB supplemented with tetracycline (20 mg/l) to select for the plasmid. The bacterial cells were collected by centrifugation, resuspended in 10 mM MgSO₄, and adjusted to a concentration of 10⁷ cfu/ml before pressure infiltration into the leaves.

The virulent *P. syringae* pv *tomato* strain DC3000 (*Pst*; Whalen *et al.*, 1991) was used for challenge inoculations. *Pst* bacteria were grown overnight in liquid KB at 28°C. After centrifugation, bacterial cells were resuspended to a final concentration of 2.5 × 10⁷ cfu/ml in 10 mM MgSO₄ containing 0.01% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands).

Cultivation of plants

Seeds of wild-type *Arabidopsis thaliana* ecotype Col-0 plants, transgenic NahG plants harboring the bacterial *NahG* gene (Delaney *et al.*, 1994), and mutant *jar1* (Staswick *et al.*, 1992), *etr1* (Bleecker *et al.*, 1988), and *npr1* plants (Cao *et al.*, 1994) were sown in quartz sand. Two-week-old seedlings were transferred to 60-ml pots containing a sand and potting soil mixture that had been autoclaved twice for 1 h. Plants were cultivated

in a growth chamber with a 9-h day (200 $\mu\text{E}/\text{m}^2/\text{sec}$ at 24°C) and 15-h night (20°C) cycle and 70% relative humidity. Plants were watered on alternate days and once a week were supplied with modified half-strength Hoagland nutrient solution (2 mM KNO_3 , 5 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM KH_2PO_4 , 1 mM MgSO_4 , and trace elements, pH 7; Hoagland and Arnon, 1938) containing 10 μM Sequestreen (Novartis, Basel, Switzerland).

Induction treatments

Plants were treated with nonpathogenic, ISR-inducing rhizobacteria by mixing a suspension of WCS417r bacteria throughout the soil to a final density of 5×10^7 cfu/kg just before the seedlings were planted as described by Pieterse *et al.* (1996).

SAR was induced 3 days before challenge inoculation by pressure infiltrating three lower leaves per plant with the avirulent pathogen *Pst*(*avrRpt2*) at 10^7 cfu/ml in 10 mM MgSO_4 by using a 1-ml syringe without a needle, as described by Swanson *et al.* (1988).

Chemical treatments were performed 3 days before challenge inoculation by dipping the leaves of five-week-old plants in a solution containing 0.01% (v/v) Silwet L-77 and either methyl jasmonate (MeJA; 100 μM), salicylic acid (SA; 5 mM), or 1-aminocyclopropane-1-carboxylate (ACC; 0.25, 0.5, 1.0, 2.5, or 5.0 mM), pH 6. Control plants were treated with 0.01% (v/v) Silwet L-77 only. MeJA was purchased from Serva, Brunschwig Chemie (Amsterdam, The Netherlands), ACC from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands), and SA from Malinckrodt Baker BV (Deventer, The Netherlands).

Challenge inoculation and disease assessment

Challenge inoculations were performed by dipping the leaves of 5-week-old plants in a bacterial suspension of the virulent pathogen *Pst* at 2.5×10^7 cfu/ml in 10 mM MgSO_4 , 0.01% (v/v) Silwet L-77. Four days after challenge, disease severity was assessed by determining the percentage of leaves with symptoms per plant (20 plants per treatment) and by examining the growth of the challenging pathogen in the leaves. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. The number of *Pst* bacteria in inoculated leaves was assessed in three sets of 20 randomly selected leaves per treatment. Leaves were weighed, rinsed thoroughly in sterile water, and homogenized in 10 mM MgSO_4 . Subsequently, appropriate dilutions were plated onto KB agar supplemented with rifampicin (50 mg/l) and cycloheximide (100 mg/l). After an incubation time of 48 hr at 28°C, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined.

Rhizosphere colonization

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

Competitive reverse transcriptase-polymerase chain reaction

Analysis of *PR-1* gene expression was performed using the competitive reverse transcriptase-polymerase chain reaction (RT-PCR) as described by Siebert and Larrick (1992). A *PR-1*-specific primer pair (5'-GTAGGTGCTCTTGTCTCTCC-3' and 5'-TTCACATAATTTCCACGAGG-3'), yielding RT-PCR products of 422 bp, was prepared based on the *Arabidopsis PR-1* cDNA sequence described by Uknes *et al.* (1992). A 900-bp heterologous competitor DNA fragment, competing for the same set of primers, was obtained as described by Siebert and Larrick (1992). Fifty nanograms of poly(A)⁺ RNA, isolated from frozen leaves, was converted into first-strand cDNA. Subsequently, equal portions of cDNA were amplified in the presence of 500 pg of competitive DNA by using the *PR-1*-specific primer pair. The products were then resolved on an agarose gel stained with ethidium bromide.

Ethylene measurement

Thirty minutes after the application of the chemicals, leaves were detached, weighed, and placed in 25-ml gas-tight serum flasks that subsequently were incubated for 24 h under climate chamber conditions. Ethylene accumulation was measured by gas chromatography as described by De Laet and Van Loon (1982).

RNA blot analysis

Total RNA was extracted from roots and leaves of 5-week-old control and ISR-expressing plants and from leaves collected 2 days after chemical application, using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). For RNA blot analysis, 15 µg of total RNA was electrophoretically separated on denaturing formaldehyde-agarose gels and blotted onto Hybond-N⁺ membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer, as described by Sambrook *et al.* (1989). RNA gel blots were hybridized and washed as described previously (Pieterse *et al.*, 1994) and exposed to a Kodak X-OMAT AR film. DNA probes were labelled with α -³²P-dCTP by random primer labelling (Feinberg and Vogelstein, 1983). Probes for the detection of *Atvsp* and *Hel* transcripts were prepared by PCR with primers based on sequences obtained from GenBank accession numbers Z18377 and U01880, respectively. Probes for *Pdf1.2* and *PR-1* were derived from an *Arabidopsis Pdf1.2* and a *PR-1* cDNA clone, respectively (Penninckx *et al.*, 1996; Uknes *et al.*, 1992).

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Simultaneous activation of a salicylic acid-dependent and a jasmonate- and ethylene-dependent defense pathway in *Arabidopsis* results in an enhanced level of systemically induced resistance

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Submitted

Abstract

Arabidopsis thaliana has been shown to exhibit at least two types of induced resistance active against a broad spectrum of pathogens: systemic acquired resistance (SAR) that is triggered upon infection by a necrotizing pathogen, and rhizobacteria-mediated induced systemic resistance (ISR) that is elicited by nonpathogenic, rhizosphere-colonizing *Pseudomonas* bacteria. ISR requires responsiveness to the plant signalling molecules jasmonate and ethylene but functions independently of salicylic acid (SA). In contrast, SAR is SA-dependent and does not require responsiveness to jasmonate and ethylene. Both the SAR and the ISR pathway are controlled by the key regulator NPR1. Here, we demonstrate that simultaneous activation of the ISR and the SAR pathway results in an additive effect on the level of induced protection against the pathogen *P. syringae* pv *tomato* (*Pst*). No enhanced level of protection was evident in *Arabidopsis* genotypes that are blocked in either the ISR or the SAR response. Expression of the SAR marker gene *PR-1* was not altered in plants simultaneously expressing ISR and SAR compared to plants expressing only SAR. Together, these observations indicate that the ISR and the SAR pathway are distinct and that there is no significant cross-talk between both pathways. Furthermore, systemic tissues expressing both types of induced resistance did not show elevated levels of *Npr1* transcripts, suggesting that the additive effect of ISR and SAR on induced protection is not based on enhanced levels of NPR1 protein. It appears that the enhanced level of induced protection in plants expressing both types of induced resistance is established through parallel activation of complementary defense responses that are active against *Pst*.

Introduction

Plants possess inducible defense mechanisms to protect themselves against pathogen attack. A classic example is systemic acquired resistance (SAR) that is activated after infection by a necrotizing pathogen and confers broad-spectrum resistance in distant, uninfected plant parts (reviewed by Ryals *et al.*, 1996). Selected nonpathogenic, rhizosphere-colonizing bacteria trigger a phenotypically similar form of resistance, that is commonly referred to as rhizobacteria-mediated induced systemic resistance (ISR; reviewed by Van Loon *et al.*, 1998). In contrast to SAR-inducing pathogens, ISR-inducing rhizobacteria do not cause any harmful symptoms on the plant.

Of various plant growth-promoting rhizobacteria (PGPR), *Pseudomonas fluorescens* WCS417r has been shown to activate ISR in several plant species including carnation (Van Peer *et al.*, 1991), radish (Leeman *et al.*, 1995), tomato (Duijff *et al.*, 1998), and *Arabidopsis thaliana* (Pieterse *et al.*, 1996). In *Arabidopsis*, WCS417r has been shown to induce ISR against the bacterial leaf pathogen *P. syringae* pv *tomato* (*Pst*), the fungal root pathogen *Fusarium oxysporum* f sp *raphani* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997), and the oomycetous leaf pathogen *Peronospora parasitica* (J. Ton and C.M.J. Pieterse, unpublished result), indicating that WCS417r-mediated ISR is effective against different types of pathogens.

In *Arabidopsis*, SAR and WCS417r-mediated ISR are regulated by distinct signalling pathways. As in many other plant species, in *Arabidopsis*, pathogen-induced SAR is associated with local and systemic increases in the level of salicylic acid (SA) and a coordinate expression of genes encoding pathogenesis-related proteins (PRs; Lawton *et al.*, 1995; Uknes *et al.*, 1993). SA is a necessary intermediate in the SAR signal transduction pathway because transgenic NahG plants, that cannot accumulate SA, are incapable of developing SAR and expressing PR genes in nontreated leaves of pathogen-inoculated plants (Lawton *et al.*, 1995). SA is also sufficient for the elicitation of SAR, because exogenous application of SA leads to both activation of PR genes and broad-spectrum resistance (Ryals *et al.*, 1996). Certain rhizobacterial strains have been shown to induce systemic resistance through stimulation of the SAR pathway by producing SA at the root surface (Maurhofer *et al.*, 1998; De Meyer and Höfte, 1997). In contrast, other rhizobacteria that induce ISR have been demonstrated to trigger a different signalling pathway, that does not require SA (Pieterse *et al.*, 1996; Press *et al.*, 1997; Van Wees *et al.*, 1997). ISR triggered by WCS417r is not associated with the activation of SA-inducible PR genes. Moreover, SA-nonaccumulating *Arabidopsis* NahG plants develop normal levels of ISR after colonization of the roots by WCS417r (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997).

The plant signalling molecules jasmonate and ethylene have been implicated in the induction of several defense responses (Boller, 1991; Wasternack and Parthier, 1997). Recently, both regulators emerged as important signalling molecules in ISR. The jasmonate response mutant *jar1* and the ethylene response mutant *etr1* of *Arabidopsis*, that express normal levels of pathogen-induced SAR (Lawton *et al.*, 1995, 1996; Pieterse *et al.*, 1998), do not express ISR upon treatment with WCS417r, indicating

that the ISR signalling pathway requires components of the jasmonate and ethylene response (Pieterse *et al.*, 1998). Plants expressing ISR do not show enhanced expression of jasmonate- and ethylene-inducible genes, such as those encoding plant defensin (*Pdf1.2*), hevein-like protein (*Hel*), or vegetative storage protein (*Atvsp*), suggesting that ISR is associated with activation of other jasmonate- and ethylene-dependent responses (Pieterse *et al.*, 1998).

Although SAR and ISR follow distinct signalling pathways, they are both blocked in the SAR regulatory mutant *npr1* of *Arabidopsis* (Cao *et al.*, 1994; Pieterse *et al.*, 1998). In the SAR signal transduction pathway, the NPR1 protein, also called NIM1 (Delaney *et al.*, 1995) or SAI1 (Shah *et al.*, 1997) is a key regulator of *PR* gene expression that acts downstream from SA (Cao *et al.*, 1994). Although the ISR response is independent of SA, it does need NPR1 to become activated. In the ISR pathway, components from the jasmonate and ethylene response have been shown to act in sequence and upstream of NPR1 (Pieterse *et al.*, 1998), demonstrating that NPR1 regulates not only SA-dependent activation of *PR* genes during SAR, but also jasmonate- and ethylene-dependent activation of so far unidentified defense responses resulting from rhizobacteria-mediated ISR. Sequence analysis of wild-type and mutant alleles revealed that NPR1 contains a functionally important ankyrin-repeat domain which may be involved in protein-protein interactions (Cao *et al.*, 1997; Ryals *et al.*, 1997). Therefore, interactions of pathway-specific proteins with NPR1 may account for the divergence of the ISR and SAR pathways downstream from NPR1.

Evidence is emerging for the existence of cross-talk between jasmonate-, ethylene- and SA-dependent signalling pathways. Jasmonate and ethylene have been shown to act in concert in activating genes encoding defensive proteins, such as proteinase inhibitors and plant defensins (O'Donnell *et al.*, 1996; Penninckx *et al.*, 1998). In most cases, SA and its functional analogs 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) have been demonstrated to suppress jasmonate- and ethylene-dependent defense gene expression (Bowling *et al.*, 1997; Doares *et al.*, 1995; Doherty *et al.*, 1988; Niki *et al.*, 1998; Peña-Cortés *et al.*, 1993; Penninckx *et al.*, 1996). Conversely, jasmonate and ethylene have been shown to stimulate SA action (Lawton *et al.*, 1994; Schweizer *et al.*, 1997; Xu *et al.*, 1994), although antagonistic effects have been described as well (Niki *et al.*, 1998). Cross-talk between different signal transduction pathways provides great regulatory potential for activating multiple resistance mechanisms in varying combinations, which may help the plant to prioritize the activation of a particular defense pathway over another (Pieterse and Van Loon, 1999; Reymond and Farmer, 1998).

The requirement of the same regulatory component NPR1 for both ISR and SAR, combined with possible cross-talk between the jasmonate-, ethylene-, and SA-dependent signalling pathways, raises the question to what extent the jasmonate- and ethylene-dependent ISR pathway and the SA-dependent SAR pathway interact. Here, we demonstrate that in *Arabidopsis*, simultaneous activation of ISR and SAR leads to an enhanced level of induced protection against infection by *Pst*. We provide evidence that this additive effect on the level of induced resistance is based on the induction of

complementary defense responses rather than on an enhancement of either the SAR or the ISR response. This indicates that there is no significant cross-talk between the ISR and the SAR pathway.

Results

Simultaneous activation of ISR and SAR leads to an enhanced level of protection

The effect of simultaneous activation of the jasmonate- and ethylene-dependent ISR pathway and the SA-dependent SAR pathway on the level of systemically induced protection was examined in *Arabidopsis* ecotype Col-0. ISR was induced by growing the plants in soil containing WCS417r bacteria for 3 weeks. SAR was elicited by pressure infiltrating three lower leaves with the necrotizing pathogen *P. syringae* pv *tomato* carrying the avirulence gene *avrRpt2* (*Pst(avrRpt2)*), three days before challenge inoculation. Plants were challenge inoculated with virulent *Pst* bacteria. Four days later, the plants had developed typical bacterial speck disease symptoms, consisting of necrotic or water-soaked spots surrounded by extensive chlorosis. Disease severity was assessed by determining the percentage of leaves with symptoms per plant. Pretreatment with either ISR-inducing WCS417r or SAR-inducing *Pst(avrRpt2)* resulted in a significant reduction in the proportion of diseased leaves compared to noninduced control plants (Figure 1A). Plants induced by a combination of the

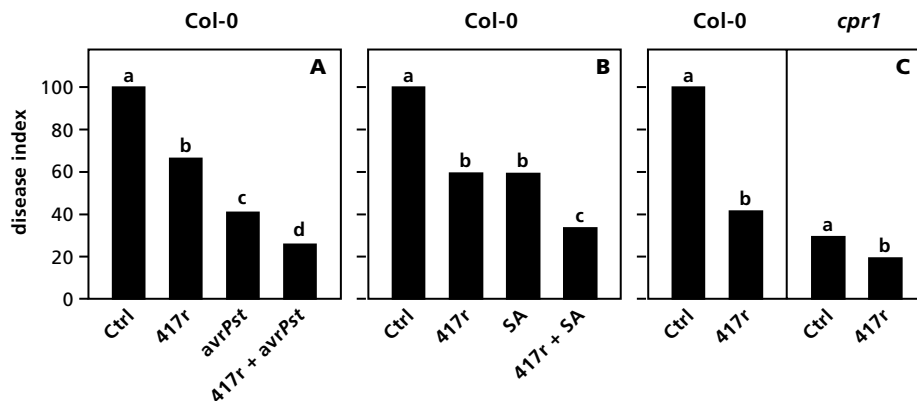


Figure 1. Quantification of protection against *Pst* in *Arabidopsis* plants expressing ISR, SAR, or both types of induced resistance.

ISR was induced by growing the plants in soil containing WCS417r (417r). SAR was induced in wild-type Col-0 plants by either preinfection with avirulent *Pst(avrRpt2)* (*avrPst*) (A), or exogenous application of 1 mM SA (B), 3 days before challenge inoculation. Mutant *cpr1* constitutively expressed SAR (C). The disease index is the mean ($n=20$ plants) of the percentage of leaves with symptoms per plant, compared to control-treated (Ctrl) Col-0 plants (set at 100%), 4 days after challenge with virulent *Pst*. The absolute proportions of diseased leaves of the control-treated Col-0 plants depicted in A, B, and C were 54.7%, 52.4%, and 70.5%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fischer's LSD test; $\alpha=0.05$). The data presented are from representative experiments that were repeated at least twice with similar results.

Table 1. Number of *Pst* bacteria in challenged leaves of control plants and plants expressing ISR, SAR, or both types of induced resistance.

Plant type	Treatment	cfu/g fresh weight ($\times 10^6$) ¹
Col-0	Control	24.0 $\pm$ 4.2 ^a
	WCS417r	15.2 $\pm$ 1.6 ^b
	<i>Pst(avrRpt2)</i>	10.7 $\pm$ 1.3 ^{bc}
	WCS417r + <i>Pst(avrRpt2)</i>	5.6 $\pm$ 1.5 ^c
Col-0	Control	16.5 $\pm$ 2.1 ^a
	WCS417r	9.9 $\pm$ 2.3 ^b
	SA	9.1 $\pm$ 2.3 ^b
	WCS417r + SA	5.2 $\pm$ 0.9 ^b
Col-0	Control	122 $\pm$ 16.8 ^a
	WCS417r	55 $\pm$ 3.0 ^b
<i>cpr1</i>	Control	29.8 $\pm$ 1.9 ^a
	WCS417r	21.6 $\pm$ 2.0 ^b

¹ Values presented correspond to the bioassays presented in Figure 1 and are average numbers of cfu/g ($\pm$ SE) from three sets of 6 plants, 4 days after challenge inoculation with virulent *Pst*. The number of *Pst* bacteria present in the leaves just after challenge did not differ between treatments or plant genotypes (data not shown). Within each set of data, different letters indicate statistically significant differences between treatments (Fischer's LSD test, $\alpha=0.05$).

WCS417r treatment and the *Pst(avrRpt2)* treatment showed a statistically significant higher reduction in disease severity than plants treated with either inducer alone. Determination of the number of *Pst* bacteria in challenged leaves revealed that proliferation of *Pst* was significantly inhibited in plants treated with either WCS417r or *Pst(avrRpt2)* (Table 1). Plants treated with both inducers showed an even more pronounced inhibition (statistically significant at $p=0.06$). These results demonstrate that simultaneous induction of rhizobacteria-mediated ISR and pathogen-induced SAR results in a higher level of protection compared to that obtained by activation of either ISR or SAR alone.

To investigate whether this enhanced level of protection could also be established by using a chemical inducer of SAR in combination with the ISR inducer WCS417r, SA was tested in a similar bioassay. SA was applied to the leaves 3 days before challenge inoculation with *Pst*. Figure 1B and Table 1 show that treatment of Col-0 with WCS417r or SA significantly reduced the proportion of leaves with disease symptoms, and inhibited growth of *Pst in planta*. Plants treated with both WCS417r and SA showed a higher reduction in disease symptoms and a greater inhibition of pathogen proliferation than plants treated with either inducer alone (statistically significant at

$p=0.15$), indicating that simultaneous activation of ISR and chemically induced SAR also leads to a higher level of protection.

The enhanced level of induced protection observed in plants expressing either biologically or chemically induced SAR in addition to rhizobacteria-mediated ISR prompted us to examine the ISR-inducibility of mutant *cpr1* of *Arabidopsis*. Mutant *cpr1* exhibits high levels of SA and PR gene expression, leading to constitutive expression of SAR (Bowling *et al.*, 1994). Compared to control-treated wild-type plants, control-treated *cpr1* plants showed a 70% reduction in leaves with symptoms after inoculation with *Pst*, and a four-fold decrease in growth of *Pst* in the challenged leaves (Figure 1C and Table 1). By growing *cpr1* plants in soil containing WCS417r, a statistically significant higher level of protection was evident in these plants (Figure 1C and Table 1). The ability of mutant *cpr1* to express ISR provides another demonstration that the level of induced resistance can be increased by concurrent expression of SAR and ISR.

Cross-talk between the ISR and SAR signalling pathway is absent

The enhanced state of induced protection after simultaneous activation of SAR and ISR may result from additive effects of two complementary defense responses, or from synergistic effects on either the ISR or SAR response. Such synergistic effects might result from cross-talk between both pathways, leading to stimulation of either the ISR or the SAR response, or both. To assess whether early signalling steps in ISR and SAR influence the expression of SAR and ISR, respectively, we investigated whether

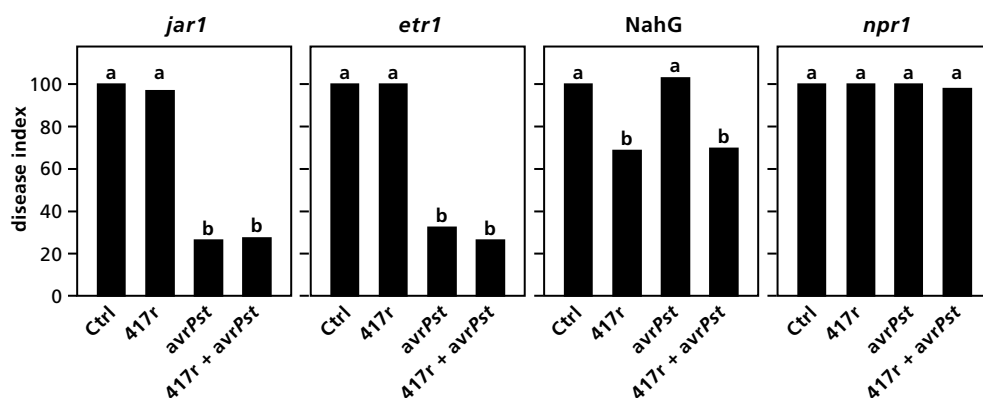


Figure 2. Quantification of protection against *Pst* in *Arabidopsis* genotypes *jar1*, *etr1*, *NahG*, and *npr1* after treatment with the ISR inducer WCS417r, the SAR inducer *Pst*(*avrRpt2*), or a combination of both inducers.

For experimental details see the text and legend to Figure 1. The absolute proportions of diseased leaves of control-treated *jar1*, *etr1*, *NahG*, and *npr1* plants were 82.3%, 75.0%, 88.9%, and 70.0%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fischer's LSD test; $n=20$, $\alpha=0.05$). The data presented are from a representative experiment that was repeated twice with similar results.

Table 2. Number of *Pst* bacteria in challenged leaves of *Arabidopsis* genotypes *jar1*, *etr1*, NahG, and *npr1* pretreated with the ISR inducer WCS417r, the SAR inducer *Pst(avrRpt2)*, or a combination of both inducers.

Treatment	cfu/g fresh weight ($\times 10^8$) ¹			
	<i>jar1</i>	<i>etr1</i>	NahG	<i>npr1</i>
Control	138 $\pm$ 5.9 ^b	116 $\pm$ 31.0 ^a	156 $\pm$ 22.2 ^a	72 $\pm$ 8.9 ^a
WCS417r	181 $\pm$ 21.0 ^a	112 $\pm$ 5.4 ^a	81 $\pm$ 9.3 ^b	80 $\pm$ 12.3 ^a
<i>Pst(avrRpt2)</i>	31 $\pm$ 3.2 ^c	54 $\pm$ 3.4 ^b	173 $\pm$ 48.0 ^a	68 $\pm$ 28.0 ^a
WCS417r + <i>Pst(avrRpt2)</i>	34 $\pm$ 4.6 ^c	60 $\pm$ 19.2 ^b	73 $\pm$ 6.7 ^b	68 $\pm$ 9.6 ^a

¹ The data presented correspond to the bioassay shown in Figure 2 and are average numbers of cfu/g ($\pm$ SE) from three sets of 6 plants, 4 days after challenge inoculation with virulent *Pst*. The number of *Pst* bacteria present in the leaves just after challenge did not differ between treatments or plant genotypes (data not shown). Within each set of data, different letters indicate statistically significant differences between treatments (Fischer's LSD test, $\alpha=0.05$).

WCS417r stimulates the SAR response in mutants that are impaired in the expression of ISR, and whether *Pst(avrRpt2)* stimulates the ISR response in plants that are impaired in the expression of SAR. Consistent with previous findings (Cao *et al.*, 1994; Lawton *et al.*, 1995, 1996; Pieterse *et al.*, 1998), WCS417r-mediated ISR was found to be blocked in the jasmonate response mutant *jar1*, the ethylene response mutant *etr1*, and the ISR and SAR regulatory mutant *npr1*, whereas SAR was abolished in the SA-nonaccumulating NahG transgenic and the *npr1* mutant (Figure 2). In contrast to wild-type plants (Figure 1A), treatment with both WCS417r and *Pst(avrRpt2)* did not result in an enhanced level of protection in *jar1*, *etr1*, NahG, and *npr1* plants (Figure 2). In the mutants *jar1* and *etr1*, the combination of treatments induced the same level of protection as *Pst(avrRpt2)* alone, whereas in NahG plants the combination of treatments protected the plants to the same extent as WCS417r alone. Mutant *npr1* that is blocked in both the ISR and the SAR pathway showed no induced protection at all. Like in wild-type plants, the observed reduction in symptoms through induction of either ISR or SAR was associated with inhibition of growth of *Pst* in challenged leaves. However, treatment with both inducers did not further reduce the number of *Pst* bacteria in the mutants (Table 2). These results indicate that components of the ISR pathway acting upstream of the jasmonate and the ethylene response have no effect on the level of SAR attained. Moreover, components of the SAR pathway acting upstream of SA do not influence the expression of ISR.

Furthermore, we studied whether the induction of ISR in addition to SAR increases SAR-associated *PR* gene expression. Therefore, we looked into the effect of WCS417r treatment on *PR-1* gene activation in SAR-expressing plants. RNA blot analysis demonstrated that the *PR-1* gene was not expressed in WCS417r-treated Col-0 plants (Figure 3). Significant amounts of *PR-1* mRNA were detected in Col-0 plants expressing either *Pst(avrRpt2)*- or SA-induced SAR, and in control-treated *cpr1*

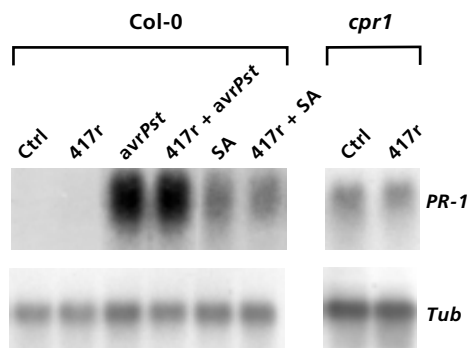


Figure 3. RNA blot analysis of the expression of the SAR response gene *PR-1* in *Arabidopsis* plants expressing ISR, SAR, or both.

Treatments were performed as described in the text and legend to Figure 1. Leaves of *Pst(avrRpt2)*- and SA-induced plants were harvested 2 days after treatment. Of the *Pst(avrRpt2)*-induced plants, the systemic, noninoculated tissue was collected. A *PR-1* gene-specific probe was used to detect *PR-1* transcripts. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β -tubulin (*Tub*).

mutants constitutively expressing SAR. In plants simultaneously expressing ISR and SAR, the level of *PR-1* transcript accumulation was similar to that observed in plants expressing SAR only (Figure 3). This indicates that the SAR pathway is neither stimulated nor suppressed in plants expressing both ISR and SAR.

Enhanced protection is not mediated through enhanced expression of the Npr1 gene

Overexpression of the SAR and ISR regulatory gene *Npr1* in *Arabidopsis* has been demonstrated to increase resistance against *P. syringae* pv *maculicola* and *Peronospora parasitica* (Cao *et al.*, 1998). To investigate whether the increased level of induced protection observed in plants expressing both ISR and SAR can be explained by an increase in *Npr1* gene expression, transcript levels of this gene were assessed in plants expressing ISR, SAR, or both. Figure 4 shows that the expression of *Npr1* was not elevated in systemic tissues expressing either WCS417r-mediated ISR, *Pst(avrRpt2)*-induced SAR or both types of induced resistance, suggesting that the enhanced level of induced resistance in plants simultaneously expressing ISR and SAR is not related to an increased availability of the NPR1 protein.

WCS417r, jasmonate, and ethylene activate the same defense pathway

Previously, we demonstrated that exogenous application of methyl jasmonate (MeJA) or the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) results in

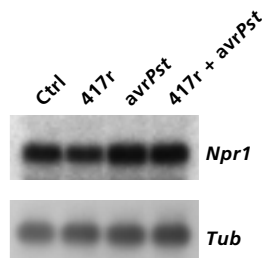


Figure 4. RNA blot analysis of the expression of the ISR and SAR regulatory gene *Npr1* in *Arabidopsis* plants expressing ISR, SAR, or both types of induced resistance.

Treatments were performed as described in the text and legend to Figure 1. Leaves of *Pst(avrRpt2)*- and SA-induced plants were harvested 2 days after treatment. Of the *Pst(avrRpt2)*-induced plants, the systemic, noninoculated tissue was collected. An *Npr1* gene-specific probe was used to detect *Npr1* transcripts. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β -tubulin (*Tub*).

induced resistance against *Pst* (Pieterse *et al.*, 1998). Like WCS417r-mediated ISR, MeJA- and ACC-induced protection are independent of SA but require NPR1 (Pieterse *et al.*, 1998). However, resistance induced by MeJA and ACC coincides with the activation of jasmonate- and ethylene-inducible genes such as *Atvsp* (Berger *et al.*, 1995), *Pdf1.2* (Penninckx *et al.*, 1996), and *Hel* (Potter *et al.*, 1993), whereas WCS417r-mediated ISR does not (Pieterse *et al.*, 1998). To investigate whether MeJA and ACC influence the WCS417r-induced pathway, we tested the effect of combinations of treatments on the level of induced protection against *Pst*. Treatment with either WCS417r, MeJA, or ACC resulted in a significant reduction of the proportion of leaves with symptoms (Figure 5) and a three- to four-fold inhibition of growth of *Pst* in the challenged leaves (Table 3). Plants induced by a combination of WCS417r with either MeJA or ACC did not show an enhanced level of induced protection, indicating that MeJA and ACC have no additive effect on WCS417r-mediated ISR. Moreover, combining MeJA and ACC treatment did not result in an enhanced level of protection either (data not shown). WCS417r neither stimulated, nor suppressed the level of MeJA- or ACC-induced expression of *Atvsp*, *Pdf1.2*, and *Hel* (Figure 6). Therefore, it is unlikely that the lack of an additive effect on the level of induced resistance is caused by competition between the WCS417r-, MeJA- and ACC-induced pathways. Apparently, WCS417r, MeJA, and ACC all activate the same signalling pathway that leads to the production of thus far unidentified defensive compounds that are effective against *Pst*.

Discussion

Plants are capable of differentially activating distinct defense pathways depending on the inducing agent. SA, jasmonate, and ethylene play an important role in this signalling network, as blocking the response to either of these signalling molecules can render plants more susceptible to pathogen infection (Delaney *et al.*, 1994; Knoester *et al.*, 1998; Staswick *et al.*, 1998; Vijayan *et al.*, 1998). Research on the mechanisms of induced disease resistance against *Pst* in *Arabidopsis* have revealed that plants possess an SA-dependent defense pathway (SAR), as well as a jasmonate- and ethylene-

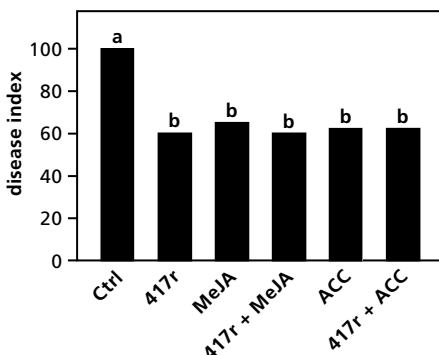


Figure 5. Quantification of protection against *Pst* in *Arabidopsis* Col-0 induced by WCS417r, MeJA, ACC, or combinations of these inducers.

ISR was induced by growing the plants in soil containing WCS417r (417r). 100 μ M MeJA and 1 mM ACC were applied 3 days before challenge with *Pst*. For experimental details see the text and legend to Figure 1. The absolute proportion of diseased leaves in control-treated plants was 66.8%. Different letters indicate statistically significant differences between treatments (Fischer's LSD test; $n=20$, $\alpha=0.05$). The data presented are from a representative experiment that was repeated twice with similar results.

Table 3. Number of *Pst* bacteria in challenged leaves of control plants and plants pretreated with WCS417r, MeJA, ACC, or combinations of these inducers.

Treatment	cfu/g fresh weight ($\times 10^6$) ¹	
Control	16.1 $\pm$ 3.5 ^a	¹ The data presented correspond to the bioassay shown in Figure 5 and are average numbers of cfu/g ($\pm$ SE) from three sets of 6 plants, 4 days after challenge inoculation with virulent <i>Pst</i> . The number of <i>Pst</i> bacteria present in the leaves just after challenge did not differ between treatments (data not shown). Different letters indicate statistically significant differences between treatments (Fischer's LSD test, $\alpha = 0.05$).
WCS417r	3.6 $\pm$ 1.2 ^b	
MeJA	5.3 $\pm$ 2.1 ^b	
WCS417r + MeJA	5.9 $\pm$ 1.3 ^b	
ACC	3.8 $\pm$ 2.1 ^b	
WCS417r + ACC	5.3 $\pm$ 1.5 ^b	

dependent defense pathway (ISR), both leading to NPR1-dependent systemic resistance (Cao *et al.*, 1994; Pieterse *et al.*, 1998). Cross-talk between SA-, jasmonate-, and ethylene-dependent pathways has been demonstrated and is thought to be involved in fine-tuning of defense gene expression (Dong, 1998; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998). To gain more insight into the extent of cross-talk between the ISR pathway and the SAR pathway, the effect of simultaneous expression of ISR and SAR on the level of induced resistance was assessed. Simultaneous activation of both pathways resulted in an enhanced level of induced protection against *Pst*. This additive effect was established irrespective of whether SAR was expressed constitutively as in *cpr1* plants, or was induced by predisposal infection with *Pst(avrRpt2)* or exogenous application of SA (Figure 1 and Table 1). A single inoculation of *Arabidopsis* with *Pst(avrRpt2)* has been shown to be sufficient for induction of the maximum level of SAR (Cameron *et al.*, 1994). Indeed, we observed no elevated levels of SAR in plants that were treated with both *Pst(avrRpt2)* and SA (S.C.M. Van Wees, data not shown). WCS417r-mediated ISR was expressed at a maximum level as well, because a 100-fold lower density of WCS417r in the rhizosphere than that used in our experiments is already sufficient to induce the maximum level of protection (S.C.M. Van Wees, data not shown). Therefore, the additive effect on the level of induced protection must be accomplished through complementary effects of ISR- and SAR-specific defense responses against *Pst*.

This enhanced level of protection was absent in *Arabidopsis* genotypes *jar1*, *etr1*, and NahG that are affected in either ISR or SAR. Mutants *jar1* and *etr1*, that are blocked in the ISR response, showed a similar level of SAR as wild-type plants when treated with both WCS417r and *Pst(avrRpt2)*, whereas transgenic NahG plants, that are impaired in the SAR response, developed similarly normal levels of ISR after receiving this combination of treatments (Figure 2 and Table 2). This indicates that, upstream of the perception of either SA, jasmonate, or ethylene, cross-talk between the pathways does not occur or, at least, has no influence on the outcome of the induced resistance effective against *Pst*. Moreover, in plants expressing SAR, either constitutively or induced by *Pst(avrRpt2)* or SA treatment, the magnitude of *PR-1* gene expression was

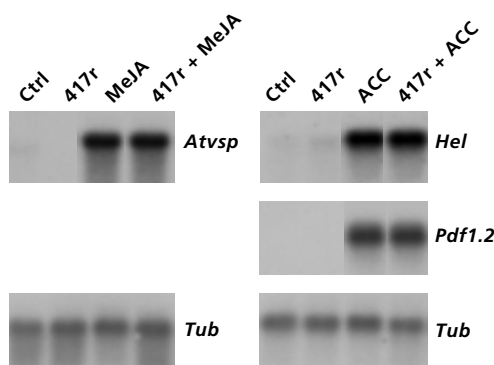


Figure 6. RNA blot analyses of the expression of jasmonate- and ethylene-inducible genes in *Arabidopsis* in response to treatment with WCS417r, MeJA, ACC, or combinations of these inducers.

Treatments were performed as described in the text and legend to Figure 5. MeJA and ACC were applied 2 days before harvest of the leaves for analysis. *Atvsp*, *Hel*, and *Pdf1.2* gene-specific probes were used. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β -tubulin (*Tub*).

unaltered when ISR was expressed as well (Figure 3). This demonstrates that components of the ISR pathway do not have a synergistic effect on the SAR response. Thus, the additive effect on the level of protection in plants expressing both ISR and SAR is unlikely to be caused by cross-talk between the ISR and the SAR pathway, but rather appears to result from a parallel activation of defense responses with complementary effects against *Pst*.

NPR1 is a key regulator of both SAR and ISR. In the SAR pathway, NPR1 regulates the SA-dependent expression of *PR* genes (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997), whereas in the ISR pathway, NPR1 regulates the jasmonate- and ethylene-dependent production of thus far unidentified defensive factors (Pieterse *et al.*, 1998). Our finding that simultaneous expression of ISR and SAR results in an enhanced level of protection indicates that the ISR and the SAR pathway do not compete for NPR1. Apparently, the pool of NPR1 protein is sufficient to allow simultaneous expression of ISR and SAR. Recently, Cao *et al.* (1998) demonstrated that elevated levels of NPR1 protein, through overexpression of the *Npr1* gene, leads to enhanced resistance against *P. syringae* pv *maculicola* and *Peronospora parasitica*. This raised the question whether the enhanced level of induced resistance observed in plants expressing both ISR and SAR is based on elevated levels of NPR1. We did not observe an increase in the expression of the *Npr1* gene in systemic tissue expressing either ISR, SAR, or both types of induced resistance (Figure 4). This strongly suggests that the constitutive level of NPR1 is sufficient to facilitate the expression of both types of induced resistance.

Previously, exogenous application of MeJA and ACC was demonstrated to induce resistance against *Pst* (Pieterse *et al.*, 1998). Like WCS417r-mediated ISR, resistance induced by MeJA and ACC is expressed in NahG plants and affected in *npr1* mutants. In contrast to plants expressing both ISR and SAR, plants that received a combination treatment with WCS417r and either MeJA or ACC did not express an enhanced level of protection (Figure 5 and Table 3). The same was true for plants that were treated with both MeJA and ACC (data not shown). WCS417r did not influence MeJA- or ACC-induced expression of the genes *Atvsp*, *Pdf1.2*, and *Hel* (Figure 6), indicating that

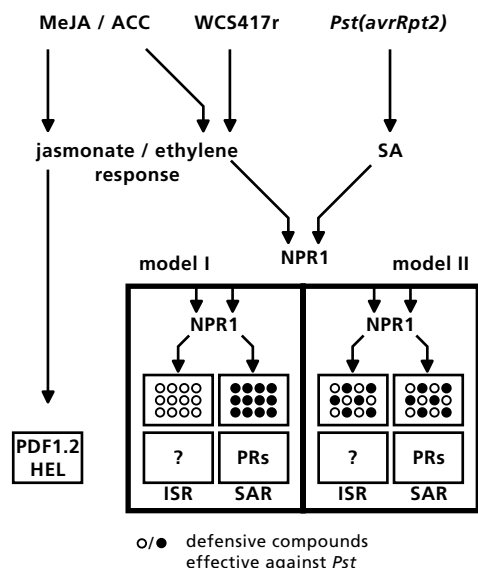


Figure 7. Signalling pathways leading to induced resistance against *Pst* in *Arabidopsis*.

Pathogen-induced SAR is dependent on SA, requires NPR1 and is associated with *PR* gene expression (according to Ryals *et al.*, 1996 and Dong, 1998). WCS417r-mediated ISR requires responsiveness to jasmonate and ethylene and is also dependent on NPR1. ISR is not associated with *PR* gene expression, indicating that downstream of NPR1 the pathways diverge (Pieterse *et al.*, 1998). PRs that accumulate in plants expressing SAR are unlikely to contribute to induced resistance against *P. syringae* pathogens (Clarke *et al.*, 1998). Cross-talk between the ISR and the SAR pathway is absent. Simultaneous activation of ISR and SAR results in an additive effect on the level of protection against *Pst*. This is not accompanied by an increase in the expression of the *Npr1* gene. Therefore, the enhanced level of induced protection against *Pst* must be accomplished through the parallel activation of thus far unidentified defense responses that are effective against *Pst*. The complementary effects on the level of protection

could be achieved through the production of distinct (model I) or more of the same antibacterial gene products (model II). In both cases, simultaneous induction of ISR and SAR leads to enhanced levels of defensive compounds that are active against *Pst*. MeJA and ACC saturate the NPR1-dependent ISR signalling pathway leading to resistance against *Pst*. This pathway is clearly different from the one leading to jasmonate- and ethylene-induced expression of *Pdf1.2* and *Hel* as the latter is NPR1-independent (Penninckx *et al.*, 1996; Thomma *et al.*, 1998). As WCS417r, MeJA, and ACC saturate the same pathway, induction of resistance against *Pst* with combinations of WCS417r, MeJA and ACC does not lead to additive effects on the level of protection. Question marks indicate unidentified defensive compounds.

the action of jasmonate and ethylene is not affected by WCS417r. Therefore, competition between WCS417r-, MeJA-, and ACC-induced pathways can not explain the absence of additive effects on the level of induced protection. Most likely, WCS417r, MeJA, and ACC saturate the same defense pathway that is active against *Pst*. This pathway must be different from the one controlling jasmonate- and ethylene-induced expression of *Pdf1.2* and *Hel*, because the latter pathway is NPR1 independent (Penninckx *et al.*, 1996; Thomma *et al.*, 1998).

In contrast to earlier experiments (Pieterse *et al.*, 1998), we found no *Pdf1.2* gene expression in MeJA-treated plants (data not shown). MeJA and ethylene have been shown to act synergistically on the induction of *Pdf1.2* (Penninckx *et al.*, 1998). Therefore, the absence of *Pdf1.2* transcripts in MeJA-treated plants might be caused by a relatively low basal level of ethylene production, as was also found by Penninckx *et al.* (1998) in sterile-grown plants.

Recently, Clarke *et al.* (1998) demonstrated that enhanced resistance against *P. syringae* pv *maculicola* in the constitutively SAR-expressing mutant *cpr6* is blocked in the *cpr6 npr1* double mutant, despite unaltered constitutive expression of *PR* genes.

This indicates that induced resistance against *P. syringae* is independent of PRs and must be accomplished through thus far unidentified antibacterial factors that are regulated through NPR1 (Clarke *et al.*, 1998). Whether the same compounds are involved in ISR against *Pst* is currently unknown.

The mechanism underlying the additive effect on the level of induced protection in plants expressing both ISR and SAR can be hypothesized in different ways. The ISR and the SAR pathway may generate distinctive defensive compounds that are both effective against *Pst* (Figure 7, model I). Alternatively, activation of both the ISR and the SAR pathway may lead to the production of the same antibacterial compounds but these compounds do not accumulate to maximal levels when only ISR or SAR is induced (Figure 7, model II). In both scenarios, concurrent activation of ISR and SAR leads to higher levels of defensive compounds that are active against *Pst*.

In conclusion, this study demonstrates that plants are capable of expressing SA-, jasmonate-, and ethylene-dependent defense responses at the same time without antagonistic effects. This leads to an elevated level of protection against pathogen attack. Therefore, simultaneous activation of ISR and SAR provides an attractive tool for the improvement of disease control.

Materials and Methods

Bacterial strains, plant material and growth conditions

Nonpathogenic, ISR-inducing *Pseudomonas fluorescens* WCS417r rhizobacteria (Van Peer *et al.*, 1991) were grown on King's medium B (KB) agar plates (King *et al.*, 1954) for 24 h at 28°C. Subsequently, bacterial cells were collected and resuspended in 10 mM MgSO₄.

The avirulent pathogen *P. syringae* pv *tomato* DC3000 carrying a plasmid with avirulence gene *avrRpt2* (*Pst*(*avrRpt2*)); Kunkel *et al.*, 1993) was used for induction of SAR. *Pst*(*avrRpt2*) bacteria were cultured overnight at 28°C in liquid KB, supplemented with tetracycline (20 mg/l) to select for the plasmid. Bacterial cells were collected by centrifugation and resuspended in 10 mM MgSO₄.

The virulent pathogen *P. syringae* pv *tomato strain* DC3000 (*Pst*; Whalen *et al.*, 1991) was used for challenge inoculations. *Pst* bacteria were grown overnight in liquid KB at 28°C. After centrifugation, the bacterial cells were resuspended in 10 mM MgSO₄, supplemented with 0.01% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands).

Seeds of wild-type *Arabidopsis thaliana* ecotype Col-0, transgenic NahG plants harbouring the bacterial *NahG* gene (Delaney *et al.*, 1994), and mutant *cpr1* (Bowling *et al.*, 1994), *jar1* (Staswick *et al.*, 1992), *etr1* (Bleecker *et al.*, 1988), and *npr1* (Cao *et al.*, 1994) plants were sown in quartz sand. Two-week-old seedlings were transferred to 60-ml pots containing a sand and potting soil mixture that had been autoclaved twice for 1 h. Before transfer of the seedlings, the potting soil was supplemented with either a suspension of ISR-inducing WCS417r rhizobacteria or an equal volume of a solution of 10 mM MgSO₄. Plants were cultivated in a growth chamber with a 9-h day (200 μE/m²/sec at 24°C) and a 15-h night (20°C) cycle at 70% relative humidity. Plants were watered on alternate days and once a week supplied with a modified half-strength Hoagland's nutrient solution, as described previously (Van Wees *et al.*, 1997).

Induction treatments

Plants were treated with ISR-inducing rhizobacteria by transferring the seedlings to soil that was mixed with a suspension of WCS417r rhizobacteria to a final density of 5×10^7 cfu/g of soil. The plants were grown in this soil for three weeks before they were challenged with *Pst*.

Pathogen-induced SAR was triggered by pressure infiltrating a suspension of the avirulent pathogen *Pst*(*avrRpt2*) at 10⁷ cfu/ml into three lower leaves of 5-week-old plants, using a 1-ml syringe without a needle. Three days later, plants were challenged with *Pst*.

Chemical treatments were performed by dipping the leaves of 5-week-old plants in a solution containing

0.01% (v/v) of Silwet L-77 and either methyl jasmonate (MeJA; 100 μ M), salicylic acid (SA; 1 mM), or 1-aminocyclopropane-1-carboxylate (ACC; 1 mM). Control plants were treated with 0.01% (v/v) Silwet L-77 only. One day before application of the chemicals, the plants were placed at 100% relative humidity. Three days after the chemicals were applied, plants were challenged with

**Rhizobacteria-mediated induced systemic resistance (ISR)
in *Arabidopsis* is not associated with a direct effect
on expression of known defense-related genes
but stimulates the expression of the jasmonate-inducible gene
Atvsp upon challenge**

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Submitted

Abstract

Selected strains of nonpathogenic rhizobacteria from the genus *Pseudomonas* are capable of eliciting broad-spectrum induced systemic resistance (ISR) in plants that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). In *Arabidopsis*, the ISR pathway functions independently of salicylic acid (SA) but requires responsiveness to jasmonate and ethylene. Here, we demonstrate that known defense-related genes, i.e. the SA-responsive genes *PR-1*, *PR-2*, and *PR-5*, the ethylene-inducible gene *Hel*, the ethylene- and jasmonate-responsive genes *ChiB* and *Pdfl.2*, and the jasmonate-inducible genes *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2* are neither induced locally in the roots nor systemically in the leaves upon induction of ISR by *Pseudomonas fluorescens* WCS417r. In contrast, plants that are infected with the virulent leaf pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) or that express SAR induced by preinfecting lower leaves with the avirulent pathogen *Pst*(*avrRpt2*) exhibit elevated expression levels of most of the defense-related genes studied. Upon challenge inoculation with *Pst*, *PR* gene transcripts accumulated to a higher level in SAR-expressing plants compared to control-treated and ISR-expressing plants, indicating that SAR involves potentiation of SA-responsive *PR* gene expression. In contrast, pathogen challenge of ISR-expressing plants led to an enhanced level of *Atvsp* transcript accumulation. The other jasmonate-responsive defense-related genes studied were not potentiated during ISR, indicating that ISR is associated with the potentiation of specific jasmonate-responsive genes.

Introduction

To combat invasions by microorganisms, plants have evolved several lines of defense. Besides preexisting physical and chemical barriers (Osbourn, 1996), inducible resistance mechanisms can be activated upon pathogen infection. An example of activated resistance is the hypersensitive response (HR) that is induced when the invading pathogen carries an avirulence (*avr*) gene of which the product is directly or indirectly recognized by the product of the corresponding resistance (*R*) gene of the host plant. In cells adjacent to the HR lesion, transcription of defense-related genes is triggered, resulting in the accumulation of compounds that are involved in cell wall reinforcement or possess antimicrobial activity (reviewed by Hammond-Kosack and Jones, 1996). When the pathogen is virulent, it is not specifically recognized by the plant. Nevertheless, nonspecific elicitors activate plant defense mechanisms, though possibly too late or too weakly to definitively restrict the pathogen, which escapes and successfully infects the plant (Van Loon, 1997).

Particularly avirulent pathogens provoke accumulation of the signalling molecules salicylic acid (SA; Malamy *et al.*, 1990; Métraux *et al.*, 1990), jasmonate (Penninckx *et al.*, 1996), and ethylene (Boller, 1991), which coordinate the activation of defense responses, and when applied exogenously can induce resistance themselves (Boller, 1991; Cohen *et al.*, 1993; Ryals *et al.*, 1996). In *Arabidopsis*, all three signalling molecules have been shown to activate specific sets of pathogen-inducible defense-related genes. SA controls the induction of genes encoding pathogenesis-related proteins (PRs) of the families PR-2 (β -1,3-glucanases), PR-5 (thaumatin-like proteins), and PR-1 with unknown biochemical properties (Uknes *et al.*, 1992). Several PRs have been shown to possess antimicrobial activity *in vitro* or *in vivo*, primarily against fungal pathogens (Kombrink and Somssich, 1997). Ethylene is involved in the expression of the pathogen-inducible genes *Hel* (encoding a hevein-like protein; Potter *et al.*, 1993), *ChiB* (encoding a basic chitinase; Samac *et al.*, 1990), and *Pdfl.2* (encoding a plant defensin; Penninckx *et al.*, 1996), all with potential antifungal activity. Jasmonate has been shown to activate the *Hel*, *ChiB*, and *Pdfl.2* genes as well (Penninckx *et al.*, 1996; Thomma *et al.*, 1998). For full expression of *Pdfl.2*, both ethylene and jasmonate are required, indicating that these hormonal signals act in concert (Penninckx *et al.*, 1998). Jasmonate is also implicated in the regulation of other pathogen-induced genes. *Lox1* and *Lox2* (encoding two lipoxygenases; Bell and Mullet, 1993; Melan *et al.*, 1993) control a feed-forward loop in jasmonate synthesis, but may also cause irreversible membrane damage leading to plant cell death (reviewed by Siedow, 1991). *Pal1* (encoding phenylalanine ammonia-lyase) controls the synthesis of phenylpropanoids such as lignin, and of SA in *Arabidopsis* (Mauch-Mani and Slusarenko, 1996), and has been demonstrated to be jasmonate inducible (McConn *et al.*, 1997). Furthermore, jasmonate has emerged as an important signal in the expression of wound-induced genes, such as *Pin* (encoding proteinase inhibitor) in tomato (Farmer and Ryan, 1992). Proteinase inhibitors help protect the plant against herbivory (Heitz *et al.*, 1999) and have been reported to be induced upon pathogen infection in tobacco plants (Linthorst

et al., 1993). Jasmonate also activates expression of the *Atvsp* gene (encoding vegetative storage protein) in *Arabidopsis* (Berger *et al.*, 1993). VSPs accumulate in the vacuoles of developing reproductive structures and young leaves, and function as temporary deposits of amino acids (reviewed by Creelman and Mullet, 1997). Upon wounding, VSPs are induced also in older plant parts (Berger *et al.*, 1993). Defense activity of VSPs has not been proven but has been suggested (Creelman and Mullet, 1997).

Resistance responses activated in primary pathogen-infected leaves are often extended to distant, noninfected tissue, conferring an elevated level of resistance. This phenomenon is commonly referred to as systemic acquired resistance (SAR; reviewed by Ryals *et al.*, 1996). SAR is effective against a broad spectrum of pathogens, and is tightly correlated with the induction of *PR* genes. SA-nonaccumulating NahG transformants of *Arabidopsis* and tobacco fail to express SAR and *PR* genes, indicating that SA is required for the SAR signalling pathway (Delaney *et al.*, 1994; Gaffney *et al.*, 1993; Lawton *et al.*, 1995). Independent mutant screens of *Arabidopsis* led to the discovery of an important SAR regulatory protein, called NPR1, NIM1, or SAI1 (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). This factor acts downstream of SA and is required for the expression of both pathogen-induced *PR* genes and SAR. Ethylene and jasmonate response mutants of *Arabidopsis* express normal levels of pathogen-induced SAR, but the systemic activation of the ethylene- and jasmonate-regulated defense-related genes *Hel*, *ChiB*, and *Pdf1.2* is blocked in these mutants. This demonstrates that SAR can function independently of ethylene- and jasmonate-mediated defense responses (Lawton *et al.*, 1995, 1996; Penninckx *et al.*, 1996; Pieterse *et al.*, 1998; Thomma *et al.*, 1998). Moreover, the pathway controlling ethylene- and jasmonate-dependent expression of the *Hel*, *ChiB*, and *Pdf1.2* genes functions independently of the SAR regulators SA and NPR1 (Penninckx *et al.*, 1996; Thomma *et al.*, 1998).

Besides pathogen infection, colonization of the rhizosphere by selected nonpathogenic *Pseudomonas* bacteria can trigger a systemic resistance as well. This form of induced resistance is referred to as rhizobacteria-mediated induced systemic resistance (ISR; reviewed by Van Loon *et al.*, 1998). In *Arabidopsis*, *P. fluorescens* WCS417r-mediated ISR has been shown to be effective against different types of pathogens, including 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 oomycetous leaf pathogen *Peronospora parasitica* (J. Ton and C.M.J. Pieterse, unpublished result). ISR signal transduction follows an SA-independent pathway that confers resistance in the absence of *PR* gene expression (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Instead, WCS417r-mediated ISR requires responsiveness to both jasmonate and ethylene (Pieterse *et al.*, 1998). However, like SAR, rhizobacteria-mediated ISR is dependent on NPR1, indicating that NPR1 is not only required for the expression of *PR* genes that are activated during SAR, but also functions in the jasmonate- and ethylene-dependent activation of thus far unidentified defense responses implicated in rhizobacteria-mediated ISR (Pieterse *et al.*, 1998).

In addition to defense reactions already apparent upon induction of resistance, other responses are manifested only after challenge inoculation. 'Potentiation' is expressed as a faster and greater activation of defense-related genes after infection of induced plants with a challenging pathogen. For instance, Mur *et al.* (1996) demonstrated that tobacco plants exhibiting pathogen-induced SAR show enhanced expression of *PR-10* and *Pal* genes upon challenge with a pathogen. SA, ethylene, and jasmonate have been shown to act as potentiating signals of defense-related gene expression. SA was reported to be a potentiator of pathogen-induced defense responses such as *Pal* gene expression and phytoalexin accumulation, that do not respond directly to SA (Kauss *et al.*, 1992, 1993; Mur *et al.*, 1996). In contrast to resistance-inducing derivatives of SA, biologically inactive analogs of SA failed to potentiate elicitor-induced *Pal* gene expression in cultured parsley cells, indicating that the resistance inducers may act in part by augmenting the activation of certain defense-related genes (Katz *et al.*, 1998). Kauss *et al.* (1994) showed that jasmonate potentiates the elicitor-induced accumulation of active oxygen species in parsley cells. In rice, jasmonate has been demonstrated to potentiate the induction of *PR-1* gene expression and resistance against the fungal pathogen *Magnaporthe grisea* by low doses of the resistance-inducing synthetic compound 2,6-dichloroisonicotinic acid (INA; Schweizer *et al.*, 1997a). Ethylene acts as a potentiator of SA- and pathogen-induced *PR-1* gene expression in *Arabidopsis* (Lawton *et al.*, 1994, 1995). Moreover, in regard to the expression of the *Pin* gene in tomato and the *PR-5* gene in tobacco, ethylene has been reported to have a potentiating effect on the action of jasmonate, and *vice versa* (O'Donnell *et al.*, 1996; Xu *et al.*, 1994). In *Arabidopsis*, a marked synergy between ethylene and jasmonate was demonstrated for the induction of *Pdf1.2* (Penninckx *et al.*, 1998).

WCS417r-mediated ISR in carnation was reported to be associated with potentiation of phytoalexin accumulation, resulting in higher phytoalexin levels after infection by the fungal pathogen *F. oxysporum* f.sp. *dianthi* (Van Peer *et al.*, 1991). The molecular mechanisms underlying rhizobacteria-mediated ISR in *Arabidopsis* are to a large extent unknown. In this study, we investigated whether the ISR-inducing rhizobacterium WCS417r activates any of a set of known defense-related genes locally in the roots and/or systemically in the leaves at different time points after treatment. Moreover, the possibility of potentiation of defense-related gene activation in ISR-expressing tissue challenged with *Pst* was assessed.

Results

Defense-related gene expression

To gain more insight into the defense mechanisms involved in rhizobacteria-mediated ISR, we studied the expression of a large set of well-characterized defense-related genes of *Arabidopsis*. Gene expression was investigated locally, as well as systemically, by analyzing mRNA accumulation in roots and leaves of ecotype Col-0 plants of which the roots were treated with the ISR-inducing rhizobacterial strain WCS417r. ISR is triggered within the first 7 days after bacterization of the roots with WCS417r (J. Ton

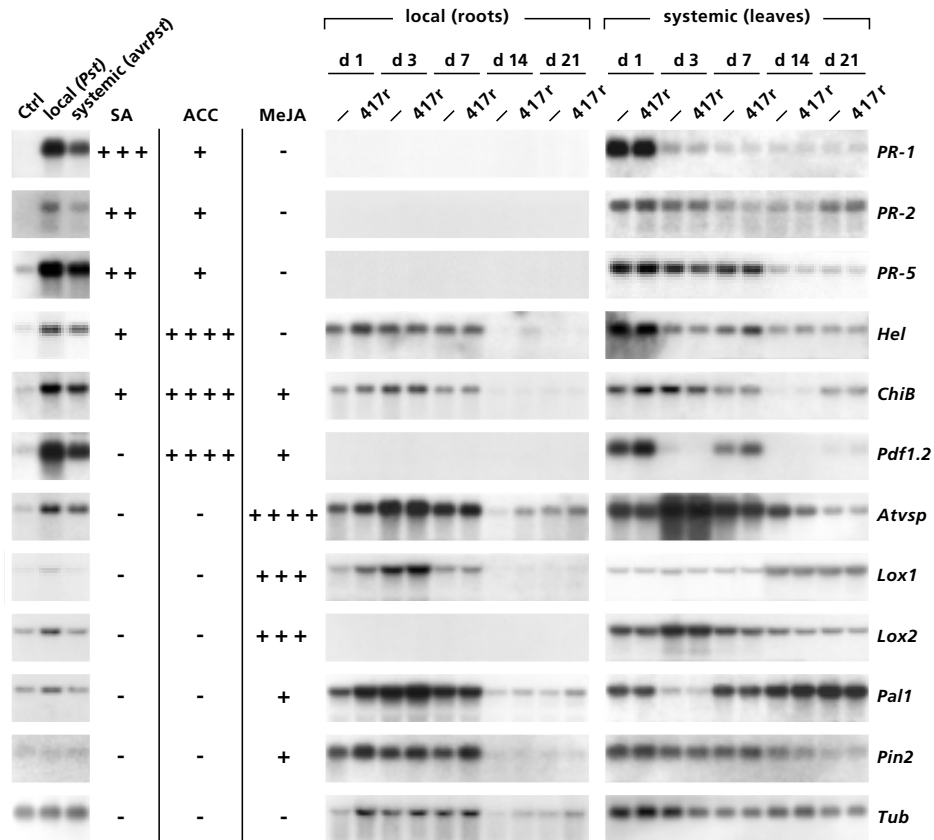


Figure 1. Local and systemic expression of defense-related genes in response to infection with *Pst*, or treatment with SA, ACC, MeJA, or WCS417r.

RNA was isolated from leaves of 5-week-old *Arabidopsis* plants harvested 2 days after either infection with *Pst* (local (*Pst*)), or application of 1 mM SA, 1 mM ACC, or 100 μ M MeJA. SAR-expressing upper leaves were harvested 2 days after infection of three lower leaves with *Pst*(*avrRpt2*) (systemic (*avrPst*)). Roots and leaves of WCS417r-treated plants (417r) were harvested at the indicated days after transferring 2-week-old seedlings to either rock wool (days 1, 3, and 7) or soil (days 14 and 21). Treatment with WCS417r bacteria consisted of application to the roots only. Control-treated plants are indicated as Ctrl or -. *Arabidopsis* gene-specific probes were used for the RNA blot analyses as indicated. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β -tubulin (*Tub*). -, no induction; + to +++, increasing degrees of induction, with +++ representing transcript levels comparable to that observed in *Pst*-infected leaves 2 days after inoculation.

and C.M.J. Pieterse, unpublished results) and lasts until at least 21 days after bacterization (Van Wees *et al.*, 1997). Therefore, leaves and roots were collected over an extended time period, between 1 and 21 days after transplanting 2-week-old seedlings to rock wool or soil, with or without WCS417r bacteria. Gene expression studies were performed with *Arabidopsis* gene-specific probes for the defense-related genes *PR-1*, *PR-2*, *PR-5*, *Hel*, *ChiB*, *Pdf1.2*, *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2*. As a

control, the expression of these genes was analyzed in *Pst*-infected leaves (local). Moreover, as a comparison, gene expression was assessed in noninfected leaves of plants that expressed SAR upon pretreatment of three lower leaves with *Pst(avrRpt2)*. Responsiveness of the genes to the defense signalling molecules SA, ethylene, and jasmonate was verified by analyzing their expression in leaves treated with SA, the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), or methyl jasmonate (MeJA).

Infection of the leaves with the virulent pathogen *Pst* resulted in local induction of nearly all of the genes, with *Pin2* as an exception (Figure 1). SAR was accompanied by a systemic accumulation of transcripts of most of the genes studied (from *PR-1* up to, and including *Atvsp* in Figure 1), indicating that SAR is associated with activation of these genes. *PR-1*, *PR-2*, and *PR-5* genes were strongly induced by SA, and slightly activated by ACC. *Hel*, *ChiB*, and *Pdf1.2* were prominently induced by ACC, and the latter two were also responsive to MeJA. The genes *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2* were all induced by MeJA only, although the level of induction varied greatly. These results are in agreement with the findings of other research groups (Bell and Mullet, 1993; Berger *et al.*, 1993; Farmer and Ryan, 1992; McConn *et al.*, 1997; Melan *et al.*, 1993; Penninckx *et al.*, 1996; Potter *et al.*, 1993; Samac *et al.*, 1990; Uknes *et al.*, 1992), and indicate that these genes indeed serve as markers for the activation of either SA-, ethylene-, or jasmonate-responsive defense pathways. Gene expression analyses of plants expressing WCS417r-mediated ISR revealed that the state of ISR differs greatly from SAR in regard to the expression of defense-related genes. Although variation in the expression of most genes was apparent, roots and leaves of WCS417r-treated plants never showed an enhanced expression of any of the genes, at any of the time points tested (Figure 1). Thus, elicitation of the ISR pathway by WCS417r has no direct effect on the local or systemic expression of the defense-related genes studied.

Induced protection in the absence of activation of known defense-related genes

Pst is the causal agent of bacterial speck disease, that is characterized by the development of necrotic or water-soaked spots surrounded by spreading chlorosis. Preinfection with an avirulent pathogen, exogenous application of SA, ACC, or MeJA, or colonization of the rhizosphere with WCS417r results in induced protection against this pathogen, as manifested by a reduction in both the percentage of leaves showing disease symptoms and growth of *Pst* bacteria in the challenged leaves (Figure 2A and Table 1). Typically, induction of SAR by preinfection with an avirulent pathogen led to the largest suppression of disease, whereas the level of protection triggered by WCS417r was comparable to that induced by SA, ACC, or MeJA. As shown in Figure 1, all these resistance-inducing agents, except for WCS417r, triggered the expression of specific sets of the defense-related genes studied. To investigate whether there is a quantitative relationship between the expression of these defense-related genes and induced protection, we tested the effect of a concentration range of SA, ACC, and MeJA on the induction of resistance against *Pst* and activation of the genes *PR-1*, *Hel*, and *Atvsp*, which are SA-, ethylene-, and jasmonate-responsive marker genes,

repectively. Resistance against *Pst* was induced maximally already at the lowest concentrations tested (Figure 2B and Table 1). Concentrations of ACC above 1 mM were significantly less effective in inducing resistance compared to the lowest concentration. This may be due to induced senescence exemplified by chlorotic spots on the leaves before challenge, resulting in a higher susceptibility to *Pst*. Although resistance was clearly induced after application of the lowest concentrations of SA, ACC, and MeJA, activation of the marker genes *PR-1*, *Hel*, and *Atvsp* was apparent only when 5- to 100-fold higher concentrations were applied (Figure 2B). Moreover, transcript levels increased in a dose-dependent manner, whereas resistance did not (Figure 2B). This indicates that similar to WCS417-mediated ISR, chemically induced resistance against *Pst* is not based on a direct activation of these defense-related genes.

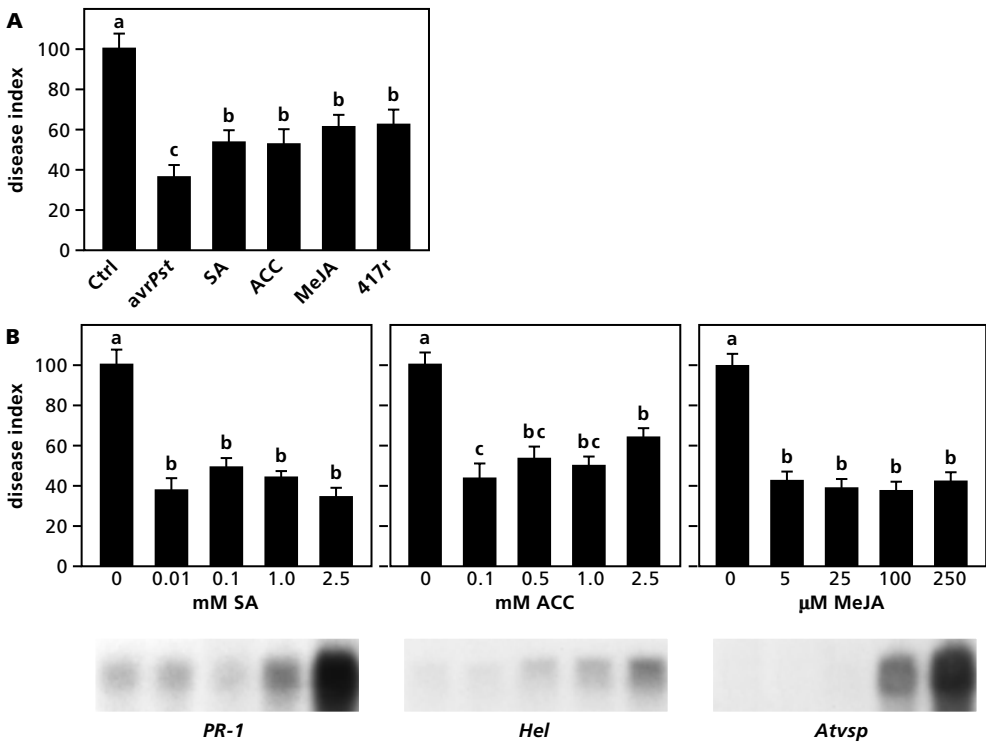


Figure 2. Quantification of induced protection against *Pst* triggered by different inducers and the effect of exogenous application of a concentration series of the chemicals SA, ACC, and MeJA on the level of protection against *Pst*, and the induction of *PR-1*, *Hel*, and *Atvsp* gene expression, respectively.

Pst(*avrRpt2*), SA, ACC, and MeJA were applied 3 days before challenge inoculation with *Pst*. ISR was induced by growing the plants in soil containing WCS417r for 3 weeks. In **A**, 1 mM SA, 1 mM ACC, and 100 μM MeJA were applied. In **B**, the chemicals were applied at the concentrations indicated. The disease index is the mean ($n=20$ plants) of the percentage of leaves with symptoms per plant, compared to control-treated plants (set at 100%), 4 days after challenge with virulent *Pst*. Within each panel, different letters indicate statistically significant differences between treatments (Fischer's LSD test; $\alpha=0.05$). The data presented are from representative experiments that were repeated at least twice. For experimental details on the RNA blot analyses see legend to Figure 1.

Table 1. Number of *Pst* bacteria in challenged leaves of plants pretreated with *Pst*(*avrRpt2*), WCS417r, or a concentration range of SA, ACC, and MeJA.

Treatment	cfu/g fresh weight ($\times 10^6$) ¹	Treatment	cfu/g fresh weight ($\times 10^6$) ¹
Control	37.02 $\pm$ 5.25 ^a	ACC (mM)	
<i>Pst</i> (<i>avrRpt2</i>)	2.38 $\pm$ 1.40 ^c	0	6.01 $\pm$ 0.35 ^a
SA (1 mM)	6.68 $\pm$ 0.72 ^b	0.25	1.56 $\pm$ 0.36 ^c
ACC (1 mM)	5.21 $\pm$ 1.07 ^b	0.5	2.63 $\pm$ 0.42 ^c
MeJA (100 μ M)	6.42 $\pm$ 1.84 ^b	1.0	2.71 $\pm$ 0.19 ^c
WCS417r	7.05 $\pm$ 2.01 ^b	2.5	4.12 $\pm$ 0.49 ^b
SA (mM)		MeJA (μ M)	
0	12.61 $\pm$ 1.43 ^a	0	6.01 $\pm$ 0.35 ^a
0.01	4.09 $\pm$ 0.92 ^b	5	2.45 $\pm$ 0.30 ^b
0.1	4.66 $\pm$ 1.66 ^b	25	2.43 $\pm$ 0.34 ^b
1.0	5.45 $\pm$ 2.29 ^b	100	1.21 $\pm$ 0.12 ^b
2.5	5.58 $\pm$ 1.23 ^b	250	2.59 $\pm$ 0.51 ^b

¹ Values correspond to the bioassays shown in Figure 2. Data sets are from different representative experiments; only ACC and MeJA concentration ranges were tested in the same experiment. Values presented are average numbers of cfu/g ($\pm$ SE) from three sets of 6 plants, 4 days after challenge inoculation with virulent *Pst*. Within each set of data, different letters indicate statistically significant differences between treatments (Fischer's LSD test, $\alpha=0.05$).

Potential of defense-related gene expression

Prior to challenge inoculation, none of the genes tested showed enhanced expression in ISR-expressing plants. Nevertheless, the expression of these genes might be potentiated, resulting in a faster or greater activation after challenge inoculation with a pathogen. To investigate this possibility, leaves from WCS417r-treated plants were analyzed for defense-related gene expression at 0, 1, and 2 days after challenge inoculation with *Pst*. For comparison, gene expression was studied in SAR-expressing leaves that were exposed to challenge with *Pst*. Figure 3A shows that challenge inoculation of SAR-expressing tissue results in enhanced expression of all SA-inducible *PR* genes in comparison to challenged control tissue. This potentiation effect was specific for the SA-inducible genes, because the transcript levels of the ethylene- and/or jasmonate-responsive marker genes *Pdf1.2* and *Atvsp* were not enhanced in SAR-expressing tissue (Figure 3A). Challenge inoculation of ISR-expressing plants was not accompanied by a greater expression of *PR* genes (Figure 3B). However, when the same RNA blots were hybridized to the *Atvsp* probe, an enhanced accumulation of *Atvsp* transcripts in challenged ISR-expressing plants was detected (Figure 3B; box with one asterisk). Potentiation of *Atvsp* by ISR was repeatedly observed in independent experiments, whereas none of the other defense-related genes showed a stimulated

expression. Thus, colonization of the rhizosphere by WCS417r can potentiate systemic expression of the jasmonate-inducible gene *Atvsp*, leading to a higher level of transcript accumulation after challenge.

Orchestration of SA- and jasmonate-responsive genes during pathogen infection
 Pathogen infection results in the production of the signalling molecules SA, ethylene, and jasmonate, which in turn activate SA-, ethylene-, and jasmonate-responsive defense-related genes, respectively. Figure 3B shows that the jasmonate-inducible genes *Atvsp*, *Pdf1.2*, *Lox2*, and *Pal1* were induced 1 day after infection with *Pst*. Although jasmonate levels continue to increase to the next day (Penninckx *et al.*, 1996; C.M.J. Pieterse, unpublished results), the expression of these jasmonate-responsive genes was strongly reduced; *Atvsp* and *Pdf1.2* transcripts were even undetectable 2 days after infection (Figure 3B; box with two asterisks). During the first 2 days after inoculation, SA levels increase dramatically (Uknes *et al.*, 1993), leading to an increase in transcript

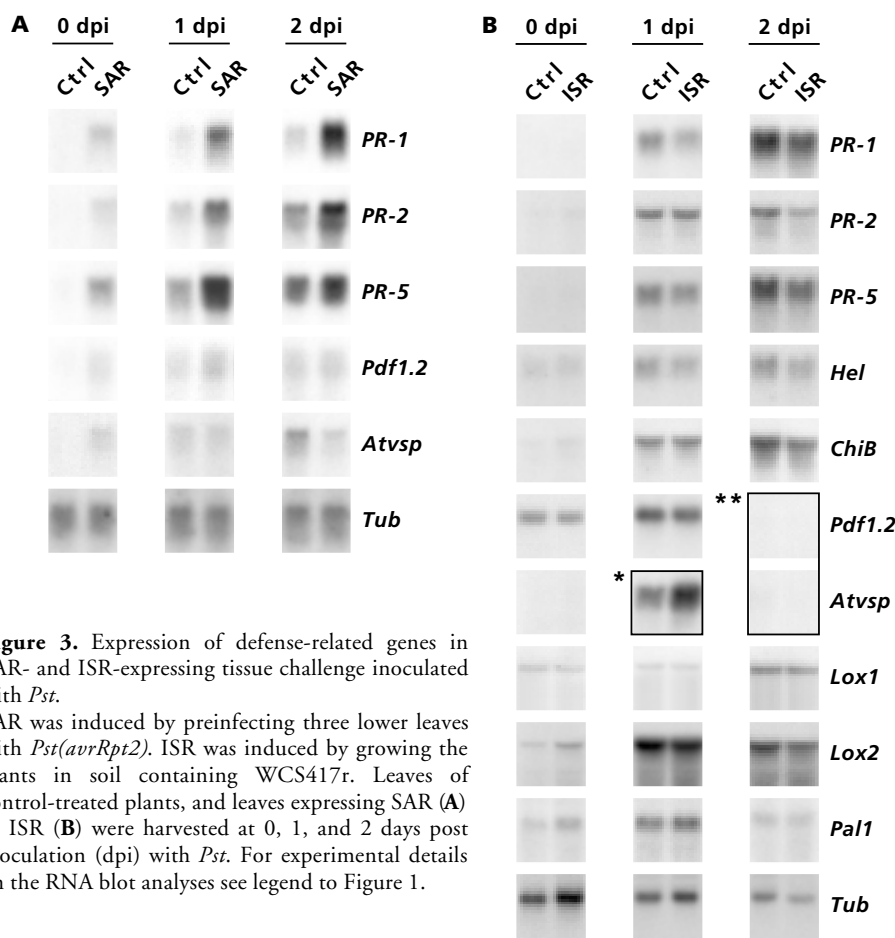


Figure 3. Expression of defense-related genes in SAR- and ISR-expressing tissue challenge inoculated with *Pst*. SAR was induced by preinfecting three lower leaves with *Pst(avrRpt2)*. ISR was induced by growing the plants in soil containing WCS417r. Leaves of control-treated plants, and leaves expressing SAR (A) or ISR (B) were harvested at 0, 1, and 2 days post inoculation (dpi) with *Pst*. For experimental details on the RNA blot analyses see legend to Figure 1.

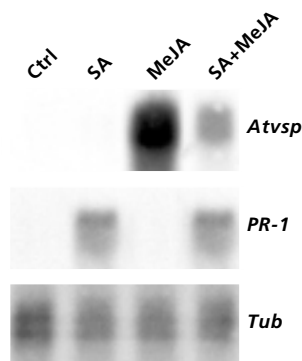


Figure 4. Expression of the jasmonate-inducible *Atvsp* gene and the SA-inducible *PR-1* gene in plants treated with SA, MeJA, or a combination of SA and MeJA.

SA (1 mM) and MeJA (100 μ M) were applied exogenously, 2 days before harvest of the leaves. For experimental details on the RNA blot analyses see legend to Figure 1.

levels of SA-inducible *PR* genes (Figure 3B). Previously, Doares *et al.* (1995) demonstrated that SA has an antagonistic effect on jasmonate-induced *Pin* gene expression in tomato, while MeJA has no effect on the expression level of the SA-induced *PR-3* gene. To investigate whether the increased SA levels in infected tissue might be responsible for the inhibition of jasmonate-responsive gene expression as observed 2 days after infection with *Pst*, we tested the effect of SA on MeJA-induced expression of *Atvsp*. Indeed, as shown in Figure 4, SA inhibited MeJA-induced expression of *Atvsp*, but MeJA did not affect the expression of the SA-inducible gene *PR-1*. This suggests that the expression of jasmonate-responsive genes is orchestrated during infection by changes in the endogenous levels of SA.

Discussion

Rhizobacteria-mediated ISR and pathogen induced SAR are phenotypically similar in that they are both active against different pathogens. However, in *Arabidopsis*, both types of biologically induced disease resistance follow a distinct signalling pathway that either requires responsiveness to jasmonate and ethylene, or is dependent on SA, respectively (Lawton *et al.*, 1995; Pieterse *et al.*, 1998). Induction of SAR by infection with a necrotizing pathogen is accompanied by local and/or systemic production of SA, ethylene, and jasmonate, leading to the activation of a large set of SA-, ethylene-, and jasmonate-responsive defense-related genes. We previously showed that rhizobacteria-mediated ISR in *Arabidopsis* is not associated with changes in the expression of *PR* genes (Pieterse *et al.*, 1996, 1998; Van Wees *et al.*, 1997). In those studies, gene expression was analyzed only in the leaves and at a single time point, i.e. just prior to challenge inoculation of 5-week-old plants that had been grown for 3 weeks in soil containing ISR-inducing WCS417r bacteria. In search for genes that are specifically activated during ISR, in the present study we analyzed the expression of 11 well-characterized *Arabidopsis* defense-related genes at different times after induction of ISR, before as well as after challenge inoculation with *Pst*. All defense-related genes studied showed responsiveness to either SA, ethylene or jasmonate, and in most cases were activated upon pathogen infection in locally infected tissue and in systemic,

SAR-expressing tissue (Figure 1). Before challenge inoculation with the pathogen, none of the genes studied were induced in ISR-expressing plants, neither locally in the roots, nor systemically in the leaves, and at none of the time points tested (Figure 1). Thus, elicitation of the ISR pathway has no direct effect on the expression of these defense-related genes.

The fact that none of the ethylene- or jasmonate-responsive genes studied was induced in ISR-expressing tissue prior to challenge inoculation, is in line with recent observations that ISR-expressing *Arabidopsis* plants do not produce enhanced levels of ethylene and jasmonate (C.M.J. Pieterse, unpublished results). Nevertheless, ISR is dependent on intact responses to both signalling compounds. This requirement for responsiveness to ethylene and jasmonate suggests that induced tissues may have become more sensitive to these regulators. Sensitization of plant cells to SA, ethylene, or jasmonate action has been described. For instance, in *Arabidopsis*, both the SA-induced expression of *PR-1* (Lawton *et al.*, 1994), and the jasmonate-induced expression of *Pdf1.2* (Penninckx *et al.*, 1998) are sensitized by ethylene. Conversely, the ethylene-induced expression of *Pdf1.2* is sensitized by jasmonate (Penninckx *et al.*, 1998). We found that in SAR-expressing *Arabidopsis* leaves, the SA-inducible *PR-1*, *PR-2*, and *PR-5* genes are potentiated, leading to enhanced expression of these genes after challenge inoculation with *Pst* (Figure 3A). The ethylene- and/or jasmonate-responsive genes *Pdf1.2* and *Atvsp* showed no potentiated expression in SAR-induced tissue after challenge (Figure 3A). In ISR-expressing leaves, none of the SA- and ethylene-responsive genes was potentiated, and neither were most of the jasmonate-responsive genes (Figure 3B). However, the jasmonate-inducible gene *Atvsp* showed increased transcript levels in challenged ISR-expressing tissue compared to challenged control leaves, indicating that the expression of *Atvsp* is potentiated (Figure 3B). The jasmonate levels in challenged ISR-expressing plants were not enhanced (C.M.J. Pieterse, unpublished results), which is in line with our observation that the other jasmonate-responsive genes studied, i.e. *Pdf1.2*, *ChiB*, *Lox1*, *Lox2*, and *Pall1*, were not potentiated and even showed a somewhat reduced level of expression in challenged, ISR-expressing plants (Figure 3B). Therefore, the potentiated expression of *Atvsp* can not be explained by accelerated jasmonate production in challenge-inoculated ISR-expressing tissue. A possible explanation for the potentiated *Atvsp* expression is that ISR-expressing tissue is sensitized only for the expression of specific jasmonate-responsive genes. The differential potentiation of jasmonate-responsive genes might be regulated by an ISR-induced transcription factor that binds to specific genes, such as *Atvsp*. In combined action with increased levels of endogenous jasmonate, as observed during pathogen attack, this could lead to an enhanced gene activation.

Vsp genes encode vegetative storage proteins that function as temporary deposits of amino acids, and they accumulate in the vacuoles of young developing leaves and reproductive structures (reviewed by Creelman and Mullet, 1997). Upon wounding, *Atvsp* gene expression is induced in local and systemic tissue of *Arabidopsis* (Berger *et al.*, 1993). VSPs have been suggested to possess antimicrobial activity, because they are

stored in vacuoles of young apical sinks, in which also large amounts of proteinase inhibitors with known defense activity are sequestered (Creelman and Mullet, 1997). The accumulation of defensive compounds in these tissues provides the plant with a preformed deterrent to herbivory and disease in regions of the plant critical for survival and reproduction. When the need for storage or defense is gone, both types of proteins are mobilized to recover amino acids. Therefore, an additional role of VSPs in plant defense is to be expected but still has to be demonstrated (Creelman and Mullet, 1997).

Although SA, ACC, and MeJA are capable of inducing both defense-related gene expression and resistance against *Pst*, we found no quantitative relationship between these two phenomena. Resistance against *Pst* was induced already to the highest level at the lowest concentrations tested, whereas the marker genes *PR-1*, *Hel*, and *Atvsp* were not activated at these concentrations of SA, ACC, and MeJA, respectively (Figure 2B). This demonstrates that the absence of enhanced levels of SA-, ethylene-, or jasmonate-responsive defense-related gene transcripts does not automatically imply that no SA-, ethylene-, or jasmonate-dependent defense responses are elicited. The resistance against *Pst* induced by low concentrations of SA, ACC, or MeJA more likely results from either the accumulation of other defensive compounds prior to challenge inoculation, and/or from potentiation of defense-related gene expression after challenge with a pathogen. These mechanisms could be the same as the ones controlling WCS417r-mediated ISR.

To date, the nature of the defense responses that contribute to the induced resistance against *Pst* is unclear. The role of *PR* genes in the expression of SAR against *P. syringae* has been questioned earlier by Clarke *et al.* (1998). They showed that the constitutively SAR- and *PR* gene-expressing mutant *cpr6* of *Arabidopsis* lost its elevated level of resistance against *P. syringae* pv *maculicola* in the mutant *npr1* background, whereas the enhanced level of *PR* gene expression was not affected by this mutation. This indicates that *PR* gene activation and bacterial resistance are not causally related (Clarke *et al.*, 1998). The increased resistance against *P. syringae* in *cpr6* mutants must therefore be accomplished through other, as yet unidentified, resistance responses that are regulated through NPR1.

Defense mechanisms of the plant are regulated by a complex network of SA-, ethylene-, and jasmonate-dependent signalling pathways. Recent investigations have proven the existence of cross-talk between these signalling pathways (reviewed by Dong, 1998; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998). In the experiment shown in Figure 3B, the expression of the jasmonate-responsive genes *Atvsp*, *Pdf1.2*, *Lox2*, and *Pall1* was reduced to insignificant or moderate levels 2 days after challenge with *Pst* compared to the expression levels induced 1 day after challenge. The jasmonate levels, however, increased continuously (C.M.J. Pieterse, unpublished results). In contrast, transcript levels of the SA-responsive *PR* genes continued to increase between 1 and 2 days after challenge (Figure 3B). SA inhibited the induction of *Atvsp* by MeJA, whereas MeJA did not affect the induction of *PR-1* by SA (Figure 4). This suggests that the strong decrease in the expression of jasmonate-responsive

genes observed 2 days after challenge inoculation might be due to elevated levels of SA, which exert an inhibitory effect on the action of jasmonate. Varying results have been presented on cross-talk between SA- and jasmonate-regulated defense pathways. In accordance with our observation, all studies to date show an inhibitory effect of SA on jasmonate action (Doares *et al.*, 1995; Niki *et al.*, 1998; O'Donnell *et al.*, 1996). In contrast, for jasmonate, different effects on SA action have been reported, i.e. stimulating, inhibiting, and no effect (Doares *et al.*, 1995; Niki *et al.*, 1998; Xu *et al.*, 1994).

Materials and methods

Bacterial cultures

Nonpathogenic, ISR-inducing *Pseudomonas fluorescens* WCS417r rhizobacteria (Van Peer *et al.*, 1991) were grown on King's medium B (KB) agar plates (King *et al.*, 1954) for 24 h at 28°C. Subsequently, bacterial cells were collected and resuspended in 10 mM MgSO₄.

The virulent pathogen *P. syringae* pv *tomato* strain DC3000 (*Pst*; Whalen *et al.*, 1991), used for challenge inoculations, was grown overnight in liquid KB at 28°C. After centrifugation, the bacterial cells were resuspended in 10 mM MgSO₄ and supplemented with 0.01% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands).

The avirulent pathogen *P. syringae* pv *tomato* DC3000 with the plasmid pV288 carrying avirulence gene *avrRpt2* (*Pst*(*avrRpt2*); Kunkel *et al.*, 1993) was used for induction of SAR. *Pst*(*avrRpt2*) bacteria were cultured overnight at 28°C in liquid KB, supplemented with 25 mg/l kanamycin to select for the plasmid. Bacterial cells were collected by centrifugation and resuspended in 10 mM MgSO₄.

Cultivation of plants

Seeds of wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) were sown in quartz sand. Two-week-old seedlings were transferred to 60-ml pots containing a sand and potting soil mixture that had been autoclaved twice for 1 h with a 24 h interval. Before transfer of the seedlings, the potting soil was supplemented with either a suspension of ISR-inducing WCS417r rhizobacteria or an equal volume of a solution of 10 mM MgSO₄. For the analysis of defense-related gene expression during the first 7 days after induction of ISR, the seedlings were transferred to rock wool drenched with nutrient solution, and WCS417r bacteria were applied in talcum to the roots, as described previously (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). This rock wool system was previously proven to be suitable for the analysis of rhizobacteria-mediated ISR against the fungal root pathogen *Fusarium oxysporum* f sp *raphani* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Plants were cultivated in a growth chamber with a 9-h day (200 µE/m²/sec at 24°C) and a 15-h night (20°C) cycle at 70% relative humidity. Plants were watered on alternate days and once a week supplied with a modified half-strength Hoagland's nutrient solution, as described previously (Pieterse *et al.*, 1996).

Induction treatments and inoculations

Plants were treated with ISR-inducing rhizobacteria by transferring 2-week-old seedlings to soil that was mixed with a suspension of WCS417r rhizobacteria to a final density of 5×10^7 cfu/g of soil. For induction of ISR in the rock wool system, the seedlings were placed horizontally on rock wool cubes and the root systems were covered with 1 ml of a 1:1 (w/v) mixture of talcum powder and a suspension of WCS417r bacteria (final density 5×10^8 cfu/g of talcum), as described previously (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997).

Pathogen-induced SAR was triggered by pressure infiltrating a suspension of the avirulent pathogen *Pst*(*avrRpt2*) at 10^7 cfu/ml into three lower leaves of 5-week-old plants, using a 1-ml syringe without a needle.

Chemical treatments were performed by dipping the leaves of 5-week-old plants in a solution containing 0.01% (v/v) of Silwet L-77 and either salicylic acid (SA), 1-aminocyclopropane-1-carboxylate (ACC), methyl jasmonate (MeJA), or a combination of these chemicals. Control plants were treated with 0.01% (v/v) Silwet L-77 only. One day before application of the chemicals, the plants were placed at 100% relative humidity. SA was purchased from Mallinckrodt Baker BV (Deventer, The Netherlands), ACC from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands), and MeJA from Serva, Brunschwig Chemie (Amsterdam, The Netherlands).

Plants were challenge inoculated with the virulent pathogen *Pst* by dipping them in a suspension of *Pst* at 2.5×10^7 cfu/ml in 10 mM MgSO₄, 0.01% (v/v) Silwet L-77. One day before challenge, the plants were placed at 100% relative humidity.

RNA blot analysis

Leaf and root tissues were collected for RNA analysis. Soil or talcum covering the root system was removed by rinsing the roots with water. Total RNA was extracted by homogenizing at least 2 g of frozen tissue in an equal volume of extraction buffer (0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS). The homogenates were extracted with phenol and chloroform and the RNA was precipitated using LiCl, as described by Sambrook *et al.* (1989). For RNA analysis, 15 µg of RNA was denatured using glyoxal and DMSO, according to 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), respectively. RNA blots were hybridized with *Arabidopsis* gene-specific probes as described previously (Van Wees *et al.*, 1997). To check for equal loading, the blots were stripped and hybridized with a probe for the constitutively expressed β-tubulin (*Tub*) gene. ³²P-labelled *Arabidopsis* gene-specific probes to detect *PR-1*, *PR-2*, *PR-5*, *Pdfl.2*, or *Pall* were synthesized by random primer labelling (Feinberg and Vogelstein, 1983) using *PR-1*, *PR-2*, *PR-5*, *Pdfl.2*, and *Pall* cDNA clones as template (Penninckx *et al.*, 1996; Uknes *et al.*, 1992; Wanner *et al.*, 1995). Probes for the detection of *Arabidopsis Hel*, *Atvsp*, *Lox1*, *Lox2*, *Pin2*, and *Tub* transcripts were prepared using templates that were generated by PCR with primers based on sequences obtained from GenBank accession numbers U01880, Z18377, L04637, L23968, X69139, and M21415, respectively. Template for the *ChiB* probe was prepared by PCR with primers based on the genomic sequence published by Samac *et al.* (1990).

Disease assessment

Four days after challenge inoculation with *Pst*, disease severity was assessed by determining the percentage of leaves with symptoms per plant, and by examining growth of the challenging pathogen in the leaves (20 plants per treatment). Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. The number of *Pst* bacteria in challenged leaves was assessed in three samples per treatment. Each sample consisted of the leaves from 6 plants. The leaf tissue was weighed and homogenized in 10 mM MgSO₄. Subsequently, appropriate dilutions were plated onto KB agar supplemented with 50 mg/l rifampicin and 100 mg/l cycloheximide. After incubation at 28°C for 2 days, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined.

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Attempted identification of genes and proteins associated with rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis*

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Abstract

Colonization of the *Arabidopsis* rhizosphere by *Pseudomonas fluorescens* WCS417r results in an induced systemic resistance (ISR) response that resembles pathogen-induced systemic acquired resistance (SAR) in that it is effective against different types of pathogens. Unlike SAR, ISR is not accompanied by the activation of genes encoding pathogenesis-related proteins (PRs). To identify proteins associated with ISR, two-dimensional polyacrylamide gel electrophoresis was performed of soluble proteins isolated from ISR-expressing and noninduced leaves. No differences in the protein patterns were apparent, indicating that, unlike SAR, ISR is not associated with substantial changes in protein abundance. To identify changes in gene expression during ISR, a cDNA library made from mRNA of ISR-expressing *Arabidopsis* leaves was differentially screened using cDNA probes that were synthesized on (i) mRNA isolated from ISR-expressing leaves and (ii) mRNA isolated from noninduced leaves, using a subtractive hybridization procedure. Out of 250.000 clones tested, only one turned out to be significantly (9-fold) upregulated in ISR-expressing tissue. Two clones were moderately (5-fold) induced, whereas ten clones showed weakly (2- to 3-fold) increased expression during ISR. These results indicate that for plants to develop a systemically increased resistance no major changes in gene expression are required.

Introduction

Selected strains of nonpathogenic *Pseudomonas* bacteria that colonize the rhizosphere have been shown to trigger an induced systemic resistance (ISR) response in distant plant parts (reviewed by Van Loon *et al.*, 1998). Rhizobacteria-mediated ISR has been demonstrated in different plant species and is effective against different types of pathogens (Van Loon *et al.*, 1998). Elicitation of ISR is dependent on the plant genotype and on the rhizobacterial strain used (Pieterse *et al.*, 1998; Ton *et al.*, 1999; Van Wees *et al.*, 1997), indicating that ISR is genetically determined. The phenomenon of ISR resembles systemic acquired resistance (SAR), that is triggered by preinfection with a necrotizing pathogen (reviewed by Ryals *et al.*, 1996). Although rhizobacteria-mediated ISR and pathogen-induced SAR are phenotypically similar, they follow distinct signalling pathways. SAR is characterized by an increase in endogenously synthesized salicylic acid (SA), that has been shown to be a necessary intermediate in the SAR signalling pathway (Delaney *et al.*, 1994; Gaffney *et al.*, 1993; Lawton *et al.*, 1995). In contrast, rhizobacteria-mediated ISR in *Arabidopsis* triggered by *P. fluorescens* WCS417r and *P. putida* WCS358r has been shown to function independently of SA (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Components of the jasmonate and the ethylene response play a crucial role in the ISR signalling pathway (Knoester *et al.*, 1999; Pieterse *et al.*, 1998).

Our ultimate goal is to get a better understanding of the molecular basis of ISR. Research on the mechanisms underlying ISR is hampered by lack of an ISR-specific molecular marker. Because ISR in *Arabidopsis* is genetically determined, specific genes must be involved in the induction and expression of ISR. Pathogen-induced SAR is associated with the activation of a large set of so-called SAR genes (reviewed by Sticher *et al.*, 1997), including genes that encode pathogenesis-related proteins (PRs; Uknes *et al.*, 1993; Ward *et al.*, 1991). In SAR-expressing tobacco plants, SAR gene products accumulate systemically to levels of 0.3 to 1% of the total mRNA and protein content (Lawton *et al.*, 1995), indicating that SAR is associated with major changes in gene expression and protein abundance. Some of the PRs have been shown to possess antifungal activity and are thought to contribute to resistance (Broekaert *et al.*, 1997; Kombrink and Somssich, 1997; Van Loon, 1997). However, a causal relationship between accumulation of SAR proteins and the broad-spectrum induced resistance characteristic for SAR has not been convincingly demonstrated (Van Loon, 1997).

In *Arabidopsis*, pathogen-induced SAR is characterized by the systemic activation of the SA-inducible genes *PR-1*, *PR-2*, and *PR-5* (Uknes *et al.*, 1993). In addition, ethylene- and jasmonate-responsive defense-related genes such as *Hel*, *Pdf1.2*, and *ChiB* are activated during pathogen-induced SAR (Penninckx *et al.*, 1996; Potter *et al.*, 1993; Thomma *et al.*, 1998). However, it is unknown to what extent the corresponding gene products contribute to the induced resistance. Clarke *et al.* (1998) demonstrated that the constitutively SAR-expressing mutant *cpr6* of *Arabidopsis* lost its elevated level of resistance against *P. syringae* pv *maculicola* when crossed with the SAR regulatory

mutant *npr1*, whereas its constitutive *PR* gene expression remained unaltered. This indicates that SAR against *P. syringae* functions independently of PRs.

Recently, we demonstrated that rhizobacteria-mediated ISR against *P. syringae* pv *tomato* (*Pst*) in *Arabidopsis* is not associated with changes in the expression of *PR* and other known defense-related genes (Chapter 6), suggesting that ISR is based on thus far unidentified defense responses. These responses could involve expression of unknown defense-related genes, as well as posttranslational modification of existing proteins. Well-known examples of modifications at the posttranslational level are phosphorylation and dephosphorylation, which are regulated by the activities of protein kinases and phosphatases, respectively, and which have been shown to play a pivotal role in signal transduction (Damman *et al.*, 1997; Yang *et al.*, 1997).

To analyze whether rhizobacteria-mediated ISR in *Arabidopsis* is associated with changes in the abundance or possible posttranslational modifications of proteins, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed of soluble proteins isolated from leaves of ISR-expressing and noninduced plants. In order to discover genes that correlate with the state of ISR, we differentially screened a cDNA library made from mRNA of ISR-expressing leaves using a subtractive hybridization procedure aimed at identifying cDNA clones that correspond to differentially induced, low-abundant mRNAs.

Results

Two-dimensional gel electrophoretic analysis of soluble proteins

In our attempt to find a molecular marker for ISR, we used 2D-PAGE to identify changes in the abundance or posttranslational modification of proteins during expression of ISR. Patterns of soluble proteins from control plants and plants grown in soil containing ISR-inducing WCS417r bacteria were compared. ISR is induced already within the first week after transfer of *Arabidopsis* seedlings to soil containing WCS417r bacteria (J. Ton and C.M.J. Pieterse, unpublished result), and the state of ISR is maintained until at least 3 weeks after induction (Van Wees *et al.*, 1997). Therefore, proteins were extracted from leaves of plants that had been grown for 1 or 2 weeks in soil supplemented with either ISR-inducing WCS417r bacteria or 10 mM MgSO₄ as a control. To check reproducibility, all extracts were prepared in duplicate from independent sets of plants of the same experiment. Figure 1 shows 2D displays of soluble proteins from one set of the leaves of control- and WCS417r-treated plants, that were harvested 1 and 2 weeks after treatment. About 1000 spots were resolved, but the patterns from WCS417r-treated leaves revealed no consistent changes. There were no newly occurring proteins or changes in abundance of existing proteins detected. Moreover, none of the visualized proteins appeared to have a shifted position. These results indicate that ISR is not accompanied by major changes in protein abundance or modification as observed during pathogen-induced SAR.

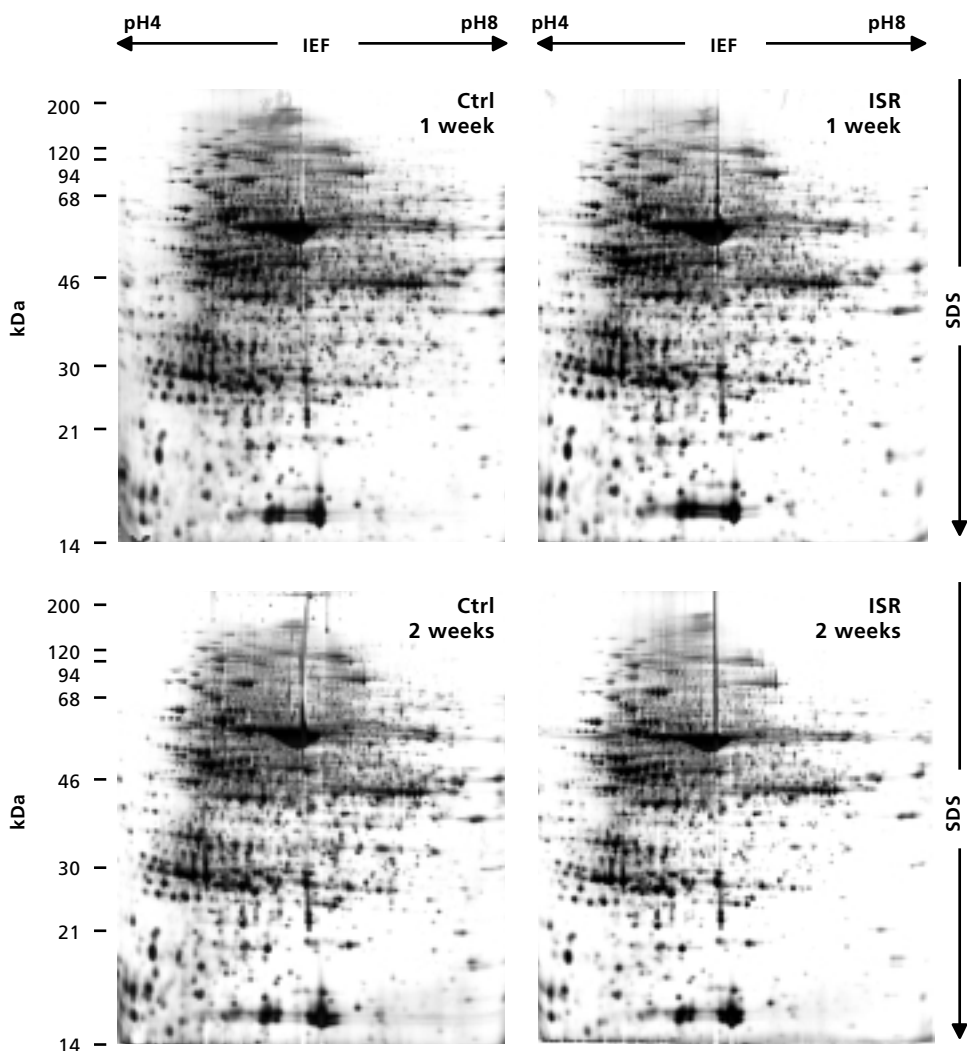


Figure 1. Silver-stained 2D-PAGE patterns of soluble proteins from noninduced control leaves and ISR-expressing leaves of *Arabidopsis*.

Proteins were extracted 1 or 2 weeks after transplanting the seedlings to soil that was supplemented with either 10 mM MgSO_4 (Ctrl) or ISR-inducing WCS417r bacteria (ISR). Five-hundred μg of soluble proteins were applied to each gel. The positions of molecular-weight markers are indicated.

Differential screening

A more sensitive method to identify possible markers for ISR is subtractive hybridization, which is commonly used for identifying cDNAs of low-abundant mRNAs of differentially expressed genes (reviewed by Wan *et al.*, 1996). We applied this method to differentially screen a cDNA library representing mRNAs from ISR-expressing leaves of *Arabidopsis* plants that had been grown in soil in the presence of WCS417r bacteria

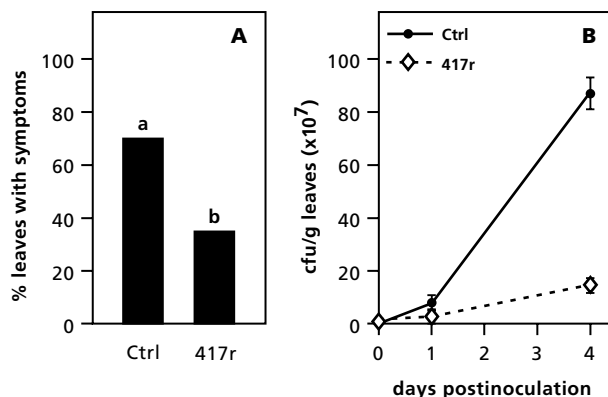


Figure 2. Quantification of rhizobacteria-mediated ISR against *Pst* in *Arabidopsis*.

ISR was induced by growing the plants for 3 weeks in soil containing WCS417r (417r) bacteria. Control (Ctrl) plants were grown in soil supplemented with 10 mM MgSO_4 . Four days after challenge inoculation with *Pst*, the percentage of leaves with symptoms per plant was determined (A). Different letters indicate a statistically significant difference between treatments (Fischer's LSD test; $n=30$, $\alpha=0.05$). The number of *Pst* bacteria in the challenged leaves was assessed at the indicated days after inoculation (B). Data points are means (cfu/g) with standard errors from two sets of 20 randomly selected leaves of plants from the same bioassay as depicted in A.

for 3 weeks. To verify that the leaf tissue used for RNA isolation was in the induced state, the expression of ISR against the leaf pathogen *Pst* was analyzed in a parallel set of plants. Figure 2 shows that these plants expressed a strong ISR, apparent as both a 50% reduction in disease symptoms (Figure 2A), and a 6-fold decrease in the proliferation of the pathogen *in planta* (Figure 2B). Just prior to challenge inoculation, mRNA was isolated from the remaining ISR-expressing plants for the construction of the cDNA library. The library was made by cloning cDNA prepared from 5 μg of the 'induced' mRNA pool into the lambda ZAP vector (Stratagene), resulting in cDNA clones with an average insert length of 1.1 kb (data not shown).

In total, 250,000 phage clones were differentially screened, potentially allowing selection of cDNAs corresponding to mRNAs with an abundance as low as 0.0004% of the total mRNA population. cDNA synthesized either on mRNAs from ISR-expressing leaves, or on mRNAs from noninduced control leaves originating from the same experiment were used as induced and noninduced probe, respectively. In the first screening, the induced probe was enriched for ISR-specific sequences by subtractive liquid hybridization, leading to the removal of up to 80% of the cDNAs that are common to both the induced and the noninduced probe (Welcher *et al.*, 1986). Out of the 250,000 phage clones, 54 gave a relatively strong signal after hybridization with the subtracted, induced probe and a relatively weak or no signal after hybridization with the noninduced probe (data not shown). These clones were rescreened using nonsubtracted cDNA probes from induced and noninduced tissue. At this stage, only 13 out of the 54 putative positive clones showed a higher

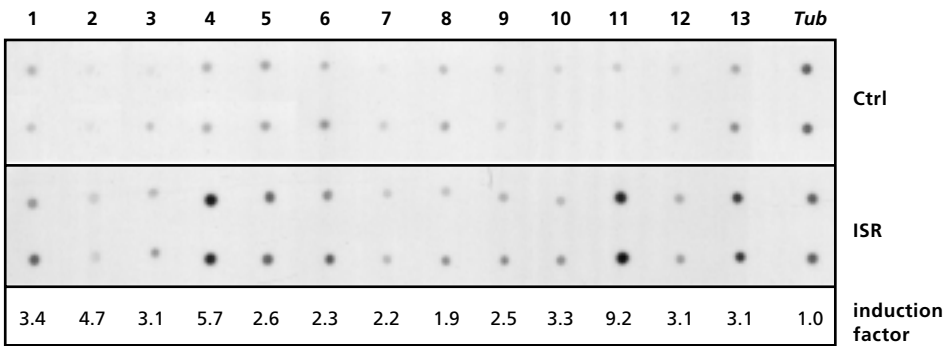


Figure 3. Relative transcript levels of *ISR1-ISR13* in control leaves and ISR-expressing leaves of *Arabidopsis*. Duplicate dot blots containing equal amounts of *ISR1-ISR13* cDNA and *Tub* cDNA as an internal standard were hybridized with the noninduced probe (Ctrl) and the nonsubtracted, induced probe (ISR). Hybridization signals were quantified using a Phosphor Imager. The table below the dot blots describes the mean of the fold induction of gene expression in ISR-expressing plants relative to control plants (induction factor).

hybridization signal with the induced probe than with the noninduced probe. From the 13 positive lambda ZAP phage clones, plasmids containing the cDNA inserts were excised *in vivo*, and isolated plasmid DNA was subsequently spotted onto two dot blots to quantify the transcript levels of the corresponding genes in induced and noninduced plants. cDNA of the constitutively expressed *Tub* gene, encoding β -tubulin, was included on each dot blot to check for equal hybridization with the induced and noninduced probe. After hybridization, the signals were quantified using a Phosphor Imager. As shown in Figure 3, the 13 clones, designated *ISR1* to *ISR13*, all hybridized to higher levels with the induced probe than with the noninduced probe. Specifically, *ISR11* was upregulated 9-fold in ISR-induced leaves compared to noninduced leaves. *ISR2* and *ISR4* were induced to approximately 5 times higher levels and a 2- to 3-fold induction was detected for *ISR1*, 3, 5-10, 12, and 13.

Discussion

Pathogen-induced SAR strongly affects gene expression, leading to the accumulation of a large set of newly synthesized proteins. The variety of these SAR proteins has been implicated in the broad-spectrum effectiveness of the SAR response (Sticher *et al.*, 1997). WCS417r-mediated ISR in *Arabidopsis* has been shown to be effective against different types of pathogens as well (Van Loon *et al.*, 1998). However, this form of induced resistance is not associated with an enhanced expression of a large set of known defense-related genes (Chapter 6).

In this study, we used nonbiased approaches to identify proteins or genes that are specifically induced after elicitation of ISR. Comparison of 2D-PAGE patterns of soluble proteins from induced and noninduced leaves demonstrated that, unlike SAR,

ISR is not associated with major changes in protein abundance/modification (Figure 1). This difference between ISR and SAR could be related to the fact that SAR is triggered by necrotizing pathogens, and thus, is associated with a stress response of the plant. In contrast, ISR is triggered by nonpathogenic micro-organisms that are beneficial to the plant, as they offer protection against pathogens and promote plant growth (Pieterse and Van Loon, 1999; Van Loon *et al.*, 1998). However, the method of 2D-PAGE allows monitoring of only the most abundant proteins (reviewed by Humphery-Smith and Blackstock, 1997). Changes in relatively low-abundant proteins cannot be discovered by this method.

As an alternative, the more sensitive method of differential screening of a cDNA library was applied. Using the procedure of subtractive hybridization, the identification of differentially expressed, rare transcripts was envisaged. A cDNA library representing mRNAs from leaves expressing WCS417r-mediated ISR was differentially screened with noninduced and subtracted, induced probes. Thirteen clones (*ISR1-ISR13*) were identified as true positives, being upregulated 2- to 9-fold in ISR-expressing leaves (Figure 3). The fact that only 13 clones turned out to be induced, confirms that induction of ISR is not associated with a massive activation of gene expression.

Our findings that induced resistance can operate in the absence of major changes in protein accumulation and gene expression sheds new light on the role of the massive production of new proteins during SAR. It is unlikely that all SAR proteins function as general defense proteins, because only a few of them have been shown to possess antipathogenic activity, which is primarily directed against specific types of fungal pathogens (Van Loon, 1997). We speculate that, like ISR, SAR might be based essentially on changes in the expression of a small group of genes as well.

The screenings for ISR-related genes/proteins performed in this study were not exhaustive. We missed the possible expression of genes that are induced either transiently at very early time points after induction of ISR, or that show a potentiated expression that is manifested as an enhanced level of expression only after challenge with a pathogen. Moreover, we did not study differential gene expression in the roots. Recently, research with promoter-less GUS lines of *Arabidopsis* led to the identification of a gene that is expressed locally in the roots 2 days after treatment with WCS417r (Léon-Kloosterziel *et al.*, 1998). In addition, RNA blot analysis of ISR-expressing plants challenged with the pathogen *Pst* revealed that the jasmonate-responsive gene *Atvsp* (encoding vegetative storage protein) shows an enhanced expression upon challenge (Chapter 6). Identification of the clones *ISR1-ISR13* extends these findings to genes that are systemically induced by WCS417r in leaves exhibiting ISR. Characterization of these *ISR* genes could provide more insight in the molecular basis underlying ISR.

Materials and methods

Bacterial cultures, cultivation of plants and induction of ISR

Pseudomonas fluorescens WCS417r rhizobacteria (Van Peer *et al.*, 1991) and *P. syringae* pv *tomato* strain DC3000 (*Pst*; Whalen *et al.*, 1991) were cultured as described before (Pieterse *et al.*, 1996). *Arabidopsis*

thaliana ecotypes Landsberg *erecta* (Ler) and Columbia (Col-0) were cultivated in 60-ml pots containing a sand and potting soil mixture that was supplemented with either a suspension of ISR-inducing WCS417r rhizobacteria to a final density of 5×10^7 cfu/g of soil, or an equal volume of 10 mM MgSO_4 . Growth conditions were as described previously (Pieterse *et al.*, 1996).

Challenge inoculation and disease assessment

Five-week-old Ler plants were challenge inoculated by dipping the leaves in a suspension of *Pst* at 1×10^8 cfu/ml in 10 mM MgSO_4 , 0.01% (v/v) Silwet L-77. One day before challenge, the plants were placed at 100% relative humidity. Four days after challenge, the percentage of leaves with symptoms per plant was determined for 30 plants per treatment. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. Multiplication of *Pst* was assessed in challenged leaves at different time points after inoculation. Two pools of randomly selected leaves (15 to 20) per treatment were weighed, rinsed thoroughly in water and homogenized in 10 mM MgSO_4 . Subsequently, appropriate dilutions were plated onto KB agar supplemented with 50 mg/l rifampicin and 100 mg/l cycloheximide. After incubation at 28°C for 2 days, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined.

Preparation of soluble proteins

Two batches of leaves from 50 plants were collected and frozen in liquid nitrogen, 1 and 2 weeks after transplanting two-week-old Col-0 seedlings to soil that was either nontreated or mixed with a suspension of WCS417r bacteria. For the isolation of proteins, a protocol based on previously described methods (Klerk and Van Loon, 1991; Rabilloud, 1996) was devised. One gram of frozen leaves was homogenized in 5 ml phenol-saturated extraction buffer (0.1 M Tris-HCl pH 9, 7 M ureum, 0.5% (w/v) SDS, 5% (v/v) β -mercaptoethanol) at room temperature. The homogenates were extracted with one volume of phenol/chloroform (pH 8) according to Sambrook *et al.* (1989). Subsequently, the phenol phase and the interphase were filtered through glass wool. The filtrate was then extracted with one volume of 0.1 M Tris-HCl pH 9, 5% (v/v) β -mercaptoethanol. The resulting phenol phase was mixed with 4 volumes of methanol containing 0.1 M ammonium acetate, and incubated overnight at -20°C to precipitate the proteins. The protein pellet was washed once with methanol, 0.1 M ammonium acetate and once with acetone. The pellet was air dried and resuspended in 1 ml isoelectric focussing (IEF) buffer (7 M ureum, 2 M thiourea, 0.4% (v/v) Pharmalytes 3-10, 0.2% (v/v) Triton X-100, 4% (v/v) CHAPS, 20 mM DTT) (Rabilloud, 1996). Protein concentrations were determined by the Bradford method (Bradford, 1976).

Two-dimensional polyacrylamide gel electrophoresis

2D-PAGE was performed essentially according to Rabilloud *et al.* (1994). For the first dimension, IEF extended from pH 4 to pH 8 using an immobilized pH gradient (IPG). The immobiline (Sigma) concentrations used to generate the pH gradient in the first dimension were calculated according to published recipes (Righetti, 1990). Five-hundred μ g of proteins were applied in a volume of 500 μ l to the entire surface of a dried IPG strip (4 $\times$ 160 $\times$ 0.5 mm), and incubated overnight to allow the strip to rehydrate and to ensure maximal diffusion of the proteins and additives from the IEF buffer into the gel. After rehydration, the strips were run using a Dry-strip Kit (Pharmacia) according to the manufacturer's protocol. Migration was carried out at 100 V for 60 min, 300 V for 510 min, and finally 3400 V overnight.

After the IEF run, the gel strips were equilibrated for 20 min in a solution containing 30% glycerol, 6 M ureum, 2.5% (w/v) SDS, 0.15 M BisTris-0.1 M HCl, and 0.8% (w/v) DTT. A second equilibration was carried out in the same solution, with DTT replaced by 4% (w/v) iodoacetamide. Subsequently, for SDS-PAGE in the second dimension, the strips were placed on top of 10% (w/v) polyacrylamide slab gels (17 $\times$ 21 $\times$ 0.15 cm) and the proteins were separated in Tris-glycine electrophoresis buffer (Sambrook *et al.*, 1989). Migration was carried out at 25 V for 1 h, followed by 150V for 4 h. The gels were stained with silver as described by Rabilloud *et al.* (1994).

Construction of the cDNA library

Using the guanidine hydrochloride method (Logemann *et al.*, 1987), total RNA was isolated from 10 g of frozen leaves of ISR-expressing *Arabidopsis* ecotype Ler plants that had been grown for 3 weeks in soil containing WCS417r bacteria. Subsequently, mRNA was isolated by oligo(dT)-cellulose chromatography as described by Sambrook *et al.* (1989). First- and second-strand cDNA was prepared from 5 μ g mRNA using Stratagene's cDNA Synthesis Kit (Stratagene, Leusden, The Netherlands) following the manufacturer's instructions. The cDNA library was constructed by ligating the double-stranded cDNA into the lambda vector Uni-ZAP XR, using Stratagene's ZAP-cDNA Synthesis Kit. Subsequently, the ligation products were

packaged into phage particles, using Stratagene's ZAP-cDNA Gigapack II Gold Cloning Kit, according to the manufacturer's protocol. The resulting cDNA library consisted of 7.4×10^6 independent phage clones with an average insert length of 1.1 kb.

Preparation of subtracted cDNA and labelling of cDNA probes

For the preparation of subtracted cDNA, Invitrogen's Subtractor Kit (Invitrogen, Leek, The Netherlands) was used. First-strand cDNA was synthesized on 1 µg of the induced pool of mRNA. The cDNA-mRNA hybrid was alkali treated to remove the template mRNA. The resulting cDNA was then hybridized to an excess (1:10 ratio) of uninduced mRNA which had been photobiotinylated using a 400W Philips HLRG-N lamp. The resulting photobiotinylated mRNA-cDNA hybrids were complexed with free streptavidin and removed from the hybridization mixture by selective phenol/chloroform extraction, leaving the unhybridized cDNAs of the induced cDNA pool behind (circa 20% according to Welcher *et al.*, 1986). This pool of subtracted cDNA was used as a template to label the subtracted induced probe with α - ^{32}P -dCTP by random primer labelling (Feinberg and Vogelstein, 1983).

Nonsubtracted cDNA probes (induced and noninduced) were prepared by random primer labelling of first-strand cDNA, that was synthesized on 1 µg of the pool of induced and noninduced mRNA, respectively, using the cDNA synthesis components from Invitrogen's Subtractor Kit.

Differential screening of the cDNA library

The 'induced' cDNA library representing mRNAs present in ISR-expressing leaves was plated on the *Escherichia coli* host strain XL1-Blue MRF', resulting in 250,000 plaques. Four replica plaque lifts were prepared using Hybond-N⁺ membranes (Amersham, 's-Hertogenbosch, The Netherlands), according to the instructions of the manufacturer. For the first screening, two plaque lifts each were differentially hybridized in 7% (w/v) SDS, 0.5 M Na₂HPO₄/NaH₂PO₄ (pH 7.2), 1 mM EDTA at 68°C for 16 hours using as probes either induced cDNA that was subtracted, or noninduced cDNA. The plaque lifts were washed twice for 30 min in 2 × SSC, 0.5% (w/v) SDS at 68°C and exposed to Kodak X-Omat AR films for 1 week at -80°C. Putative positive clones were isolated from the original agar plates using the back of a Pasteur pipette and subsequently purified in a second round of differential hybridization. This second screening was performed mainly as described above, except that a nonsubtracted induced probe was used, and the isolated phages were plated at a lower density, allowing the isolation of single phage clones. Following Stratagene's protocol of the ZAP-cDNA Synthesis Kit, a helper phage was added to the clones selected from the second screening to excise *in vivo* the pBluescript phagemid from the lambda vector, which allows characterization of the cDNA insert in a plasmid system. For a third, quantitative screening, the plasmids were isolated from the bacteria and denatured in 0.4 M NaOH after which 1 µl was spotted in duplicate onto two Hybond N⁺ membranes. As an internal standard, cDNA of the constitutively expressed β -tubulin (*Tub*) gene used. *Arabidopsis Tub*-specific cDNA was generated by PCR with primers based on the sequence obtained from GenBank accession number M21415.

The dot blots were differentially hybridized with induced and noninduced cDNA probes as described above and washed three times for 20 min at high stringency (0.1 × SSC, 0.5% (w/v) SDS at 68°C). Subsequently, the blots were exposed for autoradiography and quantified using a Molecular Dynamics Phosphor Imager with Image Quant software.

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General discussion

ISR elicited by nonpathogenic rhizobacteria is a relatively unknown resistance response implicated in the protection of different plant species against various types of bacterial, fungal, and viral pathogens (reviewed by Van Loon *et al.*, 1998). Much more knowledge is available on the phenotypically similar SAR response, that is triggered upon infection with a necrotizing pathogen (reviewed by Ryals *et al.*, 1996). Elucidation of the mechanisms underlying rhizobacteria-mediated ISR could contribute to the exploitation of ISR for controlling plant diseases. In the work described in this thesis, an *Arabidopsis*-based model system to study rhizobacteria-mediated ISR was developed, providing not only new insights into the molecular basis of ISR, but also a better understanding of the complexity of the regulation of induced disease resistance.

Rhizobacteria-mediated ISR: a plant-mediated resistance response

In Chapters 2 and 3, it was demonstrated that colonization of the *Arabidopsis* rhizosphere with the rhizobacteria *Pseudomonas fluorescens* WCS417r or *Pseudomonas putida* WCS358r resulted in an increased level of protection against both the soil-borne fungal pathogen *Fusarium oxysporum* f.sp. *raphani* (*For*) and the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*). Protection was typically manifested as a relative reduction in symptoms of 40 to 60%. The reduction in disease severity in leaves challenged with *Pst* was associated with a 5- to 20-fold decrease in proliferation of *Pst*. Infiltration of three lower leaves with WCS417r induced a similar level of ISR in upper leaves as colonization of the rhizosphere with WCS417r (Chapter 2).

Based on several observations, we conclude that this protection is plant mediated. The ISR-inducing bacteria and the challenging pathogens remained spatially separated during the entire period of the bioassays. Thus, a direct interaction between the two micro-organisms can be ruled out. The *For* bioassay was designed in such a way that the ISR inducers, covering the lower part of the root system, were isolated from the *For* pathogen that was present on the upper part of the root system (Leeman *et al.*, 1995a). In both the *For* and the *Pst* bioassay, spatial separation was verified and the inducing bacteria were not recoverable from pathogen-challenged plant parts (Chapters 2 and 3).

Moreover, it is highly unlikely that the protection was achieved through rhizobacteria-produced antimicrobial compounds, such as antibiotics, that might be transported systemically and exert a direct inhibiting effect on the pathogen *in planta*. The evidence for this is three-fold:

- 1) Under high-iron conditions, representative for the situation in the bioassays (P.A.H.M. Bakker and B.W.M. Verhagen, unpublished results), neither WCS417r nor WCS358r displayed *in vitro* antagonism against either *For* or *Pst*, suggesting that these strains do not produce antibiotics that are active against these pathogens (Chapter 3).
- 2) Resistance was induced by preparations from killed bacteria, ruling out requirement for metabolites produced by the live bacteria upon contact with roots (Chapter 3).
- 3) Ecotypes Col-0 and *Ler* expressed ISR upon treatment with WCS417r, whereas ecotype RLD did not (Chapter 3). Moreover, ISR was blocked in the mutants *jar1*, *etr1*, and *npr1* of Col-0 (Chapter 4). If transported antimicrobial compounds were involved, it is unlikely that protection would be dependent on the plant genotype.

These results strongly suggest that the elevated protection observed during ISR stems from the activity of the plant and not from a direct effect of rhizobacterial metabolites on the pathogens.

Differential induction of ISR

WCS417r, WCS358r, and *P. fluorescens* WCS374r are capable of eliciting ISR in one or more plant species. Induction of ISR is dependent on the host/rhizobacterium combination. For instance, WCS358r and WCS374r perform differently on different plant species: *Arabidopsis* is responsive to WCS358r, whereas radish and carnation are not (Chapter 3; Leeman *et al.*, 1995a; Van Peer and Schippers, 1992; Van Peer *et al.*, 1991). Conversely, radish is responsive to WCS374r, whereas *Arabidopsis* is not (Chapter 3; Leeman *et al.*, 1995a). This suggests that a specific recognition between the plant and the ISR-inducing rhizobacterium is required for the induction of ISR. This specificity might be caused by the presence of receptors on the root surface that recognize specific bacterial determinants. In radish, the strain-specific O-antigenic side chain of the LPSs has been shown to be the main bacterial determinant for the induction of ISR. The LPS of WCS374r but not that of WCS358r is recognized by radish (Leeman *et al.*, 1995b). The opposite is true for *Arabidopsis*, that recognizes the LPS of WCS358r but not that of WCS374r (Chapter 3; P.A.H.M. Bakker and I. Van

der Sluis, unpublished results). These results indicate that LPSs are important bacterial traits in determining the elicitation of ISR. The LPS of WCS417r has ISR-eliciting capacities in carnation, radish, and *Arabidopsis* (Leeman *et al.*, 1995a; Van Peer *et al.*, 1991; Chapter 3). However, one must conclude that it plays only a minor role in the induction of ISR in *Arabidopsis*, because in most experiments an O-antigen⁻ mutant of WCS417r was equally capable of triggering ISR as its wild type (Chapter 3). Apparently, other determinants of WCS417r are responsible for induction of ISR by this mutant, which implies that WCS417r produces more than a single elicitor of ISR. Other research groups, using different ISR-inducing rhizobacteria, have found that bacterially produced SA and strain-specific siderophores play a role in the elicitation of induced resistance (De Meyer and Höfte, 1997; Maurhofer *et al.*, 1994). It is unlikely that bacterially produced SA is involved in the elicitation of WCS417r- or WCS358r-mediated ISR in *Arabidopsis*, because NahG transformants, that convert SA into inactive catechol, express ISR upon induction (Chapters 2 and 3). Moreover, WCS358r does not produce SA *in vitro* (Leeman *et al.*, 1996). Purified siderophores of WCS417r and WCS358r have ISR-eliciting activity in *Arabidopsis* (P.A.H.M. Bakker and I. Van der Sluis, unpublished results) but to what extent they contribute to ISR is as yet unknown.

Also in different ecotypes of *Arabidopsis thaliana*, differential induction of ISR was demonstrated. Ecotypes Col-0 and *Ler* were responsive to treatment with WCS417r, whereas ecotype RLD was not (Chapter 3). This strongly suggests that the capacity of a plant to express rhizobacteria-mediated ISR is genetically determined. Using the progeny of a cross between Col-0 and RLD, Ton *et al.* (1999) recently demonstrated that responsiveness to WCS417r is based on a single dominant gene. This gene was shown to be involved in basal resistance against *Pst* as well, suggesting that the inability of RLD to express ISR is due to a defect in the ISR signalling pathway downstream of the recognition of the rhizobacterium.

ISR signalling

Rhizobacteria-induced defense pathways

Pathogen-induced SAR is dependent on the accumulation of the signalling molecule SA, and is characterized by the elevated expression of SAR-related *PR* genes in noninfected tissue. Increased SA production by the plant does not seem to play a role in ISR, because WCS417r- and WCS358r-mediated ISR are still expressed in SA-nonaccumulating NahG transformants of *Arabidopsis*. Moreover, *PR* gene expression is not induced in plants treated with WCS417r or WCS358r. Hence, rhizobacteria-mediated ISR in *Arabidopsis* follows an SA-independent pathway that is not associated with *PR* gene expression (Chapters 2 and 3).

These results are in full accordance with those of Hoffland *et al.* (1995, 1996) who demonstrated that WCS417r-mediated ISR in radish against *For*, *Pst*, and the fungal leaf pathogen *Alternaria brassicicola*, was not associated with the accumulation of SA-inducible PRs. Moreover, Press *et al.* (1997) showed that wild-type tobacco and its

NahG transformant were equally responsive to induction of ISR by *Serratia marcescens* 90-166 against *P. syringae* pv *tabaci*. These findings demonstrate that elicitation of an SA-independent pathway by ISR-inducing rhizobacteria occurs in other plant species as well. However, rhizobacterially produced SA has been implicated in the elicitation of the systemic resistance induced by *P. aeruginosa* 7NSK2 against *Botrytis cinerea* in bean (De Meyer and Höfte, 1997; De Meyer *et al.*, 1999), and induced by *P. fluorescens* CHAO against tobacco necrosis virus in tobacco (Maurhofer *et al.*, 1994, 1998). This suggests that other nonpathogenic rhizobacteria can elicit the SA-dependent SAR signalling pathway. One may even speculate that a particular strain triggers the SAR pathway in one plant species and the ISR pathway in another.

SA-independent signalling in ISR: involvement of jasmonate and ethylene

In Chapter 4, it was demonstrated that WCS417r-mediated ISR against *Pst* was blocked in both the jasmonate response mutant *jar1* and the ethylene response mutants *etr1*, whereas SAR was unaffected by these mutations. The latter observation confirms previous findings of Lawton *et al.* (1995, 1996). Thus, WCS417r-mediated ISR differs from SAR in that it requires components of both the jasmonate and the ethylene response. In analogy with WCS417r-mediated ISR, resistance induced against *Pst* by exogenous application of MeJA or the ethylene precursor ACC was unaffected in NahG plants and not associated with the activation of the SAR marker gene *PR-1* (Chapter 4). Based on these findings, it was postulated that MeJA and ACC trigger the ISR signalling pathway in *Arabidopsis*. This was supported by the observations that combined treatments of WCS417r and either MeJA or ACC did not result in a higher level of protection than treatment with either inducer alone, suggesting that each of the three inducers can saturate the ISR pathway (Chapter 5). Experiments designed to elucidate the sequence of signalling events during ISR revealed that components of the jasmonate response act upstream of the ethylene response in the ISR signalling pathway (Chapter 4).

A concerted action of jasmonate and ethylene has been described for other plant defense responses. For instance, jasmonate enhances ethylene-induced expression of the basic *PR-1* gene in tobacco (Xu *et al.*, 1994), whereas ethylene sensitizes tomato leaf tissue for MeJA-induced expression of the proteinase inhibitor gene *Pin* (O'Donnell *et al.*, 1996). Moreover, the jasmonate- and ethylene-responsive *Pdf1.2* gene in *Arabidopsis* is synergistically induced by a combination of both signalling molecules (Penninckx *et al.*, 1998). Similarly to WCS417r-mediated ISR, activation of the *Pdf1.2* gene requires responsiveness to both jasmonate and ethylene (Penninckx *et al.*, 1996). However, for *Pdf1.2* activation, jasmonate and ethylene must act simultaneously rather than sequentially (Penninckx *et al.*, 1998).

In Chapter 6, it was demonstrated that there is no strict correlation between induction of resistance against *Pst* and activation of the jasmonate- or ethylene-responsive genes *Atvsp* and *Hel* by MeJA and ACC, respectively. Treatment with concentrations of MeJA and ACC that were below the threshold level for induction of these genes were sufficient for elicitation of resistance. Expression analyses of a large

number of jasmonate- and/or ethylene-responsive genes in response to WCS417r treatment demonstrated that none of the genes was activated locally in the roots or systemically in the leaves at different time points after ISR induction. This made it unlikely that overall jasmonate and ethylene levels are elevated in ISR-expressing plants, although a small, localized rise, that is undetectable on the basis of whole tissue analyses of marker gene expression, can not be excluded. These findings are in agreement with other observations that show that WCS417r-mediated ISR is not associated with a local or systemic increase in jasmonate or ethylene production (Knoester *et al.*, 1999; C.M.J. Pieterse, unpublished results). The requirement of jasmonate and ethylene responsiveness for ISR could be explained by the involvement of sensitization of the plant to these signalling molecules. Alternatively, instead of increased responsiveness to jasmonate and ethylene, simply the availability of pre-existing jasmonate and ethylene signalling intermediates might be sufficient to facilitate ISR.

Recently, Knoester *et al.* (1999) tested a large set of well-characterized *Arabidopsis* mutants that are affected at different steps in the ethylene signalling pathway. None of the mutants were responsive to WCS417r, suggesting that an intact ethylene signalling pathway is required for ISR. Mutant *eir1*, which is insensitive to ethylene in the roots but not in the leaves was able to mount ISR when WCS417r was infiltrated in the leaves, but not when the bacteria were applied to the roots (Knoester *et al.*, 1999). Apparently, ethylene responsiveness is required at the site of application of the inducing agent. Perhaps, ethylene is involved in the generation or translocation of the systemically transported signal (Figure 1). This does not rule out the possibility that ethylene responsiveness is also required for the expression of ISR in tissue distant from the site of ISR induction.

A central role for NPR1 in induced disease resistance

Despite the clear differences between the ISR and the SAR signalling pathways, both ISR and SAR are dependent on the regulatory factor NPR1. Like WCS417r-mediated ISR, MeJA- and ACC-induced resistance were blocked or strongly reduced in the *Arabidopsis* mutant *npr1*, suggesting that NPR1 acts downstream of the jasmonate and ethylene response in the ISR signalling pathway (Chapter 4). Although NPR1 is involved in both ISR and SAR, the pathways must diverge downstream of NPR1. In the SAR pathway, NPR1 is a key regulator of the SA-inducible *PR* genes (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). The *PR* genes are not activated during ISR. Thus, NPR1 must also be involved in the regulation of jasmonate- and ethylene-dependent defense responses that are activated during ISR.

How can this differential regulation of defense responses by NPR1 be achieved? The *Npr1* gene is constitutively expressed (Ryals *et al.*, 1997; Chapter 5), but regulation may well occur at the protein level. Recently, Kinkema *et al.* (1998) demonstrated that upon induction of SAR, NPR1 is directed to the nucleus, leading to the activation of *PR* gene expression. In mammalian cells, the NPR1 homolog I κ B α functions in signal transduction by binding to the transcription factor NF- κ B, thereby preventing it from

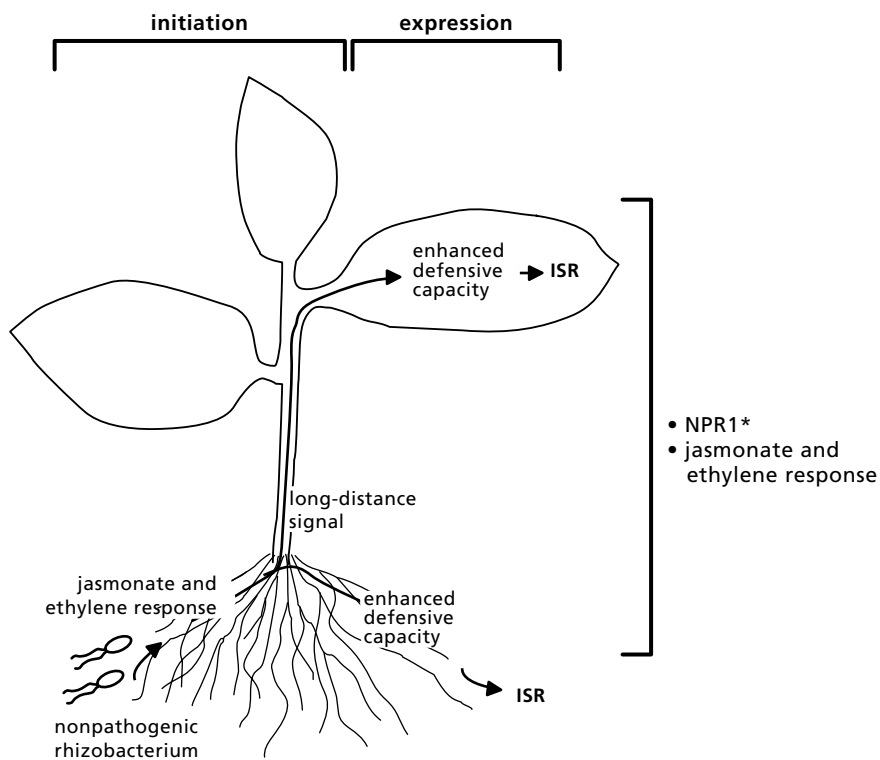


Figure 1. Proposed model for rhizobacteria-mediated ISR in *Arabidopsis*.

Colonization of the rhizosphere by selected nonpathogenic rhizobacteria triggers ISR in distant tissue. Components of the jasmonate and the ethylene response are required at the induction site of ISR, where they might be involved in either the generation or the translocation of the long-distance signal. Induction of ISR results in the activation of the regulatory factor NPR1 (indicated by an asterisk), that is required for ISR. The site of action of NPR1 in the ISR signalling pathway is unclear. The expression of ISR might consist of (1) a direct activation of defense-related gene expression, (2) potentiation of defense-related gene expression, and/or (3) posttranslational modification of existing proteins. These possibilities are contained by the term 'enhanced defensive capacity'. The work described in this thesis demonstrates that possibilities (1) and (2) might be involved in WCS417r-mediated ISR.

entering the nucleus. When the signal transduction pathway is activated, e.g. by pathogens or oxidants such as H_2O_2 (Thanos and Maniatis, 1995), $I\kappa B\alpha$ is phosphorylated and subsequently degraded, which results in a release of the NF- κ B factor to the nucleus to stimulate transcription of genes involved in inflammation and immune responses (De Martin *et al.*, 1993). It is tempting to speculate that NPR1, in analogy with the $I\kappa B\alpha$ /NF- κ B system, functions as an (indirect) activator of transcription. The NPR1 protein contains ankyrin repeats that have been implicated in protein-protein interactions (Cao *et al.*, 1997; Ryals *et al.*, 1997). Hence, activation of NPR1 might be accomplished through binding of a putative, pathway-specific protein, leading to differential activation of defense responses downstream of NPR1, i.e. *PR* genes in the case of SAR, and so far unidentified defense-related genes in the case of ISR.

The ISR and the SAR signalling pathways are compatible

Cross-talk between SA- and jasmonate/ethylene-dependent signalling pathways plays an important role in the regulation of defense responses (Dong, 1998; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998). Previously, it was shown that SA is a potent inhibitor of jasmonate-inducible gene expression (Bowling *et al.*, 1997; Doares *et al.*, 1995; Doherty *et al.*, 1988; Niki *et al.*, 1998; Peña-Cortés *et al.*, 1993; Penninckx *et al.*, 1996). This was confirmed by our finding that SA strongly inhibits MeJA-induced expression of the *Atvsp* gene (Chapter 6). This raises the question whether the jasmonate-dependent ISR pathway and the SA-dependent SAR pathway are compatible. In Chapter 5, it was shown that simultaneous activation of the ISR pathway and the SAR pathway resulted in an additive effect on the level of induced protection against *Pst*, irrespective of the sequence in which these defense responses were induced. Evidently, the SAR pathway does not inhibit the ISR pathway. The additive effect on protection was not caused by a stimulating effect of the ISR pathway on the action of the SAR pathway, because the level of *PR-1* transcript accumulation in SAR-expressing plants was not changed when ISR was expressed as well. This indicates that activation of the ISR pathway does not sensitize the tissue for SAR expression. Because simultaneous expression of ISR and SAR led to additive rather than synergistic enhancement of protection against *Pst*, both pathways appear to act independently by parallel activation of defense responses with complementary effects. This implies that the NPR1 protein is not a limiting factor in the combined effects of SAR and ISR. The defense responses activated during ISR and SAR that are active against *Pst* might be common to both ISR and SAR, but might as well be divergent (see Figure 7 in Chapter 5).

An explanation for the apparent lack of cross-talk between ISR and SAR might be that the ISR-associated components of the jasmonate and ethylene response are involved only at the site of ISR induction, which was in the roots. The SA-dependent SAR pathway was activated in the leaves and therefore could not affect this part of the ISR pathway, leaving the expression of ISR in the leaves unaffected.

Expression of ISR

Defense-related gene expression

Pathogen-induced SAR is associated with the activation of a large set of SAR genes, including *PR* genes (Uknes *et al.*, 1992; Ward *et al.*, 1991). In SAR-expressing tobacco plants, the SAR gene products accumulate systemically to levels of up to 1% of the total mRNA and protein content (Lawton *et al.*, 1995). This indicates that SAR is associated with major changes in gene expression and protein abundance. Rhizobacteria-mediated ISR and pathogen-induced SAR in *Arabidopsis* are phenotypically similar in that both lead to an enhanced level of protection against different types of pathogens (Chapters 2 and 3). However, expression analyses of a large set of known, well-characterized defense-related genes revealed that, in contrast to pathogen-induced SAR, neither one of them was associated with WCS417r-mediated ISR in *Arabidopsis* (Chapter 6). The

ability of *Arabidopsis* to express an elevated level of resistance against *Pst*, without activation of SAR-related *PR* genes indicates that PRs are not essential for systemically induced resistance. Recently, Clarke *et al.* (1998) reported that the constitutively SAR-expressing mutant *cpr6* of *Arabidopsis* had lost its enhanced level of protection against *P. syringae* pv *maculicola* (*Psm*) in the mutant *npr1* background, but the elevated level of *PR* gene expression remained unchanged. Thus, induced resistance against *Psm* is NPR1-dependent and does not require the PRs that accumulate during SAR. This observation resembles rhizobacteria-mediated ISR against *Pst*. Could the *cpr6* mutation result in constitutive expression of both SAR and ISR? If so, then *cpr6* NahG plants would still show enhanced protection against *Psm*, because ISR is not blocked by the *NahG* transgene. Unfortunately, the authors did not perform such an assay.

Nonbiased screens for gene expression and protein accumulation demonstrated that ISR is not associated with major changes in gene expression or protein abundance as observed during SAR (Chapter 7). Differential screening of a cDNA library representing mRNAs of ISR-expressing tissue yielded only 13 differentially expressed genes (*ISR1-13*). Of these, the expression was upregulated at most 9-fold. This suggests that ISR is based on subtle changes in gene expression. Further characterization of these genes might elucidate their role in ISR.

Recently, a screen of a collection of *Arabidopsis* enhancer trap lines containing the β -glucuronidase (GUS) reporter gene yielded a line that shows GUS activity in the roots upon colonization by WCS417r (Léon-Kloosterziel *et al.*, 1998). Interestingly, this line showed a similar expression pattern after treatment of the roots with ACC, indicating that the corresponding gene is ethylene inducible. Moreover, Gerrits *et al.* (1997) showed that *Arabidopsis* plants expressing WCS417r-mediated ISR accumulate an antibacterial compound prior to challenge that is active against *Pst*. This compound accumulated also in WCS417r-treated NahG plants, but not in *jar1*, *etr1*, and *npr1* plants, indicating that it might contribute to ISR against *Pst*. All together, these data indicate that the seemingly subtle molecular changes during ISR are effective in mounting resistance.

Potentiation of gene expression during ISR

Besides a direct effect on the expression of SAR genes, pathogen-induced SAR is accompanied by a faster and greater activation of defense responses after infection with a challenging pathogen, a phenomenon called potentiation (Mur *et al.*, 1996). In Chapter 6, it was demonstrated that SAR-expressing *Arabidopsis* plants indeed showed potentiation, manifested by an enhanced expression of the SA-responsive *PR* genes after challenge with *Pst*. ISR-expressing plants did not show potentiation of the expression of *PR* genes or most other defense-related genes studied (Chapter 6). However, expression of the jasmonate-responsive gene *Atvsp* was enhanced in ISR-expressing plants after challenge with *Pst* (Chapter 6). This suggests that ISR is associated with potentiation of specific jasmonate-responsive genes. The potentiation of *Atvsp* expression is unlikely to be caused by accelerated jasmonate production after

challenge, because other jasmonate-responsive genes did not show potentiated expression. This fits with the observation that jasmonate levels are not increased in challenged ISR-expressing plants compared to challenged control plants (C.M.J. Pieterse, unpublished result).

The regulation of the potentiated expression of *Atvsp* seems rather complex. In Chapter 5, we demonstrated that a combination treatment of WCS417r and MeJA did not result in an enhanced expression of *Atvsp* compared to treatment with MeJA alone. This could not be explained by a saturating effect of MeJA, because similar results were obtained when lower concentrations of MeJA were applied, inducing lower levels of *Atvsp* expression (data not shown). This demonstrates that the potentiation of *Atvsp* expression in ISR-expressing tissue is manifested as enhanced *Atvsp* expression levels only after challenge with *Pst* and not after exogenous application of MeJA. Because the *Atvsp* gene is inducible only by jasmonate and not by ethylene or SA, pathogen-induced production of jasmonate is most likely responsible for the elevated expression of *Atvsp* after challenge. Recently, Lee *et al.* (1997) demonstrated that exogenously applied MeJA and endogenously produced jasmonate activate distinct signalling pathways. These may regulate *Atvsp* induction differently, e.g. by activating different transcription factors that interact with distinct *cis* elements in the *Atvsp* promoter.

Correlation between induced resistance and basal resistance

Induced resistance has been suggested to depend on extant defense mechanisms (Van Loon, 1997) that are also triggered upon infection of noninduced plants. According to this hypothesis, the effectiveness of those basal resistance mechanisms is enhanced upon challenge of induced plants. Analysis of the *Arabidopsis* ecotype RLD, the mutants *etr1*, *jar1*, and *npr1*, and the NahG transformant showed that these genotypes all displayed an enhanced sensitivity to *Pst* infection (Chapters 2, 3, and 4). Mutant *npr1* lost the ability to express both SAR and ISR. However, RLD, *jar1*, and *etr1* plants, can express SAR but not ISR, whereas in NahG plants SAR is abolished but the ISR pathway is unaffected. Reduced basal resistance against *Pst* in these genotypes apparently does not affect both pathways. Therefore, the enhancement of extant resistance mechanisms can not explain the whole phenomenon of induced disease resistance.

Induced resistance as a mechanism to control plant diseases

The research described in this thesis demonstrates that plants are capable of differentially activating distinct defense pathways depending on the inducing agent. The signalling molecules SA, jasmonate, and ethylene play a predominant role in these pathways. Recently, Thomma *et al.* (1998) demonstrated that the SA-dependent pathway is more effective against certain pathogens, i.e. the biotrophic oomycete *Peronospora parasitica*, whereas the jasmonate-dependent pathway is directed more against other pathogens, i.e. the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea*. This suggests that the defensive compounds produced by the SA-dependent pathway, like PR-1, -2 and -5, and those produced by the jasmonate-

dependent pathways, like CHI-B (PR-3), HEL (PR-4) and PDF1.2, have different specificities. Similarly, although resistance induced by either pathogens or rhizobacteria has been shown to be effective against a large set of pathogens, the effectiveness of SAR and ISR differs with different challenging pathogens (Hammerschmidt and Kuć, 1995; Hoffland *et al.*, 1996).

The broad-spectrum efficacy of induced resistance could serve as an alternative for chemical protectants in the control of diseases in agriculture. An example of successful use of induced resistance in disease control is the chemical plant activator Bion. The active compound of Bion is benzothiadiazole (BTH), which is a functional analog of SA. In most plants studied, BTH activates the SAR pathway (Görlach *et al.*, 1996; Friedrich *et al.*, 1996; Lawton *et al.*, 1996). Bion is effective against a wide range of plant pathogens, including bacterial pathogens that are difficult to control by conventional chemical agents (B. Mantel (Novartis, Roosendaal, The Netherlands), personal communication). The observation that simultaneous activation of ISR and SAR results in a significantly enhanced level of protection (Chapter 5) offers great potential for integrating both forms of induced resistance in future agricultural practices. Combining both forms of induced resistance might even broaden the spectrum of effectiveness, as the SA- and jasmonate-dependent pathways have different specificities.

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Summary

Once a plant has been infected or otherwise stimulated, it develops an enhanced defensive capacity that is effective against different types of pathogens. This induced disease resistance is referred to as either systemic acquired resistance (SAR) when the inducer is a necrotizing pathogen, or induced systemic resistance (ISR) when the inducing agent is a nonpathogenic rhizobacterium. Rhizobacteria colonize plant roots and utilize exudates released from the roots as nutrients. Most of the rhizobacteria that trigger ISR belong to the group of the fluorescent *Pseudomonas* spp. To gain more insight into the molecular basis of rhizobacteria-mediated ISR, a model system was developed in which *Pseudomonas fluorescens* WCS417r was used as the ISR-inducing rhizobacterium and *Arabidopsis thaliana* as the host plant. The fungal root pathogen *Fusarium oxysporum* f sp *raphani* (*For*) and the bacterial leaf pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) were used as the challenging pathogens.

Similar to earlier findings in carnation and radish, WCS417r was capable of triggering ISR in *Arabidopsis*, conferring resistance to both *For* and *Pst*. The induced protection was manifested as both a reduction in symptoms of disease and inhibition of pathogen growth. The level of ISR was comparable, although usually slightly less than that of biologically induced SAR. Interestingly, *Arabidopsis* ecotypes Columbia (Col-0) and Landsberg *erecta* (*Ler*) readily expressed WCS417r-mediated ISR against both *For* and *Pst*, whereas ecotype RLD did not. This indicates that the capacity of plants to express ISR is genetically determined.

Besides WCS417r, other *Pseudomonas* spp strains were tested for ISR-inducing activity. *P. putida* WCS358r triggered ISR in *Arabidopsis*, whereas in earlier studies it was found to be incapable of inducing ISR in carnation and radish. Conversely, *P. fluorescens* WCS374r, that was effective on radish, did not trigger ISR in *Arabidopsis*. Evidently, ISR is induced when the rhizobacterium is somehow recognized by the plant. The strain-specific lipopolysaccharide (LPS) of the outer membrane of WCS417r, which was previously shown to be essential for the induction of ISR in carnation and radish, induced ISR in *Arabidopsis* as well. However, a bacterial mutant lacking the specific O-antigenic side chain of the LPS, still triggered ISR in *Arabidopsis*, indicating that the LPS is not the only bacterial determinant involved.

Elucidation of plant signalling pathways controlling disease resistance is a major objective in research on plant-pathogen interactions. The SAR signalling pathway has

been relatively well studied. SAR depends on the endogenous accumulation of salicylic acid (SA), which is responsible for the systemic activation of SAR marker genes, including genes encoding pathogenesis-related proteins (PRs) in *Arabidopsis*. Using SA-nonaccumulating NahG transformants of *Arabidopsis* that are blocked in the SAR pathway, it was demonstrated that WCS417r- and WCS358r-mediated ISR against *Pst* is independent of SA accumulation. Moreover, neither one of these rhizobacteria induced *PR* gene expression in wild-type Col-0 plants. Thus, rhizobacteria-mediated ISR and pathogen-induced SAR in *Arabidopsis* follow different pathways.

Besides SA, the plant hormones jasmonate and ethylene have been implicated in plant defense. *Arabidopsis* mutants *jar1* and *etr1*, which are affected in the jasmonate and ethylene response, respectively, expressed normal levels of pathogen-induced SAR. In contrast, these mutants were unable to mount ISR upon colonization of the roots with WCS417r. This indicates that ISR signalling requires components of both the jasmonate and the ethylene response. Exogenous application of methyl jasmonate (MeJA) or the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) was as effective as WCS417r in triggering ISR. Moreover, MeJA- and ACC-induced resistance were maintained in NahG plants and were not associated with *PR* gene expression in wild-type Col-0 plants. Furthermore, combined treatments of plants with WCS417r and either MeJA or ACC did not result in an additive effect on the level of induced protection. Therefore, MeJA and ACC appear to trigger the ISR signalling pathway in *Arabidopsis*. The protection induced by MeJA was blocked in both *jar1* and *etr1* plants, whereas ACC-induced protection was blocked only in *etr1* plants and not in *jar1* plants. This suggests that components of the jasmonate and the ethylene response act in sequence in the ISR signalling pathway. Expression analyses of a large set of known jasmonate- and/or ethylene-responsive genes revealed that induction of ISR by WCS417r in *Arabidopsis* is not accompanied by an enhanced expression of these genes. This indicates that basal or slightly elevated levels of jasmonate and ethylene are sufficient for ISR. WCS417r-mediated ISR might be based on an enhanced sensitivity for these signalling molecules, leading to an induction of unknown jasmonate- and ethylene-inducible defense responses.

The NPR1 protein is a key regulator in the SAR signalling pathway, acting downstream of SA in the elicitation of SAR and *PR* gene expression. Using mutant *npr1* of *Arabidopsis*, it was demonstrated that the ISR signalling pathway activated by WCS417r depends on NPR1 as well. The MeJA- and ACC-induced resistances were similarly affected by the *npr1* mutation, indicating that in the ISR signalling pathway, NPR1 is a regulator acting downstream of the requirement for the jasmonate and ethylene responses. In the SAR pathway, NPR1 controls the expression of *PR* genes. Since these *PR* genes are not activated during ISR, NPR1 must differentially regulate ISR- and SAR-related defense responses dependent on the pathway that is activated upstream of it.

Cross-talk between SA-, jasmonate-, and ethylene-inducible pathways is known to affect the expression level of specific genes that are activated by each of the pathways.

Combined with the fact that both ISR and SAR signalling require NPR1, this prompted us to examine in how far cross-talk between the ISR and SAR pathways might occur. Interestingly, simultaneous activation of the ISR and the SAR pathway resulted in an enhanced level of induced protection against *Pst*. In NahG, *jar1*, *etr1*, and *npr1* plants, no enhanced protection was evident. Moreover, the level of *PR-1* gene expression in SAR-expressing plants was not altered when ISR had been induced as well. These results indicate that the ISR and the SAR pathway do not influence each other. Apparently, ISR and SAR activate complementary defense responses that are active against *Pst*.

To search for changes in protein accumulation or gene expression during ISR, nonbiased screens for ISR-related proteins or genes were executed. Two-dimensional polyacrylamide gel electrophoresis of soluble proteins isolated from ISR-expressing and noninduced leaves did not yield any apparent differences in protein patterns. A cDNA library prepared from mRNA of ISR-expressing leaves was differentially screened using a cDNA probe synthesized on 1) subtracted mRNA from ISR-expressing leaves and 2) mRNA from noninduced leaves. Thirteen clones were found to be 2- to 9-fold upregulated in ISR-expressing tissue. These results indicate that, unlike SAR, ISR is not associated with major changes in gene expression and protein abundance. Instead, subtle molecular changes might account for the induction of ISR.

Studies on gene expression after challenge inoculation with *Pst* demonstrated that plants pre-induced by WCS417r contain enhanced levels of transcripts of the jasmonate-responsive gene *Atvsp* (encoding vegetative storage protein). This pattern was not found for other jasmonate-responsive genes, which indicates that only a selection of jasmonate-responsive genes is potentiated in ISR-expressing tissue. Challenge inoculation of plants expressing pathogen-induced SAR resulted in an enhanced induction of SA-responsive *PR* genes, indicating that potentiation of genes is shared between ISR and SAR, but influences the expression of distinct sets of genes.

In this thesis, molecular analyses of induction, signal transduction, and expression of rhizobacteria-mediated ISR have provided a starting point for further elucidation of the molecular basis of ISR. The finding that simultaneous activation of ISR and SAR results in an enhanced level of induced protection of a type that is effective against a broad spectrum of pathogens, long-lasting, and environmentally friendly, offers considerable potential for integrating both forms of induced resistance for crop protection.

Planten worden voortdurend bedreigd door potentiële ziekteverwekkers (pathogenen) zoals schimmels, bacteriën en virussen. In de strijd tegen oogstverliezen als gevolg van plantenziekten worden gewassen veelal behandeld met chemische bestrijdingsmiddelen die vaak giftig en milieu-onvriendelijk zijn. Tevens worden ziektebestendige (= resistente) planten geteeld die verkregen zijn via hetzij klassieke veredelings technieken (kruisen met resistente varianten), hetzij moderne genetische modificatie (op kunstmatige wijze inbrengen van een erfelijke eigenschap). Het gebruik van chemische middelen of genetisch gemodificeerde planten kan een gevaar vormen voor de volksgezondheid of het milieu en kan stuiten op ethische bezwaren. Bovendien werkt het niet in alle gevallen afdoende. Een alternatieve manier om plantenziekten te bestrijden is de van nature aanwezige resistentie van de plant te verhogen. Behandeling van zaden of wortels met bepaalde stammen van niet-pathogene *Pseudomonas* bacteriën kan de plant zodanig prikkelen dat zijn weerstand tegen verschillende pathogenen toeneemt. Deze *Pseudomonas* bacteriën worden ook wel rhizobacteriën genoemd, omdat ze de oppervlakte van het wortelstelsel (de rhizosfeer) bezetten, waar ze leven van de voedingsstoffen die door de wortels worden uitgescheiden. De rhizobacteriën bewerkstelligen een verhoging van de afweer in zowel de wortels als de bovengrondse plantendelen. Dit verschijnsel wordt geïnduceerde (= geactiveerde) systemische (= in verderop gelegen delen tot expressie komende) resistentie genoemd, afgekort ISR. ISR uit zich in een vertraagde en/of verminderde ziekte na infectie met een pathogeen. Het biedt bescherming tegen een breed spectrum aan pathogenen en blijft lange tijd behouden, soms zelfs gedurende het hele leven van de plant. Naast ISR dat geactiveerd wordt door *Pseudomonas* bacteriën is er een andere vorm van geïnduceerde resistentie bekend, namelijk systemische verworven resistentie, afgekort SAR. In het geval van SAR is de activator een pathogeen dat specifiek herkend wordt door de plant, die daarop gewoonlijk reageert met een snelle dood van de geïnfecteerde en daaraan grenzende cellen, waardoor de infectie wordt ingeperkt. Bij een volgende infectie door hetzelfde of een willekeurig ander pathogeen laat de gehele plant, net als bij ISR, een verhoogde afweer zien. In Figuur 1 van Hoofdstuk 1 (blz. 12) staan schematisch de verschijnselen ISR en SAR weergegeven.

SAR is al in 1961 gekarakteriseerd, terwijl ISR pas in 1991 voor het eerst beschreven is. Ten tijde van de start van het onderzoek beschreven in dit proefschrift was dan ook

veel meer bekend over SAR dan over ISR. Het doel van dit project was om meer inzicht te krijgen in de mechanismen die ten grondslag liggen aan de door rhizobacteriën geïnduceerde ISR. Daartoe hebben we een modelsysteem ontwikkeld met als rhizobacterie *Pseudomonas fluorescens* stam WCS417r, die een bekende ISR induceerder is op anjer en radijs; als gastheerplant hebben we *Arabidopsis thaliana* (in het Nederlands 'zandraker') gebruikt, omdat dit plantje veel voordelen biedt voor moleculair en genetisch onderzoek en vaak gebruikt wordt bij studies aan plant-pathogeen interacties. WCS417r bleek in staat in *Arabidopsis* ISR op te wekken tegen zowel het wortelinfecterende schimmelpathogeen *Fusarium oxysporum* als het bladinfesterende bacteriële pathogeen *Pseudomonas syringae*. Deze bescherming uitte zich in een reductie van de ziektesymptomen met 40 tot 60%. WCS417r bleek echter niet in staat te zijn in alle, genetisch een elkaar verwante, ecotypen van *Arabidopsis* ISR te induceren. Dit geeft aan dat er binnen *Arabidopsis* verschillen bestaan in reactie op deze rhizobacterie die waarschijnlijk genetisch bepaald zijn.

Behalve WCS417r hebben we ook andere *Pseudomonas* stammen getest op hun vermogen om ISR te induceren in *Arabidopsis*. Interessant was dat stam WCS358r ISR induceerde in *Arabidopsis* terwijl hij dat niet kon in radijs. Daarentegen was stam WCS374r effectief op radijs, maar niet op *Arabidopsis*. Hieruit blijkt dat een specifieke interactie tussen de rhizobacteriën en de plant een voorwaarde is voor inductie van ISR. Eerder onderzoek aan ISR in anjer en radijs had aangetoond dat het stamspecifieke lipopolysaccharide (LPS) van de buitenmembraan van de *Pseudomonas* bacteriën bepalend kan zijn voor de herkenning van de rhizobacterie door de plant. Ons onderzoek wees uit dat daarentegen in *Arabidopsis* het LPS niet de enige resistentie-inducerende factor is.

De mate van bescherming die bewerkstelligd wordt door het aanschakelen van ISR na behandeling van de planten met WCS417r of WCS358r, is vrijwel gelijk aan de bescherming die opgewekt wordt door een SAR-inducerend pathogeen. Echter, het verhogen van de weerstand van de plant door ISR of SAR bleek te verlopen langs van elkaar verschillende wegen, waarin diverse signaalstoffen een rol spelen (signaaltransductiewegen). Deze conclusie is voornamelijk gebaseerd op resultaten die verkregen zijn met behulp van planten die door mutatie of het inbrengen van erfelijk materiaal (gen) zodanig genetisch veranderd zijn dat ze verstoord zijn in hun reactie op, of synthese van, bepaalde signaalstoffen. Zo werd gebruik gemaakt van zogenaamde *Arabidopsis* NahG planten, die geen salicylzuur kunnen accumuleren. Salicylzuur is analoog aan de werkzame stof in aspirine en toediening ervan beschermt planten tegen ziekten. Uit eerder onderzoek is gebleken dat de NahG planten niet in staat zijn SAR tot expressie te brengen, wat bewijst dat salicylzuur een onmisbare signaalstof van de plant is bij SAR. NahG planten bleken echter niet verstoord te zijn in hun expressie van ISR, wat aangeeft dat salicylzuur geen signaalstof is bij ISR. Bovendien werden pathogenese-gerelateerde (*PR*) genen, die als merkers voor SAR beschouwd worden en waarvan de expressie onder controle staat van salicylzuur, niet geactiveerd tijdens ISR. Dit bevestigt dat de signaaltransductieweg in de plant, die geactiveerd wordt tijdens SAR, niet geactiveerd wordt tijdens ISR.

Naast salicylzuur kunnen twee andere signaalstoffen, jasmonzuur en ethyleen, een rol spelen bij afweerreacties in planten. In de *Arabidopsis* mutanten *jar1* en *etr1*, die verstoord zijn in hun reactie op respectievelijk jasmonzuur en ethyleen, werd een normaal niveau van van SAR opgewekt door pathogenen, waaruit blijkt dat jasmonzuur en ethyleen geen rol spelen bij SAR. Echter, door WCS417r geïnduceerde ISR bleek wel geblokkeerd te zijn in de *jar1* en *etr1* mutanten, wat er op duidt dat reacties op jasmonzuur en ethyleen juist cruciaal zijn in de signaaltransductieweg die leidt tot ISR. (Zie voor een schematische weergave van de ISR en SAR signaaltransductiewegen Figuur 4 van Hoofdstuk 4, blz. 62) Hiermee in overeenstemming vonden we dat toediening van jasmonzuur en ethyleen aan de planten de resistentie verhoogde. Net zoals de door WCS417r geïnduceerde ISR, waren de door jasmonzuur en ethyleen geïnduceerde resistenties onafhankelijk van salicylzuur en gingen ze niet gepaard met de activering van de SAR merker *PR* genen. Dit suggereert dat jasmonzuur en ethyleen dezelfde signaaltransductieweg activeren als WCS417r. De jasmonzuur- en ethyleen-niveaus stegen echter niet tijdens de door WCS417r geïnduceerde ISR in *Arabidopsis*. Daarom is het waarschijnlijk dat voor ISR enkel een bepaald basisniveau of een subtiele verhoging van jasmonzuur en ethyleen vereist is. De inductie van ISR zou het gevolg kunnen zijn van een verhoogde gevoeligheid van de planten voor deze signaalstoffen, waardoor toch bepaalde op jasmonzuur en ethyleen reagerende afweergenen worden geactiveerd.

Een sleutelfactor in de SAR route is het eiwit NPR1. NPR1 functioneert als schakel tussen de signaalstof salicylzuur en de daaropvolgende reacties, zoals SAR en *PR* genexpressie. *Arabidopsis* mutant *npr1*, die een niet-functioneel NPR1 eiwit maakt, bleek behalve geen SAR, ook geen ISR meer tot uiting te kunnen brengen. Bovendien waren de *npr1* mutanten ook geblokkeerd in de resistentie zoals die opgewekt wordt door salicylzuur, jasmonzuur of ethyleen. Hieruit blijkt dat activering van het NPR1 eiwit door verschillende signaalstoffen leidt tot het aanschakelen van ofwel SAR ofwel ISR (zie ook Figuur 4 van Hoofdstuk 4, blz. 62).

Planten ontvangen meerdere prikkels tegelijk. Voor optimale reacties daarop moeten de verschillende geactiveerde signaaltransductiewegen op elkaar afgestemd worden. Het was al bekend dat signaaltransductiewegen, die òf door salicylzuur, òf door jasmonzuur en/of ethyleen geactiveerd worden, elkaar in sommige gevallen kunnen beïnvloeden. De vraag die opkwam was of gelijktijdige aanschakeling van ISR (afhankelijk van jasmonzuur en ethyleen) en SAR (afhankelijk van salicylzuur) zou kunnen leiden tot een remming of juist een stimulering van één van beide afweerreacties. Bovendien zijn zowel de ISR als de SAR weg afhankelijk van het NPR1 eiwit, wat een beperkende factor zou kunnen zijn voor een gelijktijdige expressie van volledige ISR en SAR reacties. Het bleek echter dat het niveau van resistentie verhoogd werd door gelijktijdige aanschakeling van ISR en SAR. Verschillende waarnemingen duiden er op dat dit niet het gevolg is van het verhogen van de gevoeligheid van de ene weg door de andere weg. Het additieve effect op het niveau van bescherming kan het beste verklaard worden door aan te nemen dat de signaaltransductiewegen van ISR en SAR elkaar niet beïnvloeden en leiden tot elkaar aanvullende afweerreacties.

Welke afweerreacties geactiveerd worden in planten die ISR vertonen is onbekend. Een groot aantal bekende afweergenen is getest, maar geen enkele bleek geactiveerd te worden tijdens de inductie van ISR door WCS417r. Verder zijn gevoelige technieken toegepast om veranderingen te kunnen detecteren in enerzijds de concentratie/eigenschappen van ieder eiwit en anderzijds het expressieniveau van alle genen in *Arabidopsis* planten die ISR vertonen. Er werden geen veranderingen in eiwitten waargenomen. Wel werd een klein aantal genen gedetecteerd dat in sterkere mate tot expressie kwam in planten die ISR vertoonden. Mogelijk zijn deze genen betrokken bij de door ISR geboden bescherming.

Het in dit proefschrift beschreven onderzoek levert een begin van de opheldering van de moleculaire mechanismen in de plant die betrokken zijn bij door rhizobacteriën geïnduceerde ISR. De gelijktijdige aanschakeling van ISR en SAR resulteert in een verhoogd niveau van resistentie, die werkzaam is tegen een breed spectrum aan pathogenen, langdurig aanhoudt en milieuvriendelijk is. Dit toont de potentie aan van het combineren van beide vormen van geïnduceerde resistentie voor het beschermen van commerciële land- en tuinbouwgewassen tegen verschillende plantenziekten.

Na zoveel ratio is er eindelijk ruimte voor wat gevoel in dit boekje. De promotietijd bestond namelijk niet alleen uit het bedenken, uitvoeren en opschrijven van experimenten, maar ook uit het onderkennen van mijn eigen mogelijkheden en tekortkomingen, wat ik een minstens zo waardevol proces vond. Terwijl ik aan het begin van m'n OIO schap verscheidene keren sterk aan mezelf en het project twijfelde (wie herinnert zich niet mijn wanhoopsgezicht en -kreten als de frustratie toesloeg), ben ik in de loop der jaren steeds wat meer in mezelf gaan geloven. Dit was onmogelijk geweest zonder de niet-aflatende steun van Corné, die m'n coach, steun en toeverlaat, analysator, motiveerder, corrigeerder en maatje was, waarmee ik ook nog iedere dag kon gieren van het lachen. Jij gaf mij op de moeilijke momenten altijd het geloof dat er toch nog wat uit te slepen was. Heel erg bedankt, ik heb veel van je geleerd. Verder gaat mijn dank uit naar Kees, mijn promotor. Je kritische blik op het onderzoek en de manuscripten hebben mijn gedachten vaak gescherpt. Ik was niet altijd even snel te overtuigen, maar dat leverde interessante discussies en frisse inzichten op.

Yvonne, Annemiek, Femke, Erik, Mirjam en Inge, mijn studenten, jullie begeleiden was één van de leukste dingen tijdens m'n OIO schap. Het was inspirerend om een ieder van jullie, op z'n eigen manier, te zien ontwikkelen als onderzoeker en persoon. Jullie vinden allemaal wel iets van je eigen werk terug in dit proefschrift. En dan is er natuurlijk de rest van de Fyto's. Ik ben blij dat ik in zo'n gezellige groep heb kunnen werken. De lol die we met elkaar hadden en de hechte band binnen de groep maakten dat ik het onderzoek met heel veel plezier heb gedaan en dat ik het altijd heb kunnen volhouden. Ik zal de melige sfeer en het slappe gelul tijdens de pauzes, de jaarlijkse weekenden naar de Waddeneilanden en de vele etentjes met de daarmee gepaard gaande drankjes zeker missen. Het liefst zou ik aan iedereen een zinnetje wijden, maar om het geheel leesbaar (en kuis) te houden heb ik besloten dat niet te doen. Bedankt kamergenoten Marjan, Jur, Jesus, Marga, Bart, Ben, Jos, Harko en Marcel; vaste en tijdelijke medewerkers Ellis, Peter B., Joop, Boet, Karen, Bert, Long-Xian, Hans, Ientse, Joost, Debbie, Peter V. en Carlo; en natuurlijk alle (oud) studenten, die bij Fyto een stage hebben gelopen. Mijn paranimfen, Marjan en Jur, met jullie was het altijd relaxed maar ook spannend, lachen maar ook huilen, begripvol maar ook recht-door-zee, interessant maar ging het ook vaak nergens over; kortom, de AIO kamer voelde als een 'thuis'.

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Curriculum vitae

Saskia van Wees werd geboren op 30 april 1969 te Zeist. In 1987 behaalde zij het VWO-B diploma aan het Christelijk Lyceum in haar geboorteplaats. Van 1987 tot 1993 studeerde zij Biologie aan de Universiteit Utrecht. Tijdens de doctoraalfase vervulde zij twee onderzoekstages: Plantenfysiologie, onder begeleiding van Dr. A.C. Borstlap (onderzoek naar het effect van osmoticum op de protonenpompactiviteit van plasmamembraanvesicles van tabaksplanten) en Fytopathologie, onder begeleiding van Dr. E. Hoffland (onderzoek naar de rol van pathogenese-gerelateerde eiwitten in systemische resistentie opgewekt door rhizobacteriën in anjer). Na haar afstuderen was ze 8 maanden lang gastmedewerker bij de projectgroep Fytopathologie aan de Universiteit Utrecht, waar ze begon aan het ontwikkelen van het *Arabidopsis* modelsysteem ter bestudering van de moleculaire basis die ten grondslag ligt aan de door rhizobacteriën aangeschakelde systemische resistentie. Van juni 1994 tot november 1998 was ze werkzaam als OIO bij dezelfde groep. Daar werkte ze, onder begeleiding van Prof.dr. L.C. van Loon en Dr. C.M.J. Pieterse, verder aan het modelsysteem. De resultaten van het onderzoek staan beschreven in dit proefschrift.

