Early Responses of Tobacco Suspension Cells to Rhizobacterial Elicitors of Induced Systemic Resistance

Leendert C. van Loon,¹ Peter A. H. M. Bakker,¹ Walter H. W. van der Heijdt,¹ David Wendehenne,² and Alain Pugin²

¹Plant-Microbe Interactions, Institute of Environmental Biology, Department of Biology, Faculty of Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands; ²Unité Mixte de Recherche INRA 1088/CNRS 5184/Université de Bourgogne, Plante-Microbe-Environnement, 17 rue Sully, BP 86510, 21065 Dijon Cedex, France

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Colonization of roots by selected strains of fluorescent *Pseudomonas* spp. can trigger induced systemic resistance (ISR) against foliar pathogens in a plant species-specific manner. It has been suggested that early responses in cell suspension cultures in response to rhizobacterial elicitors, such as generation of active oxygen species (AOS) and extracellular medium alkalization (MA), are linked to the development of ISR in whole plants. Perception of flagellin was demonstrated to elicit ISR in *Arabidopsis*, and bacterial lipopolysaccharides (LPS) have been shown to elicit several defense responses and to act as bacterial determinants of ISR in various plant species. In the present study, the LPS-containing cell walls, the pyoverdine siderophores, and the flagella of *Pseudomonas putida* WCS358, *P. fluorescens* WCS374, and *P. fluorescens* WCS417, which are all known to act as elicitors of ISR in selected plant species, were tested for their effects on the production of AOS, MA, elevation of cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$), and defense-related gene expression in tobacco suspension cells. The LPS of all three strains, the siderophore of WCS374, and the flagella of WCS358 induced a single, transient, early burst of AOS, whereas the siderophores of WCS358 and WCS417 and the flagella of WCS374 and WCS417 did not. None of the compounds caused cell death. Once stimulated by the active compounds, the cells became refractory to further stimulation by any of the active elicitors, but not to the elicitor cryptogein from the oomycete *Phytophthora cryptogea*, indicating that signaling upon perception of the different rhizobacterial compounds rapidly converges into a common response pathway. Of all compounds tested, only the siderophores of WCS358 and WCS417 did not induce MA; the flagella of WCS374 and WCS417, although not active as elicitors of AOS, did induce MA. These results were corroborated by using preparations from relevant bacterial mutants. The active rhizobacterial elicitors led to a rapid increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, peaking at 6 min, whereas the inactive siderophores of WCS358 and WCS417 elicited a single spike at 1 min. Elicitation of the cells by cell-wall LPS of WCS358 or the siderophore of WCS374 induced a weak, transient expression of several defense-related genes, including *PAL* and *GST*. The spectrum of early responses of the suspension cells was not matched by the expression of ISR in whole tobacco plants against *Erwinia carotovora* pv. *carotovora*. Of the live bacterial strains, only WCS358 elicited significant ISR, but application of the LPS or the

siderophore of all three strains also elicited ISR. Notably, the absence of elicitation of AOS and MA in suspension-cultured cells but induction of ISR in whole plants by the siderophore of WCS358, which was lost upon treatment with the siderophore-minus mutant of WCS358, indicates that the early responses in suspension cells are not predictive of the ability to induce ISR in whole plants. Possible explanations for these discrepancies are discussed.

Plant roots support large populations of soil bacteria, notably of the genus *Pseudomonas* (Lugtenberg et al. 2001). These rhizosphere bacteria derive nutrients from root cell exudates and lysates and, in return, can promote plant growth and antagonize soilborne plant pathogens through multiple mechanisms (Handelsman and Stabb 1996; Van Loon 2007). In addition, specific bacterial strains can induce a systemic protection against both soilborne and foliar pathogens, a phenomenon known as induced systemic resistance (ISR) (Van Loon and Bakker 2005). Rhizobacteria-mediated ISR is independent of salicylic acid (SA) but requires jasmonate (JA) and ethylene signaling in the plant (Pieterse et al. 1998). This type of induced resistance confers priming of defense reactions in response to subsequent challenge with, particularly, necrotizing pathogens (Ton et al. 2002). ISR has been extensively studied in *Arabidopsis* but has also been convincingly demonstrated in salicylate nonaccumulating NahG tobacco and tomato plants that are unable to express pathogen-inducible, SA-dependent systemic acquired resistance (SAR). In contrast to SAR, ISR is not commonly associated with an accumulation of pathogenesis-related (PR) proteins, even though varying changes at the transcriptional level in reaction to several resistance-inducing rhizobacteria have been documented (Van Loon 2007).

For a number of resistance-inducing bacterial strains, the ISR-eliciting compounds have been identified and shown to comprise the cell wall outer membrane lipopolysaccharide (LPS), iron-chelating siderophores, flagella, antibiotics, quorum-sensing *N*-acylhomoserine lactones, and volatile compounds, such as 2,3-butanediol (Van Loon 2007). Bacterial LPS and flagella have been demonstrated to act as elicitors of innate immunity in animals and to function likewise as pathogen-associated molecular patterns (PAMPs) that are perceived by plants (Nürnberger et al. 2004). Recognition of these PAMPs leads to the activation of inducible plant defense responses that may confer nonhost resistance to nonhost plants and activate basal resistance and reduce disease severity in host plants (Desender et al. 2007; He et al. 2007; Jones and Dangl 2006; Li et al. 2005; Nürnberger and Lipka 2005).

Purified LPS from the bacterial leaf pathogen *Xanthomonas campestris* was shown to induce defense-related gene expression in *Brassica campestris* and to prevent the hypersensitive response in the resistant host *Capsicum annuum* in association with a more rapid induction of antimicrobial hydroxycinnamoyl-tyramine conjugates upon challenge by *X. axonopodis* (Erbs and Newman 2003). Similarly, infiltration of LPS from the bacterial wilt pathogen *Ralstonia solanacearum* or from the nonpathogens *Escherichia coli* and *Serratia marcescens* reduced hypersensitive tissue collapse when the same leaf area was subsequently infiltrated with live pathogen (Graham et al. 1977).

Protection of tobacco against the black shank-causing oomycete *Phytophthora nicotianae* by pretreatment of the leaves with LPS from the nonpathogenic bacterial endophyte *Burkholderia cepacia* was associated with an accumulation of uncharacterized PR proteins (Coventry and Dubery 2001) and phosphorylation of an ERK-like mitogen-activated protein (MAP) kinase (Piater et al. 2004). In tobacco cell suspensions, the LPS induced a rapid influx of Ca^{2+} into the cytoplasm of aequorin-transformed cells, an oxidative burst concomitant with the production of reactive oxygen and nitrogen species, alkalization of the extracellular culture medium (Gerber et al. 2004), and rapid phosphorylation of several proteins (Gerber and Dubery 2004) involved in various signaling and regulatory mechanisms (Gerber et al. 2006), as well as alterations in the expression of several genes (Sanabria and Dubery 2006). In *Arabidopsis* plants, LPS from either pathogenic or nonpathogenic bacteria induced several defense-related genes both locally and, to a much lesser extent, systemically, associated with the induction of a rapid burst of nitric oxide (NO) in cultured cells (Zeidler et al. 2004). In animals, the action of bacterial LPS is mediated by Toll-like receptors (Yang et al. 1998); however, receptors in plants have not been identified yet (Newman et al. 2007).

The mechanism by which flagella activate basal resistance responses is better understood. Conserved peptides within the major flagellar protein, flagellin, are perceived by the Toll-like receptor-like kinase FLS2 in *Arabidopsis* (Gomez-Gomez et al. 2001) and a presumably similar receptor in tomato (Robatzek et al. 2007). In *Arabidopsis*, it has been shown that flagellin perception is transduced through a MAP kinase cascade and WRKY transcription factors (Asai et al. 2002) to give rise to an enhanced resistance against invasion by the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato*, which was associated with callose deposition and activation of PR genes (Gomez-Gomez et al. 1999). Cell cultures of several species, including tobacco, reacted to bacterial flagellins through the conserved peptide flg22 with a rapid alkalization of the culture medium (Felix et al. 1999). In tobacco BY-2 cells, flagellins of nonhost pathogens induced an oxidative burst accompanied by cell death, whereas the flagellin of the host pathogen *P. syringae* pv. *tabaci* did not (Taguchi et al. 2003a), but did in non-host tomato cells (Taguchi et al. 2003b). These differences appeared to be due to post-translational modifications of the flagellin (Taguchi et al. 2003b).

Iron is a limiting nutrient for both plant and microbial growth (Robin et al. 2006). Disease-suppressing rhizobacteria can directly antagonize soilborne pathogens by competition for iron through the release and specific uptake of iron-chelating siderophores in the rhizosphere (Höfte 1993). However, bacterial siderophores can also be perceived by plants and induce systemic resistance (Bakker et al. 2007); for example, as shown by the lack of systemic protection of tobacco against black root rot by a mutant of *P. fluorescens* CHA0 lacking the ability to produce the pseudobactin siderophore (Maurhofer et al. 1994). Bacterial siderophores can influence plant iron metabolism as well (Fett et al. 1998), but a relationship between iron nutritional status and induced resistance is unclear.

All rhizobacterial *Pseudomonas* spp. and strains possess LPS and flagella and produce siderophores under iron-limiting conditions. Yet their capacity to induce systemic resistance is strongly plant species dependent. Of three well-characterized strains, *P. fluorescens* WCS417 has been demonstrated to induce resistance in *Arabidopsis*, bean, carnation, radish, and tomato but not in *Eucalyptus* spp. or rice (Ran et al. 2005; Van Loon 2007). Compared with live bacterial cells, in *Arabidopsis*, cell walls of its rifampicin-resistant derivative WCS417r induced a reduced but still significant protection against *P. syringae* pv. *tomato*, whereas cell walls of a mutant of WCS417r that lacked the O-antigenic side chain of the LPS were fully ineffective (Van Wees et al. 1997). Thus, LPS contributes to the elicitation of ISR in *Arabidopsis* by WCS417r, but other factors must also be involved. In contrast to WCS417, *P. fluorescens* WCS374 is effective in rice, as it is in radish, but not in *Arabidopsis*. In radish, both its LPS and its pseudobactin siderophore have been implicated in eliciting ISR (Leeman et al. 1995, 1996). *P. putida* WCS358 induces resistance in *Arabidopsis*, bean, and tomato but not in carnation, radish, or rice. In *Arabidopsis*, the LPS, flagella, and the siderophore all triggered ISR whereas, in tomato and bean, the LPS and the siderophore were effective but flagella were not (Meziane et al. 2005). Thus, these three bacterial components were differentially active in these plant species. These differences are likely to be the result of different structures of the elicitors. The structure of LPS varies between bacterial strains, with most of the structural heterogeneity residing in the O-antigenic polysaccharide side chain (Lerouge and Vanderleyden 2001). Similarly, flagellin gene sequence variations and post-translational modifications have been observed in the genus *Pseudomonas* (Bellingham et al. 2001; Taguchi et al. 2003b). Siderophores have highly strain-specific peptide structures to enable specific uptake of Fe-siderophore complexes (Visca et al. 2007). On the other hand, plant receptors can vary in their specificity to recognize bacterial elicitors (e.g., as evidenced from the different structural requirements for the perception of flagellin by *Arabidopsis* and tomato) (Robatzek et al. 2007).

The differential specificities of the rhizobacterial elicitors prompted us to initiate a study to compare early responses thought to represent initial signaling steps in the elicitation of ISR by comparing LPS-containing cell walls, flagella, and pseudobactin siderophores of strains WCS417, WCS358, and WCS374 in a single, well-characterized plant system (i.e., suspension-cultured cells of tobacco cv. Xanthi). These cells have been characterized extensively for their early responses to elicitors such as cryptogein from the oomycete *Phytophthora cryptogea* (Garcia-Brugger et al. 2006), which include production of active oxygen species (AOS), extracellular medium alkalization, Ca^{2+} influx and cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) elevation, cell death, and early gene expression. These responses were examined and related to the effectiveness of the three rhizobacterial strains to induce systemic resistance in intact tobacco plants against the bacterial soft rot pathogen *Erwinia carotovora*.

RESULTS

Generation of active oxygen species.

Pseudomonas fluorescens WCS417 has been demonstrated to induce systemic resistance in several plant species, including *Arabidopsis*. The O-antigenic side chain of the LPS is one of the determinants involved, but the nature of other elicitors of this bacterium has not been investigated. Addition of cell walls of WCS417 to tobacco cell suspensions provoked an almost immediate and transient burst of AOS that attained a maximum after approximately 10 min and then returned to control level within 1

h (Fig. 1A). No further increase occurred at longer times (data not shown). Re-addition of cell walls after 60 min did not lead to a renewed burst, indicating that the cells had become refractory to repeated stimulation by the cell walls. In contrast, when, instead of the bacterial cell walls, the elicitor cryptogein was added at 60 min, a strong production of AOS started after approximately 10 min, reached a peak around 30 min and remained elevated for the duration of the experiment (Fig. 1A). This oxidative burst was comparable in timing and magnitude with the cryptogein-induced burst of AOS produced by tobacco cells that had not been exposed previously to bacterial cell walls (Fig. 1B). Thus, the tobacco cells were fully capable of reacting to cryptogein after prior stimulation with cell walls of strain WCS417. When the cells were first stimulated with cryptogein and then bacterial cell walls were added at 60 min, no effect of the latter was evident, indicating that, once stimulated by cryptogein, the cells remained activated and were no longer responsive to the bacterial cell walls (Fig. 1B).

The pseudobactin siderophore of WCS417 did not lead to the production of AOS in the tobacco cells, and the effect of the flagella of this strain was negligible (Table 1), indicating that the tobacco cells reacted by an oxidative burst only in response to the cell walls of WCS417. Indeed, cell walls prepared from a flagella-minus (FL⁻) mutant of WCS417 were as active as those of the wild-type strain (Table 1).

Of *P. putida* WCS358, the cell walls, siderophore, and flagella have all been shown to induce systemic resistance in *Arabidop-*

Table 1. Production of active oxygen species by tobacco cv. Xanthi cells in response to components of *Pseudomonas putida* WCS358, *P. fluorescens* WCS374, and *P. fluorescens* WCS417 and their mutants lacking the O-antigenic side chain of the cell wall lipopolysaccharide (OA⁻), the flagella (FL⁻), or the pseudobactin siderophore (PB⁻)^a

Bacterial strain	Compound		
	Cell walls (10 µg ml ⁻¹)	Flagella (1 µg ml ⁻¹)	Pseudobactin (10 µg ml ⁻¹)
WCS358			
WT	19.0	8.5	0
OA ⁻	18.3	—	—
FL ⁻	19.7	—	—
PB ⁻	0.1	—	—
WCS374			
WT	21.0	ns	11.1
OA ⁻	21.9	—	—
FL ⁻	22.3	—	—
PB ⁻	22.0	—	—
WCS417			
WT	20.6	ns	0
OA ⁻	6.4	—	—
FL ⁻	18.2	—	—
PB ⁻	18.9	—	—

^a Maximum production rates reached at 10 to 15 min after addition of the compounds are indicated and expressed as nmol H₂O₂ per gram fresh weight of cells; WT = wild type, ns = nonsignificant, and — = not determined.

Fig. 1. Production of active oxygen species by tobacco cv. Xanthi cell suspensions in response to cell walls (10 µg ml⁻¹) from *Pseudomonas fluorescens* WCS417 (CW 417) or cryptogein (25 nM). **A**, Water control (◆), CW 417 followed at 60 min (arrow) by re-addition of CW 417 (■), or CW 417 followed by addition of cryptogein at 60 min (▲). **B**, water control (◆), cryptogein followed at 60 min (arrow) by addition of CW 417 (■), or addition of CW 417 at 60 min to a water control (▲). The data are from representative experiments out of a total of 13.

Fig. 2. Production of active oxygen species by tobacco cv. Xanthi cell suspensions in response to components of *Pseudomonas putida* WCS358 or the peptide flg22. **A**, Cell walls (10 µg ml⁻¹) (CW 358) (◆), purified flagella from WCS 358 (1 µg ml⁻¹) (FL 358) (■), 100 nM flg22 (▲), or purified pseudobactin (10 µg ml⁻¹) (PB 358) (×). **B**, CW 358 followed at 60 min (arrow) by addition of FL 358 (◆) or FL 358 followed by addition of CW 358 at 60 min (■). The data are from representative experiments out of a total of 17.

sis. When added to the tobacco cells, both the cell walls and the flagella induced an oxidative burst similar to the one induced by WCS417 cell walls. Moreover, the burst resembled the one induced by the well-characterized flg22 elicitor peptide, except that the ones induced by the bacterial compounds were much stronger (Fig. 2A). The siderophore of WCS358 proved inactive, like the siderophore of WCS417 (Table 1).

Because the bacterial cell wall preparations were derived from killed bacterial cells containing flagella, it was considered that the effect of the WCS358 cell walls might be caused by the flagella, which provoke a comparable response. This notion was supported by our observations that treatment of the tobacco cells with flagella 60 min after the addition of the cell walls caused only a slight oxidative burst, as was also the case upon restimulation by cell walls after initial stimulation by flagella (Fig. 2B). However, cell walls of an FL⁻ mutant of WCS358 were as active as those of the wild-type strain, reaching the same peak value after 10 min. The same applied to cell walls of a mutant lacking the O-antigenic side chain of the cell wall LPS (Table 1). These results suggest that WCS358 cell walls and flagella can independently provoke the same response in tobacco suspension cells.

Fig. 3. Production of active oxygen species by tobacco cv. Xanthi cell suspensions in response to components of *Pseudomonas fluorescens* WCS374 prior to or following cell wall preparations of the three strains. **A**, Cell walls (10 $\mu\text{g ml}^{-1}$) (CW 374) followed at 60 min (arrow) by addition of CW 417 (◆), pseudobactin (10 $\mu\text{g ml}^{-1}$) (PB 374) followed by addition of CW 374 at 60 min (■), or PB 374 followed by CW 358 at 60 min (▲). **B**, CW 417 followed at 60 min (arrow) by addition of PB 374 (◆) or addition of PB 374 at 60 min to water control (■). The data are from representative experiments out of a total of 19.

P. fluorescens WCS374 does not commonly induce systemic resistance in *Arabidopsis* but does so in radish, with both its cell walls and pseudobactin siderophore acting as inducing determinants. The tobacco cells reacted to WCS374 cell walls similarly as to the cell walls of WCS417 and WCS358 (Fig. 3A). To determine whether the response to the cell walls of WCS374 would make the tobacco cells fully refractory not only to the same preparation but also to the cell walls of the other bacterial strains, the cells were supplemented with those at 60 min. As shown in Figure 3A for cell walls of WCS417, a small oxidative burst ensued 10 min later, indicating that the cells could be weakly restimulated by cell walls of another strain. The same result was obtained with cell walls of WCS358, whereas a second burst upon readdition of cell walls of WCS374 was fully suppressed (data not shown).

Contrary to the siderophores of WCS417 and WCS358, the pseudobactin siderophore of WCS374 provoked an oxidative burst with kinetics similar to the one induced by cell walls (Fig. 3A). The reaction to this siderophore caused almost complete refractoriness to additional stimulation by cell walls of WCS374 but only partial refractoriness to cell walls of the other strains (Fig. 3A, shown for WCS358 only). Thus, the siderophore and cell wall preparations seemed to largely but not completely activate the same pathway leading to the production of AOS. However, when the tobacco cells were first treated with cell walls of WCS417 and, after 60 min, the siderophore of WCS374 was added, no restimulation was evident (Fig. 3B), suggesting that prior stimulation by cell walls suppresses the reaction to the siderophore. The refractoriness of the cells was diminished when the addition of the second stimulus was postponed. For instance, upon readdition of the same stimulus after 120 min, an oxidative burst with a peak value of approximately 15% of the first one ensued, indicating that the cells become responsive again with time (data not shown). Flagellar preparations of WCS374 did not cause a significant induction of AOS in the tobacco cells (Table 1).

The active cell wall preparations of WCS417, WCS358, and WCS374 and the pseudobactin siderophore of WCS374 all showed similar dose-response characteristics, as exemplified for the siderophore in Figure 4. A minimum dose of 1 $\mu\text{g/ml}$ was required to cause a significant increase in AOS, and the response was leveling off above 10 $\mu\text{g/ml}$. As shown in Figure 5A for the cell walls of WCS358, the oxidative burst was blocked by the general protein kinase inhibitor staurosporine, by the Ca^{2+} chelator EGTA as well as the inhibitor of plasma membrane Ca^{2+} -permeable channels lanthanum (LaCl_3), and by the NADPH oxidase inhibitor DPI, indicating requirements for phosphorylation events, Ca^{2+} influx, and the action of NADPH oxidase, similar to the characteristics of the oxidative burst provoked by cryptogin (Pugin et al. 1997).

Fig. 4. Concentration dependency of the production of active oxygen species by tobacco cv. Xanthi cell suspensions in response to PB 374. The data are from representative experiments out of a total of four.

To compare the relevance of the presence of the O-antigenic side chain of the bacterial LPS in the cell walls, of the flagella, and of the pseudobactin siderophore for the induction of the oxidative burst by the three types of preparations, the compounds from relevant bacterial mutants of the three strains were tested. Compared with the average peak value of 11.1 nmoles of H_2O_2 per gram fresh weight of tobacco cells for the siderophore of WCS374 (at $10 \mu\text{g ml}^{-1}$), and of 8.5 for the flagella of WCS358 (at $1 \mu\text{g ml}^{-1}$), cell wall preparations at $10 \mu\text{g ml}^{-1}$ were, on average, twice as active (Table 1). Cell walls of all FL^- mutants were as active as those of the wild-type strains. Those from the mutant of WCS417 lacking the O-antigenic side chain (OA^- mutant) had lost two-thirds of their inducing capacity, indicating that, for this strain, the O-antigenic side chain contributes greatly to the induction of the oxidative burst. In contrast, cell walls of the OA^- mutants of WCS358 and WCS374 were as active as those of their wild types (Table 1).

The siderophores are secreted compounds and, in WCS417 and WCS358, did not contribute to the induction of AOS; therefore, it was expected that cell walls of the siderophore-minus (PB^-) mutants would show the same inducing activities as their wild types. This was the case for WCS417 and WCS374 but not for WCS358, whose cell walls had lost all activity (Table 1). Presumably, the PB^- phenotype of this mutant is related to an as-yet-uncharacterized alteration in cell wall properties.

None of the bacterial compounds induced death of the tobacco suspension cells. After 24 h, the percentage of dead cells amounted to $6 \pm 1\%$, comparable with the water controls. In contrast, of cells treated with 25 nM cryptogein, approximately 50% had succumbed (data not shown).

Fig. 5. Effects of inhibitors on **A**, the production of active oxygen species and **B**, medium alkalinization by tobacco cv. Xanthi cell suspensions in response to CW 358 ($10 \mu\text{g ml}^{-1}$). Stau = 5 μM staurosporine, an inhibitor of protein phosphorylation; EGTA (2 mM), a Ca^{2+} -chelator; LaCl_3 (1 mM), a competitive inhibitor of Ca^{2+} fluxes; DPI = 10 μM diphenyleneiodonium, an inhibitor of plasma membrane-bound NADPH oxidase. The data are from representative experiments out of a total of four.

Extracellular medium alkalinization.

Those bacterial compounds that induced an oxidative burst were also active in generating a rapid alkalinization of the extracellular medium (Table 2). However, cell walls of the WCS417 OA^- mutant were almost as active and those of the WCS358 PB^- mutant were approximately half so. Moreover, flagella of WCS417 and WCS374 were also active, be it slightly and substantially less so, respectively, than flagella of WCS358 (Table 2). As exemplified in Figure 6A for the elicitors from WCS358, the cell walls caused a transient increase in extracellular pH of more than one unit within 10 min, with a peak between 10 and 20 min, followed by a gradual subsidence over the next hour, whereas the siderophore was totally inactive. In contrast, the flagella of WCS358 and the siderophore of WCS374 induced a response similar to cell walls and very different from that to cryptogein. The latter started to raise the extracellular pH only after 15 to 20 min, and a plateau was reached after 20 to 40 min (Fig. 6A).

In contrast to the refractoriness of the tobacco cells to restimulation of an oxidative burst, the cells were able to almost fully re-alkalinize the medium upon repeated stimulation (Fig. 6B), even when the same elicitor was re-added after 40 min (data not shown). It made almost no difference whether the cells were restimulated by the same or heterologous cell walls or the siderophore of WCS374 (Figs. 6B and 7). In contrast, restimulation of the tobacco cells by flagella did reduce the second alkalinization response, as exemplified by those of WCS358 shown in Figure 7.

The increase in extracellular pH was fully blocked by staurosporine but not by DPI, indicating that medium alkalinization is dependent on phosphorylation events but independent of the oxidative burst. The Ca^{2+} -influx inhibitors EGTA and LaCl_3 even caused medium acidification (Fig. 5B). These effects were again similar to the action of these inhibitors on cryptogein-stimulated cells (Tavernier et al. 1995).

Increase in $[\text{Ca}^{2+}]_{\text{cyt}}$.

Effects of the bacterial compounds on $[\text{Ca}^{2+}]_{\text{cyt}}$ concentration were monitored in cell suspensions of transgenic *Nicotiana plumbaginifolia* that constitutively expresses the Ca^{2+} -reporter protein apoaequorin in the cytosol. All compounds ac-

Table 2. Medium alkalinization by tobacco cv. Xanthi cells in response to components of *Pseudomonas putida* WCS358, *P. fluorescens* WCS374, and *P. fluorescens* WCS417 and their mutants lacking the O-antigenic side-chain of the cell wall lipopolysaccharide (OA^-), the flagella (FL^-), or the pseudobactin siderophore (PB^-)^a

Bacterial strain	Compound		
	Cell walls ($10 \mu\text{g ml}^{-1}$)	Flagella ($1 \mu\text{g ml}^{-1}$)	Pseudobactin ($10 \mu\text{g ml}^{-1}$)
WCS358			
WT	1.11	1.15	0
OA^-	1.21	—	—
FL^-	1.23	—	—
PB^-	0.50	—	—
WCS374			
WT	0.90	0.98	1.00
OA^-	0.92	—	—
FL^-	1.16	—	—
PB^-	0.96	—	—
WCS417			
WT	0.91	0.47	0
OA^-	0.88	—	—
FL^-	1.24	—	—
PB^-	1.01	—	—

^a Maximum increases in pH (ΔpH units) reached by 10 to 20 min after addition of the compounds are indicated; WT = wild type and — = not determined.

tive in inducing alkalinization in the tobacco cell culture provoked a similar transient increase in $[Ca^{2+}]_{cyt}$ in the *N. plumbaginifolia* cells, as exemplified in Figure 8A for cell walls of WCS358. After a substantial peak at approximately 6 min, $[Ca^{2+}]_{cyt}$ declined quickly for 4 min and then more slowly for another 20 min until basal levels were again reached. Cell walls of the FL⁻ mutant induced a lower response, whereas the cell walls of the PB⁻ mutant, that were ineffective in inducing an oxidative burst (Table 1), induced a still lower response (Fig. 8A), comparable with their effect on medium alkalinization (Table 2). In contrast to the siderophore of WCS374 (Fig. 8B), the siderophores of WCS358 and WCS417, that were not active in medium alkalinization, induced an identical, relatively strong peak during the first 2 min followed by an only minor elevation at approximately 6 min (Fig. 8A, shown for PB 358 only). This indicates that these siderophores did affect the tobacco cells, be it only briefly and apparently without provoking further responses.

Cryptogeiin provoked an increase in $[Ca^{2+}]_{cyt}$ comparable with the rhizobacterial cell walls but peaking slightly later (Fig. 8B). Compared with cell walls of wild-type WCS417, those from its OA⁻ mutant with reduced activity in eliciting active oxygen species (Table 1) and medium alkalinization (Table 2) were largely inactive. In contrast, flagella that did not induce an oxidative

burst but were active in provoking medium alkalinization led to a Ca^{2+} influx comparable with that caused by the cell wall preparations of the three strains or the siderophore of WCS374, as shown for the flagella of WCS374 (Fig. 8B).

Fig. 7. Medium alkalinization by tobacco cv. Xanthi suspension cells upon addition of flagella of *Pseudomonas putida* WCS358 prior to or following the crude cell walls: FL 358 followed at 120 min (arrow) by re-addition of FL 358 (◆), FL 358 followed by cell walls from the flagella-minus (FL⁻) mutant of WCS358 at 120 min (■), or addition of cell walls from the FL⁻ mutant at 120 min to a water control (▲). The data are from representative experiments out of a total of four.

Fig. 6. Medium alkalinization by tobacco cv. Xanthi suspension cells upon addition of rhizobacterial elicitors ($10 \mu\text{g ml}^{-1}$) or cryptogeiin (25 nM). **A**, Water control (◆), cell walls from the flagella-minus (FL⁻) mutant of *Pseudomonas putida* WCS358 (■), FL 358 (▲), PB 358 (×), PB 374 (*), or cryptogeiin (●). **B**, CW 358 followed at 120 min (arrow) by re-addition of CW 358 (◆), by addition of PB 374 at 120 min (■) or addition of FL 358 at 120 min (▲), PB 374 followed by re-addition of PB 374 at 120 min (×) or addition of CW 358 at 120 min (*). The data are from representative experiments out of a total of eight.

Fig. 8. Changes in cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) in suspension cells of aequorin-containing transgenic *Nicotiana plumbaginifolia* upon addition of **A**, water, CW 358 ($100 \mu\text{g ml}^{-1}$), PB 358 ($100 \mu\text{g ml}^{-1}$), and cell walls from the siderophore-minus (PB⁻) or flagella-minus (FL⁻) mutants of *P. putida* WCS358 ($100 \mu\text{g ml}^{-1}$); and **B**, cryptogeiin ($1 \mu\text{M}$), PB 374 ($100 \mu\text{g ml}^{-1}$), FL 374 ($20 \mu\text{g ml}^{-1}$), CW 417 ($100 \mu\text{g ml}^{-1}$), or cell walls from the OA⁻ mutant of *P. fluorescens* WCS417 ($100 \mu\text{g ml}^{-1}$). The data are from representative experiments out of a total of six.

Expression of defense-related genes.

To determine whether elicitor-active LPS or siderophore induced expression of defense-related genes, activation in response to cell walls of WCS358 or the siderophore of WCS374 was studied by Northern blotting (Fig. 9). Several genes showed a weak, transient induction starting before 1 h after elicitation. In all cases, induction subsided after 12 h. Upon elicitation by the cell walls, maximum expression of *PAL* and *GST* was evident after 3 h, whereas *PR-3* and *ACO* showed maximum levels already after 1 h. In response to the siderophore, expression of *PAL*, *LOX*, and *GST* peaked at 6 h and expression of *PR-3* and *ACO* appeared negligible. Thus, elicitation of the tobacco cells by the rhizobacterial compounds led to rather similar, weak, transient expression of a number of defense-related genes.

Induction of systemic resistance.

To be able to relate the observed early effects on tobacco suspension cells to induction of systemic resistance in whole plants, bioassays were conducted in which the roots of tobacco seedlings were dipped in suspensions of live bacteria or the eliciting compounds and plants were challenged 2 weeks later with either the bacterial soft rot pathogen *E. carotovora* or *Tobacco mosaic virus* (TMV). Live WCS358 bacteria significantly reduced bacterial soft rot, whereas WCS374 and WCS417 did not (Fig. 10A). The resistance-inducing activity of WCS358 was lost in the PB⁻ and the FL⁻ mutants but retained in the OA⁻ mutant (Fig. 10B; FL⁻ not shown). In contrast to the wild type and the OA⁻ and PB⁻ mutants, which colonized the tobacco roots to levels of at least 10⁶ CFU/g root, the FL⁻ mutant was impaired in root colonization. For this reason, the inability of the FL⁻ mutant to induce systemic resistance may be due to its inability to reach a sufficiently high population density on the roots.

Based on the inability of the PB⁻ mutant to induce systemic resistance, the pseudobactin siderophore of WCS358 can be implicated as a resistance-inducing determinant. Indeed, treatment with the purified pseudobactin triggered ISR, as did the cell wall preparation, to the same extent as live cells (Fig. 11). Although live cells of WCS374 and WCS417 did not trigger ISR (Fig. 10), their pseudobactins as well as their cell walls did induce a systemic protective effect against *E. carotovora* (Fig. 11). These results suggest that either the compounds produced in vitro on plate media are structurally different from those produced in vivo in the rhizosphere or, in the rhizosphere, the active structures of these components are not exposed to the root cells.

Fig. 10. Induction of systemic resistance against *Erwinia carotovora* in tobacco cv. Samsun NN plants treated with 0.1 mM MgSO₄ (Ctrl) or suspensions containing 5×10^8 CFU of live **A**, wild-type *Pseudomonas putida* WCS358, *P. fluorescens* WCS374, or *P. fluorescens* WCS417; and **B**, wild-type *P. putida* WCS358 and its mutants lacking the O-antigenic side chain of the cell wall lipopolysaccharide (OA⁻) or the pseudobactin siderophore (PB⁻). Soft rot lesion sizes were determined 3 days after inoculation and disease ratings were expressed as percentages of lesions in classes I (no lesion expansion), II (lesion expansion up to 5 mm), and III (lesion expansion >5 mm). Asterisks indicate statistically significant distributions of the disease-severity classes compared with the noninduced control treatments (χ^2 , $\alpha = 0.05$) from a total of 12 experiments.

Fig. 11. Induction of systemic resistance against *Erwinia carotovora* in tobacco cv. Samsun NN plants treated with 0.1 mM MgSO₄ (Ctrl) and either cell walls (CW) or purified pseudobactin (PB) (both at 10 μ g ml⁻¹) of *Pseudomonas putida* WCS358, *P. fluorescens* WCS374, or *P. fluorescens* WCS417. Soft rot lesion sizes were determined 3 days after inoculation and disease ratings were expressed as percentages of lesions in classes I (no lesion expansion), II (lesion expansion up to 5 mm), and III (lesion expansion >5 mm). Asterisks indicate statistically significant distributions of the disease-severity classes compared with the noninduced control treatments (χ^2 , $\alpha = 0.05$). The data are representative of three experiments.

Fig. 9. Expression of defense-related genes *PAL* (encoding phenylalanine ammonia-lyase), *LOX* (lipoxygenase), *GST* (glutathion S-transferase), *PR-3* (pathogenesis-related protein 3), and *ACO* (ACC oxidase) in tobacco cv. Xanthi suspension cells upon elicitation of CW 358 (10 μ g ml⁻¹) or PB 374 (10 μ g ml⁻¹). The data are representative of three experiments.

None of the bacterial strains induced systemic resistance to TMV (data not shown), confirming and extending previous results that WCS417-mediated ISR is not effective against viruses (Ton et al. 2002).

DISCUSSION

Production of AOS, medium alkalinization, and $[Ca^{2+}]$ influx are common reactions of plant suspension cells to biotic stresses. Notably, AOS have been associated with stress adaptation as well as programmed cell death (Gechev et al. 2006), and are known to act as signals mediating both defense gene activation and the execution of the hypersensitive reaction in response to pathogens (Torres et al. 2006). In tobacco cell suspensions, the reactions to the elicitor cryptogein have been well characterized (Garcia-Brugger et al. 2006), involving not only an oxidative burst, rise in extracellular pH, cytosol acidification, and $[Ca^{2+}]$ influx but also a rapid efflux of nitrate anions, generation of NO, activation of protein kinases, defense-related gene expression, and, finally, cell death. These reactions appear typical of microbial effectors triggering reactions that lead to a hypersensitive response.

The elicitors from nonpathogenic rhizobacteria that induce systemic resistance prime plants for enhanced defense reactions upon challenge inoculation with a pathogen but are characteristically not associated with extensive transcriptional reprogramming or with cell death (Van Loon 2007; Verhagen et al. 2004). Unlike cryptogein, which triggered a rapid and long-lasting production of AOS by the tobacco suspension cells, cell wall preparations of the rhizobacterial strains WCS358, WCS374, and WCS417; the pseudobactin siderophore of WCS374; and flagella of WCS358 all induced a rapid, but transient, oxidative burst lasting less than 30 min, similar to the one induced by the active flagellar peptide flg22. Whereas treatment with cryptogein led to cell death, cells treated with the rhizobacterial elicitors remained fully viable for at least 24 h. Their oxidative burst was also similar to the ones induced by the β -1,3-glucan laminarin in tobacco (Ménard et al. 2004) and grapevine cells (Aziz et al. 2003), which were demonstrated to be associated with increased defense-related gene expression and induced resistance in intact plants against *E. carotovora*, and *Botrytis cinerea* and *Plasmopara viticola*, respectively. Because cell walls of the FL⁻ mutants of the rhizobacterial strains WCS358, WCS374, and WCS417 were as active as those from the wild types, flagella were not required for the induction of the oxidative burst. In addition to flagella, the cell wall preparations consisted of the membranous LPS, which was shown earlier to be an inducing determinant of ISR in several plant species, including radish (Van Loon 2007). At least in radish, induction of systemic resistance requires a minimum population density of the rhizobacteria of 10^5 CFU/g of root and reaches a maximum at approximately 10^6 CFU g⁻¹ (Raaijmakers et al. 1995). Based on the yield of the bacterial compounds obtained during preparation, these numbers fit very well with the observed dose-response curve for the oxidative burst, with a threshold for cell walls at $1 \mu\text{g ml}^{-1}$ corresponding to 10^5 CFU and the response leveling off above $10 \mu\text{g ml}^{-1}$. Hence, it is most likely the LPS that is responsible for the induction of the oxidative burst.

The cell wall LPS of gram-negative bacteria consists of a lipid A moiety, a conserved core oligosaccharide, and a highly variable O-antigenic carbohydrate side chain. The detailed structures of the LPS of strains WCS358, WCS374, and WCS417 are not known; however, the observations that cell walls of OA⁻ mutants of WCS358 and WCS374 that lacked the O-antigenic side chain were as active as those from the corresponding wild types suggest that, for these strains, the lipid A

and core oligosaccharide are sufficient to trigger a full oxidative burst. This was not the case for the OA⁻ mutant of WCS417, which showed only one-third of the activity of wild-type cell walls. Thus, for WCS417, the O-antigenic side chain seems largely responsible. So far, receptors for bacterial LPS have not been identified in plants (Newman et al. 2007). How different structures can lead to an apparently identical oxidative burst remains to be established.

The ability to trigger a similar oxidative burst seems to be more common among rhizobacterial LPS, as shown for *Burkholderia cepacia* (Gerber et al. 2004). Perception of the LPS of this strain likewise triggered a rapid rise in extracellular pH, Ca^{2+} influx (Gerber et al. 2004), defense-related gene expression, and, in intact plants, protection against *Phytophthora nicotianae* (Coventry and Dubery 2001). In contrast, LPS of the pathogenic bacterium *X. campestris* pv. *campestris* triggered an oxidative burst that started later and was prolonged (Meyer et al. 2001), resembling more the reaction to cryptogein. In common with the reaction of cryptogein-treated tobacco cells, the reaction to the rhizobacterial LPS-containing cell walls was blocked by EGTA, LaCl₃, staurosporine, and DPI, indicating that the oxidative burst is commonly dependent on Ca^{2+} influx and protein phosphorylation, and involves the plasma-membrane-bound NADPH oxidase.

A second stimulation of the tobacco cells by addition of one of the active bacterial compounds after the initial oxidative burst had passed showed that, in all combinations, a state of either complete or at least substantial refractoriness was evident. In contrast, addition of cryptogein after bacterial compounds led to a full cryptogein-specific production of AOS, demonstrating that the cells had retained their full capacity to develop an oxidative burst. Hence, the refractoriness to the rhizobacterial compounds must be due to a desensitization as a result of the initial burst. The fact that the cell walls of the three strains as well as the siderophore of WCS374 and the flagella of WCS358 all induced, and were sensitive to, this desensitization strongly suggests that the same signaling pathway, or common signaling components involved in the regulation of the oxidative burst, were activated. However, it is highly unlikely that the structurally divergent compounds were all recognized by the same receptor. The small second bursts seen when flagella were employed prior to or after treatment with cell walls, when cell walls of WCS417 were added after cell walls of WCS374 and, notably, when cell walls followed treatment with the pseudobactin of WCS374, indicate that quantitative variations occurred that were more pronounced as the bacterial components were more dissimilar. Hence, it is most likely that signaling upon perception by different receptors converges rapidly into a common response pathway.

Extracellular medium alkalinization was induced with identical kinetics, involving a rise of more than one pH unit within 10 min, by the cell walls of the three rhizobacterial strains and the siderophore of WCS374 but not the pseudobactins of WCS358 or WCS417. A short, transient depolarization measured on roots of intact radish plants 10 min after dipping in a suspension of live cells of WCS374 (H. Steijl and A. van Bel, *unpublished*) indicates that a kinetically similar response is evident in intact roots. Medium alkalinization is the common parameter employed to monitor the action of the bacterial PAMP flagellin (Felix et al. 1999). Our observations agree with this notion, in that the flagella of not only WCS358 but also WCS374 and, to a slightly lesser extent, WCS417 led to a rise in extracellular pH. However, our observations that the flagella of WCS374 and WCS417 did not induce any significant oxidative burst in the tobacco cells indicate that the production of AOS and medium alkalinization are not necessarily correlated. As is evident from the use of the inhibitors, medium alkalinization was independent

of the action of the H₂O₂-producing NADPH oxidase, although it did require protein phosphorylation and Ca²⁺ influx. Moreover, stimulated cells became hardly refractory to medium alkalization upon repeated stimulation.

From the literature, it is evident that the kinetics of changes in extracellular pH are different for elicitors that induce a comparable oxidative burst. The alkalization of the medium of tobacco cells upon treatment with the LPS from *B. cepacia* was much smaller and was maintained for at least 60 min (Gerber et al. 2004), whereas the reaction to laminarin was completely terminated by 30 min (Klarzynski et al. 2000). Moreover, [Ca²⁺]_{cyt} increased only slowly in aequorin-transformed tobacco cells treated with LPS from *B. cepacia* (Gerber et al. 2004). In contrast, the increase in response to the cell walls of WCS358, WCS374, and WCS417; the siderophore of WCS374; and flagella of the three strains was much more rapid and transient, proving rather similar to the one induced by applying flg22 (Lecourieux et al. 2005) or LPS of pathogenic *X. campestris* pv. *campestris* bacteria (Meyer et al. 2001) and dissimilar from the one induced by laminarin (Lecourieux et al. 2006). The siderophores of WCS358 and WCS417 were perceived by the tobacco cells, as evidenced by the strong spike of [Ca²⁺]_{cyt} 1 min after their addition. This response was very different from the one induced by the other rhizobacterial compounds. These results demonstrate that these compounds induced partly similar, partly different responses in the tobacco suspension cells compared with published data on other bacterially derived elicitors. Such differences may be related to the use of different cell lines varying in their reactions and sensitivities to similar compounds. Moreover, during signaling, different responses can result from differences in the timing and the intensity of similar responses.

Treatment of tobacco suspension cells with laminarin or LPS from *B. cepacia* was shown to result in an accumulation of PR proteins (Coventry and Dubery 2001; Klarzynski et al. 2003) and an induction in intact plants of systemic resistance against *E. carotovora* and *P. nicotianae*, respectively. Similarly, infiltration of leaves on *Arabidopsis* plants with flg22 or LPS preparations from the nonpathogenic bacteria *Escherichia coli* and *Pseudomonas aeruginosa* increased SA levels and induced PR gene expression, as well as both local and systemic acquired resistance against *P. syringae* pv. *maculicola* (Mishina and Zeier 2007), similar to the flg22-triggered ISR against *P. syringae* pv. *tomato* (Zipfel et al. 2004). In contrast, WCS417 ISR-elicited in *Arabidopsis* against several pathogens (Ton et al. 2002) and insects (Van Oosten et al. 2008) is associated neither with increases in SA, JA, or ethylene (Pieterse et al. 2000), nor with PR-gene expression in the leaves (Verhagen et al. 2004). In the tobacco suspension cells, at least the cell walls of WCS358 and the siderophore of WCS374 induced a transient expression of a range of defense-related genes over the first 24 h, including SA-inducible PR-3, JA-regulated LOX, and ethylene-stimulated ACO. Apparently, the active rhizobacterial elicitors stimulate defense-related gene expression only temporarily at an early stage, and this may or may not reflect the primed state that results from the induction of ISR.

Induction of systemic resistance was tested by challenging treated plants with either the bacterial pathogen *Erwinia carotovora* or the virus TMV. *E. carotovora* has been shown to be sensitive to SA-independent but ethylene-dependent defenses (Geraats et al. 2003; Vidal et al. 1998) and, therefore, should be sensitive to the JA- and ethylene-dependent ISR induced by the large majority of nonpathogenic rhizobacteria (Ton et al. 2002; Van Loon and Bakker 2005). In contrast, TMV is resisted by SA-dependent defenses and sensitive to SAR (Ryals et al. 1996). In agreement with these different characteristics,

none of the bacterial treatments induced resistance against TMV, whereas ISR against *E. carotovora* was induced by one of the three bacterial strains and by all of the purified cell wall and siderophore preparations.

The expression of ISR against *E. carotovora* in treated plants did not correlate with the reactions of the tobacco suspension cells to the rhizobacterial compounds. Whereas, in the cell suspensions, the cell wall LPS of WCS358, WCS374, and WCS417 and the siderophore of WCS374 all induced the production of AOS, medium alkalization and an increase in [Ca²⁺]_{cyt} in whole plants, live bacteria of only strain WCS358 triggered significant ISR. Its cell wall LPS and pseudobactin siderophore were similarly active, in spite of the fact that the latter did not provoke an oxidative burst or a rise in extracellular pH in the cell suspension. In accordance with these results, the OA⁻ mutant could still trigger ISR through its siderophore, whereas the PB⁻ mutant appeared to be impaired in its cell-wall-dependent activity also: its cell walls did not induce an oxidative burst in the suspension-cultured cells. Apparently, the PB⁻ mutant had lost both its LPS and siderophore activity to elicit ISR. The results obtained with WCS374 and WCS417 are more difficult to interpret. In spite of the absence of ISR elicited by live cells of these two strains, their cell wall LPS and siderophores were all active in eliciting ISR against *E. carotovora*. This discrepancy may be explained by differences in the structure or the expression of these determinants between bacteria grown under different conditions (Newman et al. 2007).

These results clearly demonstrate that the ability of a rhizobacterial elicitor to trigger early signaling events commonly related to defense responses in tobacco suspension cells does not necessarily correspond to its resistance-inducing properties in intact tobacco plants. Suspension cells differ physiologically from cells in intact plant tissues, and the chlorophyll-containing tobacco cells used do not visibly resemble the chlorophyll-lacking root cells that must be the site of perception of the bacterial compounds in vivo. Additionally, the reactions measured in suspension cells are local reactions, whereas ISR is a systemic response that must be mediated by distinct signaling compounds that are generated locally and transported throughout the plant. Verhagen and associates (2004) showed that, in *Arabidopsis*, strain WCS417 induced distinct changes in gene expression locally in the roots but only primed the leaves for defense without any obvious alterations in gene expression—which occur merely after challenge inoculation. Similarly, sulfated laminarin elicited an oxidative burst and PR-gene expression in tobacco but protected against TMV only locally, not systemically (Ménard et al. 2004, 2005).

The poor correlation between the defense-related reactions in suspension-cultured cells and the elicitation of ISR in whole plants indicates that the relationship between early reactions, such as the production of AOS, the extracellular alkalization, and the signature of the Ca²⁺ signal, is not straightforward, and that their interpretation needs caution as well as further consideration. The parameters are obviously useful in analyzing the perception and early signal transduction of rhizobacterial compounds. However, their relationship to the generation and establishment of ISR remains far from clear.

MATERIALS AND METHODS

Cultivation of bacteria and preparation of bacterial components and cryptogoin.

Bacterial strains and mutants are listed in Table 3. *P. putida* WCS358, *P. fluorescens* WCS374, and *P. fluorescens* WCS417 and their mutants were grown for 24 h on King's medium B

(KB) agar plates (King et al. 1954) at 28°C. The cells were collected in 10 mM MgSO₄, washed twice by centrifugation for 5 min at 5,000 × g, and finally resuspended in 10 mM MgSO₄.

All bacterial compounds were prepared at least twice from batches cultured at different times. Lipopolysaccharide-containing cell walls (crude LPS) were isolated essentially as described previously (Leeman et al. 1995). Bacterial cells were resuspended in 10 mM phosphate-buffered saline (PBS), pH 7.2. The suspension was centrifuged for 10 min at 8,000 × g, and the pellet washed again and lyophilized. Lyophilized cells (100 mg) were resuspended in 10 ml of 50 mM Tris-HCl, 2 mM EDTA, pH 8.5, and sonicated 20 times at resonance amplitude for 15 s at 0 to 4°C. The translucent sonicated suspension was centrifuged for 20 min at 600 × g to remove intact cells, and the supernatant was centrifuged again for 60 min at 8,000 × g to obtain the broken cell walls as a pellet. The pellet was lyophilized and the dry powder was stored at 0 to 4°C.

For the preparation of flagella, bacterial cells were resuspended in 20 mM K-phosphate buffer, pH 7.4, washed by centrifugation, and treated in a blender (Ultra-Turrex T25, Janke & Kunkel IKA Labortechnik) at 21,000 rpm to shear off the flagella. Intact cells and cell debris were removed by centrifugation for 1 h at 23,400 × g at 4°C. The supernatant was centrifuged for 4 h at 60,000 × g at 4°C to obtain the flagella as a pellet. The pellet was lyophilized and stored at –20°C.

Purified pseudobactin siderophores were isolated from culture supernatants of bacteria grown for 4 days in 2 liters of standard succinate medium, pH 7, at 28°C. Cells were removed by centrifugation for 20 min at 5,700 × g, the supernatant was lyophilized in darkness, and the pseudobactin was purified as described by Raaijmakers and associates (1994). After chromatography, using PD10 columns (Pharmacia), the pseudobactin-containing fractions were collected, lyophilized, and stored as a dry powder in darkness at 0 to 4°C. Purity was checked based on the molar extinction coefficient of 13,670 at 400 nm and pH 7 of pseudobactin 358 and by analytical high-performance liquid chromatography using purified pseudobactin 374 as a reference (Djavanheri 2007).

For testing, all preparations were suspended in sterile distilled water to a concentration of 1 mg ml^{–1}. To saturate pseudobactin preparations with iron, solutions were brought to 0.9 mM FeCl₃ (approximately 110% Fe). The addition of Fe to the siderophore preparations had no significant effect on the production of AOS. Furthermore, none of the preparations possessed any catalase activity as measured by a change in absorbance at 240 nm upon addition of H₂O₂, ruling out quenching in the determination of AOS-eliciting activity.

The pathogen *E. carotovora* pv. *carotovora* was grown for 24 h in liquid Luria-Bertani (LB) medium (Miller 1972) at 28°C.

Cells were collected by centrifugation and the supernatant re-centrifuged for 20 min at 8,000 × g. The pellets were resuspended in 10 mM MgSO₄, whereas the supernatant was filtered through a 0.2-μm polycarbonate filter and the filtrate checked for the absence of bacterial cells by plating on LB agar plates. A 50% dilution of the cell-free supernatant was used as a source of auto-inducers to stimulate the virulence of the pathogen on tobacco (Smadja et al. 2004).

Cryptogein was purified according to Bonnet and associates (1996) and dissolved in water as a 100-μM stock solution.

Plant material.

Cell suspensions of tobacco (*N. tabacum* cv. Xanthi) and transgenic aequorin-expressing *N. plumbaginifolia* were grown as described previously (Lecourieux et al. 2002; Pugin et al. 1997) and subcultured weekly. Cells were maintained in the exponential phase of growth and subcultured 1 day prior to utilization.

Tobacco plants (cv. Samsun NN) were grown from seed in a greenhouse under controlled conditions (Geraats et al. 2003) for 6 to 8 weeks. After inoculation with the pathogen, plants were kept under a glass cover to maintain relative humidity near 100%.

Chemicals.

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The chemicals were dissolved in water except for staurosporine, which was prepared as a stock solution in dimethyl sulfoxide.

Determination of AOS.

The production of AOS by the cells was assayed by chemiluminescence, using luminol as reagent (Pugin et al. 1997). Briefly, cells were washed by filtration and resuspended to 0.1 g fresh weight ml^{–1} in buffer (10 mM HEPES, 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, pH 5.75), and then equilibrated for 2 h at 24°C on a rotary shaker (150 rpm) before treatments. Typically, 3 × 10⁵ cells are present per 0.1 g fresh weight in the suspensions. Upon addition of test compounds, samples of 250 μl were withdrawn every 5 min and added to 350 μl of 50 mM HEPES, 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, pH 6.5, and luminol (final concentration 25 μM). Chemiluminescence was measured using a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) for 10 s and expressed in nanomoles of H₂O₂ per gram fresh weight of cells, based on a standard calibration curve obtained by H₂O₂ addition to tobacco cell suspension aliquots.

Inhibitors were added 5 min before the addition of the test compounds.

Table 3. Bacterial strains and mutants used in this study

Strain	Origin and relevant properties	Reference
<i>Erwinia carotovora</i> pv. <i>carotovora</i> SCC3193	Isolated from infected potato	Pirhonen et al. 1988
<i>Pseudomonas fluorescens</i> WCS374	Isolated from potato rhizosphere	Geels and Schippers 1983
<i>P. fluorescens</i> WCS374r	Spontaneous rifampicin-resistant derivative of WCS374	Geels and Schippers 1983
WCS374 LWP74 30b	Spontaneous O-antigen-negative mutant of WCS374	De Weger et al. 1989
WCS374 L29	WCS374 Tn5 mutant lacking flagella	De Weger et al. 1987
WCS374 Mut2	WCS374 Tn5 mutant lacking pseudobactin	Weisbeek et al. 1986
<i>P. fluorescens</i> WCS417	Isolated from wheat rhizosphere	Lamers et al. 1988
<i>P. fluorescens</i> WCS417r	Spontaneous rifampicin-resistant derivative of WCS417	Van Peer et al. 1990
WCS417 UP17 B4	Spontaneous O-antigen-negative mutant of WCS417	Leeman et al. 1996
WCS417 Mob-10	WCS417 Tn5 mutant lacking flagella	P. A. H. M. Bakker, unpublished
WCS417 S680	WCS417 Tn5 mutant lacking pseudobactin	Duijff et al. 1993
<i>P. putida</i> WCS358	Isolated from potato rhizosphere	Geels and Schippers 1983
<i>P. putida</i> WCS358r	Spontaneous rifampicin-resistant derivative of WCS358	Glandorf et al. 1992
WCS358 43B	Spontaneous O-antigen-negative mutant of WCS358	De Weger et al. 1987
WCS358 GMB6	WCS358 Tn5 mutant lacking flagella	L. A. de Weger, unpublished
WCS358 JM218	WCS358 Tn5 mutant lacking pseudobactin	Marugg et al. 1985

Determination of medium pH.

Extracellular pH was measured with a glass combination electrode every 10 min on pre-equilibrated 1-g aliquots of cells in 2 mM HEPES, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 175 mM mannitol, pH 5.75.

Measurements of [Ca²⁺]_{cyt}.

Aequorin-expressing *N. plumbaginifolia* cells were suspended in 2 mM HEPES, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 175 mM mannitol, pH 5.75. In vivo reconstitution of aequorin was performed by addition of 1 µM coelenterazine to cells in suspension buffer for at least 3 h in the dark (150 rpm, 24°C). Coelenterazine is a prosthetic group of aequorin required for its full Ca²⁺-binding activity. The bioluminescence of 250-µl aliquots of cells (maintained in a cuvette) was recorded continuously at 1-s intervals by digital luminometry. At the end of the experiments, residual functional aequorin was quantified by adding 300 µl of lysis buffer (10 mM CaCl₂, 2% Nonidet P40 [vol/vol], 10% ethanol [vol/vol]) and monitoring the resulting increase in luminescence. Luminescence data transformation into cytosolic Ca²⁺ concentration was performed as described previously (Lecourieux et al. 2002).

Monitoring of cell death.

Cell viability was assayed using the vital dye Neutral Red as described by Binet and associates (2001). To 2 ml of cell suspension in 10 mM HEPES, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 175 mM mannitol, pH 5.75, the test compound, and 20 µl of 1% Neutral Red solution were added and the cells were incubated on a rotary shaker at room temperature. Dead cells that had not accumulated the dye were counted 24 h later.

Determination of defense-related gene expression.

Upon addition of LPS-containing cell walls of strain WCS358 or the pseudobactin siderophore of WCS374 to the tobacco cell suspension maintained in the culture medium, 2.5-ml samples were withdrawn at intervals, filtered, and immediately frozen. Total RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD, U.S.A.) according to the supplier's instructions. Total RNA (10 µg per lane) was separated on 1.2% agarose gels (wt/vol) containing 1.1% formaldehyde (wt/vol), blotted to a nylon membrane (Hybond-XL; Amersham Biosciences, Piscataway, NJ, U.S.A.), and cross-linked by UV light. The cDNA clones used as probes for hybridization were labeled by random priming (Ready-To-Go DNA Labelling Beads -dCTP; Amersham Biosciences). Membrane hybridization was performed at 65°C as described by Church and Gilbert (1984). The membrane was washed with 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) twice for 5 min at room temperature, with 0.5% sodium dodecyl sulfate (wt/vol) and 2× SSC twice for 30 min at 65°C, and subsequently with 0.1% SSC twice for 30 min at room temperature. The membrane was then exposed to X-Omat AR film (Kodak, Rochester, NY, U.S.A.).

Bioassay for ISR.

Four-week-old tobacco seedlings were uprooted and treated by dipping the root system in a 200-ml suspension of live bacteria (10⁸ CFU/ml) or bacterial component (25 µg ml⁻¹) in 10 mM MgSO₄ for 15 s before transplanting into a sand-potting soil mixture that had been autoclaved twice with a 24-h interval. After 14 days, the plants were challenge inoculated with *E. carotovora* by infiltrating three leaves with 50 to 75 µl of a suspension of the pathogen (10⁸ CFU ml⁻¹ in 50% cell-free culture filtrate), using a syringe without a needle, and kept for 3 days at 100% relative humidity. During this period, soft rot lesion development was monitored daily. Lesion sizes were de-

termined after 3 days, and disease ratings were expressed as percentages of lesions in classes I (no lesion expansion), II (lesion expansion up to 5 mm), and III (lesion expansion > 5 mm). Data were analyzed statistically by SPSS version 14.0 (χ^2 , α = 0.05).

Alternatively, plants were challenged by inoculation with purified TMV and lesion sizes were determined 7 days later, as described previously (Knoester et al. 2001).

Root colonization by *Pseudomonas* strains.

To determine the ability of the three rhizobacterial strains to colonize tobacco roots to high levels, rifampicin-resistant derivatives of strains WCS358, WCS374, and WCS417 were employed. Rhizosphere population densities were determined 1 week after transplanting and treatment of the roots with 10⁸ CFU ml⁻¹. The roots of two plants per treatment were harvested, weighed, rinsed briefly in water, and shaken vigorously for 1 min in glass tubes containing 5 ml of 10 mM MgSO₄ and 0.5 g of glass beads (0.17 mm). Appropriate dilutions were plated on KB agar supplemented with cycloheximide (100 mg liter⁻¹), ampicillin (40 mg liter⁻¹), chloramphenicol (13 mg liter⁻¹), and rifampicin (150 mg liter⁻¹). After overnight incubation at 28°C, the number of CFU per gram of root fresh weight was determined.

All experiments were conducted at least twice with similar results. All data reported are from representative experiments. Variation within experiments was negligible; variation between experiments was due entirely to minor differences in responsiveness between individual batches of tobacco suspension cells.

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