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Analysis of determinants of *Pseudomonas fluorescens* WCS374r involved in induced systemic resistance in *Arabidopsis thaliana*

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Abstract: The role of iron-regulated determinants of *Pseudomonas fluorescens* WCS374r in mediating induced systemic resistance (ISR) against *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) and *Turnip crinkle virus* (TCV) was studied in *Arabidopsis thaliana*. Under conditions of iron limitation, WCS374r produces large amounts of salicylic acid (SA), the fluorescent siderophore pseudobactin (Psb), and pseudomonine (Psm), a siderophore with an SA moiety. The biosynthesis of SA and Psm in WCS374r is closely related, since mutants impaired in SA biosynthesis are also defective in the production of Psm. Mutants affected in the production of one or more of these metabolites were used to unravel their role in ISR by WCS374r against Pst and TCV. WCS374r-mediated ISR against Pst does not rely on the production of any of the iron-regulated metabolites, because all mutants impaired in the biosynthesis of one or several of these metabolites elicited ISR. In contrast, SA biosynthesis by WCS374r is a prerequisite to effectively trigger ISR against TCV, and simultaneous biosynthesis of Psb appears to be required. Our data suggest that different determinants of WCS374r are required for ISR against different pathogens.

Key words: *Pseudomonas fluorescens* WCS374r, *P. syringae* pv. *tomato*, pseudobactin, pseudomonine, salicylic acid, siderophores, *Turnip crinkle virus*

Introduction

Bacterial determinants of rhizobacteria-mediated induced systemic resistance (ISR) that have been identified include cell surface components, such as outer membrane lipopolysaccharides (LPS) or flagella, iron-regulated metabolites, volatile compounds, antibiotics, and cyclic lipopeptides (Bakker et al., 2007; Iavicoli et al., 2003; Meziane et al., 2005; Ongena et al., 2008; Ryu et al., 2004; Tran et al., 2007). *Pseudomonas fluorescens* strain WCS374r can elicit ISR in radish against *Fusarium oxysporum* (Leeman et al., 1995). These authors provided evidence that LPS, the pseudobactin siderophore (Psb), and (an) unknown iron-regulated metabolite(s) are involved in inducing resistance in radish. Particularly salicylic acid (SA), an iron-regulated metabolite produced in relatively large quantities by WCS374r, was suggested to be involved (Leeman et al., 1996). However, strain WCS374r did not induce resistance in Arabidopsis against *P. syringae* pv. *tomato* (Pst), whereas application of the chemical SA did (Van Wees et al., 1997). These observations can be explained by hypothesizing that this bacterium does not exude free SA in the Arabidopsis rhizosphere. WCS374r produces the additional siderophore pseudomonine (Psm), which contains a SA moiety (Mercado-Blanco et al., 2001) and, thus, in the rhizosphere all SA might be incorporated in Psm. However, when applied at a low inoculum density *P. fluorescens* strain WCS347r does trigger ISR against Pst in Arabidopsis. Moreover, WCS374r elicited ISR in

Arabidopsis against *Turnip crinkle virus* (TCV). We investigated the involvement of the iron-regulated metabolites SA, Psm and Psb in ISR against both these pathogens, by comparing the abilities of the parental strain and selected mutants to elicit ISR.

Material and methods

Bacterial strains and growth conditions

P. fluorescens WCS374r was originally isolated from the rhizosphere of potato (Geels &Schippers, 1983) and is resistant to chloramphenicol, ampicillin and rifampicin. The Tn*5* insertion mutants defective in Psb production, 374-02 and 374-08, were described by Weisbeek et al. (1986), and Mercado-Blanco et al. (2001), respectively. Mutants 4A-1 (Psb^+, Psm^-, SA^+), AT-12 (Psb^-, Psm^-, SA^+), 4B-1 (Psb^+, Psm^-, SA^-) and BT-1 (Psb^-, Psm^-, SA^-) were described by Djavaheri (2007). The bacteria were grown on King's medium B (KB) agar plates for 24 h at 28˚C. Bacterial cells were scraped off the plates and suspended in 10 mM $MgSO_4$. The suspension was centrifuged (10 min at 8000 x *g*) and the pellet was re-suspended in 10 mM $MgSO_4$.

Cultivation and bacterial treatment of plants

Arabidopsis was grown as described by Pieterse et al. (1996). Briefly, seeds of *A. thaliana* accession Col-0 were sown in quartz sand and two-week-old seedlings were transferred to 60-ml pots containing a sand/potting soil mixture that was autoclaved twice for 20 min with a 24 h interval. Treatments with *P. fluorescens* were done by inoculation of the soil with a bacterial suspension to final densities of 10^3 or 5×10^7 colony-forming units (CFU) per g of soil, prior to transplanting the seedlings. The ISR bioassays were performed by challenging five-week-old plants with either Pst or TCV. Rhizosphere population densities of the introduced bacteria were determined by dilution plate counts on KB agar containing the appropriate antibiotics, as described by Van Wees et al. (1997).

Pst inoculation and disease rating

Pst was cultured O/N at 28˚C in liquid KB. Suspensions of the pathogen were prepared and challenge inoculations were performed as described by Pieterse et al. (1996). Three to four days after challenge the percentage of leaves per plant showing necrotic or water-soaked lesions surrounded by chlorosis were scored.

TCV inoculation and disease rating

Bioassays with TCV were performed as described previously (Ton et al., 2002). Eleven days after challenge inoculation the percentage of leaves per plant with symptoms of chlorosis and curling was scored.

Results and discussion

When applied at low cell densities WCS374r elicits ISR against Pst

In ISR bioassays *P. fluorescens* WCS374r was applied to soil at densities of 10^3 or 5×10^7 CFU per g. When the bacterial strain was introduced at the high density, its level in the Arabidopsis rhizosphere remained fairly constant, around 10^7 CFU per g root. However, when applied at low density, rhizosphere populations of WCS374r rapidly increased from 10^3 to 10^7 CFU per g root within three weeks. As shown previously by Van Wees et al. (1997), application at the high density did not result in elicitation of ISR against Pst. However, when applied at the low density of 10^3 CFU per g of soil, WCS374r reduced disease severity by almost 40% as compared to the control treatment. Apparently, active multiplication of WCS374r is required for production of bacterial elicitors of ISR in the rhizosphere of *Arabidopsis*.

WCS374r-mediated ISR against TCV is independent of initial cell densities
Both at the high and the low initial inoculation density, WCS374r significantly reduced the percentage of diseased leaves. Thus, in contrast to ISR against Pst, WCS374r-mediated ISR against TCV does not depend on the initial density at which the rhizobacterium was applied to the soil. These results suggest that ISR against the bacterial and the viral pathogen depends on different bacterial determinants. Using mutants of WCS374r defective in the production of one or more iron-regulated metabolites, we tested this hypothesis.

WCS374r-mediated ISR against Pst is independent of Psb, Psm or SA production
In these experiments, WCS374r and mutants 4A-1 (Psb^+, Psm^-, SA^+), AT-12 (Psb^-, Psm^-, SA^+), 4B-1 (Psb^+, Psm^-, SA^-), BT-1 (Psb^-, Psm^-, SA^-), and 374-02 and 374-08 (Psb^-, Psm^+, SA^+) were all applied at 10^3 CFU per g of soil. WCS374r and all its mutants reduced the relative percentage of leaves with symptoms of Pst significantly. Thus, production of the iron-regulated metabolites Psb, Psm or SA by WCS374r is not required for its elicitation of ISR against Pst in Arabidopsis.

SA and Psb are both required for WCS374r-mediated ISR against TCV
The relative percentage of leaves showing symptoms of TCV was significantly reduced by WCS374r (introduced at 10^3 CFU per g of soil) as compared to the non-treated control. The Psb^+, Psm^-, SA^- mutant 4B-1 did not reduce disease incidence, whereas the Psb^+, Psm^-, SA^+ mutant 4A-1 controlled disease as effectively as the wild-type strain, suggesting that SA is a key metabolite in ISR against TCV. However, all Psb mutants, including those that can produce SA (AT-12, 374-02 and 374-08), did not reduce disease symptoms. Collectively these data suggest that both SA and Psb are necessary for WCS347r to elicit ISR against TCV.

Conclusions

When applied at low, but not at high, population densities, *P. fluorescens* WCS374r can elicit ISR in Arabidopsis against the bacterial pathogen Pst, with neither of the iron-regulated metabolites Psb, Psm or SA being required. In contrast, WCS374r-mediated ISR against TCV depends on production of both SA and Psb, and WCS374r is effective when applied at either low or high population densities. These results suggest that different elicitors of WCS374r trigger signal-transduction pathways that are differentially effective against the bacterial and the viral pathogen used in this study.

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