Induction and expression of PGPR-mediated induced resistance against pathogens

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Abstract

Treatment of plants with selected strains of plant growth-promoting rhizobacteria (PGPR) can induce systemic resistance in carnation, cucumber, radish, tobacco, and Arabidopsis as evidenced by an enhanced defensive capacity upon challenge inoculation with a pathogen. In the induction of resistance by Pseudomonas spp. in carnation, radish, and Arabidopsis, the O-antigenic side chain of the bacterial outer membrane lipopolysaccharide acts as an inducing determinant, but other bacterial traits are also involved. Siderophores have been implicated in the induction of resistance in tobacco and Arabidopsis, and a novel type of siderophore, fluorebactin, may explain induction of resistance associated with salicylic acid (SA) in radish. Although some bacterial strains are equally effective in inducing resistance in different plant species, others show specificity which suggests specific recognition between bacteria and plants at the root surface. Moreover, genetic variation for inducibility by specific PGPR strains is present in carnation and Arabidopsis.

In contrast to the phenotypically similar systemic acquired resistance (SAR) induced by pathogens, PGPR-mediated induced systemic resistance (ISR) does not always require SA. SAR-associated SA production induces pathogenesis-related proteins (PRs), but no accumulation of PRs was detectable in radish and Arabidopsis expressing ISR. In addition, ISR is fully expressed in Arabidopsis plants transformed with the NahG gene and unable to accumulate SA. In contrast, Arabidopsis mutated in the Etr1 gene and insensitive to ethylene, or in the jar1 gene and insensitive to jasmonic acid, were no longer inducible. These results demonstrate that compared to pathogens inducing SAR, non-pathogenic rhizobacteria inducing ISR trigger a different signal-transduction pathway not dependent on the accumulation of SA and activation of PR-genes, but dependent on perception of ethylene and jasmonic acid.

Introduction

Rhizobacteria-mediated induced systemic resistance. Plant growth-promoting rhizobacteria (PGPR) can suppress diseases through antagonism between the bacteria and soilborne pathogens, as well as by inducing a systemic resistance in the plant against both root and foliar pathogens (Schippers 1988). In enhancing the defensive capacity throughout the plant, rhizobacteria seem to activate a signal-transduction pathway leading to the well-studied phenomenon of systemic acquired resistance (SAR) (Hammerschmidt and Kuc 1995, Ryals et al. 1996). However, SAR is induced by limited pathogen infection, avirulent pathogens or cultivar-nonpathogenic races of pathogens causing local necrosis, such as a hypersensitive reaction. PGPR are non-pathogenic and generally considered to not cause harmful effects in the host. SAR is dependent on the production of salicylic acid (SA) in response to infection (Gaffney et al. 1993) and is associated with the accumulation of pathogenesis-related proteins (PRs) both in the inoculated and in distant leaves (Ryals et al. 1996, Van Loon and Van
Kammen 1970). Because exogenous application of SA induces both SAR and PRs, the latter are commonly taken as markers of the induced state.

SAR is non-specific with respect to both the inducing and the challenging pathogen. Thus, a primary infection of cucumber with the fungus *Colletotrichum lagenarium* or with tobacco necrosis virus (TNV) leads to enhanced resistance against various foliar and root diseases caused by fungi, bacteria, and viruses (Kuc 1982). Selected rhizobacterial PGPR strains similarly protected cucumber against anthracnose caused by the fungus *C. orbiculare* (Wei et al. 1991), Fusarium wilt caused by the fungus *Fusarium oxysporum* f.sp. *cucumerinum* (Liu et al. 1995a), bacterial angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* (Liu et al. 1995b), cucurbit wilt disease caused by the bacterium *Erwinia tracheiphila* (Kloepper et al. 1993), and mosaic disease caused by cucumber mosaic virus (Liu et al. 1992). This type of protection was named induced systemic resistance (ISR) as a more general term encompassing SAR (Kloepper et al. 1992).

To conclude that the mechanism responsible for protection is ISR requires that disease suppression be shown to be plant-mediated and that it extends to plant parts that are not in contact with the inducing agent. Accumulation of PRs could additionally be used as an indication that SAR had been induced, although by itself it provides insufficient evidence that induced resistance is the sole mechanism involved. To protect cucumber plants, PGPR strains have been applied by seed treatment, cotyledon injection, or as a soil drench. Under these conditions, the bacteria could invade the plant vascular system and be carried to the aerial parts of the plants (Kluepfel 1993). In the case of protection against Fusarium wilt, it was shown that the inducing bacteria applied to one part of a split root system did not move to the part inoculated with the pathogen (Liu et al. 1995a), indicating protection through ISR. The other studies did not address this question in detail, but it was mentioned that bacteria inducing systemic resistance were not translocated and did not colonize challenged leaves (Liu et al. 1992, 1995b). It cannot be ruled out, however, that antimicrobial metabolites are produced by rhizobacterial strains and transported through the plant in doses sufficient to reduce pathogen activity. In none of these studies were protected plants analyzed for accumulation of PRs, leaving the question whether the protection induced by the PGPR strains was similar to SAR.

Systemic induction of the major PRs of tobacco was associated with ISR against TNV in tobacco treated with the PGPR strain *Pseudomonas fluorescens* CHA0 (Maurhofer et al. 1994). The level of resistance attained was similar to the SAR developing in plants by prior inoculation with TNV. Strain CHA400, a siderophore (pyoverdin)-negative mutant of CHA0, induced partial resistance against the virus, suggesting the involvement of pyoverdin in elicitation of ISR (Table 1). Root colonization of tobacco plants with CHA0 caused an increase in SA in leaves, as did leaf infection with TNV. These results indicate that in tobacco rhizobacteria-mediated ISR and pathogen-induced SAR are both phenotypically and mechanistically similar, with ISR, at least partly, being determined by the siderophore(s) of CHA0. However, the transposon insertion generating the pyoverdin-minus mutation in CHA400 was not localized, and it is not clear whether the loss of pyoverdin is the only mutation in CHA400. Besides pyoverdin, CHA0 produces several metabolites with potentially toxic effects on micro-organisms and plants, including SA (Meyer et al. 1992). Therefore, it is not clear whether the increase in SA in bacterized plants is the result of induction by the bacteria of the synthesis of SA in the plant, or whether the plant takes up bacterial SA and translocates it to the leaves.
Induction of systemic resistance by specific *Pseudomonas* strains has likewise been shown in carnation (Van Peer et al. 1991), radish (Leeman et al. 1995a), *Arabidopsis* (Pieterse et al. 1996) and bean (De Meyer and Höfte 1997). In carnation, *P. fluorescens* strain WCS417 remained confined to the roots and protected plants that were subsequently stem-inoculated with *F. oxysporum* f.sp. *dianthi* from Fusarium wilt. Radish was protected against *Alternaria brassicicola* and different isolates of *F. oxysporum*, as well as *P. syringae* pv. *tomato* (Hoffland et al. 1996). *Arabidopsis* similarly developed ISR against *Peronospora parasitica* (J. Ton unpublished data), *F. oxysporum* f.sp.*raphani* and *P. syringae* pv. *tomato* (Pieterse et al. 1996, Van Wees et al. 1997). To demonstrate ISR against root pathogens, a separate inoculation system was developed (Leeman et al. 1995a) in which roots are placed horizontally on rockwool cubes. Adjoining rockwool cubes are compartmentalized through enclosure in plastic bags with only a small incision in the bags allowing protrusion of the radicle into the next compartment. The lower part of the root in one compartment is treated with a bacterial suspension in talcum emulsion. After a few days the upper part of the root in the other compartment is inoculated with the challenge pathogen. In no case were inducing bacteria found to be present in the compartment harboring the challenge pathogen. When the challenger was a leaf pathogen, bacteria were mixed through the soil in which seedlings were planted, and leaves were inoculated at various times afterwards. Induction of resistance against *Botrytis cinerea* was demonstrated in bean upon seed treatment with *P. aeruginosa* strain 7NSK2 (De Meyer and Höfte 1997). Under none of these conditions, were inducing bacteria recovered from the above-ground parts, suggesting that it was highly likely that reduced symptom expression upon challenge with foliar pathogens was due to ISR.

**Table 1.** Bacterial determinants of induced systemic resistance.

<table>
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<th>Bacterial strain</th>
<th>Plant species: bacterial determinant</th>
<th>Reference</th>
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| *Pseudomonas aeruginosa* strain 7NSK2 | Tobacco: salicylic acid  
Bean: salicylic acid | De Meyer et al. 1996  
De Meyer and Höfte 1997 |
| *P. fluorescens* CHAO WCS374  | Tobacco: siderophore  
Radish: lipopolysaccharide siderophore  
iron-regulated factor  
Carnation: lipopolysaccharide  
Radish: lipopolysaccharide iron-regulated factor  
*Arabidopsis*: lipopolysaccharide | Maurhofer et al. 1994  
Leeman et al. 1995b  
Leeman et al. 1996  
Leeman et al. 1996  
Van Peer and Schippers 1992  
Leeman et al. 1995b  
Leeman et al. 1996  
Van Wees et al. 1997 |
| *P. putida* WCS358       | *Arabidopsis*: lipopolysaccharide siderophore | Bakker and Van der Sluis unpublished data  
Bakker and Van der Sluis unpublished data |
**Bacterial determinants of induced systemic resistance.** Of three bacterial strains tested in our group, *P. fluorescens* WCS417 and WCS374 induced systemic resistance in radish against *F. oxysporum* f.sp. *raphani*, whereas, *P. putida* WCS358 did not (Leeman et al. 1995a). All three strains colonized radish roots to the same extent, indicating that both WCS417 and WCS374 possess inducing determinants that are lacking in WCS358. Heat-killed cells were similarly effective and the component responsible was identified as the outer membrane lipopolysaccharide (LPS) (Leeman et al. 1995b) (Table 1). Phage-resistant mutants of WCS417 and WCS374 that lacked the O-antigenic side-chain of the LPS (OA-minus), but colonized radish roots to the same extent as the wild-type strains, were not protective, demonstrating that it is the O-antigenic side-chain of the LPS that was responsible for the induction. Similarly, purified LPS of WCS417 was as effective as live cells in eliciting ISR in carnation (Van Peer and Schippers 1992), establishing the LPS as the main bacterial determinant responsible.

In assays in which radish plants in the separate inoculation system received nutrient solution containing EDDHA as an iron chelator to establish low-iron conditions, possible inducing effects of bacterial siderophores (pseudobactins) were tested. WCS358 and its siderophore-minus mutant did not induce resistance, but WCS374, its siderophore-minus and also its OA-minus mutant did induce resistance. Although the effect was not statistically significant, the same effects were observed for WCS417 and its mutants. Thus, under iron-limited conditions both WCS374 and WCS417 expressed an additional determinant that elicits ISR in radish (Leeman et al. 1996).

Testing the purified pseudobactins of the three strains revealed that the siderophores of WCS358 and WCS417 did not induce resistance, but the one of WCS374 did induce resistance. Even so, the latter was not necessary for the induction by live bacteria, because the WCS374 siderophore-minus mutant was as effective in eliciting ISR as the wild type under low-iron conditions. Therefore, a different iron-regulated metabolite must be responsible, as must also apply to WCS417 (Table 1). Like pseudobactins, SA is produced by several bacteria and acts as an additional iron-chelating siderophore under low-iron conditions (Meyer et al. 1992). Tests for the possible production of SA by the three strains revealed that in vitro at low iron availability strains WCS374 and WCS417 produced approximately 50 and 10 μg SA/ml in standard succinate medium, respectively, whereas, WCS358 did not produce SA. The production of SA by WCS374 and WCS417 decreased concomitantly with the production of pseudobactin when iron availability was increased. SA itself induced resistance in radish at concentrations as low as 100 fg per root tip when applied in talcum emulsion in the separate inoculation system (Leeman et al. 1996). Thus, SA production appeared to fulfill the criteria for the additional bacterial determinant responsible for the induction of systemic resistance under low-iron conditions.

Induction of resistance by *P. aeruginosa* 7NSK2 in bean is likewise iron-regulated (De Meyer and Høfte 1997). Strain 7NSK2 produces three siderophores under iron limitation, pyoverdin, pyochelin, and SA. By using mutants deficient in one or more siderophores, it was demonstrated that SA production was essential for induction of resistance against *B. cinerea*. As SA is itself part of bacterial siderophores of the pyochelin type, a role for this siderophore was not excluded. SA produced by 7NSK2 appeared similarly required for its elicitation of ISR in tobacco against tobacco mosaic virus (De Meyer et al. 1996) (Table 1). In contrast, iron-regulated induction of systemic resistance in cucumber and tobacco by the rhizobacterial strain *Serratia marcescens* 90-166 was not primarily determined by SA, even though strain 90-166 is capable of producing SA in vitro (Press et al. 1997). These observations suggest that
SA-containing siderophores rather than free SA might act as inducers.

To investigate the possible role of SA in the elicitation of ISR in radish under low-iron conditions, the SA-biosynthetic genes from WCS374 were cloned. By mobilizing a gene bank of WCS374 into the WCS358 siderophore-minus mutant, a 28 kb cosmid clone was identified. A 5 kb EcoRI fragment of this clone was sufficient to convert WCS358, as well as *E. coli*, into SA producers (Mercado-Blanco et al. 1998). This region appeared to be part of a larger operon containing enzymes for the synthesis of an enterobactin type siderophore. Indeed, when the complete 28 kb cosmid clone was transferred into the WCS358 siderophore-minus recipient, a novel siderophore fluorescing blue-green under UV light was produced. This novel siderophore has been designated fluorebactin. Secretion of large quantities of SA by WCS374 in vitro may be an artefact resulting from lack of substrate(s) required for fluorebactin synthesis. If so, synthesis of fluorebactin rather than secretion of SA could explain induction of resistance in radish under low-iron conditions.

Both WCS374 and WCS417, but not WCS358, induced resistance in radish. In contrast, in *Arabidopsis* WCS374 was ineffective, whereas both WCS417 and WCS358 elicited ISR (Van Wees et al. 1997). Apparently, none of the determinants of WCS374 capable of inducing systemic resistance in radish is expressed on, or recognized by *Arabidopsis* roots. Since the O-antigenic side chain of WCS417 acts as the major bacterial determinant in the induction of systemic resistance in carnation and radish, crude cell wall preparations of WCS417 and WCS358 were tested for their capacity to elicit ISR in *Arabidopsis*. Cell walls of both bacterial strains induced systemic resistance (Table 1), but the level attained commonly fell short of that effected by live bacteria (Van Wees et al. 1997). Testing of the OA-minus mutants revealed that these were still inducive, whether tested under low- or under high-iron conditions. However, cell walls of the WCS417 OA-minus mutant no longer induced resistance. These results demonstrated that induction of systemic resistance in *Arabidopsis* by either WCS417 or WCS358 depends on the action of at least two bacterial determinants: the O-antigenic side-chain of the LPS and a metabolite(s) produced by living cells. Like the OA-minus mutant of WCS358, its siderophore-minus mutant still protected *Arabidopsis* against *P. syringae* pv. *tomato*. Moreover, purified pseudobactin of WCS358 induced resistance. These observations are suggestive of iron limitation occurring in the rhizosphere of *Arabidopsis*. Thus, at least for WCS358, both the O-antigenic side-chain of the LPS and the siderophore produced act as factors eliciting ISR (P.A.H.M. Bakker and I. van der Sluis unpublished data).

**Plant responses to induction of systemic resistance by rhizobacteria.** In both pathogen-induced SAR and rhizobacterially-mediated ISR a signal is generated upon induction that is transported systemically throughout the plant, and leads to the state of induced resistance. Although SA can induce both PRs and SAR (Ryals et al. 1996), it is not the translocated signal. Rather SA is required in signal transduction (Vernooij et al. 1994). PRs were associated with the induction of systemic resistance in tobacco by *P. fluorescens* CHA0 (Maurohofer et al. 1994), suggesting that SA might be involved. However, so far this is the only report linking ISR with PRs. No accumulation of proteins cross-reacting with antibodies against PRs was apparent upon induction of ISR in radish by WCS417 (Hoffland et al. 1995) or WCS374 (unpublished data), whereas, such accumulation was readily apparent upon induction of SAR by either *P. syringae* pv. *tomato* or SA. Also in *Arabidopsis*, induction of systemic resistance by the rhizobacterial strains was not associated with the accumulation of PRs (Pieterse et al. 1996).
To determine whether SA is required for the establishment of ISR, experiments were conducted using *Arabidopsis* transformed with the *NahG* gene from *P. putida*. The *NahG* gene encodes a salicylate hydroxylase, that converts SA into catechol, preventing SA action. Both development of SAR and accumulation of PRs in response to necrotizing infection are impaired in this transformant (Gaffney et al. 1993). However, WCS417 and WCS358 induced the same extent of protection in untransformed *Arabidopsis* and in the *NahG* transformant, indicating that the induction of systemic resistance by these rhizobacterial strains is independent of SA accumulation (Pieterse et al. 1996, Van Wees et al. 1997). WCS358 is not capable of producing SA and, thus, any ISR dependent on SA would require the plant to produce SA endogenously. However, eliminating the inducing action of SA in the plant still allowed the rhizobacteria to induce resistance. Often, the ISR elicited by rhizobacteria was as strong as pathogen-induced SAR. Hence, whereas, SA is required for the induction of SAR by necrotizing pathogens, it is not required for the phenotypically similar ISR induced by selected strains of non-pathogenic rhizobacteria. Any SA that may be produced by those rhizobacteria likewise does not seem to be involved in generating the induced state.

Within the species *A. thaliana*, differences in inducibility by rhizobacteria were found among ecotypes (Ton et al. 1998, Van Wees et al. 1997). Thus, ecotypes Columbia and Landsberg *erecta* were inducible, as were most other ecotypes tested. In contrast, ecotypes RLD and Ws-O were not. The roots of the latter were colonized to similar extents as those of Columbia and Landsberg *erecta*, suggesting that these ecotypes lack receptors for the bacterial inducing determinants, or are impaired in the subsequent signal-transduction pathway. Both RLD and Ws-O display SAR upon induction with either *P. syringae* pv. *tomato* or SA, indicating that at least the signal-transduction pathway leading to SAR is unimpaired.

Because PRs have been repeatedly suggested to be markers of the induced state (Ryals et al. 1996), and at least some of the PRs have antifungal and antimicrobial activities, PRs have been considered to play a major role in the enhanced defensive capacity of induced plants that is expressed upon challenge inoculation (Van Loon 1997). In spite of repeated attempts, we have been unable to specifically link the induced state in carnation, radish, or *Arabidopsis* to alterations in enzyme activities, inhibitory compounds, electrophoretic protein patterns, or gene expression. However, we recently demonstrated that *Arabidopsis* mutated in the *etr* gene, and as a result insensitive to the plant hormone ethylene (Chang et al. 1993), is no longer inducible by rhizobacteria (Pieterse et al. 1998). Induction of SAR by pathogens or SA occurs normally (Lawton et al. 1995). Using an *Arabidopsis* mutant insensitive to jasmonic acid (JA), *jar1* (Staswick et al. 1992), similar results were obtained (Pieterse et al. 1998). Exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid or of jasmonic acid induced protection against *P. syringae* pv. *tomato* similar to ISR. These observations indicate that our inducing rhizobacteria activate a signal-transduction pathway different from the one leading to SAR, requiring perception of both ethylene and JA rather than SA, even though both pathways result in a phenotypically similar enhanced defensive capacity expressed upon challenge inoculation. Both ethylene and JA are produced by and act as hormones in plants. Our results suggest that recognition of the inducing bacteria by the roots may result in a change in ethylene and JA production or metabolism in the plant.

The availability of bacterial strains that differentially induce ISR and plant ecotypes that are differentially inducible as well as defined *Arabidopsis* mutants with defects in various signal-transduction pathways and defensive activities now offers an excellent starting point for elucidating the physiological, biochemical, and molecular mechanisms involved in PGPR-mediated ISR.
Literature


