Microbial Siderophores Exert a Subtle Role in Arabidopsis during Infection by Manipulating the Immune Response and the Iron Status

Alia Dellagi*, Diego Segond, Martine Rigault, Mathilde Fagard, Clara Simon, Patrick Saindrenan, and Dominique Expert

Laboratoire des Interactions Plantes-Pathogènes, UMR 217, INRA/AgroParisTech/Université Pierre et Marie Curie, 75231 Paris cedex 05, France (A.D., D.S., M.R., M.F., D.E.); and Institut de Biotechnologie des Plantes, UMR 8618, Centre National de la Recherche Scientifique-Université Paris-Sud, 91405 Orsay cedex, France (C.S., P.S.)

Siderophores (ferric ion chelators) are secreted by organisms in response to iron deficiency. The pathogenic enterobacterium Erwinia chrysanthemi produces two siderophores, achromobactin and chrysobactin (CB), which are required for systemic dissemination in host plants. Previous studies have shown that CB is produced in planta and can trigger the up-regulation of the plant ferritin gene AtFER1. To further investigate the function of CB during pathogenesis, we analyzed its effect in Arabidopsis (Arabidopsis thaliana) plants following leaf infiltration. CB activates the salicylic acid (SA)-mediated signaling pathway, while the CB ferric complex is ineffective, suggesting that the elicitor activity of this siderophore is due to its iron-binding property. We confirmed this hypothesis by testing the effect of siderophore structurally unrelated to CB, including deferrioxamine. There was no activation of SA-dependent defense in plants grown under iron deficiency before CB treatment. Transcriptional analysis of the genes encoding the root ferrous ion transporter and ferric chelate reductase, and determination of the activity of this enzyme in response to CB or deferrioxamine, showed that these compounds induce a leaf-to-root iron deficiency signal. This root response as well as ferritin gene up-regulation in the leaf were not compromised in a SA-deficient mutant line. Using the Arabidopsis-E. chrysanthemi pathosystem, we have shown that CB promotes bacterial growth in planta and can modulate plant defenses through an antagonistic mechanism between SA and jasmonic acid signaling cascades. Collectively, these data reveal a new link between two processes mediated by SA and iron in response to microbial siderophores.

Iron is essential for most forms of life. It is required for the catalytic activity of proteins mediating electron transfer and redox reactions, such as those involved in respiration, photosynthesis, DNA synthesis, and defense against reactive oxygen species. However, it is often unavailable because it is present as insoluble ferric hydroxide complexes in aerobicosis and at neutral pH. In its ferrous form, iron is more soluble and catalyzes the Fenton reaction in the presence of hydrogen peroxide, which leads to the formation of hydroxyl radicals, resulting in protein denaturation, DNA breaks, and lipid peroxidation (Pierre and Fontecave, 1999). Therefore, iron acquisition, utilization, and storage are subject to different levels of homeostatic regulation.

In plants, iron is assimilated from the soil through the roots (Briat et al., 2007; Kim and Guerinot 2007). Under iron deficiency, Arabidopsis (Arabidopsis thaliana) activates processes described as strategy I based on the acidification of the soil by H+-ATPases, iron reduction by a ferric chelate reductase (FRO2; Robinson et al., 1999), and Fe3+ transport across the plasma membrane of root epidermal cells via the iron transporter IRT1 (Eide et al., 1996). Iron is then transported to plant organs essentially as citrate and nicotianamine complexes (Briat et al., 2007). Storage and buffering in dedicated compartments including apoplast and organelles (vacuole, plastids) avoid iron toxicity (Briat et al., 2007). In plastids, ferritins represent the major iron-containing proteins. In Arabidopsis, the ferritins AtFER1 to AtFER4 are mainly involved in buffering iron and protect the plant cells against oxidative stress (Ravet et al., 2009). Vacuolar iron stores can be mobilized to the cytosol via the divergent metal transporters AtNRAMP3 and AtNRAMP4 during seedling development (Lanquar et al., 2005).

Microorganisms have developed powerful iron acquisition systems based on the production of siderophores, which are selective ferric ion chelators secreted...
in response to iron deficiency (Andrews et al., 2003; Winkelmann, 2007). Siderophores have low molecular weights and very diverse chemical structures that can contain one or a combination of several types of iron-binding moieties: hydroxamate, catecholate, and hydroxycarboxylate. Once loaded with iron, siderophores are specifically transported through the bacterial envelope via protein transporters; in the cytosol, iron is reduced and distributed to iron-containing molecules. During microbial infection, a competition for iron between the host and the microorganism may take place. Phytopathogenic bacteria and fungi can use siderophores to multiply in the host and to promote take place. Phytopathogenic bacteria and fungi can use siderophores to multiply in the host and to promote infection (Expert, 1999; Haas et al., 2008). Oide et al. (2006) demonstrated that in four ascomycete species, 
*Cochliobolus miyabeanus*, *Cochliobolus heterostrophus*, *Fusarium graminarium*, and *Alternaria brassicicola*, siderophores are required for resistance to hydrogen peroxide and for full pathogenicity on their respective hosts maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), and Arabidopsis. Likewise, the fire blight-causing agent *Erwinia amylovora* takes advantage of its siderophore deferrioxamine (DFO) for the infection of apple (*Malus domestica*) and ornamentals like *Saintpaulia* plants (Pérombelon, 2002). The bacterial cells invade the intercellular spaces of parenchymatous tissues and secrete large quantities of plant cell wall-degrading enzymes, leading to tissue disorganization (Murdoch et al., 1999). Under iron deficiency, *E. chrysanthemi* releases two siderophores: the hydroxycarboxylate achronomobactin, which is produced when iron becomes limiting (Münzinger et al., 2000), and the catecholate chryso-bactin (CB; Persmark et al., 1989), which prevails under severe iron deficiency. CB and achronomobactin production are required for the systemic progression of maceration symptoms on the hosts (Enard et al., 1988; Dellagi et al., 2005; Franza et al., 2005). Neema et al. (1993) showed that the production of CB enables bacterial cells to compete with plant cells for iron, preventing sequestration of this metal by the plant ferritins. Consistently, the low availability of iron in the apoplastic of infected *Saintpaulia* leaves induces the expression of the *ftc* gene, encoding the bacterial outer membrane ferric-chryso-bactin (Fe-CB) transporter, which is up-regulated under iron depletion (Masclaux and Expert, 1995). Unlike in mammals, where the iron-sequestrating proteins of the transferrin family are able to reduce extracellular iron availability upon infection (for review, see Schaible and Kaufmann, 2004; Weinberg, 2009), there is no evidence that these proteins exist in plants. In Arabidopsis, the genes encoding the ferritin AtFER1 and the vacuolar metal transporters AtNRAMP3 and AtNRAMP4 are involved in basal resistance to *E. chrysanthemi*, indicating that changes in plant iron trafficking occur during infection (Dellagi et al., 2005; Segond et al., 2009).

Interestingly, *AtFER1* gene expression can be activated by the purified siderophore CB and not by Fe-CB in Arabidopsis leaves (Dellagi et al., 2005). This observation led us to suppose that the siderophore could act as a modulator of plant defense responses, since *AtFER1* is part of the defense reactions triggered by *E. chrysanthemi*. Thus, we investigated the role of CB in the activation of Arabidopsis defense responses triggered by *E. chrysanthemi* upon infection, namely the salicylic acid (SA), the jasmonic acid (JA), and the ethylene (ET) pathways, which are three major signaling pathways involved in the plant’s immune network (Glazebrook, 2005; Fagard et al., 2007).

In this work, we show that CB activates the SA-dependent defense pathway and that this process is dependent on iron availability in the plant. Not only CB but other types of siderophores could be elicitors, revealing a new function for these iron ligands in plant-microbe interactions. We also show that, when infiltrated into leaves, siderophores provoke iron deficiency in the roots. This work describes a new link between iron and immunity, which appears to be more complex than a simple nutritional competition.

**RESULTS**

**CB Triggers the Signaling Cascade Mediated by SA**

In Arabidopsis, *E. chrysanthemi* triggers defenses mediated by the major signaling molecules SA, JA, and ET, as revealed by marker gene expression analysis 24 h after bacterial inoculation (Fagard et al., 2007). We thus investigated whether the siderophore CB is able to activate similar responses. We monitored the expression of SA-, ET-, and JA-dependent defense genes by reverse transcription (RT)-PCR after water or CB infiltration in Arabidopsis leaves, using 0.25, 0.5, and 1 mM CB. All concentrations gave similar results (Supplemental Fig. S1), but the reproducibility of the data was best with 1 mM CB. Therefore, we used 1 mM CB in all the following experiments. We found that 24 h post infiltration (hpi), CB strongly activates the expression of the SA marker gene *PR1* (Fig. 1A). We did not find any significant modification in the expression of *PDF1.2*, which is a good marker for the ET/JA pathway (Penninckx et al., 1998). Thus, we focused on the SA pathway. We then used Arabidopsis lines expressing *GUS* fusions to the *PR1* promoter. We observed a strong *GUS* staining 24 h following infiltration of CB, which was not detected after water infiltration (Fig. 1B). The intensity of *GUS* staining in leaves treated with CB was similar to that observed in SA-treated leaves, used as positive controls. To determine whether the activation of *PR1* expression correlated with an accumulation of SA, we measured the SA
content by HPLC in Arabidopsis leaves 24 h after CB treatment. Figure 1C shows that siderophore treatment results in a 2- to 3-fold increase in SA content 24 hpi compared with control leaves. Altogether, these data show that CB triggers the SA defense pathway when infiltrated into Arabidopsis leaves.

SA can be synthesized in Arabidopsis through two distinct pathways, involving either Phe ammonia-lyase or isochorismate synthase (ICS1/SID2). Because it was previously found that the ICS1/SID2 pathway is involved in the up-regulation of PR1 after E. chrysanthemi infection (Fagard et al., 2007), we proceeded on the hypothesis that this pathway could be also required for the CB-induced response. Therefore, we monitored the accumulation of SA in ecotype Columbia (Col-0) and in a sid2 mutant (Nawrath and Métraux, 1999). While CB infiltration resulted in a 2- to 3-fold accumulation of total SA in Col-0 leaves compared with control leaves 24 hpi, no significant accumulation of this hormone was observed in sid2 leaves (Fig. 2A). We can conclude that the SID2 gene is necessary for the biosynthesis of SA in response to CB.

In order to check whether up-regulation of the PR1 gene in response to CB is dependent on SA biosynthesis, we monitored by RT-PCR the expression of its transcript in Col-0 and sid2 leaves treated with water or CB. PR1 expression was strongly activated by the siderophore 24 hpi in Col-0 leaves. By contrast, the presence of PR1 transcripts was hardly detected in sid2 leaves infiltrated with the siderophore (Fig. 2B). The up-regulation of PR1 by CB is thus dependent on the accumulation of SA in the leaves via the SID2 gene activity. We also monitored the expression of PAD4 and EDS5 genes known to act upstream of PR1 in the SA-mediated response. PAD4 encodes a protein similar to lipases and is required for resistance and accumulation of SA following infection with Pseudomonas syringae pv maculicola and Hyaloperonospora parasitica (Glazebrook et al., 1996, 1997; Zhou et al., 1998). EDS5 encodes a MATE-type multidrug efflux pump presumably involved in SA efflux from the chloroplast and is required for resistance to P. syringae and H. parasitica and the accumulation of SA in response to P. syringae (Nawrath and Métraux, 1999; Nawrath et al., 2002). We found that both PAD4 and EDS5 are up-regulated between 7 and 24 h after CB treatment (Fig. 2B). This response is independent of SA accumulation, since it was similar in Col-0 and in the sid2 deficient lines (Fig. 2B).

To determine whether the SA-mediated response activated by CB requires functional PAD4 and EDS5 genes, as well as the NPR1 gene encoding the SA sensor protein (Mou et al., 2003), CB or water was infiltrated onto the leaves of Col-0, eds5, pad4, and npr1 plants (Fig. 2C). No up-regulation of PR1 was observed in eds5 and npr1 mutants, indicating that the corresponding genes must be functional to mediate the response to CB. In the pad4 mutant, the expression of PR1 was still up-regulated. Collectively, these results indicate that CB activates a signaling pathway leading to PR1 up-regulation that is independent of PAD4 but dependent on SA production via SID2 and EDS5 and on the perception of this hormone via NPR1.

As CB infiltration in Arabidopsis leaves activates the expression of the ferritin-encoding gene AtFER1 (Dellagi et al., 2005), we asked whether this response requires the integrity of the SA pathway. We monitored the expression of this gene in Col-0, sid2, eds5, and npr1 leaves treated with CB. AtFER1 up-regulation was observed in all lines in response to the siderophore (Fig. 2, B and C). Similar results were obtained with the pad4 mutant line. These results indicate that AtFER1 up-regulation by CB is independent of the SA-mediated signaling pathway.

The Iron-Chelating Property of Siderophores Is Required for the Activation of the SA-Mediated Signaling Pathway

AtFER1 gene transcription is not activated by Fe-CB (Dellagi et al., 2005). Siderophore iron-binding activity is measured by calculating the pFe [defined as $-\log[Fe^{3+}]$], where [Fe$^{3+}$] = free [Fe$^{3+}$] in solution calculated at deter-
mined concentrations of ligand and Fe(III) and pH; for CB, pFe = 14.5 [Tomisic´ et al., 2008]}. Thus, we analyzed the expression of the SA pathway in response to leaf infiltration with CB or Fe-CB (Fig. 3A). We found that the PR1, EDS5, and PAD4 genes were not up-regulated by Fe-CB. These results indicate that the elicitor activity of the siderophore is related to its chemical state.

To determine whether the activation of the SA pathway is specific to CB, we tested the activity of a structurally unrelated siderophore, DFO. DFO is produced by the bacterial plant pathogen E. amylovora (Kachadourian et al., 1997) and is able to activate the transcription of AtFER1 in Arabidopsis leaves (Dellagi et al., 2005). Compounds of the DFO family harbor three hydroxamate groups that can bind Fe$^{3+}$ very efficiently (pFe = 24.2; Tomisic´ et al., 2008). We found that, like CB, DFO infiltrated onto Arabidopsis leaves results in transcript accumulation of genes from the SA pathway (Fig. 3B). Ferrioxamine (Fe-DFO) did not induce this response. The same results were obtained with ferrichrome, another hydroxamate-type siderophore (data not shown).

Collectively, these results suggest that the presence of siderophores in intercellular spaces of Arabidopsis leaves, when they are iron free, induces an SA-mediated response similar to that activated by pathogens. This process is not specific to the siderophore structure, as it can be activated by either catecholates or hydroxamates.

**Activation of the SA-Mediated Signaling Pathway by CB Depends on Iron Availability to the Plant**

When present in the plant tissue, a siderophore should rapidly take up iron from iron-containing molecules, suggesting that this metal plays a critical role in activation of the SA-dependent process, depending on whether it is bound or not to the ligand. To check whether the nutritional iron status of the plant influences the SA-mediated response, we compared the effect of CB on plants grown under iron-sufficient and iron-deficient conditions. We used hydroponically grown plants for which nutritional iron was adjusted as described in “Materials and Methods” and analyzed the expression of PR1. The results (Fig. 4) indicate that PR1 is up-regulated in plants grown under iron sufficiency, while this was not the case in iron-deficient plants. We measured the amounts of SA in leaves treated with CB from plants grown under both conditions. In agreement with PR1 expression profiles, we found that iron-deficient plants do not accumulate significant amounts of SA (data not shown). These results show that iron present in the plant growth medium is necessary for up-regulation of the SA-mediated pathway in response to CB.

Up-regulation of AtFER1 is observed in the presence of iron (Gaymard et al., 1996). As expected, the up-regulation of AtFER1 occurring in response to CB treatment (Dellagi et al., 2005) was not detected with plants grown under iron-deficient conditions (Fig. 4).

**Siderophores Trigger an Iron Deficiency Response in the Roots**

As the presence of siderophores in the plant leaves can lead to iron withholding, we investigated whether these ligands are able to trigger an iron deficiency reaction in the plant. We analyzed the expression of IRT1 and PRO2 genes, encoding the iron transporter IRT1 (Eide et al., 1996) and the ferric chelate reductase...
FRO2 (Robinson et al., 1999), known to respond to iron deficiency in the root. Both genes appeared to be up-regulated in roots 7 h after CB leaf treatment compared with control plants (Fig. 5A). Fe-CB infiltration in leaves did not activate the expression of these genes, as expected (Fig. 5A). We also observed a similar response to that observed with CB after infiltration of DFO (Fig. 5B).

We then determined the enzymatic activity of FRO2 in roots from plants treated with CB. Figure 5C shows that 24 h after siderophore infiltration in leaves of hydroponically grown plants, the FRO2 activity in roots was three times higher than in control plants. These data indicate that the presence of a siderophore in Arabidopsis leaves causes an iron deficiency in the roots, suggesting the propagation of a leaf-to-root signal.

The SA- and ET-Mediated Signaling Pathways Are Dispensable for IRT1 and FRO2 Up-Regulation by CB

As the SA pathway is induced by CB and ET is involved in the up-regulation of FRO2 and IRT1 genes in response to iron deficiency (Lucena et al., 2006), we investigated the role of SA and ET in expression of the root response induced by CB. We used the sid2 and ein2 mutants, the latter being affected in ET perception (Alonso et al., 1999). CB infiltration in the leaves of these mutants led to the activation of IRT1 and FRO2, and notably, expression levels of these genes were higher in the sid2 mutant (Fig. 6). Thus, the SA and ET pathways are not required to mediate the iron deficiency root response induced by CB.

CB Manipulates Plant Defenses and Promotes in Planta Bacterial Growth

The data presented above indicate that siderophores act as elicitors of plant defense controlled by the SA hormone. As E. chrysanthemi triggers a set of defenses in Arabidopsis during infection, including the SA-mediated signaling pathway (Fagard et al., 2007), we wondered if the activation of this pathway was reduced after inoculation of a siderophore-deficient mutant compared with the wild-type strain. Thus, we used the E. chrysanthemi CB-deficient mutant affected in the cbsE gene (Franza et al., 2005). Expression of the PR1 gene was monitored 3, 7, and 24 h after infiltration of Arabidopsis leaves with wild-type cells or the siderophore-deficient mutant. As observed previously, the PR1 gene was strongly up-regulated by the wild-type bacteria compared with the control plants (Fig. 7A). Infection by the siderophore-deficient mutant resulted in reduced expression of this gene. This result indicates that CB, during bacterial infection, contributes to the activation of the SA pathway, although it is not the only elicitor of this process. Interestingly, expression of PDF1.2, the gene marker of the ET/JA pathway that is not activated by wild-type bacteria 24 h after infiltration, was strongly up-regulated in response to the siderophore-deficient mutant (Fig. 7A). This result suggests that CB represses the expression of PDF1.2. As PDF1.2 expression is known to be activated by JA, we analyzed the expression of PDF1.2 in leaves treated with JA or with both JA and CB (Fig. 7B). We observed an accumulation of PDF1.2 transcripts in response to JA, which was not detected after coinfiltration of CB and the hormone. This result confirms that CB can repress the expression of PDF1.2.
Previous studies using bacterial mutants unable to produce CB or a chromatobactin have shown that these siderophores promote the infection process in host plants (Enard et al., 1988; Dellagi et al., 2005; Franza et al., 2005). We thus asked whether infiltration of CB onto Arabidopsis leaves prior to *E. chrysanthemi* inoculation could affect the bacterial growth. We infiltrated water or 1 mM CB onto Arabidopsis leaves 48 h before bacterial challenge. Bacterial populations were determined over 2 d post inoculation (Fig. 7C). In the control leaves preinfiltrated with water, *E. chrysanthemi* grew by less than 1 order of magnitude after 2 d of infection. In the leaves pretreated with CB, we observed a much faster growth and an increase in bacterial counts by 1 order of magnitude. These data indicate that preinfiltration of CB stimulates bacterial growth.

**DISCUSSION**

The plant pathogenic bacterium *E. chrysanthemi* requires the production of siderophores for systemic progression in host tissues (Enard et al., 1988; Dellagi et al., 2005; Franza et al., 2005). Production of siderophores and pectinases is controlled by iron availability, indicating that high-affinity iron uptake by this bacterium is a critical factor during pathogenesis (Franza et al., 2002). In order to know the role of siderophores in the infection process more precisely, we need to understand how these compounds are perceived in the host. In this work, we have investigated the plant’s response to the siderophore CB. We found that two physiological functions are modulated by this molecule: plant defense and iron assimilation.

**Role of CB in the Activation of the SA-Mediated Signaling Pathway**

We found that CB in Arabidopsis activates the SA-mediated signaling pathway leading to *PR1* gene expression. Our results using the *sid2* mutant show that CB activates SA biosynthesis in Arabidopsis. The structural similarity between SA and CB allowed the hypothesis that CB or its potential degradation products could act as precursor(s) in SA biosynthesis. However, since other siderophores with no structural relationship to SA are also able to trigger the SA pathway, we excluded this hypothesis. CB also requires *NPR1* to activate the expression of *PRI*. The NPR1 protein is an important player in SA signaling and in systemic acquired resistance (Dong, 2004). In the cytosol, it is present as disulfide-bound oligomers that monomerize following reduction consecutive to SA-controlled redox changes (Mou et al., 2003). The monomers are translocated to the nucleus, where they interact with TGA transcription factors that recognize cis elements in *PR* gene promoters (Johnson et al., 2003). This means that the response induced by the siderophore could also result in a cellular redox change involving SA and leading to the activation of the *PRI* gene via NPR1. We also show that siderophore-mediated *PRI* up-regulation does not require *PAD4*. *PAD4* encodes a triacyl-glycerol lipase acting upstream...
of SA (Jirage et al., 1999) and is necessary for SA accumulation and amplification of SA-dependent defense responses (Zhou et al., 1998). It is not required for PR1 up-regulation during the hypersensitive response observed in an incompatible interaction involving resistant plants. However, PAD4 is required for full PR1 up-regulation in compatible interactions involving susceptible plants (Zhou et al., 1998). In light of these data, it is possible that the SA response triggered by a siderophore is strong enough and is comparable to an incompatible interaction, except that there is no reaction of cell death. Indeed, we never observed necrosis after siderophore treatment at the macroscopic level or at the microscopic level after trypan blue staining (data not shown).

Our results indicate that CB activates the AtFER1 gene independently of the SA-mediated signaling pathway (Fig. 8). Thus, it may be possible that regulation of the ferritin gene by CB takes place upstream of the SA response. It would be helpful to determine whether AtFER1 contributes to the responses induced by CB in an atfer1 mutant. As an iron-buffering molecule, ferritin could contribute to changes in the cellular iron status and activate downstream signals.

Role of CB in the Activation of the Iron Deficiency Root Response

We recently reported that infection of Arabidopsis by *E. chrysanthemi* triggers an iron deficiency response in the roots (Segond et al., 2009). This work shows that this response is also induced by infiltration of a siderophore on the leaf, suggesting that this ligand is responsible for the root reaction when it is released by bacterial cells during infection.

The elicitor activity of siderophores is likely due to their strong iron-chelating capacity rather than to recognition by a plant receptor. Indeed, we found that the siderophores induce a reaction similar to iron

---

**Figure 7.** Effects of CB on the expression of PR1 and PDF1.2 genes during *E. chrysanthemi* infection. A, Col-0 leaves were infiltrated with 10 mM MgSO₄ or 10⁷ colony-forming units mL⁻¹ bacterial suspension of *E. chrysanthemi* wild type (*E.ch*) or CB negative mutant (*E.ch cbs*). Leaves were harvested at the indicated times after treatment. Expression patterns of PR1 (SA pathway) and PDF1.2 (ET/JA pathway) were monitored by RT-PCR. The constitutive EF1α gene was used as a control. B, RT-PCR using RNAs extracted from leaves 24 h after treatment with 0.05% (w/v) methanol (cont), JA, or JA + CB. C, Plants were infiltrated with water or CB 48 h before inoculation with a bacterial suspension of wild-type *E. chrysanthemi* cells. Leaves were harvested at the times indicated after bacterial infiltration, and then bacterial counts were performed as indicated in “Materials and Methods.”

---

**Figure 8.** Diagram showing the responses of Arabidopsis to microbial siderophores. Leaf infiltration of iron-free siderophores (CB or DFO) activates the SA-mediated signaling pathway leading to PR1 up-regulation, the basal defense marker PAD4, ferritin accumulation via AtFER1, and root iron uptake via IRT1 and FRO2. Up-regulation of IRT1 and FRO2 appeared to be partially repressed by SA. Activation of the SA pathway and AtFER1 up-regulation depends on iron availability to the plant (indicated with dashed arrows). Further details are discussed in the text.
deficiency consisting of IRT1 and FRO2 expression and production of the FRO2 enzymatic activity. It is tempting to speculate that the iron taken up by the roots is rapidly translocated to the leaves, a process that may cause an oxidative stress (Fig. 8). This oxidative stress could activate the SA pathway and AtFER1 gene expression, as these two responses are known to be inducible by reactive oxygen species (Leon et al. 1995; Gaymard et al., 1996; Petit et al., 2001). Two observations are in agreement with this hypothesis. First, under iron deficiency, CB treatment does not induce the up-regulation of PR1 or that of AtFER1, indicating that iron is required for activation of the SA response and confirming that this metal is essential to AtFER1 up-regulation. Second, the expression of IRT1 is rapid (7 hpi) but decreases between 7 and 24 h, indicating that the iron deficiency signal disappears during this period, likely because of a negative feedback due to iron uptake via IRT1. The timing of activation of the various SA marker genes (7–24 hpi) is compatible with this interpretation. In addition, the protein IRT1 can transport other cations than Fe2+, including Zn2+, Mn2+, and Cd2+ (Korsunova et al., 1999), and it is conceivable that some of these metals are taken up by the plant after IRT1 induction and contribute to the responses observed.

We investigated whether SA or ET is involved in the activation of the iron deficiency response by CB. Our data show that the ein2 mutation does not affect IRT1 and FRO2 up-regulation following CB treatment, suggesting that ET is not involved in this process. On the other hand, we found that IRT1 and FRO2 transcripts accumulate to higher levels in the sid2 mutant compared with the wild-type ecotype. This observation suggests that SA could exert a negative control of the iron deficiency response. This is consistent with the iron-binding capacity of SA (pFe = 12.1; Nurchi et al., 2009), a property that might confer onto this molecule a cellular iron-sensing function, as suggested in bacteria (Adilakshmi et al., 2000).

Role of CB in the Control of E. chrysanthemi Pathogenesis

CB pretreatment enhances the multiplication of E. chrysanthemi cells in the leaf (Fig. 7), which is in agreement with the fact that siderophore-deficient mutants are affected in their aggressiveness (Enard et al., 1988; Dellagi et al., 2005; Franzia et al., 2005). The weaker activation of PR1 after inoculation of the CB-deficient mutant compared with the wild-type strain indicates that CB produced during infection is likely responsible for activation of the SA pathway. However, under this condition, the PR1 gene is still expressed, indicating the existence of other elicitors of the SA pathway. Achromobactin, the second E. chrysanthemi siderophore, could contribute to this response, and oligogalacturonides generated by pectinases are likely to be involved (Fagard et al., 2007). We also found that, unlike wild-type bacteria, the CB-deficient mutant activates the expression of a marker of the JA/ET pathway, PDF1.2, 24 hpi. This suggests that CB represses this pathway that is involved in Arabidopsis resistance to E. chrysanthemi (Fagard et al., 2007). By activating the biosynthesis of SA via CB, the bacteria modulate the plant defense responses and take advantage of the antagonism between the SA and JA pathways. Furthermore, as siderophores activate iron uptake in the roots, the plant iron content must increase, thus explaining the beneficial effect of CB on E. chrysanthemi growth in the leaves.

Some siderophores secreted by soil-borne Pseudomonas species (pyoverdin and pyocyanin) can promote systemic plant protection against soil and foliar pathogens, a phenomenon known as induced systemic resistance (Audenaert et al., 2002; Haas and Défago, 2005). Induced systemic resistance is known to be dependent on the ET and JA pathways and independent of the SA pathway (Pieterse et al., 1998). In this work, we show that the elicitor activity of the siderophore CB that we observed is SA dependent, indicating that this process is different from induced systemic resistance.

In conclusion, this work shows that microbial pathogens can modulate the activity of the plant iron acquisition system via the modulation of siderophore production during infection and that this process can lead to changes in the expression of plant immune responses. These changes may be to the advantage of the pathogen or may help the plant to resist the pathogen. This could explain why in a number of plant-pathogen interactions, no role for siderophores was found in virulence, while in others, siderophores are important pathogenicity factors. The future challenges now are to better understand the molecular mechanisms by which siderophores activate the SA pathway and the root iron deficiency response.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) seeds from the Col-0 ecotype were obtained from the Institut National de la Recherche Agronomique Versailles collection. The sid2-1 mutant was kindly donated by J.-P. Métraux. Seeds of ein2-1 (Guzman and Ecker, 1990), npr1-1 (Cao et al., 1994), pad4-1 (Glazebrook et al., 1997), and eds5-1 (Glazebrook et al., 1996) were provided by the Nottingham Arabidopsis Stock Center (Scholl et al., 2000). The PR1::GUS lines were kindly provided by F. Ausubel. Plants were grown as described by Fagard et al. (2007). Hydroponic cultures were performed as described by Segond et al. (2009) and were used for all experiments where roots were used for RNA extraction or FRO2 activity monitoring. Experiments with iron-starved plants were performed as follows. Plants were first grown under the above-described conditions for 5 weeks and then transferred to iron-deficient medium after washing the roots for 5 min with medium containing the reductant sodium dithionite (5.7 ms) and the chelator bathophenanthroline-disulfonic acid (0.3 ms), both from Sigma. The plants were kept under high-moisture conditions during the experiments.

Chemical Preparations

Leaf treatment with the following chemicals was performed by infiltration of the solutions at the indicated concentrations in the intercellular leaf space.
using a syringe without a needle. CB was a gift from J. Buyer (Lu et al., 1996), and Fe-CB was prepared as described by Rauscher et al. (2002). DFO and Fe-DFO (a gift from R. Kachadorian) were prepared as described by Kachadorian et al. (1997). JA and SA were purchased from Sigma-Aldrich. Siderophores and SA were used at a concentration of 1 mM diluted in distilled water. JA was used at 0.1 mM in 0.05% (w/v) methanol.

**Bacterial Strains and Culture Conditions**

The wild-type strain, *Erwinia chrysanthemi* 3937 (our collection), was isolated from *Saintpaulia ionantha* (African violet). The CB-deficient mutant PPV11 is derived from strain 3937 that contains an insertional element inactivating the biosynthetic CB gene *cbEL* (cb-E1; Franza et al., 2005). Growth conditions were as described by Dellagi et al. (2005).

**Plant Inoculations and Determination of Bacterial Growth**

To monitor bacterial growth after siderophore treatment, we first infiltrated water or CB on the entire leaf. Forty-eight hours later, a small hole was made with a needle within the leaf, and then 5 μL of a bacterial suspension at a density of *5 × 10⁶* colony-forming units mL⁻¹ made up in 50 mM phosphate buffer (pH 7) was spotted on the hole. Leaves were harvested in 0.9% NaCl and ground using a pestle and sterile sand. The resulting suspensions were used for serial dilutions followed by plating on an appropriate medium. For RNA extractions and GUS fusions, we used a syringe without a needle to infiltrate the entire leaf or a portion of the leaf with SA, siderophore solution, or bacterial suspensions at *5 × 10⁶* colony-forming units mL⁻¹ in 10 mM MgSO₄ (half a leaf was infiltrated for GUS staining).

**RNA Extraction, Northern Blotting, and RT-PCR**

Northern-blot hybridization was carried out as described by Dellagi et al. (2009). *IRT1* and *FRO2* probes were prepared as described by Segond et al. (2009). For RT-PCR analysis, reverse transcription was performed as described by Fagard et al. (2007). PCR runs were of 94°C for 4 min, 26 to 30 cycles, and each cycle consisting of 94°C for 30 s, 54°C to 58°C for 30 s, and 72°C for 1 min, with a final step of 72°C for 10 min to complete polymerization. Primers for *EF1a*, *PRI1*, *PAD4*, *CHIB*, and *ED55* were described by Fagard et al. (2007). The other gene-specific primers were as follows: *PDF1.2-F* (5'-TCATGGCTAAGTTTGCTTCCATCATCACCC-3') and *PDF1.2-R* (5'-GTA-GATTAAACATCGGAGAC-3'). Equal cDNA amounts were checked by performing different PCR cycles with *EF1a* primers (Supplemental Fig. S2). Experiments were repeated at least three times. Representative data are shown.

**In Planta GUS Expression Detection**

In planta GUS expression detection was performed as described by Dellagi et al. (2005). Experiments were repeated three times with similar results.

**Root FRO Assays**

Root FRO activity was performed as described by Yi and Guerinot (1996). Briefly, roots from control plants or plants treated with the siderophore were incubated in a solution containing 0.1 mM Fe(III)-EDTA and 0.3 mM ferrozine in distilled water in the dark. After 20 min, the absorbance of the solution was measured at 562 nm, using the same solution without roots as a control.

**SA**

Treated leaves were harvested and then weighed before freezing in liquid nitrogen. They were ground in a frozen state in Eppendorf tubes using TissueLyser II (Qiagen). 7.2-10⁶ SA (1 μCi, 54 mM) mol⁻¹ (New England Nuclear) was used for recovery determination. Total SA was extracted and analyzed as described by Baillieul et al. (1995) with a Nova-Pak 4-μm C-18 column (150 × 3.9 mm; Waters) as part of the Waters system (1525 Binary HPLC Pump, 2475 Multi λ Fluorescence Detector, 2996 Photodiode Array Detector, 717 Autosampler; Waters). Data were analyzed using Empower Pro Software (Waters).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Defense gene expression in Arabidopsis leaves infiltrated with CB.

**Supplemental Figure S2.** Validation of the RT-PCR approach using *EF1a* as a constitutively expressed gene.

**ACKNOWLEDGMENTS**

We thank Floriant Belvert for help with SA quantification. We thank J.-P. Metraux for kindly providing the seeds of sid2 mutants and F. Ausubel for seeds of the *PR1::GUS* line. We are grateful to S. Davenport for English editing of the manuscript.

Received March 24, 2009; accepted May 5, 2009; published May 15, 2009.

**LITERATURE CITED**


Gaymard F, Bouchereau J, Briat JF (1996) Characterization of a ferritin mRNA from Arabidopsis thaliana accumulated in response to iron...
through an oxidative pathway independent of abscisic acid. Biochem J 318: 67–73


