

2. Theory

2.1. Bacterial elicitors and plant signalling in induced systemic resistance

2.2. Experimental procedures: “Induced Systemic Resistance Bioassays in *Arabidopsis thaliana*”

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2.3. Abstract

Plant root colonizing, fluorescent *Pseudomonas* spp. have been studied for decades for their plant growth promoting properties and their effective suppression of soil borne plant diseases. The modes of action that play a role in disease suppression by these bacteria include siderophore-mediated competition for iron, antibiosis, and induced systemic resistance (ISR). The involvement of ISR is typically studied in systems in which the *Pseudomonas* bacteria and the pathogen are inoculated and remain spatially separated on the plant, e.g. the bacteria on the root and the pathogen on the leaf, or the use of split root systems. Since no direct interactions are possible between the two populations, suppression of disease development has to be plant mediated. We discuss bacterial traits and the plant signal transduction pathways involved in *Pseudomonas* mediated ISR, in particular for the model plant *Arabidopsis thaliana*.

2.4. Text of theory

2.4.1. Background

Plant growth promoting rhizobacteria (PGPR) have been studied in detail since the early eighties of the twentieth century, stimulated by reports on increased plant growth after root colonization by these bacteria (Burr *et al.*, 1978; Geels and Schippers, 1983; Kloepper *et al.*, 1980). Increased plant growth by PGPR in most cases is due to suppression of pathogenic micro-organisms (Schippers *et al.*, 1987; Weller, 1988). The mechanisms underlying PGPR-mediated disease suppression include competition for nutrients with the pathogen, siderophore-mediated competition for iron, direct inhibition by production of antibiotics, and production of lytic enzymes, but also induced resistance (Bakker *et al.*, 1991).

Induced resistance is a state of enhanced defensive capacity developed by a plant reacting to specific biotic or chemical stimuli (Van Loon *et al.*, 1998). In 1991, two research groups discovered independently that induced systemic resistance (ISR) is a mode of action of plant growth-promoting rhizobacteria (PGPR) in suppressing diseases (Van Peer *et al.*, 1991; Wei *et*

al., 1991). The ISR-eliciting PGPR were inoculated in the plant root environment (rhizosphere) and remained spatially separated from the pathogen that was inoculated on the aboveground plant parts. It was demonstrated that ISR is also effective against root-infecting pathogens by creating and maintaining spatial separation between the PGPR bacteria and the pathogen in the rhizosphere, for instance by a split root system (Leeman *et al.*, 1995a; Zhou and Paulitz, 1994). Especially the group of fluorescent *Pseudomonas* spp. has received attention with regard to elicitation of ISR (Van Loon *et al.*, 1998; Bakker *et al.*, 2003; Bakker *et al.*, 2007). A threshold population density of 10^5 colony-forming units per gram of root was required for an effective elicitation of ISR against fusarium wilt on radish by *Pseudomonas fluorescens* WCS374r (Raaijmakers *et al.*, 1995). *P. fluorescens* strain WCS374r significantly protected radish from fusarium wilt under commercial greenhouse conditions, leading to average yield increases of 40 % (Leeman *et al.*, 1995c).

ISR is phenotypically similar to systemic acquired resistance (SAR) that is triggered by necrotizing pathogens and results in reduced disease caused by a challenging pathogen. For SAR, accumulation of salicylic acid (SA) in the plant is required (Sticher *et al.*, 1997). This was demonstrated in transgenic plants incapable of SA accumulation due to constitutive expression of the *nahG* gene, a salicylate hydroxylase gene from *P. putida*, as these plants no longer express SAR (Gaffney *et al.*, 1993). Applying SA exogenously can also trigger the SA-dependent SAR pathway. In the model plant *Arabidopsis thaliana*, ISR by *P. fluorescens* WCS417r is still operative in NahG plants (Pieterse *et al.*, 1996). Using mutants of *A. thaliana* that are non- or less responsive to ethylene (ET) or jasmonic acid (JA) it was concluded that an intact response to these plant hormones is required for expression of ISR (Pieterse *et al.*, 1998). In *Arabidopsis*, SAR is effective against pathogens that in non-induced plants are resisted through SA-dependent defenses, whereas ISR is effective against pathogens that are resisted through JA/ET-dependent defenses (Ton *et al.*, 2002). *P. syringae* pv. *tomato* is a pathogen against which both SA and JA/ET responses are effective. When ISR and SAR were simultaneously activated, enhanced disease suppression occurred against this pathogen (Van Wees *et al.*, 2000). Therefore there are opportunities to enhance the effectiveness of induced resistance. Detailed knowledge of the bacterial traits that elicit ISR and the plant signal transduction pathways involved in induced resistance will be instrumental in developing this phenomenon for commercial applications. The focus of this chapter will be on ISR elicited by fluorescent *Pseudomonas* strains in the model plant *Arabidopsis thaliana*.

2.4.2. Bacterial elicitors of ISR

Lipopolysaccharides

The lipopolysaccharides (LPS) of *Pseudomonas* strains were the first ISR elicitors studied. Reports that components of the cell surface of plant pathogenic bacteria can induce resistance (Graham *et al.*, 1977; Minardi *et al.*, 1989) stimulated a study on the role of LPS in ISR elicited in carnation by *P. fluorescens* WCS417r against *Fusarium oxysporum* f.sp. *dianthi* (Van Peer and Schippers, 1992). Both heat-killed cells and purified LPS of strain WCS417r elicited ISR to a level similar to that obtained by treatment with viable bacterial cells. Accumulation of phytoalexins after challenge inoculation with *F. oxysporum* f.sp. *dianthi* of plants treated with either viable or heat-killed cells or with LPS of WCS417, was significantly higher than in fusarium challenged control plants. LPS was also demonstrated to be of importance in ISR against *F. oxysporum* f.sp. *raphani* in radish elicited by *P. fluorescens* strains WCS374r and WCS417r (Leeman *et al.*, 1995b). In this study, both extracted LPS and mutants of the strain that lack the O-antigenic side chain of the LPS

were used. Whereas application of LPS did reduce fusarium wilt on radish to a level comparable to treatment with the wild-type bacteria, the O-antigen minus mutants did not reduce disease incidence. Similar results were observed for *P. putida* WCS358 on bean and tomato. In those studies, a mutant of WCS358 lacking the O-antigen no longer elicited ISR whereas application of LPS induced resistance (Meziane *et al.*, 2005). In *Arabidopsis*, LPS of *P. fluorescens* WCS417r and *P. putida* WCS358 appears to be involved in ISR against *P. syringae* pv *tomato*. Extracted LPS of the strains did elicit ISR but mutants lacking the O-antigen were as effective as the parental strains, suggesting redundancy in ISR elicitors (Van Wees *et al.*, 1997; Bakker *et al.*, 2003; Meziane *et al.*, 2005).

Flagella

It has been well established that bacterial flagellins, the main protein component of flagella, can elicit defense responses in plants (Gomez-Gomez and Boller, 2000; Zipfel *et al.*, 2004). The involvement of flagella in ISR has so far only been studied in the *P. putida* strain WCS358 in *Arabidopsis*, bean and tomato (Meziane *et al.*, 2005). In this study, isolated flagella and a non-motile mutant that lacks flagella were used to evaluate their importance as elicitors of ISR. Application of flagella did not induce resistance in bean or tomato, and the mutant was as effective as the wild type. It was concluded that flagella do not play a role in ISR elicited by WCS358 in bean and tomato. However, in *Arabidopsis* application of WCS358 flagella elicited ISR against *P. syringae* pv *tomato*. The non-motile mutant that lacks flagella was as effective as the wild-type WCS358 in eliciting ISR in *Arabidopsis*. Therefore, it was concluded that there are additional determinants in strain WCS358 that can induce resistance (Meziane *et al.*, 2005). These results suggest that flagella can be involved in ISR, but they are not the main ISR elicitor of this *Pseudomonas* strain.

Salicylic acid

It is well known that exogenous application of SA to plants leads to induced resistance (Sticher *et al.*, 1997). Production of significant amounts of SA has been reported for specific strains of PGPR that elicit ISR. Therefore, production of SA was suggested to be involved in ISR induced by fluorescent *Pseudomonas* spp. (Leeman *et al.*, 1996; De Meyer and Höfte, 1997; Maurhofer *et al.*, 1998). However, from studies on the role of bacterially produced SA in induced resistance, it was concluded that it is not SA itself that is the microbial signal (Press *et al.*, 1997; Audenaert *et al.*, 2002; Ran *et al.*, 2005a). SA biosynthesis is often linked to the production of SA containing siderophores, like pyochelin in *P. aeruginosa* 7NSK2 (Audenaert *et al.*, 2002) or pseudomonine in *P. fluorescens* WCS374r (Mercado-Blanco *et al.*, 2001). Therefore, these bacteria may well produce only the SA-containing siderophore instead of excreting SA into the rhizosphere. Strains that were genetically modified to produce SA but not the SA-containing siderophore, appear to elicit induced resistance through the production of SA. For instance, mutant KMPCH of *P. aeruginosa* 7NSK2 produces SA but not pyochelin. Audenaert *et al.* (2002) clearly demonstrated that production of SA is the main eliciting determinant for this mutant. Likewise, SA biosynthetic genes expressed in a non-SA-producing *P. fluorescens* strain resulted in improved ISR (Maurhofer *et al.*, 1998). Production of SA by *Pseudomonas* spp. strains is dependent on the availability of iron and it is produced only upon iron limitation. Siderophores are also produced by fluorescent pseudomonads under conditions of iron limitation and their role in ISR has been investigated by several research groups.

Siderophores

Most aerobic and facultative anaerobic micro-organisms, including fluorescent *Pseudomonas* spp., produce low-molecular-weight Fe³⁺-specific chelators, so-called siderophores under conditions of low iron availability. The siderophores sequester ferric ions in the environment and the ferrated siderophores are taken up by the microbial cells through specific recognition by membrane proteins (Höfte, 1993). Several studies have suggested that siderophores can be bacterial signals that elicit ISR. In those studies purified siderophores could elicit ISR (Leeman *et al.*, 1996; Meziane *et al.*, 2005; Ran *et al.*, 2005b), and mutants defective in siderophore production were less or non-effective compared to the wild-type. In some cases purified siderophores did trigger ISR but siderophore mutants were as effective as the wild-type strain (Leeman *et al.*, 1996; Meziane *et al.*, 2005). Apparently multiple determinants of one *Pseudomonas* strain can elicit ISR, and this redundancy becomes clear when one trait is knocked out but the other still leads to effective ISR (Bakker *et al.*, 2003). On one hand such redundancy hampers mutant studies on bacterial elicitors of ISR, but on the other hand presence of multiple inducing traits leads to robustness of the system. An iron-regulated N-alkylated benzylamine derivative was also reported to play a role in *Pseudomonas*-mediated ISR (Ongena *et al.*, 2005). For *Serratia marcescens* 90-166, mutant analysis revealed that catechol siderophore biosynthesis genes are associated with ISR in cucumber (Press *et al.*, 2001).

In earlier studies the effect of siderophores was postulated to be confined to direct interactions between the pathogen and the biocontrol agents (Loper and Buyer, 1991), but in many cases the iron-regulated metabolites of pseudomonads are involved in eliciting ISR. Similarly, antibiotics produced by biocontrol agents have a direct inhibitory effect on microbial pathogens, but may also have a role in triggering ISR.

Antibiotics

The antibiotic 2,4-diacetylphloroglucinol (DAPG) produced by fluorescent pseudomonads plays a key role in take-all decline (Raaijmakers and Weller, 1998; Weller *et al.*, 2002). The mode of action of DAPG is direct inhibition of the take-all pathogen *Gaeumannomyces graminis* var. *tritici* (Mazzola *et al.*, 1995). However, also for DAPG a role in ISR was demonstrated. In *Arabidopsis*, DAPG is the key compound in ISR elicited by *P. fluorescens* CHA0 against *Hyaloperonospora parasitica* (Iavicoli *et al.*, 2003), and by *P. fluorescens* Q2-87 against *P. syringae* pv. *tomato* (Weller *et al.*, 2004). The importance of DAPG production in ISR was supported by observations that DAPG mutants do not induce resistance and that the ability to elicit ISR is restored in complemented mutants. Moreover, the pure compound DAPG elicited ISR (Iavicoli *et al.*, 2003; Weller *et al.*, 2004). For *P. aeruginosa* 7NSK2 it is now apparent that the phenazine antibiotic pyocyanin is involved in ISR against *B. cinerea* in tomato (Audenaert *et al.*, 2002). A very interesting recent finding on the strain 7NSK2 is that the pyocyanin can have both positive and negative effects on disease development in rice, depending on the pathogen. The pyocyanin production by 7NSK2 elicits ISR against *Magnaporthe grisea* whereas it actually enhances susceptibility for *Rhizoctonia solani*. The latter was concluded from observations that the wild type could not protect the host against *R. solani* and the pyocyanin mutant could induce resistance (De Vleeschauwer *et al.*, 2006).

Additional determinants that influence ISR by fluorescent *Pseudomonas* spp. have been reported, like bacterial volatiles (Ryu *et al.*, 2004), production of *N*-acyl-l-homoserine lactone (Schuhegger

et al., 2006), 2,3-butanediol (Han *et al.*, 2006b), and gene products involved in diverse functions like a methyl-accepting chemotaxis protein, biosynthesis of purines, phospholipase C, transport of branched-chain amino acids, an ABC transporter, and gene products of unknown function (Han *et al.*, 2006a). A mutation in the *edd* gene, encoding 6-phosphogluconate dehydratase, of *P. chlororaphis* 06 resulted in less efficient tobacco root colonization and reduced elicitation of ISR against *Erwinia carotovora* (Kim *et al.*, 2007). Recently surfactin and fengycin lipopeptides produced by *Bacillus subtilis* have been implicated in elicitation of ISR in bean and tomato against *Botrytis cinerea* (Ongena *et al.*, 2007). The multitude of determinants of ISR described in a relatively short time period suggests that many more bacterial determinants will be discovered.

2.4.3. Plant defense signaling

Effectiveness of ISR and SAR

Upon challenge with a pathogen, expression of rhizobacteria-mediated ISR is similar to the classical systemic acquired resistance (SAR) elicited by a necrotizing pathogen, in that disease severity is reduced (Van Loon *et al.*, 1998). Both SAR and ISR are effective against a broad range of pathogens, however, there are some differences in their effectiveness. In *Arabidopsis*, ISR mediated by *P. fluorescens* WCS417r and SAR are effective against the bacterial leaf pathogens *P. syringae* pv. *tomato* and *Xanthomonas campestris* pv. *armoraciae*, the oomycetous leaf pathogen *H. parasitica*, and against the fungal soil-borne pathogen *F. oxysporum* f.sp. *raphani* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997; Pieterse *et al.*, 2002; Ton *et al.*, 2002). Whereas SAR is effective against Turnip crinkle virus (TCV), WCS417r-mediated ISR did not reduce the symptoms caused by TCV or accumulation of the virus, and conversely, ISR is effective against the fungal pathogen *Alternaria brassicicola* whereas SAR is not (Ton *et al.*, 2002). These differences between SAR and ISR implicate the involvement of different mechanisms. Van Wees *et al.* (2000) observed enhanced protection against *P. syringae* pv. *tomato* when SAR and ISR were activated simultaneously, which supports the hypothesis that different signal transduction pathways are induced.

ISR is SA-independent but requires an intact response to jasmonate and ethylene

The endogenous plant signal SA plays a key role in SAR as became evident from studies with plants expressing the *nahG* gene. This gene encodes a *P. putida* salicylate hydroxylase, an enzyme that converts SA into catechol. Transgenic tobacco and *Arabidopsis* plants expressing the *nahG* gene did no longer accumulate SA nor did they establish SAR upon attack by a pathogen (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). A first difference between SAR and ISR became apparent when NahG *Arabidopsis* was tested for expression of *P. fluorescens* WCS417r-mediated ISR. Whereas in *Arabidopsis* SAR requires SA signaling, ISR is independent of SA accumulation and *PR* gene expression (Pieterse *et al.*, 1996; Pieterse and Van Loon, 1999). Using the jasmonate (JA) response mutant *jar1* and the ethylene (ET) response mutant *etr1* a further distinction between SAR and ISR could be made. Intact responses to both JA and ET are required for expression of ISR but not for SAR (Pieterse *et al.*, 1998). Finally, use of the SAR regulatory mutant *npr1* (Cao *et al.*, 1994) revealed that both ISR and SAR require NPR1 (Pieterse *et al.*, 1998).

ISR is associated with priming for enhanced defense

An early increase in SA levels and expression of pathogenesis related (PR) proteins are observed at

the onset of SAR (Ryals *et al.*, 1996). Developing such markers for ISR would be most instrumental for studies on both bacterial elicitors and plant signaling. Studies intended to identify effects of the state of ISR on expression of known defense-related genes were all negative (Van Wees *et al.*, 1999). This suggested that the resistance attained was not associated with major increases in the levels of either JA or ET. Indeed, analysis of JA and ET levels in leaves of ISR-expressing plants revealed no changes in the production of these signal molecules (Pieterse *et al.*, 2000; Hase *et al.*, 2003). Therefore, it had to be assumed that the JA and ET dependency of ISR is based on an enhanced sensitivity to these hormones, rather than on an increase in their production.

To identify ISR-related genes, the transcriptional response of over 8,000 Arabidopsis genes was monitored during WCS417r-mediated ISR (Verhagen *et al.*, 2004). However, systemically in the leaves, none of the ~8,000 genes tested showed a consistent change in expression in response to effective colonization of the roots by WCS417r, indicating that the onset of ISR in the leaves is not associated with detectable changes in gene expression. However, after challenge inoculation of WCS417r-induced plants with the bacterial leaf pathogen *P. syringae* pv. *tomato*, 81 genes showed an augmented expression pattern in ISR-expressing leaves compared to inoculated control leaves, suggesting that ISR-expressing plants are primed to respond faster and/or more strongly upon pathogen attack. The majority of the primed genes was predicted to be regulated by JA and/or ET signalling, confirming earlier findings that colonization of the roots by WCS417r primed Arabidopsis plants for augmented expression of the JA- and/or ET-responsive genes *AtVSP2*, *PDF1.2* and *HEL* (Hase *et al.*, 2003; Van Wees *et al.*, 1999). Priming is a phenomenon associated with different types of induced resistance (Conrath *et al.*, 2002; Conrath *et al.*, 2006). It provides the plant with an enhanced capacity for rapid and effective activation of cellular defense responses once a pathogen is contacted, and allows the plant to react more effectively to any invader encountered, by boosting the defenses activated in the host. This mechanism could also explain the broad-spectrum action of induced resistance.

Other ISR-inducing rhizobacteria have been demonstrated to enhance the plant's defensive capacity by priming for potentiated defense-related gene expression (e.g. De Meyer *et al.*, 1999; Ahn *et al.*, 2002; Kim *et al.*, 2004; Tjamos *et al.*, 2005). This indicates that priming is a common feature in rhizobacteria-mediated ISR. Priming for defense may combine advantages of enhanced disease protection with low metabolic costs. Recently, Van Hulten *et al.* (2006) examined the costs and benefits of priming in comparison to activated defense in Arabidopsis. The study revealed that the benefits of priming-mediated resistance outweigh the costs under conditions of pathogen pressure, suggesting an evolutionary advantage of this mechanism of induced resistance over constitutive activation of defense responses.

2.4.4. Concluding remarks

Identification of bacterial elicitors of ISR relies on time-consuming bioassays in which suppression of disease symptoms and population dynamics of the pathogen are used as parameters. Development of indicator plants that contain a reporter gene that is expressed when ISR occurs, would be instrumental in identifying additional elicitors in known bacterial strains and for selecting new ISR inducers. Discovery of genes that have elevated expression after root colonization by *P. fluorescens* WCS417r (Verhagen *et al.*, 2004; Léon-Kloosterziel *et al.*, 2005) provided potential candidates for reporter gene constructs.

Improved efficacy of biocontrol by fluorescent *Pseudomonas* spp. may be established by combining SA-dependent and SA-independent induced systemic resistance (Van Wees *et al.*, 2000). Several *Pseudomonas* strains are potentially able to induce the SA-dependent signal transduction pathway because they can produce relatively high amounts of SA (in the 10-100 μ M range) when grown under conditions of low iron availability. However, ISR by SA-producing strains seems independent of SA, and it is speculated that in most cases the bacterially produced SA is not excreted but is incorporated into siderophores (Audenaert *et al.*, 2002; Mercado-Blanco *et al.*, 2001; Ran *et al.*, 2005b). By either uncoupling SA production from the biosynthesis of SA-containing siderophores (Audenaert *et al.*, 2002) or by transfer of SA biosynthesis genes into non-SA-producers (Maurhofer *et al.*, 1998), a more effective ISR could be established. Production of SA by strains that also elicit SA-independent ISR could induce both signal transduction pathways simultaneously, leading not only to enhanced protection against certain pathogens (Van Wees *et al.*, 2000), but possibly also to protection against a wider range of pathogens (Ton *et al.*, 2002). The observed redundancy in ISR-eliciting determinants in PGPR may guarantee the robustness of ISR. If one determinant fails to elicit ISR or is not produced under certain conditions, other elicitors can still be effective. In such cases it would be advantageous if the different traits were also differentially regulated. Increased knowledge on the variety of bacterial determinants of ISR and their regulation in the rhizosphere will not only increase our fundamental understanding of plant-microbe interactions in this highly dynamic environment, but also provide opportunities to exploit this mode of action of PGPR in crop protection strategies.

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