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Impact of induced systemic resistance on the bacterial microbiome of *Arabidopsis thaliana*

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Abstract: Induced systemic resistance (ISR) has been described for several strains of fluorescent pseudomonads in a variety of crop plants and is effective against a wide range of pathogens. Using the model plant *Arabidopsis thaliana*, progress has been made in understanding signal transduction pathways involved in induced resistance. Perception of ethylene and jasmonic acid is important for ISR. The traits of *Pseudomonas* spp. that can trigger ISR appear to be diverse, and include iron regulated metabolites, antibiotics, and lipopolysaccharides. Since pathogen growth is restricted on plants that are in the state of ISR, we hypothesized that the indigenous microflora could also be affected by ISR. Using cultivation dependent techniques, effects of plant defense signaling on the total bacterial and the *Pseudomonas* spp. microflora of *Arabidopsis* were studied and related to susceptibility of *Arabidopsis* genotypes to bacterial speck caused by *Pseudomonas syringae* pv. *tomato*.

Key words: fluorescent *Pseudomonas* spp., induced systemic resistance, phyllosphere, root colonization

Introduction

Induced systemic resistance (ISR) triggered by selected plant growth-promoting *Pseudomonas* bacteria is effective against a wide range of plant pathogens (Bakker et al., 2007). Activation of ISR in plants leads to an enhanced defensive capacity enabling plants to respond faster and/or more effectively to microbial attackers (Van Loon et al., 1998; Conrath et al., 2002; Verhagen et al., 2004). ISR requires an intact response to jasmonic acid (JA) and ethylene (ET), although it is not associated with increased production of these hormones, and neither with increased expression of known defense-related genes (Van Wees et al., 1999; Pieterse et al., 2000; Verhagen et al., 2004). *Arabidopsis* mutants defective in expression of ISR and/or altered in salicylic acid (SA) signaling, and exogenous application of methyl-JA and SA were used to study the possible impact of defense signaling on the rhizosphere microflora. Denaturing gradient gel electrophoresis (DGGE) revealed that mutants of *Arabidopsis thaliana* affected in the JA and/or SA responsive signal transduction pathway developed a bacterial rhizosphere microflora that differed from the one on the wild-type control, however, application of JA or SA did not affect the bacterial community structure (Doornbos et al., 2011). In the present study possible effects of selected *Arabidopsis* mutants that differ in their susceptibility to *P. syringae* pv. *tomato* DC300 (*Pst*) on rhizosphere and phyllosphere bacterial and *Pseudomonas* spp. populations were evaluated.

Material and methods

Cultivation of plants

Arabidopsis genotypes used are listed in table 1. Seeds were sown in autoclaved (20 min at 121°C) sand in shallow plastic containers. The containers were covered and kept at 4°C in the dark for 2 days, after which the seeds were allowed to germinate in a greenhouse conditioned as described below, at 100% relative humidity. Routinely, 2-week-old seedlings were transferred individually to 60ml pots containing a potting-soil/sand mixture (12:5 v/v) that had been autoclaved twice for 20 min with a 24 h interval. However, plants used for analysis of abundance and diversity of indigenous *Pseudomonas* populations in the rhizosphere were transplanted into non-autoclaved potting soil-sand mixture. After transplanting, plants were grown in the greenhouse with an 8 h-day (200µE m^{-2} sec^{-1}) at 24°C and 16 h night cycle at 20°C and 70% relative humidity, and watered with half-strength Hoagland nutrient solution once a week and with tap water as required.

Table 1: Listing and relevant characteristics of *Arabidopsis* lines used.

Genotype	Relevant characteristics
Col-0	Wild-type accession Colombia-0
cpr1-1	constitutive expressor of PR* genes 1, SA overproducer
etr1-1	ethylene response 1, ET insensitive
jar1-1	jasmonate resistance 1, JA insensitive
NahG	Transformant expressing bacterial SA hydroxylase, does not accumulate SA
npr1-1	non-expressor of PR-genes, impaired in SA and JA/ET-dependent defense responses

*PR: pathogenesis related

Disease induction and assessment

Five-week-old plants were inoculated with *Pst* as described by Pieterse *et al.* (1996). Briefly, plants were placed at 100% relative humidity one day before inoculation. *Pst* was cultured overnight in liquid KB medium (King et al., 1954) at 28°C while shaken at 180rpm. Bacterial cells were washed by centrifugation for 5 min at 1.200 x *g* and resuspended in 10mM $MgSO_4$. Leaves were dipped in a bacterial suspension of $2.5 \cdot 10^7$ cfu/ml *Pst* supplemented with 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands), and kept at 100% relative humidity. Four days after inoculation, disease severity was quantified by determining the fraction of leaves per plant showing necrotic lesions and/or chlorosis. From ten plants of each genotype, leaves of five replicates consisting of two pooled plants were ground in a mortar and pestle in 10mM sterile $MgSO_4$. Population densities of *Pst* were quantified by plating appropriate dilutions on KB agar (King et al., 1954) supplemented with 100µg/ml

natamycin and 50µg/ml rifampicine. Numbers of colony forming units (cfu) were determined after incubation for 48 h at 28°C (Pieterse et al., 1996).

Quantification of bacterial populations in the rhizosphere

Roots with tightly adhering soil were harvested and shaken three times in 10mM $MgSO_4$ with glass beads (0.6-0.8mm) on a vortex at maximum speed for 1 min. Population densities of aerobic heterotrophic bacteria were determined by plating serial dilutions on 1/10 strength TSA^+ [3g/l tryptic soy broth (Difco Laboratories, Detroit, Mi, USA), 13g/l agar technical (Oxoid Ltd, Basingstoke, UK) and 100µg/ml natamycin (Delvocid, DSM, Delft, NL)] and counting colony forming units (cfu) after 7 days incubation at 20°C. Quantification of *Pseudomonas* spp. was performed by plating appropriate dilutions on KB^+ agar [KB agar (King et al., 1954), supplemented with 13µg/ml chloramphenicol, 40µg/ml ampicillin and 100µg/ml natamycin (Delvocid, DSM, Delft, NL) and counting cfu after incubation for 48 h at 28°C.

Analysis of bacterial phyllosphere communities

From ten 5-week-old plants, three leaves were removed with sterile scissors and placed in an Eppendorf vial. Weighed samples were ground in 10mM sterile $MgSO_4$ with a sterile Eppendorf pestle (Eppendorf, Hamburg, Germany). Population densities of aerobic heterotrophic bacteria were assessed by plating serial dilutions on 1/10 strength TSA^+ [3g/l tryptic soy broth (Difco Laboratories, Detroit, Mi, USA), 13g/l agar technical (Oxoid Ltd, Basingstoke, UK) and 100µg/ml natamycin (Delvocid, DSM, Delft, NL)]. Numbers of cfu per gram of leaf were determined after incubation for 7 days at 20°C. Quantification of predominantly *Pseudomonas* spp. was performed by plating appropriate dilutions on KB^+ agar [KB agar (King et al., 1954), supplemented with 13µg/ml chloramphenicol, 40µg/ml ampicillin and 100µg/ml natamycin (Delvocid, DSM, Delft, NL). Numbers of cfu were determined after incubation for 48 h at 28°C.

Results and discussion

Disease severity and Pst population densities in Arabidopsis mutants affected in defense signaling

A significantly higher percentage of diseased leaves was observed in the mutants *etr1* and *npr1*, and in the NahG transformant as compared to the wild type. Population densities of *Pst* were determined by selective plating and varied between 10^4 and 10^6 cfu per gram of leaf. Although no significant differences in *Pst* populations were observed, their numbers were approximately 10-fold lower in the *cpr1* mutant, which constitutively expresses SA-dependent defenses. In line with this observation, the genotypes unable to express SA-dependent defense responses, *npr1* and NahG, displayed 8- and 6-fold higher population densities of the pathogen, respectively. However, a significant correlation between disease severity and population densities of the pathogen was not apparent for the different genotypes.

Populations of culturable bacteria and Pseudomonas spp. in the phyllosphere and rhizosphere of Arabidopsis genotypes differing in Pst susceptibility

To assess the effect of altered defense signaling on the indigenous bacterial microflora in the phyllosphere, the same genotypes were assayed for their bacterial microflora in the absence of *Pst*. Population densities of aerobic bacteria and *Pseudomonas* spp. were quantified by selective plating. Compared to Col-0, higher population densities of culturable bacteria were

found in the phyllospheres of *etr1*, *npr1*, and NahG. For the *Pseudomonas* spp. it was observed that not only *etr1*, *npr1*, NahG, but also *jar1* harbored significantly higher population densities. Apparently, increased susceptibility of a genotype to *Pst* is correlated with higher population densities of indigenous bacteria in the phyllosphere.

Population densities of total culturable bacteria in the rhizospheres of the different Arabidopsis genotypes ranged from $2 \cdot 10^7$ to $1 \cdot 10^9$ cfu per gram of rhizosphere soil. The JA-response mutant *jar1*, the ET-response mutant *ein2* and the constitutive SA-producing *cpr1* showed significantly lower numbers of culturable bacteria compared to the Col-0 wild type. Numbers of cfu of *Pseudomonas* spp. in the rhizosphere were between $5 \cdot 10^5$ and $5 \cdot 10^7$ per gram root and demonstrated tendencies similar to total bacteria, except for *ein2*. However, *Pseudomonas* populations seemed more sensitive to SA-dependent defenses, indicated by a decreased abundance in *cpr1* and a tendency of increased bacterial numbers in the NahG rhizospheres.

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