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Biocontrol Agents in Signaling Resistance

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I. DISEASE SUPPRESSION THROUGH INDUCED SYSTEMIC RESISTANCE

The mechanisms by which biological control agents suppress disease comprise competition for nutrients, notably iron, production of antibiotics, and secretion of lytic enzymes, as well as inducing resistance in the plant [1,2]. The former three mechanisms act primarily on the pathogen by decreasing its activity, growth, and/or survival and require the biocontrol agent and the pathogen to be in close proximity. Because microorganisms with biocontrol properties and soilborne pathogens are both attracted to the rhizosphere, where root exudates and lysates provide a nutritious environment, antagonism between biocontrol agents and pathogens occurs locally. Such interactions may be favored by both the biocontrol agent and the pathogen growing preferentially over anticlinal walls of root epidermal cells [3]. However, pathogenic fungi such as *Fusarium oxysporum* f.sp. *lini* and f.sp. *raphani* grow towards root apices and penetrate through the tips that emerge virtually sterile from beneath the root cap (4; H. Steijl, T. van Welzenis, J. van den Heuvel, and L. C. van Loon, unpublished). Hence, those Fusarium wilts are among the diseases most difficult to control effectively and reliably by antagonistic microorganisms.

Induced resistance is a plant-mediated mechanism and can only be demonstrated unequivocally when the biocontrol agent and the pathogen never contact each other. Thus, induced resistance acts at a distance and can protect plants systemically. This elicitation of a systemically enhanced defense capacity is variously denoted as systemic acquired resistance (SAR) or induced systemic resis-

tance (ISR) and can result from stimulation by either biotic or abiotic agents. SAR/ISR is relatively easily demonstrable with rhizobacteria as biocontrol agents that remain confined to the roots but protect plants against disease caused by a foliar pathogen. It is most difficult to prove when biocontrol fungi tend to colonize plant tissues systemically, and special measures are necessary to ensure that contact between the biocontrol agent and the pathogen is avoided. Systemic resistance induced by nonpathogenic, root-colonizing bacteria was first conclusively demonstrated in 1991 by van Peer et al. [5] in carnation and Wei et al. [6] in cucumber. Carnation cuttings were rooted on a substratum of rock wool and bacterized by pouring a suspension of a rifampicin-resistant derivative of *Pseudomonas fluorescens* strain WCS417 (WCS417r) over the roots. One week later, plants were stem-inoculated with *F. oxysporum* f.sp. *dianthi* between the first and second pair of leaves. As a result of root colonization by WCS417r, the number of wilted plants of the carnation cv. Pallas was reduced on an average from about 50% to 20% and in a single experiment with cv. Lena from 69% to 38%. Strain WCS417r could not be isolated from stem tissue, indicating that protection was plant-mediated [5]. Cucumber was protected against anthracnose, caused by *Colletotrichum orbiculare*, after seeds had been bacterized with 6 of 94 rhizobacterial strains tested. Treatment with these strains reduced both the number and the size of anthracnose lesions after challenge inoculation of the foliage with the fungus. None of the bacterial strains was recovered from surface-disinfected petioles on the day of challenge with *C. orbiculare*, clearly suggesting that systemic resistance had been induced [6].

In cucumber, systemic resistance against anthracnose could also be induced by several isolates of nonpathogenic fungi that were isolated from zoysiagrass rhizospheres. The fungal isolates were introduced into autoclaved potting medium in which surface-disinfected seeds were sown. After 21 days the seedling leaves were inoculated with *C. orbiculare*. The rhizosphere fungi did not colonize the aerial portions of the plant, indicating that the disease suppression observed was the result of induction of systemic resistance [7,8]. In other investigations ISR was demonstrated by using plants with split-root systems in which the biocontrol agent and the pathogen remain physically separated from each other. Colonization of one part of the root system of watermelon by selected isolates of nonpathogenic *F. oxysporum* protected the plants against Fusarium wilt when the other part was challenge inoculated with *F. oxysporum* f.sp. *niveum* [9]. Under similar conditions, nonpathogenic *F. oxysporum* isolate Fo47 and *Penicillium oxalicum* suppressed Fusarium wilt in tomato by inducing systemic resistance against pathogenic *F. oxysporum* f.sp. *lycopersici* [10,11]. Cordier et al. [12] likewise demonstrated that the arbuscular mycorrhizal fungus *Glomus mosseae* induces systemic resistance in tomato against *Phytophthora parasitica*. Also, the fungal biocontrol agents *Trichoderma harzianum* and *Trichoderma hamatum*, which are capable of antagonizing sensitive pathogenic fungi by producing antibi-

otics and lytic enzymes, have been reported to induce systemic resistance in tomato, lettuce, pepper, bean, and tobacco against grey mold, caused by *Botrytis cinerea* [13], and in radish against leaf spot caused by *Xanthomonas campestris* pv. *armoraciae* [14].

Most research on microbially induced systemic resistance has been conducted with rhizobacteria, however. Apart from carnation and cucumber, rhizobacteria-mediated ISR has been conclusively demonstrated in Arabidopsis, bean, radish, tobacco and tomato and shown to be effective against fungi, bacteria, viruses, and insects. Where investigated, ISR was shown to require a threshold population of the biocontrol agent, above which little further increase in protection is evident. Moreover, a time interval is necessary between application of the inducer and the onset of protection of the plant, indicating that the plant needs time to reach the induced resistant state [2].

The ability of nonpathogenic rhizobacteria and fungi to induce systemic resistance appears to be fairly common but by no means general. Resistance-inducing biocontrol agents must express a specific determinant that is recognized by plant roots and elicits a response that culminates in an enhanced defensive capacity throughout the entire plant. Such systemic induction requires not only local perception of the stimulus, but also its transport or the transport of a mobile signal that is generated as a result of local stimulus perception. Moreover, the mobile signal, in turn, must be transduced at the sites where resistance is expressed (Fig. 1). Phenomenologically, the situation resembles both the wound response and pathogen-induced SAR [15,16], except that in those responses plant tissues start reacting to various forms of cellular damage, whereas in biocontrol agent–induced systemic resistance generally no deleterious effects of the inducing microorganism on the plant are visible.

II. HISTOLOGY OF THE INDUCED RESISTANT STATE

Histological investigations at the light and electron microscopic levels have attempted to relate the induced resistant state to tissue alterations that might provide clues as to the mechanism of ISR. Adventitious roots from *Agrobacterium rhizogenes*–infected pea (Ri T-DNA transformed roots) were inoculated in vitro with *Bacillus pumilis* strain SE34, a bacterium that protects cotton roots against *F. oxysporum* f.sp. *vasinfectum* [17]. Whereas in nonbacterized roots the pea pathogen, *F. oxysporum* f.sp. *pisi*, multiplied abundantly through much of the tissue including the vascular stele, in prebacterized roots pathogen growth was restricted to the epidermis and the outer cortex. In these prebacterized roots typical host reactions included strengthening of epidermal and cortical cell walls and deposition of wall appositions containing large amounts of callose as well as phenolic compounds. No induction of visible host defense reactions was evident in bacte-

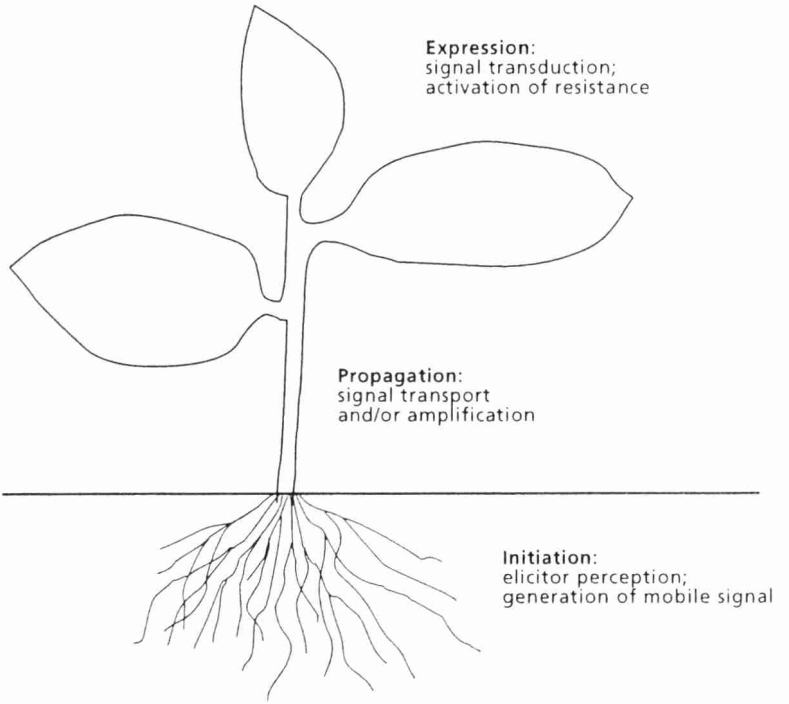

Figure 1 Representation of the stages in systemic resistance induced by rhizobacteria or nonpathogenic soilborne fungi.

rized pea roots before challenge with the pathogen, when a large number of bacteria had grown on the root surface and displayed the ability to colonize some intercellular spaces in the epidermis and the outer cortex. Because differences in defensive responses became evident only after challenge, it appears that the induced state constitutes a sensitization of the plant to respond more effectively to pathogen attack [18,19].

Essentially similar results were obtained when in vitro Ri T-DNA transformed pea roots were treated with the biocontrol bacterium *P. fluorescens* strain 63-28R and challenged with *Pythium ultimum* [20] or when in vivo tomato plants were bacterized with the same bacterial strain and subsequently infected with *F. oxysporum* f.sp. *radicis-lycopersici* [21]. Noninfected, 63-28R–treated tomato plants showed no symptoms, and their root systems appeared healthy. Bacteria grew actively at the root surface and colonized some host epidermal cells and intercellular spaces in the outermost root tissues. Apparent preservation of cell wall architecture, as shown by regular patterns of cellulose and pectin over the

walls adjacent to the areas of bacterial colonization, provided evidence that wall-degrading lytic enzymes were not, or only very slightly, produced by the endophytic bacteria once inside the plant. A slight accumulation of electron-opaque substances along the epidermal cell walls and/or within the invaded intercellular spaces, as well as slight deposition of β-1,3-glucan in the host cell walls, pointed to some discrete host reactions that resemble defense responses. It thus appears that the perception of the bacteria by the roots leads to a minimal activation of wall-associated defenses, effectively priming the plant to respond faster and to a higher extent upon subsequent challenge inoculation.

Similar observations suggested that the mycoparasitic fungus *Pythium oligandrum* also has the potential to induce plant defense reactions in tomato roots challenged with *F. oxysporum* f.sp. *radicis-lycopersici* [22,23]. *Pythium oligandrum* was able to penetrate the root epidermis without extensive host wall degradation and, subsequently, ramified in all root tissues by inter- and intracellular growth. This implied that at least small amounts of cell wall hydrolytic enzymes, such as pectinases and cellulases, were produced to locally weaken or loosen the host cell walls, thereby facilitating spread into root tissues. However, the invading hyphae were structurally altered as evidenced by the frequent occurrence of empty fungal shells in the root tissues. Moreover, *Pythium* ingress in the root tissues was associated with host metabolic changes, leading to the elaboration of structural barriers at sites of potential fungal penetration. These observations indicate that the plant reacted defensively to invasion by *Pythium oligandrum*.

Similar formation of physical and chemical barriers at sites of potential fungal entry were detected in cucumber plants that reacted more rapidly and more efficiently to infection by pathogenic *Pythium ultimum* when pretreated with the endophytic biocontrol bacterium *Serratia plymuthica* strain R1GC4 [24]. A non-pathogenic strain of *F. oxysporum*, able to induce systemic resistance in tomato, likewise triggered typical defense reactions, such as wall appositions, intercellular plugging, and intracellular osmiophylic deposits [25]. Both *Pseudomonas corrugata* strain 13c and *Pseudomonas aureofaciens* strain 63-28 increased levels of phenylalanine ammonia-lyase (PAL), peroxidase, and polyphenoloxidase in cucumber roots, which peaked 2–4 days after root treatment. Similar experiments were done with split-root systems, which demonstrated that the induction of higher enzyme levels was systemic. Analysis of the peroxidase isoenzymes showed that the bacteria specifically induced an increase in one acidic isoform [26]. Similarly, upon root inoculation with the biocontrol agent *Trichoderma harzianum*, peroxidase and chitinase activities increased in both the roots and the leaves of treated cucumber seedlings, indicating that the fungus stimulated defense reactions in the plant [27]. In contrast, no histological differences were apparent in tomato stems in which resistance was induced by *Penicillium oxalicum*, as evidenced by reduced cambial loss upon challenge inoculation with *F. oxysporum* f.sp. *lycopersici* [28]. Similarly, no cell reactions were observed in

mycorrhizal tomato root systems, but upon challenge with *Phytophthora parasitica*, accumulation of phenolics and plant cell defense responses were augmented [12].

III. ROLE OF SALICYLIC ACID IN INDUCED RESISTANCE

A. Pathogen-Induced SAR

Phenotypically, biocontrol agent–induced systemic resistance and pathogen-induced SAR are similar in that both develop as a result of the interaction of the plant with a microorganism and the resulting enhanced defensive capacity is expressed both locally and systemically against a broad spectrum of attacking organisms. Pathogen-induced SAR is a general phenomenon that occurs as a result of limited infection by a pathogen, notably in incompatible interactions that lead to a hypersensitive reaction. However, necrosis is not a prerequisite for pathogen-induced resistance, indicating that the signaling pathways leading to tissue necrosis, on the one hand, and induced resistance, on the other hand, are distinct. Nevertheless, hypersensitive necrosis contributes to the level of SAR attained [29]. Pathogen-induced SAR is triggered by elicitors that are involved either in specific gene-for-gene resistance or in the nonspecific elicitation of defense reactions. As a result, salicylic acid (SA) is produced as a signal and induces the resistant state. The role of SA has been established on the grounds that exogenous application of SA or its functional analogues 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) mimic pathogen-induced SAR by inducing resistance in many plant species towards the same broad spectrum of pathogens [16,30]. Endogenous accumulation of SA occurs both locally and, at lower levels, systemically concomitant with the development of SAR. Moreover, plants transformed with the *NahG* gene do not accumulate SA and do not develop SAR in response to biological or chemical inducers of SAR. The *NahG* gene encodes SA-hydroxylase, which converts SA into catechol, a product that does not induce resistance. The experiments with *NahG*-transformed plants indicate that SA is an essential signaling molecule in SAR induced by avirulent pathogens. Moreover, NahG-containing plants are more susceptible to a variety of fungal, bacterial, and viral pathogens [31].

SA can be synthesized from either cinnamic acid [32] or chorismic acid [33], and the regulation of its synthesis has not been clarified. Neither its mode(s) of action nor the molecular mechanism of resistance induction has been elucidated [34]. Although SA may be transported from locally infected leaves, it does not appear to be the primary long-distance signal for systemic induction [35,36]. Nevertheless, its presence is required for SAR to be expressed. Indeed, local application of SA to individual plant leaves does not lead to systemic induction of resistance unless SA can be transported out of the leaf to other plant parts

[37]. However, when applied to roots SA appears to be readily absorbed and transported throughout the plant, and acquired resistance is manifested systemically. Exceedingly low concentrations of SA applied to the roots of radish and bean have been reported to induce systemic resistance against Fusarium wilt and grey mold, respectively [38,39]. The higher levels of SA in plants expressing SAR suggest that an amplifying step is required for induced resistance to be manifested. Although exogenous application of SA can mimic the induction of resistance by pathogens, additional plant signals must play a role and the nature of the endogenous mobile signal is still unknown.

Associated with SAR triggered by either endogenous regulators or exogenous application of SA is the accumulation of pathogenesis-related proteins (PRs). PRs have been defined as plant proteins that are induced in pathological and related situations [40] and currently comprise 14 protein families [41]. Induction of at least some families of PRs occurs invariably in plants with necrotizing infections. Particularly appearance of the SA-inducible PR-1 type proteins is generally taken as a marker of the induced resistant state. Some of the PR families are β-1,3-glucanases and chitinases and are capable of hydrolyzing fungal cell walls. Other PRs have less well characterized antimicrobial activities or unknown functions. The association of PRs with SAR suggests an important contribution of these proteins to the enhanced defensive capacity of induced tissues. However, none seem to act against viruses, which, nevertheless, are also effectively protected against.

B. Rhizobacterium-Mediated ISR

To determine whether biocontrol agent–induced resistance is not only phenotypically but also mechanistically similar to pathogen-induced SAR, induced plants can be analyzed for the presence of specific PRs, and induction of resistance can be compared in untransformed and *NahG*-transformed plants. As described above, some biocontrol agents elicit increases in PR-type enzymic activities, suggestive of a pathway involving SA similar to that triggered by avirulent pathogens. At least 8 of the 10 major PRs induced in tobacco in response to pathogens causing hypersensitive necrosis were found in plants grown in soil containing *Pseudomonas fluorescens* CHA0, which suppresses necrotic lesion formation on leaves inoculated with tobacco necrosis virus (TNV) [42]. Similarly, when transgenic tobacco plants were assayed for induction of β-glucuronidase (GUS) activity under the direction of the tobacco PR-1a promoter, out of 10 rhizobacterial strains previously demonstrated to induce systemic resistance in cucumber, *Bacillus pumilis* strain T4, *Pseudomonas putida* strain 89B-61, *Serratia marcescens* strain 90-166, and *Burkholderia gladioli* strain IN-26 significantly enhanced GUS activity. The ability to enhance GUS activity was associated with reduction of symptoms of wildfire disease caused by *Pseudomonas syringae* pv. *tabaci*

upon root bacterization by any of these four strains. Neither enhanced GUS activity nor reduction of symptoms of wildfire disease were noted with three control bacterial strains [43]. However, increased GUS activity was observed locally when the four inducing strains were infiltrated in leaves, and only strain T4 increased GUS activity in the leaves when it was applied as a soil drench. In contrast, *Enterobacter asburiae* strain JM-22, which did not induce resistance in cucumber when applied as a soil drench, did induce protection against wildfire disease in tobacco and also induced GUS activity in the leaves. Thus, the systemic induction of resistance against wildfire disease by application of any of the five strains to plant roots was associated with systemically enhanced GUS expression by two of these strains only.

Local induction of plant defense responses by rhizosphere bacteria or fungi has been observed upon leaf infiltration or stem inoculation, whereas upon root treatment such metabolic changes are often not apparent. Thus, a *Pseudomonas aureofaciens* strain induced symptoms of a hypersensitive response on bean cotyledons [44]. Similarly, out of 15 rhizobacteria tested, 9 strains of the genus *Pseudomonas* and two *Serratia* strains induced a hypersensitive response and the production of phytoalexins in wounded bean cotyledons and hypocotyls [45]. Inoculation of bean hypocotyls with nonpathogenic binucleate *Rhizoctonia* species induced systemic resistance against pathogenic *Rhizoctonia solani* or *Colletotrichum lindemuthianum* but also increased peroxidase, β-1,3-glucanase, and chitinase activities [46]. Such observations indicate that at least some rhizobacterial and fungal species have limited pathogenic activity when applied to aboveground plant parts.

No accumulation of PRs was found in either leaves or roots of radish with systemic resistance induced by *P. fluorescens* WCS417r [47,48], and neither were PR mRNAs or proteins apparent in Arabidopsis leaves upon root bacterization with the same rhizobacterial strain [49,50]. Similarly, *Pseudomonas aeruginosa* strain 7NSK2–induced resistance in tobacco was not associated with PR-1 expression [51]. Together, these results suggest that some rhizobacterial strains induce resistance by a SA-dependent mechanism, whereas others act independently of SA. This conclusion has been corroborated by experiments with SA-nonaccumulating NahG plants, even though the situation has turned out to be complex. *P. fluorescens* WCS417r, as well as *Pseudomonas putida* WCS358r, induced systemic resistance in NahG Arabidopsis to the same level as in untransformed plants, demonstrating that SA is not involved in ISR elicited by these strains [49,50]. In contrast, *P. aeruginosa* 7NSK2–induced systemic resistance was abolished in NahG tobacco [51] and tomato [52], indicating that ISR triggered by this rhizobacterial strain does depend on in planta SA accumulation, even though the marker gene for SA-dependent SAR, PR-1, is not activated. Conversely, *Serratia marcescens* 90-166, which activated the PR-1a promoter in the leaves of transgenic PR-1a-GUS tobacco [43], induced systemic resistance

against wildfire disease equally well in NahG and in untransformed tobacco, ruling out an involvement of SA in the induction [53]. These results clearly indicate that the presence or absence of *PR* gene activation by these rhizobacterial strains is not a reliable parameter for determining whether the mechanism of resistance induction is SA-dependent or SA-independent.

Direct measurements of SA in leaves of tobacco plants grown in soil containing resistance-inducing *P. aeruginosa* 7NSK2 mutant KMPCH (which is deficient in production of the siderophores pyoverdin and pyochelin but can produce SA [see below]), increased free SA levels about 50% over the level in control plants, while amounts of bound SA were similar. A similar increase in free SA was detected in the rhizosphere of bean plants grown in soil with KMPCH. These increases seem sufficient to activate the SAR pathway [39], even though they are too low to activate PR gene expression [49,54]. In cucumber split-root systems, when *P. corrugata* 13 and *P. aureofaciens* 63–28 induced systemic resistance against *Pythium aphanidermatum*, SA accumulated up to sixfold in bacterized root parts and up to fourfold in distant roots on the opposite side. However, in this system exogenously applied SA failed to induce local or systemic resistance against a challenge infection by *Pythium* in planta [55]. This suggests that SA is not a primary causative factor in ISR in cucumber against *Pythium*.

IV. BACTERIAL DETERMINANTS OF ISR

A. Structural Components and Metabolic Compounds

On the one hand, the rhizobacterial strain should produce one or more ISR-eliciting compounds; on the other hand, the plant should possess a matching receptor and an inducible defense pathway downstream of it that activates the induced resistant state upon recognition. In the systemic resistance induced by *P. fluorescens* strains WCS374 and/or WCS417 in carnation, radish, tomato, and Arabidopsis, heat-killed bacteria, bacterial cell walls, or purified outer membrane lipopolysaccharide (LPS) were as effective in inducing systemic resistance as were live bacteria [50,56–58]. These observations provided original proof that ISR was not the result of a bacterial metabolite that might be transported through the plant and affect the activity of a pathogen upon contact. Rather, specific bacterial components must be perceived by the plant. In radish, the bacterial LPS appeared to act as an inducing determinant. In agreement with this finding, bacterial mutants that lacked the O-antigenic side chain of the LPS (OA^-) did not induce resistance in radish. Cell wall preparations of WCS417r likewise induced resistance in Arabidopsis. However, WCS417 OA^- still induced resistance, indicating that another determinant of WCS417r also acts as an inducer of resistance in Arabidopsis. Similarly, *P. putida* WCS358, as well as its LPS, triggered ISR, but WCS358 OA^- retained the capacity to induce systemic resistance. Therefore,

like WCS417r, WCS358r must possess additional inducing determinants. Because LPS may be contaminated by flagella, purified flagella of WCS358r were also tested. Like LPS, the flagella induced resistance, and so did a mutant lacking flagella (P. A. H. M. Bakker, B. W. M. Verhagen, and I. van der Sluis, unpublished). Collectively, these results indicate that flagella, while capable of eliciting ISR, were unlikely to be the sole determinant in LPS preparations, and the O-antigen of LPS must be perceived by the plant independently from the perception of the flagella.

The O-antigenic side chain of LPS consists of repeated oligosaccharide moieties. Such oligosaccharides may resemble fungal elicitors that activate plant defenses [59] and may be perceived by similar types of receptors that are likely to be present in the plasma membrane of the root cells. So far, putative receptors for bacterial LPS have not been characterized in plants, and the mechanism of perception and coupling to induced resistance signaling remains unclear. In contrast, plant cells have been shown to possess a highly sensitive perception system for bacterial flagellins, the major structural protein of the flagella. Recognition occurs through perception by the plant of the most highly conserved domain within the N-terminus of the protein [60]. Sensitivity to flagellin in Arabidopsis is associated with the expression of a putative receptor kinase containing leucine-rich repeats and sharing structural and functional homologies with known plant resistance genes and with components involved in the innate immune system of mammals and insects [61]. Such results imply that the perception of bacterial flagella by plant roots might directly activate a signaling pathway involved in activating resistance responses. Bacterial components involved in antagonizing pathogens in the rhizosphere might also act in inducing resistance. Lytic enzymes, particularly glucanases, can liberate endogenous elicitors from plant cell walls, as has been shown for, e.g., β-1,3-glucanase in soybean [62]. The biocontrol strains *P. fluorescens* 89B-61 and *Enterobacter asburiae* JM22 were endophytic in cotton, and although they did not induce marked cellular alterations upon internal colonization, they did hydrolyze wall-bound cellulose [63]. However, no bacteria that trigger ISR and remain confined to the root surface have been shown to act through this mechanism.

Antibiotics can be toxic not only to pathogenic fungi and bacteria, but also to plant cells. Such toxicity may cause localized necrosis, leading, in turn, to systemically induced resistance. A transformant of *P. fluorescens* CHA0 that overproduced pyoluteorin and 2,4-diacetylphloroglucinol protected tobacco roots significantly better against black root rot, caused by *Thielaviopsis basicola*, than the wild-type strain, but drastically reduced the growth of the tobacco plants and was also toxic to sweet corn and cress [42,64]. There was no correlation between the sensitivity of the pathogens to the antibiotics and the degree of disease suppression by the overproducing strain. It seems, therefore, that resistance was induced, indicating that plants that are sensitive to antibiotics may be induced as

a result of their toxic action. Rhizobacteria and fungi may also produce plant hormones than can affect plant growth and development as well as plant responses [65]. Notably, ethylene reduces root elongation while stimulating production of phytoalexins, synthesis of PRs, and strengthening of plant cell walls upon elicitation of plant defense responses [66,67].

B. Iron-Regulated Metabolites

Iron availability not only influences microbial antagonism by determining the extent of competition through siderophore production, but has also been found to be a major factor in the induction of systemic resistance by rhizobacteria. Pyoverdin siderophore production was implicated in the induction of systemic resistance against TNV in tobacco by *P. fluorescens* CHA0. The level of resistance induced was partially abolished when plants were bacterized with the siderophore-minus (sid$^-$) mutant CHA400 instead of with CHA0 [42]. Purified pseudobactin siderophore of *P. putida* WCS358 induced resistance in Arabidopsis, but its sid$^-$ mutant was still as effective as the wild type, confirming the presence of multiple resistance-inducing determinants in this strain. In radish, the pseudobactin siderophore of *P. fluorescens* WCS374r, but not of *P. putida* WCS358r or *P. fluorescens* WCS417r, induced resistance against *F. oxysporum* f. sp. *raphani*. The sid$^-$ mutant of WCS374 still induced resistance, however, allegedly as a result of LPS acting as the inducing determinant. In the presence of ample available iron, when siderophore production is suppressed, the OA$^-$ mutant did not induce resistance. In contrast, under conditions of low iron availability the OA$^-$ mutant induced the same level of systemic resistance as the wild-type strain, and the level of resistance attained was greater than under iron-sufficient conditions (Fig. 2). This enhanced resistance induction was also evident in the sid$^-$ mutant, suggesting that an additional iron-regulated factor might be involved. Similar results were obtained for strain WCS417, suggesting that the same situation might apply [38].

Under iron-limited conditions, certain rhizobacterial strains such as *P. fluorescens* CHA0, can produce SA as an additional siderophore [68,69]. Thus, it has to be considered that resistance-inducing bacteria may either activate SA-dependent induced resistance by triggering the SAR pathway in the plant, or themselves secrete the signaling compound SA in the rhizosphere. The induction of systemic resistance in tobacco to TNV by strain CHA0 might be fully explained by the production of SA by this strain, the more so because CHA0 also induced PRs in tobacco. Moreover, root colonization of the plants by either the wild-type strain or its sid$^-$ mutant CHA400 caused up to fivefold increases in SA in the leaves [70]. Introduction of the SA-biosynthetic gene cluster *pchDCBA* from *P. aeruginosa* strain PAO1 [33] under the control of a strong constitutive promoter into strain CHA0 increased the production of SA in the rhizosphere of

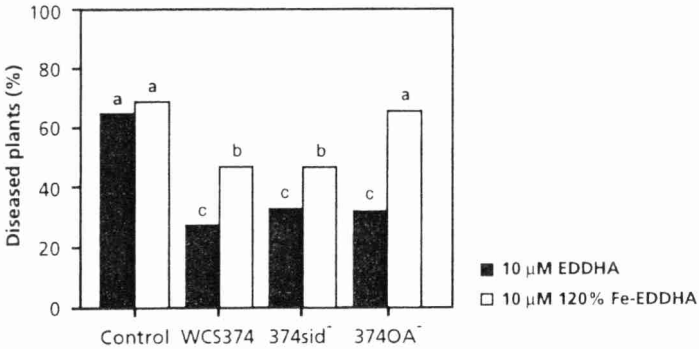

Figure 2 Disease incidence in radish grown in soil containing *Fusarium oxysporum* f.sp. *raphani* without rhizobacteria (control) or in the presence of *Pseudomonas fluorescens* WCS374 or its pseudobactin siderophore-minus (sid⁻) or O-antigenic side chain lacking (OA⁻) mutants, under iron limitation (10 μM EDDHA added) or with ample iron supply (10 μM EDDHA complexed with excess Fe). Different letters indicate statistically significant differences of $p \leq 0.05$.

tobacco but did not further increase protection against TNV. In contrast, introduction of the genes into the non–SA-producing strain P3 made this poor biocontrol agent an effective inducer. Although NahG plants were not tested, these results are consistent with bacterially produced SA acting as the inducing factor [70].

The induction of systemic resistance in bean against *Botrytis cinerea* and in tobacco against tobacco mosaic virus (TMV) by *P. aeruginosa* 7NSK2 was found to be iron-regulated. Under iron limitation 7NSK2 produces three siderophores: pyoverdin, pyochelin, and SA [71]. Both pyoverdin-negative mutants and mutants lacking both pyoverdin and pyochelin induced resistance in bean and tobacco, whereas mutants deficient in SA production did not. Bacterization of NahG tobacco plants with either the wild-type strain or its mutants did not induce systemic resistance to TMV, demonstrating that the resistance induced by 7NSK2 in tobacco, and probably also in bean, is dependent on bacterially produced SA [51,72]. It appeared that nanogram amounts of SA produced by the rhizobacteria are already sufficient to trigger the plant-mediated resistance response, probably through the induction of SA biosynthesis in the plant [39]. However, 7NSK2-induced resistance was not associated with PR-1 expression [51]. Because SA is a precursor of pyochelin, it cannot be excluded that bacterially produced SA is converted to pyochelin and that pyochelin acts as the inducing determinant.

A comparable situation may exist in the systemic resistance induced by *P. fluorescens* WCS374r against *F. oxysporum* f.sp. *raphani* in radish. Both *P. fluorescens* WCS374r and WCS417r are capable of producing SA in vitro under iron-limiting conditions, whereas *P. putida* WCS358r is not. These capacities correlate well with their ability to induce systemic resistance in radish [38,73].

However, no accumulation of PRs was apparent in either roots or leaves of bacterized plants. Cloning of the SA-biosynthetic locus of WCS374r revealed it to contain four open reading frames with homologies to bacterial isochorismate synthase, 2,3-dihydroxybenzoate AMP-lyase, histidine decarboxylase, and chorismate pyruvate-lyase (*pmsCBAE*), respectively. This SA-biosynthetic locus was found to be co-expressed with genes involved in the synthesis of the siderophore pseudomonine, which contains both a salicylate and a histamine moiety [74]. It is possible that in the rhizosphere histidine is provided in root exudates, whereupon SA is quickly incorporated into pseudomonine. Because under those conditions no free SA would be secreted by the bacterium, it can be assumed that SA is not involved in the induction of systemic resistance in radish by WCS374r. Similarly, SA produced by the biocontrol strain *Serratia marcescens* 90-166 is not the primary determinant of ISR in cucumber and tobacco. This strain, while capable of producing SA under low-iron conditions, induced resistance to wildfire disease in both untransformed and NahG tobacco. Bacterial mutants that did not produce detectable amounts of SA retained ISR-eliciting activity against *C. orbiculare* in cucumber, and an ISR-minus mutant still produced SA in vitro [53]. A siderophore may be involved, because fertilization of the plants with ferric iron significantly reduced the level of ISR in cucumber to *C. orbiculare*.

V. *ARABIDOPSIS THALIANA* AS A MODEL TO STUDY RHIZOBACTERIA-MEDIATED INDUCED SYSTEMIC RESISTANCE

A. Signal Transduction

1. SA-Independent Signaling

At least in those instances where no accumulation of PRs is associated with ISR and ISR was fully maintained in NahG plants, an SA-independent pathway must be operative. The existence of such a SA-independent pathway was first demonstrated in Arabidopsis, which was adopted as a model to study the signaling pathway(s) involved in rhizobacteria-mediated ISR [49]. In this model *P. fluorescens* WCS417r was used as the inducing agent, because this strain had been demonstrated to trigger ISR in several plant species, e.g., carnation [5], radish [73], and tomato [11]. Colonization of Arabidopsis roots by WCS417r bacteria was found to protect against different types of pathogens, including the bacterial leaf pathogens *Pseudomonas syringae* pv. *tomato* DC3000 and *Xanthomonas campestris* pv. *armoraciae*, the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani*, and the oomyceteous leaf pathogen *Peronospora parasitica*. WCS417r induced ISR without systemic activation of *PR* genes (49,50; J. Ton and C. M. J. Pieterse, unpublished) and without a concomitant increase in SA levels [75].

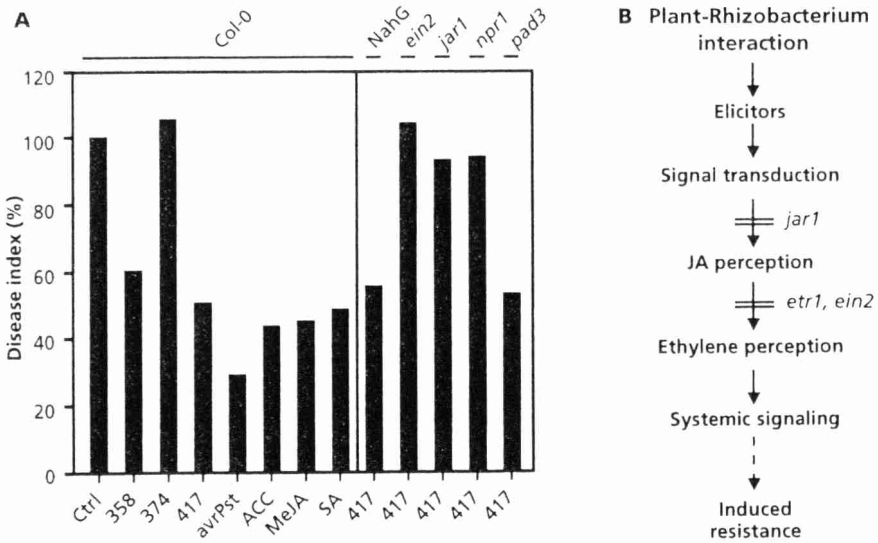

Figure 3 (A) Relative disease index of Arabidopsis ecotype Columbia (Col-0), transformant NahG and mutants treated with different bacterial strains and chemical inducers of systemic resistance and challenged with virulent *Pseudomonas syringae* pv. tomato. (B) Signal-transduction pathway leading to rhizobacteria-mediated induced systemic resistance in Arabidopsis.

Moreover, NahG plants developed normal levels of ISR after colonization of the roots by ISR-inducing WCS417r [49] (Fig. 3A) or WCS358r [50]. Similarly, the SA induction–deficient mutants *sid1-1* and *sid2-1* [76] expressed normal levels of induced resistance in response to ISR-inducing rhizobacteria (C. M. J. Pieterse, unpublished), providing compelling evidence that, in contrast to pathogen-induced SAR, rhizobacteria-mediated ISR in Arabidopsis functions independently of SA. As a consequence, SA-dependent resistance responses were not activated upon bacterization of the roots with WCS417r [54]. Therefore, ISR-expressing plants are unlikely to be protected against pathogens that are resisted exclusively by an SA-dependent defense mechanism. Indeed, WCS417r-mediated ISR was found to be ineffective against turnip crinckle virus (J. Ton, unpublished), which is a pathogen that is resisted by Arabidopsis ecotype Dijon through a defense response that is dependent on SA but not on jasmonic acid (JA) and ethylene [77].

2. Jasmonic Acid– and Ethylene-Dependent Signaling

Studies on mutants and transgenics of Arabidopsis and other plant species, such as tobacco and soybean, revealed that besides SA, JA and ethylene play a key

role in the regulation of plant defenses, because blocking the response to either of these hormonal compounds can render plants more susceptible to certain pathogens and even insects. For instance, mutants that are affected in JA biosynthesis or signaling are more susceptible to pathogens such as *Pythium mastophorum* [78], *Pythium irregulare* [79], and *P. syringae* pv. *tomato* [80], as well as to insect herbivory [81,82]. Similarly, ethylene-insensitive tobacco plants transformed with a mutant *etr1-1* gene from Arabidopsis lost their ability to resist the soilborne pathogen *Pythium sylvaticum* [83]. Similar results were obtained with ethylene-insensitive soybean plants, which developed more severe symptoms in response to *Septoria glycines* and *Rhizoctonia solani* [84]. Furthermore, ethylene-insensitive Arabidopsis mutants exhibit enhanced susceptibility to the necrotrophic fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* [85,86] and to the bacterial leaf pathogens *Erwinia carotovora* pv. *carotovora* [87] and *P. syringae* pv. *tomato* [80].

To investigate the possible role of JA and ethylene in the signaling pathway controlling rhizobacteria-mediated ISR in Arabidopsis, Pieterse et al. [80] tested the JA response mutant *jar1-1* and the ethylene response mutants *etr1-1* and *ein2* for their ability to express ISR. Both types of mutants were unable to develop ISR against *P. syringae* pv. *tomato* in response to bacterization of the roots with WCS417r (Fig. 3A), indicating that both JA and ethylene are involved in the ISR signaling pathway. Like treatment with WCS417r, application of methyl jasmonate (MeJA) or the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) was effective in inducing resistance against *P. syringae* pv. *tomato* in wild-type (Fig. 3A), as well as NahG plants. MeJA-induced protection was blocked in *jar1-1* and *etr1-1* plants, whereas ACC-induced protection was affected in *etr1-1* plants, but not in *jar1-1* plants, indicating that components from the JA response act upstream of the ethylene response in the ISR pathway [80] (Fig. 3B). Whereas in rhizobacterially mediated ISR JA and ethylene appear to act sequentially, a concerted action of JA and ethylene has been described for other defense-related responses. For instance, JA and ethylene are acting together in activating proteinase inhibitor gene expression in tomato in response to wounding [88]. Similarly, induction of the plant defensin gene *Pdf1.2* in Arabidopsis requires concomitant activation of both the JA- and ethylene-response pathway [89].

To further investigate the roles of JA and ethylene in the ISR signaling pathway, the levels of these signaling molecules were determined in plants upon root bacterization. In plants grown in soil containing WCS417r, neither the JA content nor the level of ethylene evolution was altered in systemically resistant leaves [75,90]. Also, at the site of application of ISR-inducing rhizobacteria the levels of JA and ethylene did not change [75,90], indicating that rhizobacteria-mediated ISR is not based on the induction of changes in the biosynthesis of either of these signal molecules.

By using the *Lox2* co-suppressed transgenic line S-12, additional evidence

was obtained that an increase in JA production is not required for the induction or expression of ISR. Transgenic S-12 plants, which are affected in the production of JA in response to wounding [91] and pathogen infection [75], developed normal levels of ISR in response to treatment with WCS417r [75], demonstrating that plants are capable of expressing ISR in the absence of increased JA levels. These results seem to suggest that the JA and ethylene dependency of ISR is based on an enhanced sensitivity to these hormones, rather than on an increase in their production.

3. The Role of NPR1 in ISR Signaling

Although pathogen-induced SAR and rhizobacteria-mediated ISR follow distinct signaling pathways in Arabidopsis, they are both blocked in the regulatory mutant *npr1-1* (for *n*onexpresser of *PR* genes) [80,92] (Fig. 3A). NPR1 (also called NIM1 or SAI1) was originally discovered as a key regulatory protein that functions downstream of SA in the SAR pathway [92–94]. Recently, several research groups provided evidence that, upon induction of SAR, NPR1 activates *PR-1* gene expression by physically interacting with a subclass of basic leucine zipper protein transcription factors that bind to promoter sequences required for SA-inducible *PR* gene expression [95–97]. Elucidation of the sequence of ISR-signaling events revealed that NPR1 also functions downstream of the JA and ethylene response in the ISR pathway [80]. This suggests that NPR1 is required not only for the SA-dependent expression of *PR* genes that are activated during SAR, but also for the JA- and ethylene-dependent activation of so far unidentified enhanced defensive responses resulting from rhizobacteria-mediated ISR. The mechanism underlying the divergence of the SAR and the ISR pathway downstream of NPR1 is not known. Possibly, interactions of pathway-specific proteins with NPR1 are involved.

4. Application of Rhizobacteria to Roots or Leaves Triggers the Same ISR Pathway

Similar to root application, infiltration of leaves with ISR-inducing WCS417r bacteria induces protection against *P. syringae* pv. *tomato* in noninfiltrated leaves [49]. To test whether infiltration of leaves with ISR-inducing rhizobacteria triggers the same signaling pathway as root application, Arabidopsis genotypes Columbia (Col-0), NahG, *jar1-1*, *etr1-1*, and *npr1-1* were tested for their ability to express ISR against *P. syringae* pv. *tomato* after pressure infiltrating three lower leaves with ISR-inducing WCS417r bacteria. Leaf infiltration and root application of WCS417r were similarly effective in eliciting ISR in wild-type Col-0 plants. SA-nonaccumulating NahG plants also developed a statistically significant level of ISR after leaf induction. In contrast, mutants *jar1-1*, *etr1-1*, and *npr1-1* did not express ISR after infiltration of the leaves with ISR-inducing WCS417r

bacteria [75]. Moreover, infiltration of three lower leaves per plant with WCS417r or WCS358r resulted in a significant level of protection against *P. syringae* pv. *tomato* in the nontreated leaves, whereas WCS374r did not induce resistance. These results are in full agreement with those obtained after application of WCS417r, WCS358r, or WCS374r bacteria to the roots [50,80] (Fig. 3A) and demonstrate that ISR-inducing rhizobacteria trigger the same systemic signaling pathway when applied to either roots or leaves.

5. ISR Requires Ethylene-Dependent Signaling at the Site of Induction

Knoester et al. [90] tested a set of well-characterized Arabidopsis mutants that are affected at different steps in the ethylene-signaling pathway for their ability to express ISR. None of the mutants developed ISR against *P. syringae* pv. *tomato* after treatment of the roots with WCS417r, confirming that an intact ethylene-signaling pathway is required for the expression of ISR. Mutant *eir1-1*, which is insensitive to ethylene in the roots but not in the shoots, was able to mount ISR when WCS417r was infiltrated into the leaves, but not when the bacteria were applied to the roots. If ethylene signaling were required only for the systemic expression of ISR at the site of challenge inoculation, *eir1-1* plants should develop normal levels of ISR in the leaves after application of WCS417r to the roots. However, this was not the case. Therefore, one can postulate that ethylene signaling is required at the site of application of the inducer and may be involved in the generation or translocation of the systemically transported ISR signal [90] (Fig. 3B).

B. Molecular-Genetic Analysis

1. Search for ISR-Related Genes in *Arabidopsis*

The state of pathogen-induced SAR is characterized by the concomitant activation of a set of *PR* genes. In SAR-expressing plants, *PR* gene products accumulate systemically to levels from 0.3 to 1% of the total mRNA and protein content [98]. However, although some PRs possess antimicrobial activity, a causal relationship between accumulation of PRs and the broad-spectrum resistance characteristic of SAR has never been convincingly demonstrated [99]. Of many defense-related genes tested in Arabidopsis (e.g., the well-characterized SA-inducible genes *PR-1*, *PR-2*, and *PR-5*, and JA- and/or ethylene-responsive genes *Lox1*, *Lox2*, *Atvsp*, *Pdf1.2*, *Hel*, *ChiB*, and *Pal1*, none were found to be upregulated in plants expressing ISR, either locally in the roots, or systemically in the leaves [54] (Fig. 4). Moreover, neither standard differential screening of a cDNA library of WCS417r-induced plants nor 2D-gel analysis of proteins from induced and noninduced plants yielded significant differences [100]. Thus, in contrast to SAR, the onset

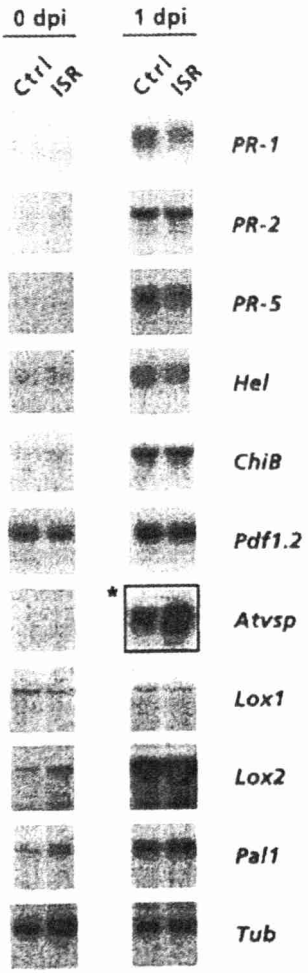

Figure 4 Expression of defense-related genes in ISR-expressing Arabidopsis leaf tissue after challenge inoculation with *P. syringae* pv. *tomato*. ISR was induced by growing the plants in soil containing WCS417r. Leaves of control-treated plants and leaves expressing ISR were harvested 0 and 1 day after inoculation (dpi). The box with the asterisk shows potentiation of the *Atvsp* gene in ISR-expressing tissue.

of ISR is not associated with major changes in gene expression. Nevertheless, ISR-expressing plants are clearly more resistant to different types of pathogens. Therefore, plants must possess as yet undiscovered defense-related gene products that contribute to their broad-spectrum disease resistance.

In a search for ISR-related genes, a large collection of Arabidopsis lines containing enhancer-trap *Ds* transposons with a promoterless β-glucuronidase (GUS) reporter gene [101] was screened. One enhancer-trap line showed local GUS activity in the roots upon colonization by WCS417r (K. M. Léon-Kloosterziel, unpublished). The roots of this line showed a similar expression pattern after treatment with the ethylene precursor ACC, indicating that this line contains a transposon insertion in the vicinity of an ethylene-inducible gene that is upregulated in the roots upon colonization by WCS417r. There are several candidate genes in the vicinity of the enhancer-trap *Ds* transposon, one of which encodes a thaumatin-like protein with homology to tobacco PR-5 and tomato osmotin (K. M. Léon-Kloosterziel, unpublished). However, the role of this gene in ISR remains to be elucidated.

2. Potentiation of JA-Dependent Responses in Plants Expressing ISR

Potentiation is expressed as a faster and stronger activation of defense responses of induced plants after infection with a challenging pathogen and can make the plant react more effectively to an invading pathogen. This phenomenon has been observed in different plant systems. For instance, carnation plants expressing WCS417r-mediated ISR produce significantly more phytoalexins upon stem inoculation with *F. oxysporum* f.sp. *dianthi* [5]. This enhanced phytoalexin production may contribute to limiting ingress of the pathogen in ISR-expressing plants.

To investigate whether phytoalexins are involved in ISR in Arabidopsis, plant mutants impaired in the biosynthesis of the Arabidopsis phytoalexin camalexin were tested for their ability to express WCS417r-mediated ISR. Mutants *pad1-1*, *pad2-1*, *pad3-1*, and *pad4-1* showed wild-type levels of ISR against *P. syringae* pv. *tomato* after root bacterization with WCS417r (Fig. 3A), indicating that at least in this particular plant-pathogen combination phytoalexins do not contribute to the enhanced level of resistance (C. M. J. Pieterse, unpublished).

Potentiation has been demonstrated at the level of gene expression as well. For instance, tobacco plants exhibiting pathogen-induced SAR showed enhanced expression of *PR-10* and *Pal* genes upon challenge with a pathogen [102]. Similarly, SAR-expressing Arabidopsis plants accumulated enhanced levels of *PR-1*, *PR-2*, and *PR-5* transcripts after challenge [54]. Because rhizobacteria-mediated ISR is not associated with increased production of either JA or ethylene, potentiation of JA- or ethylene-dependent defense responses points to an important role in ISR. If the JA and ethylene dependency of ISR is based on enhanced sensitivity

to these signal molecules, one would expect that ISR-expressing plants react faster or more strongly to pathogen-induced JA or ethylene production.

To clarify this point, van Wees et al. [54] studied the expression of the JA-responsive genes *Atvsp*, *Pdf1.2*, *Lox2*, and *Pal1* and the ethylene-responsive genes *Hel* and *ChiB* in control and ISR-expressing plants after challenge with *P. syringae* pv. *tomato*. In noninduced control plants, pathogen infection induced the expression of all genes tested. In challenged, ISR-expressing plants, only *Atvsp* displayed an enhanced level of expression in comparison to challenged control plants (Fig. 4). The expression of the other JA-responsive genes and the expression of the ethylene-responsive genes was not potentiated, suggesting that ISR is associated with the potentiation of a specific set of JA-responsive genes. The mechanism and the significance of this phenomenon in induced broad-spectrum resistance is still unknown.

3. Identification of a Novel Locus (*ISR1*) Controlling Rhizobacteria-Mediated ISR

Previously, Leeman et al. [73] and van Wees et al. [50] provided evidence that the expression of rhizobacteria-mediated ISR varies with the host/rhizobacterium combination by showing that radish and Arabidopsis plants respond differentially to a set of ISR-inducing *Pseudomonas* spp. strains: radish developed ISR against *F. oxysporum* f.sp. *raphani* after colonization of the roots by WCS417r or WCS374r, but not in response to WCS358r, whereas Arabidopsis was responsive to WCS417r and WCS358r, but not to WCS374r (see Fig. 3A). Although both radish and Arabidopsis possess the machinery to express rhizobacteria-mediated ISR and both WCS374r and WCS358r are able to trigger this response, they clearly fail to do so in the radish/WCS358r and the Arabidopsis/WCS374r combinations. Also within plant species, differential induction of ISR has become evident. In Arabidopsis most ecotypes, e.g., Columbia and Landsberg *erecta*, develop ISR in response to treatment with WCS417r, but ecotypes RLD and Wassilewskija (Ws) are nonresponsive [50,103]. This suggests that specific recognition between the plant and the ISR-inducing rhizobacterium is required for the elicitation of ISR.

In a genetic approach to identify ISR-related genes, 10 ecotypes of Arabidopsis were screened for their potential to express ISR and SAR against *P. syringae* pv. *tomato* [103]. All ecotypes tested developed SAR. However, of the 10 ecotypes RLD and Ws did not develop ISR after treatment of the roots with WCS417r. This WCS417r-nonresponsive phenotype was associated with a relatively high susceptibility to *P. syringae* pv. *tomato*, which was apparent as both a higher proliferation of the pathogen in the leaves and more severe disease symptoms. Genetic analysis of the F_1, F_2, and F_3 progeny of a cross between the WCS417r-responsive ecotype Col-0 and the WCS417r-nonresponsive ecotype

RLD revealed that both the potential to express ISR and the relatively high level of basal resistance against *P. syringae* pv. *tomato* are monogenic, dominant traits that are genetically linked. The corresponding locus, designated *ISR1*, was mapped between CAPS markers *B4* and *GL1* on chromosome III. Neither responsiveness to WCS417r nor the relatively high level of basal resistance against *P. syringae* pv. *tomato* was complemented in the F_1 progeny of crosses between RLD and Ws, indicating that both ecotypes are affected in the same locus.

Interestingly, mutants *jar1-1* and *etr1-1*, which are affected in their response to JA or ethylene, respectively, show the same phenotype as ecotypes RLD and Ws in that they are both unable to express WCS417r-mediated ISR and show enhanced susceptibility to infection by *P. syringae* pv. *tomato* [80]. Analysis of the ethylene responsiveness of RLD and Ws revealed that both ecotypes show a reduced sensitivity to ethylene that co-segregates with the recessive alleles of the *ISR1* locus [104]. Therefore, it was hypothesized that the Arabidopsis *ISR1* locus encodes a novel component of the ethylene-response pathway that plays an important role in disease-resistance signaling.

VI. PLANT PHYSIOLOGICAL AND ECOLOGICAL ASPECTS OF INDUCED RESISTANCE

A. Plant Growth and Defense Against Different Types of Attackers

Constitutive expression of pathogen-induced SAR and PRs in Arabidopsis *cpr* mutants has invariably been associated with reduced plant growth. This suggests that maintenance of the state of SAR imparts a metabolic burden on the plant. The induction of systemic resistance in tobacco by *P. fluorescens* CHA0, which is also associated with the accumulation of PRs, likewise reduces plant growth [42]. In contrast, many other rhizobacterial strains that can induce resistance promote plant growth and are hence called plant growth–promoting rhizobacteria (PGPR). Thus, *P. fluorescens* WCS417 was found to stimulate growth of Arabidopsis by 33% [105]. The mechanisms of plant growth promotion by rhizobacteria are only poorly understood [65] and may, or may not, be linked to disease-suppressing properties. However, the absence of major changes in gene expression of plants induced by most PGPR implies only minimal metabolic costs as compared to plants expressing SAR. The level of resistance induced by rhizobacteria is always quantitatively less than SAR induced by necrotizing pathogens [103]. The general absence of PRs in the former and their presence in the latter type of induced resistance, together with the established antipathogen activities of at least some among the PRs, indicates that PRs could be responsible for the higher level of induced resistance with SAR and tissue necrosis [99].

Evidence is accumulating that components from SA-, JA-, and ethylene-

dependent defense pathways can affect each other's signaling. SA and its functional analogs 2,6-dichloroisonicotinic acid and benzothiadiazole suppress JA-dependent defense gene expression [54,106–110], possibly through the inhibition of JA synthesis and action [111]. SA inhibits the JA-dependent wound response, whereas JA has been reported to both stimulate and reduce SA-dependent resistance responses [110,112–114]. Cross-talk between different signal transduction pathways is thought to provide great regulatory potential for coordinating multiple resistance mechanisms in varying combinations and may help the plant to prioritize the activation of a particular defense pathway over another [15,105,115–117]. Such cross-talk between signaling pathways leads to conflicting outcomes with respect to induced resistance to pathogens, which depends primarily on the SA pathway, and to herbivorous insects, which activate the JA pathway [116,118,119].

Since ISR and SAR share the regulatory factor NPR1, the question was raised as to what extent the JA-dependent ISR pathway and the SA-dependent SAR pathway interact. Recently, van Wees et al. [120] investigated possible antagonistic interactions between both pathways. Simultaneous activation of both pathways resulted in an additive effect on the level of induced protection against *P. syringae* pv. *tomato*. No enhanced level of protection was evident in Arabidopsis genotypes that are blocked in either the ISR or the SAR response. Expression of the SAR marker gene *PR-1* was not altered in plants expressing both ISR and SAR compared to plants expressing SAR alone, indicating that the SAR and the ISR pathway are compatible and that there is no significant cross-talk between these signaling pathways. Furthermore, plants expressing both types of induced resistance did not show elevated levels of *Npr1* transcripts. Apparently, the constitutive level of NPR1 is sufficient to facilitate simultaneous expression of both SAR and ISR. It was hypothesized that the enhanced protection in plants expressing both types of induced systemic resistance is established through parallel activation of complementary, NPR1-dependent defense responses that are both active against *P. syringae* pv. *tomato*.

B. ISR and Crop Protection

The level of protection afforded by rhizobacteria-mediated ISR is seldomly more than 50% reduction in disease severity or the numbers of diseased plants, which corresponds to at most a 50% increase in crop yield. Chemical crop protection is usually cheaper and far more effective [121,122]. Therefore, for biocontrol to become economically competitive, its reliability and effectiveness must be improved. Such improvements can be envisaged when ISR is employed in combination with other strategies for suppressing disease. For instance, reducing the activity of a pathogen by microbial antagonism will weaken its pathogenic potential and inhibit it even more when it encounters a host plant in which the defensive

capacity is enhanced through the induction of systemic resistance. In several cases, application of combinations of rhizobacterial strains with different mechanisms improves disease suppression. Moreover, under conditions in which single strains may fail to reduce disease, combinations are likely to afford at least protection by the other strain(s). In the protection of radish against *F. oxysporum* f.sp. *raphani*, strains *P. putida* RE8 and *P. fluorescens* RS111a each suppressed the percentage of wilted plants by about 50%. Both strains are able to induce systemic resistance in radish, but may also possess additional antagonistic mechanisms. Disease suppression by the combination of RE8 and RS111a was significantly better as compared to the single strains [123]. Similar results were obtained in the combination of the resistance-inducing strain RE8 and *P. putida* WCS358 [124]. Suppression of Fusarium wilt of radish by WCS358 does not involve ISR [73] but occurs through siderophore-mediated competition for iron [125]. Similar additive effects have been found in the suppression of damping-off of tobacco seedlings inoculated with *Pythium torulosum*, *Pythium aphanidermatum*, or *Phytophthora parasitica*, when treatment with *Bacillus cereus* strain UW85 was combined with SA-induced SAR [126]. Combination of ISR-inducing rhizobacteria with biocontrol fungi is another option for improving crop protection. Indeed, co-inoculations of rhizobacteria and antagonistic fungi suppressed Fusarium wilt in flax, tomato, and radish under conditions in which their efficacies or population densities were too low to do so on their own [127,128].

Relatively little attention has been given to combining biocontrol agents with low doses of chemical crop protectants. However, when a resistance-inducing biocontrol agent is not sensitive to the chemical compound used, in combination they may act synergistically, such that substantially lower doses of the chemical are needed to achieve a similar level of disease control. Such approaches may substantially decrease the inputs of toxic chemicals into the environment while maintaining adequate levels of crop protection.

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