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Understanding the involvement of rhizobacteria-mediated induction of systemic resistance in biocontrol of plant diseases

P.A.H.M. Bakker, L.X. Ran, C.M.J. Pieterse, and L.C. van Loon

Abstract: Specific strains of nonpathogenic rhizobacteria can induce systemic resistance that is effective against a range of plant pathogens. To exploit induced systemic resistance, detailed knowledge of the triggering bacterial traits involved and on signal transduction pathways in the plant is necessary. Possibilities to improve effectiveness of induced resistance by rhizobacterial strains are discussed.

Key words: induced systemic resistance, lipopolysaccharides, *Pseudomonas fluorescens*, *Pseudomonas putida*, salicylic acid, siderophores, signaling pathways.

Résumé : Des souches spécifiques de rhizobactéries non pathogènes peuvent induire, chez les plantes, une résistance systémique efficace contre un ensemble d'agents pathogènes. Afin de pouvoir tirer profit de la résistance systémique induite, une connaissance détaillée des caractéristiques bactériennes liées au déclenchement et des voies de transmission du signal dans la plante est nécessaire. Les possibilités d'amélioration de l'efficacité de la résistance induite par des souches rhizobactériennes sont discutées.

Mots clés : résistance systémique induite, lipopolysaccharides, *Pseudomonas fluorescens*, *Pseudomonas putida*, acide salicylique, sidérophores, voies de signalisation.

Introduction

The estimated number of prokaryotic cells in our planet's soil is 2.6×10^{29} , providing an enormous capacity for genetic diversity (Whitman et al. 1998) and a great potential for exploitation. One of the uses of prokaryotes from soil is for biological control of soilborne plant diseases (Handelsman and Stabb 1996). Particularly, strains of plant-root-inhabiting fluorescent *Pseudomonas* spp. have been studied in detail for their disease suppressive properties. In a recent review, Weller et al. (2002) described the importance of these bacteria in soil suppressiveness against *Gaeumannomyces graminis* (Sacc.) Arx. & D. Olivier var. *tritici* J. Walker. Active and long-term colonization of the root surface of the plant (Lugtenberg et al. 2001), and effective expression of disease-suppressive traits (Handelsman and Stabb 1996), are generally considered prerequisites for successful suppression of soilborne diseases by rhizobacteria. The mechanisms involved in disease suppression

are diverse and include competition for iron, production of antibiotics, and induction of systemic resistance (Handelsman and Stabb 1996).

Induced systemic resistance

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (van Loon et al. 1998). In 1991, two research groups independently described induced systemic resistance (ISR) as the mode of action of disease suppression by nonpathogenic rhizosphere bacteria (Van Peer et al. 1991; Wei et al. 1991). Since then, the involvement of ISR in disease suppression has been studied for a wide range of biological control microorganisms and, in many cases, ISR was found to be involved. Phenotypically, ISR is similar to systemic acquired resistance (SAR) that is triggered by necrotizing pathogens. Although the terms SAR and ISR are synonymous, for convenience in the present study, we wish to distinguish between pathogen- and rhizobacteria-induced resistance, by using SAR for the pathogen-induced type and ISR for the rhizobacteria-induced type. Whereas SAR requires accumulation of salicylic acid (SA) in the plant (Sticher et al. 1997), in *Arabidopsis thaliana* (L.) Heynh., ISR does not and, instead, is dependent on intact responses to ethylene and jasmonic acid (JA) (Pieterse et al. 1998). When these different signal transduction pathways are triggered simulta-

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neously in *A. thaliana*, disease suppression is enhanced (Van Wees et al. 2000). This suggests that combining bacterial traits that trigger either the SA-, or the ethylene- or JA-dependent response can improve biological control. Elucidating the nature of bacterial triggers of the different pathways will enable us to utilize ISR in a sensible way.

Distinguishing between ISR and direct effects

Arabidopsis mutants that are insensitive to ethylene (*etr1*) or JA (*jar1*) and the *Arabidopsis* mutant expressing no pathogenesis-related proteins for resistance to *Pseudomonas fluorescens* Migula WCS417r (Pieterse et al. 1998). Use of the *etr1*, *jar1*, and *npr1* mutants can enable us to determine whether direct interactions between biocontrol agents and the pathogen are effective in disease suppression. When the biocontrol bacteria and the pathogen are coinoculated into the rhizospheres of these mutants, there will be protection only if the bacteria can directly inhibit the development of the pathogen. When the bacteria fail to suppress disease in the mutants, but are effective on the wild-type plants, ISR must be the main mode of action.

Role of salicylic acid

A rhizobacterial metabolite that was suggested to trigger the SA-dependent signal transduction pathway is SA itself (De Meyer and Höfte 1997; Leeman et al. 1996; Maurhofer et al. 1998). Salicylic acid production has been observed for several bacterial strains, and exogenously applied SA can induce resistance in many plant species. For example, *P. fluorescens* strain WCS374 produces relatively large quantities of SA under conditions of iron limitation. Moreover, WCS374 is more effective, in radish, in ISR against fusarium wilt under iron-limited conditions compared with conditions of sufficient iron availability (Leeman et al. 1995). Pseudobactin siderophores, low-molecular-weight molecules, are also produced by WCS374 under iron-limited conditions. However, a pseudobactin mutant of WCS374 was as effective as the parental strain in disease suppression. Therefore, it was concluded that SA was probably responsible for the increased effectiveness of WCS374 under conditions of iron limitation. However, no indications for activation of SA-dependent signaling in radish after WCS374 treatment were observed (Hoffland et al. 1995). Moreover, treatment with strain WCS374 cannot induce systemic resistance in *A. thaliana*, whereas the application of SA does (Van Wees et al. 1997). Possibly, the iron-regulated ISR by WCS374 in radish is mediated by the SA-containing siderophore pseudomonine (Mercado-Blanco et al. 2001). Pseudomonine is a nonfluorescent siderophore, produced by WCS374, that was discovered when analyzing nonfluorescent mutants of this strain (Figs. 1 and 2). Whether pseudomonine is active in ISR remains to be elucidated. The plant-root-colonizing *Pseudomonas aeruginosa* (Schroeter) Migula strain 7NSK2 induces resistance in tobacco, bean, and tomato. Its ability of ISR has been linked to production of SA (De Meyer and Höfte 1997). However, recent evidence strongly suggests that SA is not the inducing compound from 7NSK2, but

that the compounds pyochelin and pyocyanin produced by this strain are a prerequisite for the induction of resistance (Audenaert et al. 2002). It was postulated that 7NSK2 does not produce SA in the rhizosphere, but instead, that the SA is channeled into the siderophore pyochelin (Audenaert et al. 2002). Thus, for several bacterial strains, a role of bacterially produced SA in induced resistance has been suggested, but upon careful examination, SA seems not to be involved directly (Audenaert et al. 2002; van Loon et al. 1998).

Role of siderophores

Siderophores are low-molecular-weight molecules that are secreted by microorganisms to take up iron from the environment (Höfte 1993), and their modes of action in suppression of disease were thought to be solely based on competition for iron with the pathogen (Bakker et al. 1993; Duijff et al. 1999; Loper and Buyer 1991). Interestingly, siderophores can induce systemic resistance (ISR) (Leeman et al. 1996). One may therefore consider whether the mode of action of other bacterial metabolites that have been implicated in disease suppression also involves triggering of systemic resistance mediated by rhizobacteria.

Role of antibiotics

Production of antibiotics has been described as a powerful mode of action in disease suppression by which development and (or) activity of the pathogen is thought to be directly inhibited (Handelsman and Stabb 1996). The involvement of antibiotic production in ISR has not been investigated in detail. However, from studies that utilized strains of pathogens varying in their sensitivity to the antibiotics produced by the biocontrol bacteria, ISR does not seem to be involved. Isolates of *G. graminis* var. *tritici* that varied in their sensitivity to the antibiotics phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol were also differentially sensitive to biological control by *Pseudomonas* spp. strains producing these same antibiotics (Mazzola et al. 1995). These results demonstrated that the effect of these antibiotics on the pathogen was direct and not mediated by the plant. However, antibiotics do have direct effects on plants and, therefore, might induce systemic resistance mediated by rhizobacteria. For example, the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol reduced growth of sweet corn, cress, and cucumber (Maurhofer et al. 1992) and, in turn, the stress caused by these antibiotics may also trigger resistance. To further study the possible involvement of antibiotics in ISR, strains of biocontrol bacteria that produce specific antibiotics should be applied spatially separated from the pathogen on the plant surface, to prevent direct interactions between their populations (van Loon et al. 1998). If these strains induce systemic resistance, their mutants lacking antibiotic production should be tested. Conversely, the purified compound can be tested in an experimental setup for resistance induction.

Fig. 1. Production of fluorescent and nonfluorescent siderophores by fluorescent *Pseudomonas* spp. *Pseudomonas fluorescens* WCS374 (1), a nonfluorescent transposon mutant of WCS374 (2), a *P. putida* strain (3), and its nonfluorescent mutant (4) were grown on King's medium B (King et al. 1954) (A) and on chrome azurol sulfonate (CAS) medium (Schwyn and Neilands 1987) (B). On King's medium B, the pseudobactin-type siderophore fluoresces under UV and, on CAS medium, siderophore activity leads to production of an orange halo. Whereas the nonfluorescent mutant of *P. putida* does not produce a halo on CAS, the mutant of WCS374 still does, suggesting the presence of a second, nonfluorescent siderophore in WCS374.

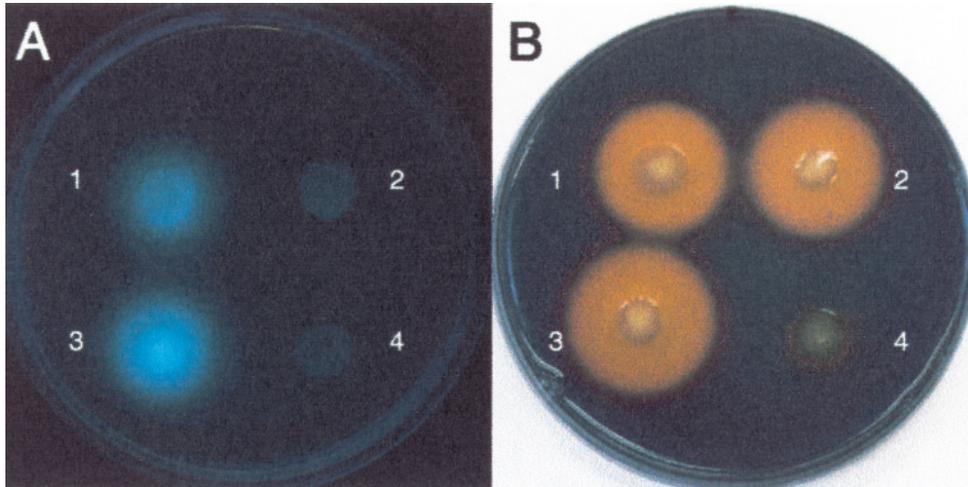
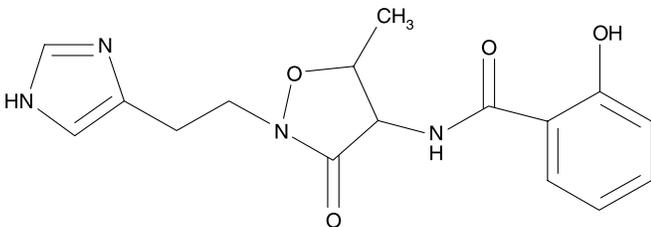


Fig. 2. Structure of pseudomonine, the nonfluorescent siderophore produced by WCS374 (Mercado-Blanco et al. 2001). The molecule contains histamine, a cyclothreonine moiety in the middle, and, interestingly, a SA group. It has been postulated that, in the rhizosphere, WCS374 does not produce SA, but instead, the SA is channeled into pseudomonine.



Role of lipopolysaccharides

Lipopolysaccharides (LPS) have been implicated in ISR triggered by fluorescent pseudomonads in carnation (Van Peer and Schippers 1992), radish (Leeman et al. 1995), and *A. thaliana* (Van Wees et al. 1997). In the case of ISR against *Globodera pallida* (Stone) Behrens on potato by *Rhizobium etli* Segovia et al., the LPS also plays a major role (Reitz et al. 2002). In these studies, either purified LPS was used to induce systemic resistance, or mutants that lack part of the LPS were compared with the parental strain regarding their abilities to induce resistance. Another bacterial determinant that was suggested to be involved in ISR is flagellin, the protein subunit of the flagella.

Research approach

It appears that in most inducing bacteria, more than one determinant is operative in triggering systemic resistance (ISR) in the plant. The classical approach of studying the mode of action of biocontrol bacteria by generating specific

mutants is, therefore, not very informative for studying traits involved in ISR. If one trait is knocked out in the bacterium, the other trait(s) still induce resistance and the mutant is as effective as the parental strain in suppressing disease. In studying induced resistance mediated by *P. putida* WCS358 on *A. thaliana*, we have experienced this problem. The fluorescent pseudobactin siderophore, the O-antigen of the LPS, and flagellin all induce resistance when they are isolated and applied to the plant roots. Mutants that are defective in one of these three traits are as effective as the parental strain in disease suppression. Even double mutants are still effective in ISR. An alternative approach is to mobilize a gene bank from a strain with ISR ability into a strain with no such ability and, in such a way, identify genes that encode bacterial traits for ISR. Also, the use of indicator plants that contain a reporter gene expressed when ISR occurs would be very helpful in studies for identifying the inducing bacterial determinants. A major problem that has hampered the development of such reporter lines is the lack of known, defense-related genes responding to the bacterial treatments (Van Wees et al. 1999). The development of gene chip technology is a powerful tool to study gene expression patterns in response to ISR. Currently, transcript profiles of *Arabidopsis* genes are analyzed using DNA microarrays (Pieterse et al. 2002). It appears that several genes are up or down regulated when ISR occurs (B.W.M. Verhagen and C.M.J. Pieterse, personal communication).

Role of population densities

In a dose-response study of ISR mediated by *P. fluorescens* WCS374 in radish, it was revealed that population densities of 10^5 colony forming units per gram of root are required for significant suppression of disease (Raaijmakers et al. 1995). In situations where no bacteria are introduced, population densities of one single bacterial genotype probably never reach such high densities, making

it unlikely that, in the field, plants are triggered sufficiently by bacteria to express ISR. *Pseudomonas fluorescens* strain RS111 and a derivative of this strain, RS111a, have been studied mainly for their abilities to suppress disease when applied in combination with other fluorescent *Pseudomonas* spp. strains (De Boer et al. 1999). In dose-response studies, it was demonstrated that RS111a was effective in suppressing fusarium wilt of both radish and flax at much lower initial population densities than RS111 (K.C. Zhang, L.C. van Loon, and P.A.H.M. Bakker, unpublished data). Moreover, RS111a suppressed fusarium wilt significantly better than RS111 when applied in a bioassay specific for disease suppression through ISR (De Boer 2000). These results suggest that the level of disease control through ISR by a specific strain can be improved. Further studies to elucidate the determinants of ISR of these strains are ongoing.

Combining mechanisms of disease suppression

Since ISR and antibiosis seem to be two separate mechanisms of disease suppression by fluorescent pseudomonads, the combination of these modes of action may well lead to improved efficacy of biological control. The antibiosis would first weaken or even partly kill the population of the pathogen and, subsequently, this weakened population would be confronted with a plant that is in a state of enhanced defensive capacity, thus resulting in much less disease development. One could also argue that use of strains that trigger both the JA- or ethylene-dependent and the SA-dependent pathway may increase the efficacy of biological control. The latter strategy may require manipulation of bacterial metabolites that trigger the SA-dependent signal transduction pathway. A way to manipulate the production of SA by bacteria such that it can contribute to induction of systemic resistance is to uncouple SA production from the biosynthesis of the SA-containing siderophore. In mutant KMPCH of *P. aeruginosa* 7NSK2, which is unable to synthesize pyochelin and therefore accumulates SA, induction of resistance is indeed mediated by SA (Audenaert et al. 2002). Transfer of the SA-biosynthetic genes of *P. aeruginosa* to *P. fluorescens* strain P3 led to induced resistance in tobacco against tobacco necrosis virus after treatment with the SA-producing derivative of P3 (Maurhofer et al. 1998). The latter strategy requires use of genetically modified bacteria for field release and, before such releases, the impact of the modified bacteria on resident microbial communities has to be examined. It has also been questioned if induced resistance may affect the belowground community of plants (Sonnemann et al. 2002). Use of recently developed techniques that take into account the total microflora, including those that are nonculturable, will be valuable in this respect (Glandorf et al. 2001). Also, the state of enhanced defense capacity may negatively affect plant growth because of fitness costs (Heil 2001). In contrast, a significant growth stimulation was observed in *A. thaliana* plants treated with the *P. fluorescens* WCS417r inducing systemic resistance (Pieterse and van Loon 1999).

In conclusion, ISR is a powerful mode of action in biological control of both soilborne and aerial plant diseases. Since ISR by one rhizobacterial strain can be effective

against a range of plant pathogens, including bacteria, oomycetes, and fungi (Ton et al. 2002), this technology may contribute significantly to reducing the input of plant protecting chemicals.

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