Induced Systemic Resistance in *Arabidopsis thaliana* in Response to Root Inoculation with *Pseudomonas fluorescens* CHA0

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Root inoculation of *Arabidopsis thaliana* ecotype Columbia with *Pseudomonas fluorescens* CHA0r partially protected leaves from the oomycete *Peronospora parasitica*. The molecular determinants of *Pseudomonas fluorescens* CHA0r for this induced systemic resistance (ISR) were investigated, using mutants derived from strain CHA0: CHA400 (pyoverdine deficient), CHA805 (exoprotease deficient), CHA77 (HCN deficient), CHA660 (pyoluteorin deficient), CHA631 (2,4-diacetylphloroglucinol [DAPG] deficient), and CHA89 (HCN, DAPG- and pyoluteorin deficient). Only mutations interfering with DAPG production led to a significant decrease in ISR to *Peronospora parasitica*. Thus, DAPG production in *Pseudomonas fluorescens* is required for the induction of ISR to *Peronospora parasitica*. DAPG is known for its antibiotic activity; however, our data indicate that one action of DAPG could be due to an effect on the physiology of the plant. DAPG at 10 to 100 μ M applied to roots of *Arabidopsis* mimicked the ISR effect. CHA0r-mediated ISR was also tested in various *Arabidopsis* mutants and transgenic plants: NahG (transgenic line degrading salicylic acid [SA]), *sid2-1* (nonproducing SA), *npr1-1* (non-expressing NPR1 protein), *jar1-1* (insensitive to jasmonic acid and methyl jasmonic acid), *ein2-1* (insensitive to ethylene), *etr1-1* (insensitive to ethylene), *eir1-1* (insensitive to ethylene in roots), and *pad2-1* (phytoalexin deficient). Only *jar1-1*, *eir1-1*, and *npr1-1* mutants were unable to undergo ISR. Sensitivity to jasmonic acid and functional NPR1 and EIR1 proteins were required for full expression of CHA0r-mediated ISR. The requirements for ISR observed in this study in *Peronospora parasitica* induced by *Pseudomonas fluorescens* CHA0r only partially overlap with those published so far for *Peronospora parasitica*, indicating a great degree of flexibility in the molecular processes leading to ISR.

Plants are protected from microbial infection by a combination of preformed and inducible barriers. Systemic acquired resistance (SAR) is a widely described induced defense mechanism that has been demonstrated for a large number of plant-pathogen interactions (Hammerschmidt 1999; Sticher et al. 1997). SAR is expressed locally and systemically after a local-

ized infection by a necrotizing pathogen and is associated with an early increase in endogenously synthesized salicylic acid (SA) (Sticher et al. 1997) and the subsequent activation of a large number of genes coding for pathogenesis-related proteins (PRs) (Van Loon 1997). The induced genes associated with SAR are likely to include genes other than those for PRs only. For example, genome-wide analyses of *Arabidopsis thaliana* undergoing SAR revealed a number of genes besides those for known PRs that are likely to function in the induced resistance response (Maleck et al. 2000).

Colonization of roots with plant growth-promoting rhizobacteria (PGPR) can also lead to systemic resistance in parts of the plant that are spatially separated from the inducing microorganism. The protection is typically manifested as both a reduction in disease symptoms and inhibition of pathogen growth and appears to be phenotypically similar to pathogen-induced SAR. This effect of rhizobacteria is referred to as induced systemic resistance (ISR) and has been demonstrated in different plant species, e.g., bean, carnation, cucumber, radish, tobacco, tomato, and in the model plant *Arabidopsis thaliana* (Van Loon et al. 1998).

Selected biocontrol rhizobacteria provide protection to soil-borne pathogens by antagonistic interactions between the biocontrol agent and the pathogen, as well as by stimulation of the host defense. This control can involve the production of various antibiotic compounds or iron chelators by the biocontrol strain, competition between the pathogen and biocontrol strain for colonization sites at the root surface, and induced resistance in the host (Baker et al. 1985). A well-studied example is *Pseudomonas fluorescens* WCS417r-mediated ISR in *Arabidopsis thaliana*. *Pseudomonas fluorescens* WCS417r applied on roots protects leaves from *Pseudomonas syringae* pv. *tomato* DC3000 and *Fusarium oxysporum* f. sp. *raphani*. Resistance is induced independently of SA and PR accumulation before infection but requires an intact response to the plant hormones jasmonic acid (JA) and ethylene (ET) (Pieterse et al. 1998). In particular, root inoculation of *Peronospora parasitica* with *Pseudomonas fluorescens* WCS417r does not lead to an accumulation in the roots or in the leaves of the SA-responsive genes *PR-1*, *PR-2*, and *PR-5*, of the ET-inducible gene *Hel*, of the ET- and JA- responsive genes *ChiB* and *Pdf1.2*, or of the JA-inducible genes *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2*. A change could only be observed in the potentiation of the expression of JA-dependent *Atvsp* after pathogen challenge of ISR-expressing plants (van Wees et al. 1999). Further studies using *Peronospora parasitica* have shown that ISR is effective against those pathogens that, in a noninduced state, are resisted through JA- and ET-dependent defenses (Ton et al. 2002). The bacterial determinants responsible for the induction of resis-

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tance in the aerial parts of the plant are not yet characterized but seem to depend on multiple traits (Pieterse and van Loon 1999; Pieterse et al. 2001; Van Loon et al. 1998).

In this article, we describe an *Arabidopsis*-based model system in which the biocontrol strain *Pseudomonas fluorescens* CHA0 (Stutz et al. 1986) is used to induce ISR in leaves to pathogenic strains of *Peronospora parasitica*. It is the first report of a typical PGPR-induced ISR in *A. thaliana* besides the studies using strain WCS417r (Pieterse et al. 1996, 1998, 2000; Ton et al. 2002). We have examined the spectrum of resistance induced by strain CHA0 in *Arabidopsis* and have explored the physiological and molecular basis for CHA0-induced ISR. Our findings only partially overlap with those observed for ISR induced by *Pseudomonas fluorescens* WCS417r, indicating that it is difficult to derive a general model for PGPR-induced ISR.

RESULTS

Pseudomonas fluorescens CHA0r-mediated ISR against *Peronospora parasitica*.

We first tested if root colonization by a rifampicin-resistant mutant of *Pseudomonas fluorescens* CHA0 could be consistently obtained with the root-inoculation method used here. The amount of bacteria recovered from root surfaces was determined three weeks after inoculation of 1-week-old plants with *Pseudomonas fluorescens* CHA0r. A single inoculation of a 30-ml pot with 5×10^7 CFU per g of soil of *Pseudomonas fluorescens* CHA0r was used for the pretreatments of plants, and colonization of roots was reflected by a bacterial titer of 1.8×10^7 CFU per g of root fresh weight (FW) ($\pm 1.2 \times 10^6$; $n = 7$). This titer was consistently obtained throughout all experiments carried out in this study. We also tested if a clean spatial separation of the challenging leaf pathogen and the inducing agent could be obtained. A suspension of strain CHA0r was applied to the roots and, two weeks later, the leaf material was tested for the presence of CHA0r. Leaves were excised and ho-

mogenized, and aliquots of the homogenate were plated out on King's medium B (KB) agar plates amended with rifampicin. No bacterial colonies could be observed, indicating that CHA0r remained confined to the root zone (data not shown). The protective effect of CHA0r against *Peronospora parasitica* was then tested in young *Arabidopsis* seedlings (1-week-old plants at the start of the treatment with CHA0r). Colonization of the rhizosphere by *Pseudomonas fluorescens* CHA0r resulted in a decrease in fungal sporulation (Fig. 1A) and a decreased fungal colonization (Figs. 1B and 2). Typical hallmarks of resistance, such as callose deposits or trailing necroses, were not observed. The correspondence between fungal sporulation and host colonization is an indication of the protection induced by the treatment with *Pseudomonas fluorescens*. We used fungal sporulation as a parameter in the rest of this study to quantify the level of protection; this method is both rapid and quantitative. No differences in appearance, size, or weight of plants could be observed as a result of the treatment with CHA0r (data not shown). Furthermore, no changes in the content of 73 ions (including K, Ca, S, Mg, P, Cl, Na, Mn, Fe, Al, Sr, Zn, Si, and Ti, i.e., the 14 more abundant elements) after treatment of plants with CHA0r could be observed, using inductively coupled plasma mass spectrometry (ICP-MS) analysis (Matera and Le Hécho 2001; data not shown). Thus, under the experimental conditions used here, root treatment with CHA0r did not lead to detectable effects on plant growth that could have affected the sporulation of the pathogen.

ISR mediated by mutants of *Pseudomonas fluorescens* CHA0.

In order to find bacterial factors involved in ISR against *Peronospora parasitica*, the potential of *Pseudomonas fluorescens* CHA0r to induce ISR was compared with that of some mutants affected in the expression of factors known to be associated with biocontrol. *Pseudomonas fluorescens* WCS417r was included in these experiments as a positive control, since it has

Fig. 1. Protection against *Peronospora parasitica* as a result of root treatment with *Pseudomonas fluorescens* CHA0r in wild-type *Arabidopsis* Col-0 plants. ISR was triggered by growing 1-week-old plants for 2 weeks in soil containing *Pseudomonas fluorescens* CHA0r at 5×10^7 CFU per g of soil. The 3-week-old plants were challenge-inoculated by spraying a spore suspension of compatible *Peronospora parasitica* NOCO at 2.5×10^4 spores per ml. Five days after challenge inoculation, the disease was rated by **A**, the sporulation or **B**, the hyphal development after microscopic inspection of stained single leaves. Letters indicate statistically significant differences between the control and CHA0r-treated plants (Fisher test; $\alpha = 0.05$; $n > 50$). Data presented are means ($\pm$ SE) from at least three independent experiments. **B**, Five days after challenge inoculation. Disease rating classes were as follows: 0 = no hyphae, I = less than 50% of the leaf area colonized by hyphae, II = 50 to 80 percent of leaf area colonized by hyphae, and III = leaf completely colonized by hyphae. Induced plants have a significantly different distribution than controls (Chi-square test; $\alpha = 0.05$; $n > 150$).

Fig. 2. Tissue colonization by *Peronospora parasitica* NOCO in leaves of *Arabidopsis* Col-0 protected by a root treatment with strain CHA0r. Typical leaves from 3-week-old plants that were challenge-inoculated by spraying with a spore suspension of *Peronospora parasitica* NOCO (2.5×10^4 spores per ml) were used to localize the fungus. **A**, Uninfected control plants. **B**, Less than 50% of the leaf area covered by hyphae. **C**, Leaf area (50 to 80%) covered by hyphae. **D**, Leaf completely colonized by hyphae. Bars represent 1 mm.

already been reported to induce ISR against *Peronospora parasitica* and other pathogens (Ton et al. 2002). All strains were applied as described for CHA0r. Figure 3 shows that the pyoverdine-deficient mutant *Pseudomonas fluorescens* CHA400 induced resistance in *Arabidopsis* against *Peronospora parasitica* to an extent similar to that induced by strain CHA0r. Strains CHA805 (exoprotease deficient), CHA77 (HCN deficient), CHA660 (pyoluteorin deficient), and WCS417r caused a reduction of symptoms compared with the control treatment, but reductions were smaller than those observed after treatment with CHA0r. No statistically significant differences in sporulation could be observed between treatments with CHA631 (2,4-diacetylphloroglucinol (DAPG) deficient) or CHA89 (HCN-, DAPG- and pyoluteorin deficient) and control plants.

The role of DAPG in CHA89 and CHA631 in ISR.

Since strains CHA89 and CHA631 were both impaired in ISR, the implication of DAPG in ISR was examined further. A deficiency in root colonization could be ruled out, since bacterial counts in the rhizosphere of plants inoculated with strains CHA89 or CHA631 were comparable to those of CHA0r-treated plants (data not shown). The importance of DAPG was further tested in a complementation experiment. For this experiment, we selected *Pseudomonas fluorescens* CHA631 characterized by a single mutation in the *phlA* gene that affects DAPG production. Strain CHA89 is impaired in the global activator (GacA) of antibiotic and cyanide production. The plasmid used for complementation of CHA89 is too unstable

Fig. 3. Protection against *Peronospora parasitica* in *Arabidopsis* Col-0 plants induced by wild-type *Pseudomonas fluorescens* CHA0r, WCS417r, and mutants. Plants were grown for 2 weeks in soil containing bacteria at 5×10^7 CFU per g of soil. Three-week-old plants were challenge-inoculated by spraying a spore suspension of compatible *Peronospora parasitica* NOCO at 2.5×10^4 spores per ml. After challenge inoculation (5 days), the sporulation was evaluated and expressed relative to challenged control plants. Letters indicate statistically significant differences between samples (Fisher test; $a = 0.05$; $n > 50$). Data presented are means ($\pm$ SE) from at least three independent representative experiments. Mutants derived from strain CHA0 have the following characteristics: CHA400 (*pvd*⁻ nonproducing pyoverdine), CHA805 (*apr*⁻, nonproducing exoprotease), CHA77 (*hcnA*⁻, nonproducing HCN), CHA660 (*plr*⁻, nonproducing pyoluteorin), CHA89 (*gacA*⁻, impaired in global antibiotics synthesis), and CHA631 (*phlA*⁻, nonproducing 2,4-diacetylphloroglucinol).

when bacteria grow in soil (Laville et al. 1992), making complementation of this particular mutant very difficult. Strain CHA631 complemented with plasmid pME6261 carrying the wild-type *phlA* gene presented a fully restored ability to produce DAPG in vitro on malt agar medium (data not shown), could colonize roots, and could induce ISR to *Peronospora parasitica* to the same extent as did the CHA0r strain (Fig. 4). Since DAPG is known for its antibiotic properties (Keel et al. 1992), we tested whether root inoculation with the CHA0r strain leads to an increase in DAPG in the sap and the leaves. However, DAPG and its degradation product monoacetylphloroglucinol (MAPG) remained undetected in the sap using GC-MS analyses (detection limit: <10 ng per g of FW). Treating roots with DAPG further assessed the possible involvement of DAPG in ISR. Protection was reproducibly observed at 10, 50, and 100 μ M DAPG (Fig. 5). No signs of phytotoxicity were observed in leaves of plants after soil drench with DAPG at any of the concentrations tested. DAPG also remained undetected in leaves of plants treated on the roots with DAPG (data not shown). Taken together, these results indicate that DAPG is required for the induction of ISR by *Pseudomonas fluorescens* CHA0r.

ISR mediated by *Pseudomonas fluorescens* CHA0r in *Peronospora parasitica* mutants impaired in signaling for defense.

Plant determinants involved in ISR were characterized by studying effectiveness of strain CHA0r in various *Peronospora parasitica* mutants or transgenic plants impaired in signaling for defense. All experiments were carried out according to the procedure described before using CHA0r as inducer of ISR and *Peronospora parasitica* as challenge organism. ISR to *Perono-*

Fig. 4. Protection against *Peronospora parasitica* in *Arabidopsis* Col-0 plants induced by various mutants of *Pseudomonas fluorescens*. CHA0 = wild-type *Pseudomonas fluorescens* CHA0 strain; CHA631 = a 2,4-diacetylphloroglucinol (DAPG) mutant of strain CHA0; and CHA631/pME6261 = strain CHA631 complemented with the plasmid pME6261, restoring DAPG production. Plants were grown for 2 weeks in soil containing fluorescent pseudomonads at 5×10^7 CFU per g of soil. Plants (3-week-old) were challenge-inoculated by spraying a spore suspension of compatible *Peronospora parasitica* NOCO at 2.5×10^4 spores per ml. Five days after challenge inoculation, the sporulation was evaluated and expressed relative to challenged control plants. Letters indicate statistically significant differences between samples (Fisher test; $a = 0.05$; $n > 50$). Data presented are means ($\pm$ SE) from at least three independent representative experiments.

spora parasitica was unaffected in plants unable to accumulate SA (NahG and *sid2-1*), phytoalexins (*pad2-1*), or in mutants insensitive to ET (*ein2-1* and *etr1-1*) (Fig. 6). In contrast, the mutants *npr1-1*, *jar1-1*, and *eir1-1* were unable to mount CHA0r-induced ISR against *Peronospora parasitica* and showed statistically significant differences with the wild-type control (Fig. 5). Since ISR does not take place in *A. thaliana* accession Wassilewskija (WS-0) using *Pseudomonas fluorescens* WCS417r as inducer (Ton et al. 2002), we included this ecotype in our experiments. Our results show that ISR could not be induced by strain CHA0r in *Peronospora parasitica* ecotype WS-0 (Fig. 6). Summarizing, induction of ISR in *Peronospora parasitica* Col-0 against *Peronospora parasitica* by strain CHA0r requires functional NPR1, JAR1, and EIR1 proteins.

The effect of DAPG applied to roots was tested in the mutants *npr1-1*, *jar1-1*, and *eir1-1*. DAPG was mostly effective in *npr1-1*, less effective in *jar1-1*, and ineffective in *eir1-1* (Fig. 7). Thus, proteins EIR1-1 and, to a lesser extent, JAR1-1 are required for the action of DAPG.

Accumulation of SA, JA, and ET during ISR.

SA and JA were measured in *Arabidopsis* Col-0 plants at day 0, 1, 3, and 5 after challenge with *Peronospora parasitica*. No differences between control and CHA0r-treated plants were observed during the period when the pathogen interacted with its host. ET measurements were also carried out following the same time course as for SA and JA, but ET remained undetected. However, ET production was detectable after treatment of the plants with the ET precursor 1-aminocyclopropane-1-carboxylate (data not shown).

DISCUSSION

Certain strains of *Pseudomonas fluorescens* have been extensively described as plant growth-promoting biocontrol agents (Cronin et al. 1997a and b; Laville et al. 1992; Maurhofer et al. 1994; Thomashow et al. 1996). In this study, we have analyzed ISR in *Peronospora parasitica* induced by

the biocontrol agent *Pseudomonas fluorescens* CHA0r and challenged with *Peronospora parasitica*. An experimental advantage of this biological system is the existence of various defined mutants of *Pseudomonas fluorescens* CHA0r and *Peronospora parasitica*, making it possible to gain insight into the molecular requirements for ISR.

The protection of the plant was characterized by the extent of sporulation of *Peronospora parasitica* in relation to the FW of plants. Since potential changes in FW induced by strain CHA0r could potentially have affected this spores per g of FW ratio, it was important to determine if the growth of the plant was increased during the course of the experiments. Under the conditions described here, no plant growth-promoting effect could be detected (data not shown). At this stage, *Arabidopsis* plants seem to require little additional input. Moreover, the content in various ions of both CHA0r-treated and nontreated plants was similar (data not shown). This is in agreement with the absence of growth-promoting effects induced by CHA0r treatments. Thus, ISR to *Peronospora parasitica* induced by *Pseudomonas fluorescens* CHA0r expressed as a decrease in spores per g of FW reflects a true effect on the pathogen rather than an increase in plant growth. We have also tested ISR induced by strain CHA0r against the fungus *Botrytis cinerea* and the bacterium *Pseudomonas syringae*. In both cases, no protection could be observed (data not shown).

The effect of various mutants of CHA0r on ISR makes an involvement of pyoverdine, pyoluteorin, exoprotease, and HCN unlikely. Results obtained with mutants CHA89 and CHA631 indicate that DAPG might play an important function in the induction of ISR. Additional support is provided by complemen-

Fig. 5. 2,4-diacetylphloroglucinol (DAPG)-mediated protection against *Peronospora parasitica* in *Arabidopsis* Col-0. Plants (3-week-old) were soil drenched with DAPG at different concentrations (1, 10, 50, 100, and 500 μM); 2 days later, they were challenge-inoculated by spraying a spore suspension of compatible *Peronospora parasitica* NOCO at 2.5×10^4 spores per ml. After challenge inoculation (5 days), the sporulation was evaluated and expressed relative to challenged control plants. Letters indicate statistically significant differences between samples (Fisher test; $a = 0.05$; $n > 50$). Data presented are means ($\pm$ SE) from at least three independent representative experiments.

Fig. 6. CHA0r-mediated protection against *Peronospora parasitica* in *Arabidopsis* Col-0, WS-0, and various mutants or transgenic lines derived from Col-0. Protection was induced by growing plants for 2 weeks in soil containing fluorescent pseudomonads at 5×10^7 CFU per g of soil. Plants (3-week-old) were challenge-inoculated by spraying a spore suspension of compatible *Peronospora parasitica* NOCO at 2.5×10^4 spores per ml. After challenge inoculation (5 days), the sporulation was evaluated and expressed relative to challenged control plants. Letters indicate statistically significant differences between samples (Fisher test; $a = 0.05$; $n > 50$). Data presented are means ($\pm$ SE) from at least three independent representative experiments. Col-0-derived plants are NahG (transgenic lines degrading SA), *ein2-1* (insensitive to ethylene), *etr1-1* (insensitive to ethylene), *sid2-1* (nonproducing SA), *pad2-1* (phytoalexin deficient), *npr1-1* (nonexpressing NPR1 protein), *jar1-1* (insensitive to JA and MeJA), and *eir1-1* (insensitive to ethylene in roots).

tation experiments, as well as by the effect obtained by root treatment with DAPG (Figs. 4 and 5). DAPG plays an essential role in the biocontrol of black root rot of tobacco (Keel et al. 1990, 1992; Schnider-Keel et al. 2000), take-all of wheat (Thomashow 1996), damping-off of sugarbeet caused by *Pythium* spp. (Fenton et al. 1992), bacterial soft rot of potato (Cronin et al. 1997a), and resistance to potato cyst nematodes (Cronin et al. 1997b). This biocontrol may be explained by a direct effect of DAPG on these soil-borne pathogens, since DAPG is well known for its antifungal, antibacterial, and phytotoxic activity (Keel et al. 1992). Nevertheless, the biocontrol of *Pseudomonas fluorescens* CHA0r against the leaf pathogen *Peronospora parasitica* observed in the present study might be based on another mode of action. Firstly, neither DAPG nor MAPG could be detected in the sap or the leaves of protected plants treated on the roots with strain CHA0r or DAPG. Secondly, assuming that DAPG exerts a direct antibiotic effect in the leaves against *Peronospora parasitica*, it is difficult to explain why this direct effect does not take place in all mutants (Figs. 6 and 7). A more likely explanation might be based on an indirect effect of DAPG. Recent studies on pea plants indicate that DAPG can act also as a plant hormone-like substance, inducing physiological and morphological changes that enhance nodulation by *Rhizobium* (De Leij et al. 2002). DAPG was also reported to enhance root exudation of organic molecules (Barber and Martin 1976). In the experimental system presented here, we propose that DAPG produced by *Pseudomonas fluorescens* CHA0r is detected by the roots, in which it leads to physiological changes that subsequently induce ISR.

The results obtained from studies with various *Peronospora parasitica* mutants impaired in signaling pathways for induced resistance show that NPR1-1, JAR1, and EIR1 are necessary for CHA0r-mediated ISR in *Peronospora parasitica* Col-0. Furthermore, EIR1-1 was found to be necessary for the protection by root treatments with DAPG, suggesting that it is placed in the pathway for DAPG action. The results obtained with the *jar1-1* and *npr1-1* mutants reveal additional complexity in the defense signaling pathways and suggest that NPR1-1 and JAR1 might, in part, act independently of DAPG during CHA0r-induced resistance. The observation that both NahG

and *sid2-1* impaired in SA accumulation were able to mount ISR against *Peronospora parasitica* makes it unlikely that SA is involved in the onset of ISR in our system. In addition, no changes in SA levels were observed between CHA0r-treated and untreated plants and *Peronospora parasitica* infection-induced SA production in *Arabidopsis* to the same extent as in control and root-colonized plants. Furthermore, the expression of *PR-1*, a marker gene of the SA-dependent pathway, showed no difference in CHA0r-treated plants compared with untreated plants (data not shown).

CHA0r-mediated ISR does not operate in *jar1*, a mutant insensitive to JA, but no increase in JA was detected in CHA0r-treated plants compared with controls. Moreover, the expression of *Pdfl.2*, a marker gene of the JA signaling pathway, was not induced in root-inoculated plants (data not shown). Thus, the sensitivity to JA is necessary for the establishment of ISR, but the induction of ISR by CHA0r is not accompanied by an increase in JA.

In comparison with the study by Pieterse and associates (2000), only the mutation in EIR1 affected CHA0r-mediated ISR. Other mutants affecting ethylene perception (*etr1-1*, *ein2.1*) had no effect. EIR1 is a membrane-bound protein localized exclusively in roots and was proposed to have a root-specific role in the transport of auxin (Luschnig et al. 1998). Since other mutants affected in ET perception do not affect CHA0r-mediated ISR, it is possible that the mutation in EIR1 acts independently of ET signaling. The precise function of EIR1 in CHA0r-mediated ISR thus remains to be determined.

In summary, while the results presented here point to an interesting role played by DAPG in ISR, they only partially overlap with those obtained for ISR in *Arabidopsis* treated with the *Pseudomonas fluorescens* WCS417r (Pieterse and Van Loon 1999). ISR induced by strain WCS417r or CHA0r both depend on NPR1 and JAR1 and differ with respect to the sensitivity to ET (Pieterse et al. 1998; van Wees et al. 1999). In this study, we show that the action of DAPG is likely to depend on EIR1 but not on NPR1 and JAR1 that might be involved somewhere else in the network for defense signaling.

It is therefore difficult to assign a definitive signaling pathway for ISR. Rather, it appears that plants exhibit a remarkable flexibility in the perception and subsequent transduction of stimuli generated by root-colonizing bacteria.

MATERIALS AND METHODS

Origin of seeds and bacteria.

Seeds of *Arabidopsis* accessions Columbia (Col-0) and Wasilewskija (Ws-0) were purchased from Lehle Seeds (Round Rock, TX, U.S.A.). Col-0 accession mutants *ein2-1* (Guzman and Ecker 1990), *eir1-1* (Roman et al. 1995), and *etr1-1* (Bleecker et al. 1988) were obtained from the Nottingham *Arabidopsis* Stock Center; *npr1-1* (Cao et al. 1997), *jar1-1* (Staswick et al. 1992) were provided, respectively, by X. Dong (Duke University, Durham, NC, U.S.A.) and P. E. Staswick (University of Nebraska, Lincoln, U.S.A.), and *pad2-1* (Glazebrook and Ausubel 1994) by J. Glazebrook (Novartis Agricultural Discovery Institute Inc., San Diego). The transgenic line NahG (Lawton et al. 1995) was provided by J. Ryals (Novartis, Research Triangle Park, NC, U.S.A.). Rifampicin-resistant *Pseudomonas fluorescens* strains CHA0r (Natsch et al. 1994) and WCS417r (Pieterse et al. 1996) were provided by D. Haas (University of Lausanne, Switzerland) and C. Pieterse (University of Utrecht, The Netherlands), respectively, and strains CHA400 (Keel et al. 1989), CHA660 (Maurhofer et al. 1994), CHA89 (Laville et al. 1992), CHA805 (Blumer et al. 1999), CHA77 (Laville et al. 1998), and CHA631 (Schnider-Keel et al. 2000) by D. Haas (University of Lausanne, Switzerland).

Fig. 7. 2,4-diacetylphloroglucinol (DAPG)-mediated protection of *Arabidopsis* Col-0, *eir1-1*, *jar1-1*, and *npr1-1* against *Peronospora parasitica*. Plants (3-week-old) were soil-drenched with DAPG (50 μ M) 2 days before challenge-inoculation by spraying a spore suspension of compatible *Peronospora parasitica* NOCO at 2.5×10^4 spores per ml. After challenge inoculation (5 days), the sporulation was evaluated and expressed relative to challenged control plants. Letters indicate statistically significant differences between samples (Fisher test; $a = 0.05$; $n > 50$). Data presented are means ($\pm$ SE) from at least three independent representative experiments.

Plant growth conditions.

All seeds were stored in a cold room at 5°C. Seeds were sown in 60 ml of soil mixture consisting of four parts sphagnum peat with added nutrients (Substrate 1, Klasmann, 8404 Winterthur, Switzerland), two parts vermiculite 0.4 to 1.2 mm (GVZ-Bolltec AG, 8022 Zürich, Switzerland), and one part quartz sand 0.3 to 0.9 mm (Carlo Bernasconi AG, 8114 Dänikon, Switzerland). After sowing, the pots were randomly positioned in trays and were kept for 5 days at 100% relative humidity (RH) until germination. Plants were cultivated in a growth chamber with a 12-h day (23°C) and night (18°C) cycle and 60% RH. Plants were watered twice a week with distilled water.

Cultivation of rhizobacteria and pathogens.

Rifampicin-resistant *Pseudomonas fluorescens* strains CHA0r (Natsch et al., 1994) and WCS417r (Pieterse et al. 1996) were grown on KB agar plates overnight at 28°C. Bacterial cells were collected by centrifugation, were washed, and were resuspended in distilled water to a final density of 10^9 CFU per ml.

P. syringae pv. *tomato* DC3000 (*Pst*) (Whalen et al. 1991) was grown overnight at 28°C on KB agar plates supplemented with 50 µg of rifampicin per ml. Bacterial cells were collected by centrifugation, were washed, and were resuspended in 10 mM MgCl₂ to a final density of 2.5×10^7 CFU per ml.

Peronospora parasitica (Thomma et al. 1998) isolates NOCO and EMWA were maintained on *Arabidopsis* accessions Col-0 and WS-0, respectively. Sporulating leaves were collected in tap water, were shaken gently but thoroughly, and were counted with a hemocytometer. Appropriate dilutions were made and applied on compatible plants.

B. cinerea isolate BMM (Thomma et al. 1998) was grown on 1/2 potato dextrose agar (PDA) medium for 2 weeks at 22°C. Subsequently, the conidia were collected and transferred in 3 ml of 1/2 potato dextrose broth (PDB). The density of conidia was determined with a hemacytometer.

Pathogen inoculation and disease rating.

Peronospora parasitica. Seedlings were challenge-inoculated by spraying a conidial suspension of *Peronospora parasitica* (2.5×10^4 spores per ml), using a glass sprayer for thin-layer chromatography (TLC). Inoculated plants were kept at 100% RH for 6 h. Thereafter, the lids were removed. After 6 days, the sporulation was determined by counting the number of spores per g of FW of leaves. Briefly, leaves of plants from at least 12 pots per treatment were collected in tubes, and their FW was determined. An appropriate volume of tap water was added to each tube, which was subsequently shaken. The number of spores was determined microscopically, using a haemocytometer. Leaf colonization was assessed by microscopic inspection of lactophenol- and trypan blue-stained leaves, as described by Zimmerli and associates (2000). A visual rating was used that reflected the degree of infection: I = less than 50% of the leaf area colonized by hyphae; II = 50 to 80% of the leaf area colonized by hyphae; and III = leaf completely colonized by hyphae.

B. cinerea. To infect plants with *B. cinerea*, a piece of agar containing sporulating hyphae was gently mixed in 1/2 PDB. The concentration of spores was adjusted to obtain 50 to 100 spores per 3-µl drop. Droplets were deposited on leaves of plants at the rosette stage. High humidity is required during all the infection time. After 2 days, the first symptoms appeared as yellow necrotizing haloes. Within 5 to 6 days, whole leaves were necrotized. Usually, the size of the necrosis was measured at the fourth day.

P. syringae pv. *tomato* DC3000. Five-week-old plants were challenged by spraying a suspension of virulent *P. syringae* pv.

tomato DC3000 bacteria in 10 mM MgCl₂ and 0.01% (vol/vol) Silwet L-77, using a TLC glass sprayer. One hour before challenge inoculation, the plants were placed at 100% relative humidity. After challenge, the plants were kept at 100% relative humidity for 2 h, and then, the lids were completely removed. Once the plants were dry, leaves were collected from five pots (three replicate leaves per pot). A second sampling was carried out three days later. Leaves were weighed and homogenized in 10 mM MgCl₂. Serial dilutions were plated on selective KB agar plates supplemented with 100 µg of cycloheximide and 50 µg of rifampicin per ml. After incubation at 28°C for 2 days, the number of rifampicin-resistant CFU per g of infected leaf tissue was determined, and bacterial proliferation over the 3-day time interval was calculated.

Induction of ISR in *Arabidopsis* with root treatments.

Induction of ISR was performed routinely by soaking 30-ml pots of 1-week-old seedlings in a bacterial suspension to obtain a final density of 5×10^7 CFU per g of soil. Plants remained in contact with the rhizobacteria and were used two weeks later for pathogen challenge. Control seedlings were supplemented with an equal volume of distilled water. Each pot contained between 40 and 60 seedlings. Alternatively, ISR was induced by DAPG (Maybridge, Tintagel, England) applied 2 days before challenge.

Evaluation of root colonization by *Pseudomonas fluorescens*.

Roots from Col-0 plants inoculated with the different CHA0 strains were collected at the end of the bioassay, and their FW was determined. Roots were resuspended in 10 mM MgSO₄ and were strongly swirled in the presence of fine glass beads. Aliquots of this homogenate were plated on KB agar plates containing 13 µg of chloramphenicol, 100 µg of cycloheximide, and 50 µg of ampicillin per ml for all strains, 25 µg of kanamycin per ml for CHA400, CHA660, and CHA89, or 50 µg of rifampicin per ml for CHA0r and WCS417r, and colonies were diluted appropriately.

Evaluation of the effect of CHA0r on the growth of *Arabidopsis* Col-0.

Col-0 plants (1-week-old) were root-inoculated with CHA0r as indicated above. The FW was determined at the following timepoints: day 0 (time of bacterial inoculation), 14 (time of challenge with pathogen), and 20 (time of symptom assessment).

Determination of SA, JA, and ET.

Control or CHA0r-treated plants were collected at day 0, 1, 3, and 6 after challenge with *Peronospora parasitica*. The content of free SA and JA was determined according to Meuwly and Métraux (1993) and Schweizer and associates (1997), respectively. For ET determinations, small plastic vials containing 70 3-week-old control or CHA0r-treated plants were transferred to individual 105-ml glass containers closed with a septum. The volume in the glass container was reduced to 60 ml with quartz sand (0.1 mm mesh size). Vials were left closed for 24 h, and ET levels were measured by GC (Hewlett-Packard 5890, Palo Alto, CA, U.S.A.). ET was determined 1, 3, and 6 days after challenge-inoculation with *Peronospora parasitica*. The detection limit of ethylene was 35 ng/plant or 33 ng/ml.

ICPMS.

Arabidopsis plants (1-week-old) were pretreated with *Pseudomonas fluorescens* CHA0. Two weeks after root colonization, control and treated plants were collected and dried at 60°C for 24 h. When fully dried, 300 mg was ground and used for ICP-MS analysis, according to Matera and Le Hécho (2001).

Detection of DAPG and MAPG.

Col-0 plants (1-week-old) were root-inoculated with CHA0r, as indicated above. After two weeks, leaves were excised, and the slowly exuding sap was collected 2 h later, using a micropipette. The collected sap (approximately 500 µl) was evaporated to dryness in vacuo and was suspended in water (2 ml). Alternatively, 1-week-old Col-0 plants were drench-treated once with DAPG (0.1 mM in the soil volume). Two weeks later, leaves (approximately 1 g) were excised and were homogenized in methanol, using a Polytron homogenizer (2 × 2 ml, 1 min, full speed). The soluble material was recovered by centrifugation (5 min, 3,000 g), evaporated in vacuo to dryness, and suspended in water (2 ml). Organic substances from the aqueous sap or leaf suspensions were extracted with ethyl acetate (2 × 2 ml), and the extract was evaporated in vacuo to dryness. The extract was solubilized in pyridine (100 µl), to which 100 µl of N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane was added, and the mixture was heated (80°C) in a sealed tube. Samples were analyzed by GC/MS (Hewlett-Packard MSD 5973), as described in Sieber and associates (2000).

Transformation of *Pseudomonas fluorescens* CHA631.

Competent cells of *Pseudomonas fluorescens* were obtained by growing an overnight preculture in Luria broth (LB) to an optical density at 600 nm of 0.5 at 35°C. Then, bacteria were washed twice in an ice-cold sterile solution containing 15% glycerol and 1 mM MOPS. After the last wash, bacteria were resuspended in 1% of the initial volume. Competent bacteria were either freshly used or kept at -80°C. Thirty nanograms of the plasmid pME6261 (10 kb) carrying the *phlA* gene (Schnider-Keel et al. 2000) was used for the transformation of strain CHA631 by electroporation (5 ms, 25 mF, 200 W, 2.5 kV/cm). Bacteria were transferred to fresh LB and were inoculated at 35°C for 1.5 h. Bacteria were plated on a selective LB medium containing 100 µg of tetracycline per ml and were incubated overnight at 30°C. Single colonies of transformants were isolated, and the presence of the plasmid was checked by polymerase chain reaction. Bacteria strongly express DAPG when grown on malt agar (Difco, Detroit). This molecule confers a brown color to the medium (Laville et al. 1992).

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