

Mechanisms of PGPR-induced resistance against pathogens

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Summary : Plant growth-promoting rhizobacteria 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. Specific *Pseudomonas* strains induce systemic resistance in carnation, cucumber, radish, tobacco and *Arabidopsis*, as evidenced by an enhanced defensive capacity upon challenge inoculation. 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 SA induces phenotypically similar systemic acquired resistance, it is not necessary for the systemic resistance induced by most rhizobacterial strains, because this induced resistance is not associated with the accumulation of pathogenesis-related proteins and is fully expressed in *Arabidopsis* transformants unable to accumulate SA. Although some bacterial strains are equally effective in inducing resistance in different plant species, others show specificity, indicating specific recognition between bacteria and plants at the root surface.

Growth promotion and disease suppression by *Pseudomonas* spp.

Plant growth promotion by rhizobacteria can result from physical and chemical stimulation of plant roots, resulting in more rapid emergence, higher chlorophyll levels, and increased stature (Lynch, 1976). Growth promotion can also depend on suppression of either deleterious micro-organisms in the soil, that reduce plant growth and development, and/or of soilborne pathogens that cause diseases such as damping-off, rots and wilts (Schippers *et al.*, 1987). Notably in suppressive soils, PGPR may reduce the activities of a pathogen to such an extent that a susceptible crop remains healthy. Among the PGPR, *Pseudomonas* spp. have received much attention (Schippers, 1988; Bakker *et al.*, 1991; Lemanceau and Alabouvette, 1993). Several bacterial strains have been shown to possess disease-suppressive activity. Among the mechanisms involved are competition for nutrients, particularly iron, antibiosis and secretion of lytic enzymes (Sivan and Chet, 1992; Handelsman and Stabb, 1996). In most micro-organisms the limited bioavailability of iron induces the production of iron-chelating siderophores. *Pseudomonas* strains produce highly specific siderophores that cannot be utilized by other micro-organisms. Thus, siderophore-mediated competition for iron can deprive the pathogen of sufficient iron, thereby decreasing disease incidence. Competition for iron is the mechanism by which *Pseudomonas putida* strain WCS358r antagonizes *Fusarium oxysporum* f.sp. *dianthi* (Fod) in the rhizosphere of carnation (Duijff *et al.*, 1993, 1994) and *F. oxysporum* f.sp. *raphani* (For) in radish (Raaijmakers *et al.*, 1995). In carnation, under low-iron conditions WCS358r reduced the number of plants suffering from *Fusarium* wilt by up to 40%, whereas its siderophore-deficient (sid⁻) mutant JM218 was ineffective. Moreover, increasing [Fe³⁺] to 200 μ M abolished the suppressive action of wild type WCS358r, a condition which abolished siderophore production in vitro (Duijff *et al.*, 1993). Also in combinations with the nonpathogenic *F. oxysporum* strain Fo47 WCS358r reduced disease incidence on

carnation, whereas JM218 did not (Lemanceau *et al.*, 1992). No significant difference was observed between the rhizosphere population of WCS358r and that of mutant JM218, excluding the possibility that the lack of disease suppression by the mutant was caused by impaired root colonization. Using the same strain and mutant Raaijmakers *et al.* (1995) demonstrated siderophores to be responsible for suppression of *Fusarium* wilt in radish. The siderophore, pseudobactin (PSB) 358, inhibited conidial germination and growth of Fod and For in vitro, whereas JM218 was not inhibitory at all. Siderophore-mediated competition for iron thus appears to be the sole mechanism by which WCS358r suppresses *Fusarium* wilt of carnation and radish.

Pseudomonas fluorescens strain WCS417r likewise reduced *Fusarium* wilt in carnation in an iron-independent manner, and was similarly inhibitory to fungal conidial germination and mycelial growth. However, of two sid⁻ mutants, S680 and M634, neither was impaired in disease suppression or fungal antagonism. Again, the mutants, on an average, did not show differences in root colonization compared to wild-type WCS417r. Thus, competition for iron does not seem to play a role in the protective effect of WCS417r on carnation against Fod, even though protection was stronger at low than at high [Fe³⁺] (Duijff *et al.*, 1993). In in vitro tests no evidence was obtained for the production of antibiotics or lytic enzymes by WCS417r. When plants were bacterized on the roots and Fod was spatially separated from the introduced bacteria by inoculation of the fungus directly in the stem, *Fusarium* wilt was substantially reduced in the moderately resistant carnation cv. Pallas, whereas the same isolate failed to reduce disease incidence significantly in the susceptible cv. Lena. The sid⁻ mutant S680 was as effective as wild-type WCS417r, whereas WCS358r was ineffective in this assay (Duijff *et al.*, 1993).

Because none of the inducing bacterial strains could be reisolated from the stem surface or interior tissue of carnation, spatial separation of the inducing rhizobacteria and the fungal pathogen was maintained for the duration of the experiments.

Table 1. Bacterial determinants of induced systemic resistance

Bacterial strain	Plant species: bacterial determinant	Reference
<i>Pseudomonas aeruginosa</i> strain 7NSK2	Tobacco: salicylic acid	De Meyer <i>et al.</i> , 1996
	Bean: salicylic acid	De Meyer and Höfte, 1997
<i>Pseudomonas fluorescens</i> CHAO WCS374 WCS417	Tobacco: siderophore	Maurhofer <i>et al.</i> , 1994
	Radish: lipopolysaccharide siderophore iron-regulated factor	Leeman <i>et al.</i> , 1995b
		Leeman <i>et al.</i> , 1996
		Leeman <i>et al.</i> , 1996
	Carnation: lipopolysaccharide Radish: lipopolysaccharide iron-regulated factor	Van Peer and Schippers, 1992
Leeman <i>et al.</i> , 1995b		
<i>Arabidopsis</i> : lipopolysaccharide	Leeman <i>et al.</i> , 1996	
<i>Pseudomonas putida</i> WCS358	<i>Arabidopsis</i> : lipopolysaccharide siderophore	Van Wees <i>et al.</i> , 1997
		Bakker and Van der Sluis, unpublished
		Bakker and Van der Sluis, unpublished

Nonetheless, fungal activity was suppressed, indicating that a plant-mediated process had to be involved that resulted in an enhanced defensive capacity of distant tissues. This phenomenon is phenotypically similar to systemic acquired resistance (SAR), in which a plant is able to exhibit an increased resistance as a result of stimulation by limited pathogenic attack (Ross, 1961; Kuć, 1982). However, the rhizobacteria did not cause any symptom on carnation, suggesting a different way of plant stimulation. Because the inducing action extends systemically, the term "induced systemic resistance" (ISR) was adopted (Kloepper *et al.*, 1992). Van Peer *et al.* (1991) demonstrated that upon inoculation of carnation with Fod, phytoalexins accumulate around the site of infection. Inducing rhizobacteria by themselves did not induce phytoalexins. However, challenge inoculation of bacterized plants with Fod led to an increased accumulation of the phytoalexins. Thus, (a) signal(s) provided by WCS417r at the root system, induced in the stem sensitization of defense responses against Fod, as evidenced by the effect on phytoalexin production. In accordance with the disease suppressive activity, phytoalexin accumulation was more pronounced in the moderately resistant cv. Pallas than in the sensitive cv. Lena, indicating that sensitization of the plant becomes effective only when resistance reactions are triggered by the invading pathogen.

Either heat-killed bacterial cells or purified, protein-free outer membrane lipopolysaccharide (LPS) preparations were as effective as living cells in eliciting ISR (Van Peer and Schippers, 1992). Thus, cell surface components present in the LPS of the bacterium appear to be the inducing factor (Table 1). Probably, part of the LPS is recognized by a receptor at the root surface, most likely the O-antigenic side-chain polysaccharide. However, neither the detailed structure of the LPS of WCS417r, nor a binding entity on roots of carnation, have been identified.

A similar induced systemic resistance was described by Wei *et al.* (1991). By treatment of cucumber seeds with either of six PGPR strains, they obtained significant reduction in lesion size after challenge inoculation with the foliar pathogen *Colletotrichum orbiculare*. None of the bacterial strains was

recovered from surface-disinfested petioles on the day of challenge, indicating that the reduced symptom expression was caused by plant-mediated protection rather than competition or antagonism.

Characteristics of bacterially-induced systemic resistance in radish

Radish and carnation are similarly affected by *Fusarium* wilt, caused by For and Fod, respectively. In contrast to the time required for symptoms to develop in carnation, which may take over three months, bioassays for ISR in radish can be completed in three weeks. Diseased plants eventually die, but internal symptoms of vascular discoloration and internal browning can be scored from 10 days after challenge inoculation onwards, and the percentage of diseased plants increases progressively with time. Stem inoculation of radish plants is not possible, however. Therefore, a separate inoculation system was developed, in which 5-days-old radish seedlings are laid horizontally on rockwool cubes (Leeman *et al.*, 1995a). Adjoining rockwool cubes are compartmented through enclosure in plastic bags, only a small incision in the bags allowing the lower part of the root to contact the cubes in one compartment and the upper part the ones in the adjacent compartment. Routinely, the part containing the root tip is treated with a bacterial suspension in talcum emulsion. After a few days, the upper part of the root is covered with pathogen inoculum in peat. In this bioassay, the inducing rhizobacterium and the challenging pathogen were confirmed to remain confined to their spatially separate locations on the plant root throughout the experiments. Thus, disease suppression as a result of bacterization must be due to ISR.

An interval of two days between bacterization of the root tip and inoculation of the pathogen on the root base was optimal for the induction of systemic resistance (Leeman *et al.*, 1995a), indicating that ISR needs time to develop. Indeed, the bacteria must be perceived by the plant at the root surface and a signal must be generated and transported to elicit the induced

state in other plant parts. That ISR is truly systemic was borne out by using different leaf pathogens for challenge. Induction of systemic resistance by WCS417r was effective against the avirulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Pst), as well as the fungal pathogens *Alternaria brassicicola* and an isolate of *F. oxysporum* from *Arabidopsis* leaves (Hoffland *et al.*, 1996). The resistance induced was as strong as SAR induced in upper leaves by treating the lower leaves of the plant with the pathogen Pst. These observations demonstrate again the phenotypic similarity between SAR and ISR as to both the extent of protection attained, and the non-specificity of the induced resistance with regard to the challenging pathogen. Pst was ineffective when applied to the roots, presumably because this bacterium is unable to maintain itself in the root environment. In contrast, the rhizobacterium WCS417r was as effective when applied to lower leaves as when present on the roots. Unexpectedly, when WCS417r and the fungal root pathogen *Rhizoctonia solani* were inoculated in the different compartments of the separate inoculation system, no ISR against *R. solani* was manifested (Hoffland *et al.*, 1996). However, later experiments have indicated that ISR against *R. solani* becomes evident when the dose of challenge inoculum is lowered sufficiently to not rapidly kill the plants (P.A.H.M. Bakker, P.G.J. Vogel and L.C. van Loon, in preparation).

Similar observations were made when radish cultivars differing in genetic resistance against For were tested for elicitation of ISR by *Pseudomonas fluorescens* strain WCS374 (Leeman *et al.*, 1995a). Significant suppression of Fusarium wilt was generally observed only when disease incidence in the nonbacterized control treatments, dependent on pathogen inoculum density, ranged between approximately 40 and 80%. At lower disease incidence, protection became insignificant, whereas at higher disease pressure, defenses appeared to be overwhelmed by the relatively large doses of the fungus applied.

Of three bacterial strains tested, WCS374 and WCS417 induced systemic resistance in radish against For, whereas WCS358, like in carnation, did not (Leeman *et al.*, 1995a). All three strains colonized radish roots to the same extent, indicating that both WCS374 and WCS417 possess inducing determinants that are lacking in WCS358. WCS374 was as effective as WCS417 in inducing resistance and was used to determine the dose-response relationship of ISR in radish. A significant non-linear asymptotic relationship was demonstrated between the initial rhizosphere population density of the bacterium and the percentage of diseased plants. At 10^5 cfu.g⁻¹ root, almost optimal protection was already achieved, whereas none was apparent when roots were colonized to a lesser extent (Raaijmakers *et al.*, 1995). No relationship was apparent between disease incidence and the rhizosphere population density of WCS374 determined 24 days after bacterization. It may be assumed, therefore, that the initial triggering of the plant leads to its induced state and, once reached, subsequent protection is independent of the remaining bacterial population in the rhizosphere. Also in this respect, ISR appears similar to SAR, in that induced resistance, once triggered, is maintained often for the life of the plant (Bozarth and Ross, 1964).

In standard assays in which radish plants in the separate inoculation system received half-strength Hoagland's nutrient solution containing 10 μ M Fe-EDDHA as an iron source, Fusarium wilt disease incidence was reduced by up to 50% by either WCS374 or WCS417 (Leeman *et al.*, 1995a). Heat-killed cells were similarly effective (Leeman *et al.*, 1995b), suggest-

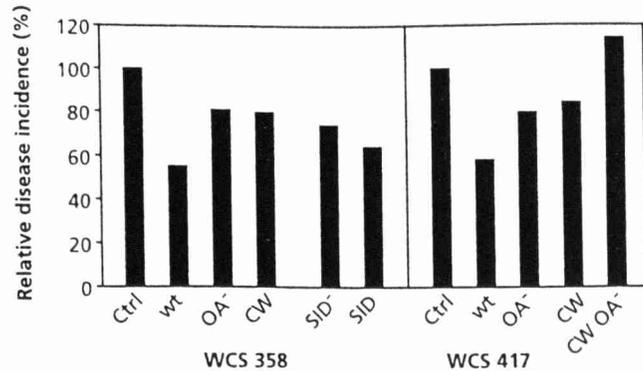


Figure 1. Relative disease incidence of *Pseudomonas syringae* pv. *tomato*-induced leaf chlorosis in *Arabidopsis* upon inoculation 3 weeks after bacterization of the roots with *Pseudomonas putida* WCS358 or *Pseudomonas fluorescens* WCS417 and their mutants (OA⁻ = lacking the O-antigenic sidechain of the outer membrane lipopolysaccharide; SID⁻ = lacking pseudobactin production) or with crude cell wall preparations (CW) or purified siderophore (SID).

ing that, as in carnation, bacterial LPS acts as an inducing determinant (Table 1). Indeed, purified LPS (consisting of lipid A - inner core - Oantigenic side-chain) of both strains induced systemic resistance, whereas crude cell wall preparations or purified LPS of WCS358 did not. Moreover, phage-resistant mutants of WCS374 and WCS417 were isolated that lacked the O-antigenic side-chain of the LPS but colonized radish roots to the same extent as the wild-type strains. Neither these live mutants, nor their crude cell walls or purified lipid A - inner core complex reduced disease incidence, demonstrating that it is the O-antigenic side-chain of the LPS that was responsible for the induction. Unlike wild type WCS374, the O-antigenic side-chain lacking (OA⁻) mutant did not induce systemic resistance when applied on the cotyledons of radish. Thus, the resistance-inducing O-antigen of WCS374 was recognized not only by the roots, but also by the shoot. Disease suppression and yield increases obtained in commercial greenhouse trials (Leeman *et al.*, 1995c) were reproduced in a bioassay with greenhouse soil naturally infested with For. In these assays root colonization by WCS374, but not its OA⁻-mutant, prior to infection by the pathogen suppressed the disease, suggesting that induced resistance is responsible for the protection of radish by WCS374 under greenhouse conditions.

Induced systemic resistance as affected by iron-regulated factors

Pseudomonas fluorescens strain CHAO is an effective biocontrol agent of different diseases caused by soilborne pathogens and has been found to induce systemic resistance in tobacco against tobacco necrosis virus (TNV) (Maurhofer *et al.*, 1994). The level of resistance attained was similar to the SAR developing in plants by prior inoculation with the same virus. Strain CHA400, a siderophore (pyoverdine)-negative mutant of CHAO, induced partial resistance against TNV, implicating the involvement of the pyoverdine in the elicitation of ISR against TNV in tobacco by CHAO (Table 1). The ISR elicited by CHAO and the SAR induced by TNV were both associated with the systemic induction of the major pathogenesis-related proteins (PRs) of tobacco. PRs characteristically accumulate during a hypersensitive reaction and are often used as markers of the

state of induced resistance (Ryals *et al.*, 1996; Van Loon, 1997). Their induction is dependent on the accumulation of salicylic acid (SA) (Gaffney *et al.*, 1993) and root colonization of tobacco plants with CHA0, as well as leaf infection with TNV, caused an increase in SA in leaves (Maurhofer *et al.*, 1994). These observations point to ISR and SAR in tobacco being not only phenotypically, but also mechanistically similar, with ISR, at least partly, being determined by the siderophore of CHA0. However, the transposon insertion generating the pyoverdininus mutation in CHA400 was not localized, and it is not clear whether the loss of pyoverdinin is the only mutation in CHA400.

Besides pyoverdinin, CHA0 produces several metabolites with potentially toxic effects on micro-organisms and plants, among which 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. The production of SA by the bacteria is iron-regulated and in the type of soil bioassay used by Maurhofer *et al.* (1994), information on iron availability in the rhizosphere is lacking. Similar experiments in tobacco using *Pseudomonas aeruginosa* strain 7NSK2 as the inducing rhizobacterium and tobacco mosaic virus (TMV) as the challenging leaf pathogen suggest that bacterially-produced SA does contribute to the induction of systemic resistance (De Meyer *et al.*, 1996) (Table 1). By using mutants deficient in the production of the siderophores pyoverdinin, pyochelin and/or SA, De Meyer and Höfte (1997) demonstrated that SA production by 7NSK2 was essential for induction of resistance in bean to *Botrytis cinerea*. A role for pyoverdinin could not be demonstrated, but one for pyochelin was not excluded. While we have confirmed the induction of systemic resistance against TMV in tobacco by 7NSK2, also other *Pseudomonas* strains, not capable of producing SA, appear able to systemically reduce TMV lesion expansion in some assays, indicating that also other bacterial traits can be involved.

To investigate the possible involvement of iron-regulated metabolites in the induction of systemic resistance in radish, the effectiveness of the protection against Fusarium wilt afforded by the three bacterial strains, their OA⁻-mutants, and their sid⁻-mutants was assessed under low-iron conditions (Leeman *et al.*, 1996). Iron limitation was imposed by the addition of 10 μ M of the uncomplexed iron chelator EDDHA to the nutrient solution. The mutation did not affect the abilities of the strains to colonize the roots, or to induce resistance. WCS358 and its sid⁻-mutant colonized radish roots to the same extent but did not induce resistance, whereas WCS374 and WCS417 and their sid⁻-mutants did both. However, WCS374 and its sid⁻-mutant gave substantially greater disease control under low-iron than under high-iron conditions. Although the effect was not statistically significant, the same tendency was observed for WCS417 and its mutant. Moreover, whereas the OA⁻-mutants of WCS374 and WCS417 did not induce resistance at high iron availability, they did so under low-iron conditions, and to an extent similar to the protection afforded by the wild-type strains. Thus, under iron-limited conditions both WCS374 and WCS417 express an additional determinant that elicits ISR in radish.

Testing the purified pseudobactins of the three strains revealed that at 70 μ g per root tip the siderophores of WCS358 and WCS417 did not induce resistance, but the one of WCS374 did. Even so, the latter is not necessary for the induction by live bacteria, because the WCS374 sid⁻-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. 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 μ g SA.ml⁻¹ standard succinate medium (SSM), 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 systemic resistance in radish at concentrations as low as 100 fg per root tip when applied in talcum emulsion in the separate inoculation system. Thus, SA production appeared to fulfil the criteria for the additional bacterial determinant responsible for the induction of systemic resistance under low-iron conditions.

Induction of SAR in radish by either SA or the bacterial leaf pathogen Pst induced accumulation of proteins cross-reacting with antibodies against the tobacco PRs 1, 2 and 5. In contrast, no accumulation of such proteins was apparent upon induction of ISR by WCS417r (Hoffland *et al.*, 1995) or WCS374 (unpublished), casting doubt as to the possible involvement of bacterially-produced SA in vivo. To be able to determine if SA plays any role in inducing systemic resistance under our assay conditions, we set out to obtain a WCS374 Tn5 mutant specifically impaired in SA production. Because SA, like pseudobactins, acts as an iron-chelating siderophore under low-iron conditions (Meyer *et al.*, 1992), lack of iron chelation on CAS-agar upon mutagenization of a WCS374 sid⁻ (pseudobactin)-mutant was used for screening. However, no mutants were recovered. Instead, a gene bank of WCS374 was constructed and mobilized into the WCS358 sid⁻-mutant JM218. This led to the identification of a 28 kb cosmid clone, a 5 kb EcoRI fragment of which was sufficient to convert WCS358, as well as *E. coli*, into SA producers (Mercado-Blanco *et al.*, submitted). Sequencing of the 5 kb fragment revealed the presence of four open reading frames with homologies to isochorismate synthase (ICS), 2,3-dihydroxybenzoate AMP ligase (DHBAL), histidine decarboxylase (HD), and the *pchB* gene of *P. aeruginosa*, respectively. For *P. aeruginosa* it has been shown that the ICS and the *pchB* gene are both necessary and sufficient for SA production (Serino *et al.*, 1995). The DHBAL gene is likely to be involved in a pathway from isochorismate to (a) siderophore(s) of the enterobactin type.

The potential relationship with siderophores was further strengthened by the presence of the HD gene, that shows strong similarity with enzymes involved in the biosynthesis of histamine-containing siderophores. SA itself is part of bacterial siderophores of the pyochelin-type, suggesting that this gene cluster in WCS374 functions in the synthesis of an additional siderophore. Indeed, when the complete 28 kb cosmid clone was transferred into the WCS358 sid⁻ recipient, a novel siderophore fluorescing bluegreen under UV light was produced. This novel siderophore has been designated fluorebactin (Mercado-Blanco *et al.*, submitted). The emerging view is that both SA and (a) histamine (analog) are incorporated into fluorebactin and that fluorebactin is produced by WCS374 in addition to pseudobactin at low iron availability in vivo. As shown by reverse transcriptase-PCR, the four characterized genes form a single transcriptional unit, whose expression is iron-regulated. It appears to be part of a larger operon contained within the 28 kb cosmid clone, that gives rise to fluorebactin production. Secretion of large quantities of SA by WCS374 in vitro in SSM may be an artefact resulting from lack of substrate(s) required for fluorebactin synthesis. The possible role of fluorebactin as an inducer of systemic resistance under

low-iron conditions is now being further investigated.

Characteristics of bacterially-induced systemic resistance in *Arabidopsis*

A different way to assess the possible role of bacterially-produced SA is the use of plants in which SA action is abolished. This is the case in e.g. *Arabidopsis* transformed with the *NahG* gene from *P. putida*. The *NahG* gene encodes a salicylate hydroxylase, that converts SA into catechol. Both development of SAR and accumulation of PRs in response to necrotizing infection are impaired in this transformant (Gaffney *et al.*, 1993), and expression of resistance in response to different pathogens is reduced (Delaney *et al.*, 1994). Since radish and *Arabidopsis* are both crucifers, it was expected that *Arabidopsis* would be similarly inducible by our rhizobacterial strains.

Arabidopsis proved similarly susceptible to For and, using the same separate inoculation system, induction of systemic resistance against For was demonstrated upon bacterization of the root tips (Van Wees *et al.*, 1997). However, whereas both WCS374 and WCS417, but not WCS358, were inducive in radish, in *Arabidopsis* WCS374 was ineffective, but both WCS417 and WCS358 elicited ISR. WCS374 colonized *Arabidopsis* roots to slightly lower levels than the other two strains, but still reached numbers well above 10^5 cfu.g⁻¹ root, the threshold population needed to elicit ISR in radish (Raaijmakers *et al.*, 1995). Apparently, none of the determinants of WCS374 capable of inducing systemic resistance in radish is expressed on or recognized by *Arabidopsis* roots. SA, when applied to *Arabidopsis* roots, induced SAR, in accordance with other observations (Uknes *et al.*, 1992). Because of the three bacterial strains tested WCS374 has the largest potential to produce SA, it can be excluded that SA was produced in the rhizosphere of *Arabidopsis* by WCS374 at a level sufficient for inducing systemic resistance.

Similar ISR was evident when, instead of the fungal root pathogen For, the bacterial leaf pathogen Pst was used for challenge inoculation. As a result of root bacterization with either WCS417 or WCS358, the spreading chlorosis progressing into necrosis on Pst-infected leaves was substantially reduced, the percentage of leaves with symptoms being lessened by up to 50% (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). When tests were conducted for the possible involvement of SA, the same extent of protection was obtained in *Arabidopsis* transformed plants expressing the *NahG* gene. This clearly demonstrated that the induction of systemic resistance by rhizobacteria is independent of SA accumulation. 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 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.

The latter conclusion has been corroborated by recent results with a non-SA producing mutant of the ISR-PGPR strain *Serratia marcescens* 90-166. The SA-mutant still induced resistance to *C. orbiculare* in cucumber and to *P. syringae* pv. *tabaci* in tobacco (Press *et al.*, 1996). However, this contrasts with the results of De Meyer and Höfte (1997) demonstrating

that SA is the determinant responsible for the induction of resistance in bean against *B. cinerea*.

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 the three bacterial strains were tested for their capacity to elicit ISR. Cell walls of both WCS417 and WCS358 induced systemic resistance (Table 1), but the level attained commonly fell short of that effected by live bacteria (Fig. 1). Testing of the OA-mutants revealed that these were still inducive, whether tested under low- or under high-iron conditions. However, cell walls of the WCS417 OA-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-mutant of WCS358, the sid-mutant JM218 still protected *Arabidopsis* against Pst. Moreover, purified pseudobactin of WCS358 induced resistance (Fig. 1). These observations suggest that iron limitation occurs in the rhizosphere of *Arabidopsis* and that, 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 observations).

Within the species *Arabidopsis thaliana*, differences in inducibility by rhizobacteria were found among ecotypes. Thus, ecotypes Columbia (Col) and Landsberg *erecta* (La-er) 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 Col and La-er, 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 Pst or SA, indicating that at least the signaltransduction pathway leading to SAR is unimpaired.

Like in radish, induction of systemic resistance by the rhizobacterial strains was not associated with the accumulation of PRs (Pieterse *et al.*, 1996). 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). PRs were associated with the induction of systemic resistance in tobacco by CHA0 (Maurhofer *et al.*, 1994). However, so far this is the only report linking ISR with PRs. 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, whereas induction of SAR by pathogens or SA occurs normally (Pieterse *et al.*, in preparation). Similar results were obtained using an *Arabidopsis* mutant insensitive to jasmonic acid (JA), *jar1* (Staswick *et al.*, 1992). These observations indicate that our inducing rhizobacteria activate a signal-transduction pathway different from the one leading to SAR (Fig. 2), requiring perception of both ethylene and JA rather than SA. Both ethylene and JA are produced by and act as hormones in plants and our results suggest that recognition of the inducing bacteria by the roots may result in a change in ethylene and JA production or metabolism

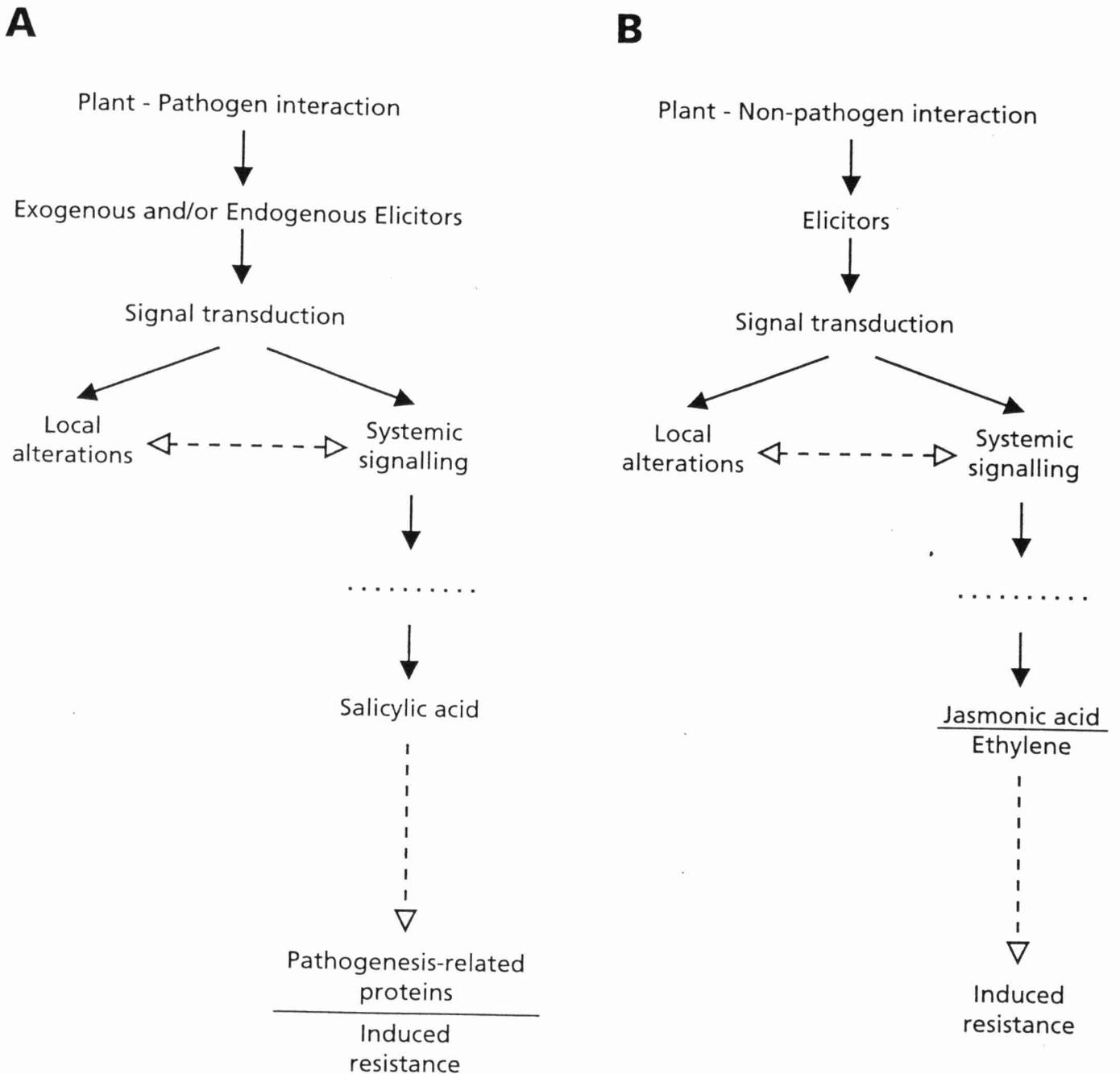


Figure 2. Signal-transduction pathways leading to (A) pathogen-induced systemic acquired resistance and (B) rhizobacteria-elicited induced systemic resistance.

in the plant.

The availability of, on the one hand, bacterial strains that differentially induce ISR and plant ecotypes that are differentially inducible and, on the other hand, 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.

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