Microbial Siderophores Exert a Subtle Role in Arabidopsis during Infection by Manipulating the Immune Response and the Iron Status[1][W]

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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 siderophores 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 aerobiosis 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 Fe^{2+} 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 divalent 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

[1] This work was supported by the Institut National de la Recherche Agronomique, the Centre National de la Recherche Scientifique (D.E.), and the Ministère de l'Enseignement Supérieur et de la Recherche (D.S. and C.S.).

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[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.109.138636

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 infection (Expert, 1999; Haas et al., 2008). Oide et al. (2006) demonstrated that in four ascomycete species, *Cochliobolus miyabeanus*, *Cochliobolus heterostrophus*, *Fusarium graminareum*, 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*) seedlings and flowers and for the resistance to hydrogen peroxide (Dellagi et al., 1998).

The importance of iron homeostasis in plant disease has been largely documented in the case of the bacterial pathogen *Erwinia chrysanthemi* (Expert, 1999). *E. chrysanthemi* is an enterobacterium causing soft rot on economically important crops including potato (*Solanum tuberosum*) and chicory (*Cichorium intybus*) 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 achromobactin, which is produced when iron becomes limiting (Münzinger et al., 2000), and the catecholate chrysobactin (CB; Persmark et al., 1989), which prevails under severe iron deficiency. CB and achromobactin 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 apoplasm of infected *Saintpaulia* leaves induces the expression of the *fct* gene, encoding the bacterial outer membrane ferric-chrysobactin (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

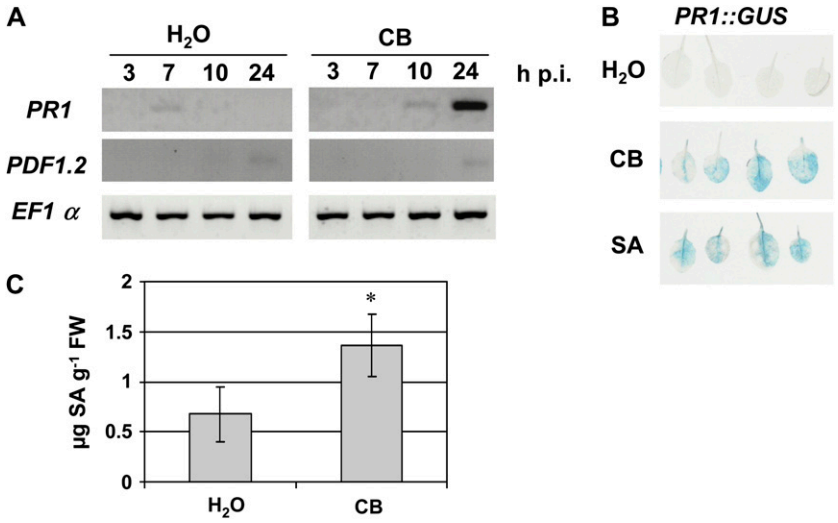

Figure 1. *PR1* gene expression and SA production in Arabidopsis leaves following CB treatment. A, Expression patterns of *PR1* (SA pathway) and *PDF1.2* (ET/JA pathway) were monitored by RT-PCR using RNAs extracted from Col-0 leaves at the indicated times after infiltration of distilled water or CB. The constitutive *EF1α* gene was used as a control. B, GUS staining of leaves from transgenic *PR1*::*GUS* plants 24 h after infiltration of water, CB, or SA. C, Total SA content was measured by HPLC in Col-0 leaves 24 h after the indicated treatments (μg SA g^{-1} fresh weight [FW]). n = 6, error bars indicate SD, and the asterisk indicates a significant difference from the control by the Mann-Whitney test ($P < 0.01$).

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 accumulation 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-

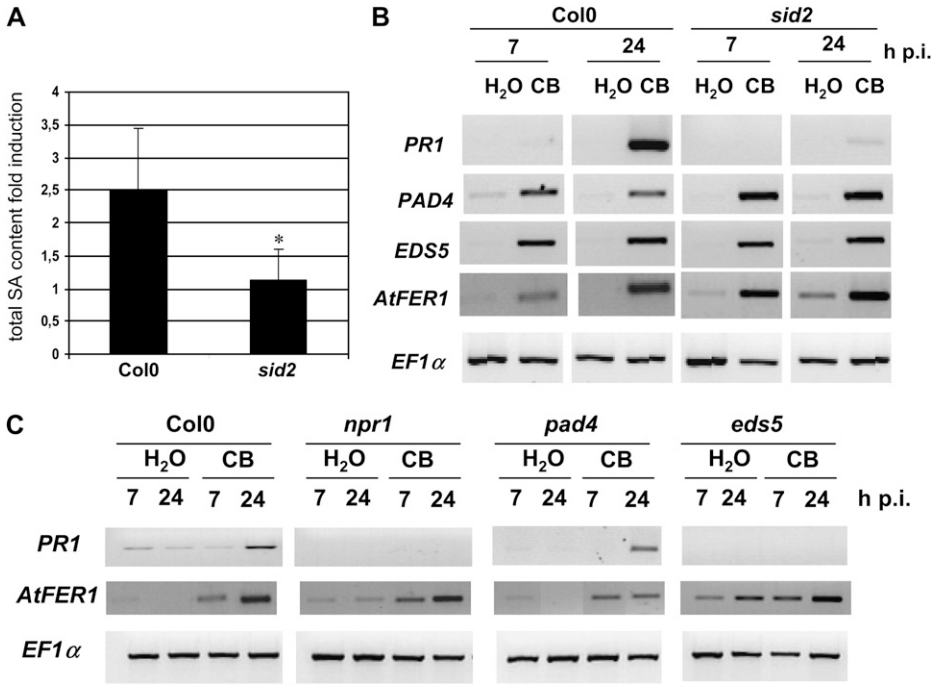

Figure 2. Roles of genes of the SA pathway in the signaling cascade triggered by CB. A, Total SA content (measured as in Fig. 1) fold induction (i.e. ratio of SA content in CB-treated leaves to SA content in water control). $n = 6$, error bars indicate SD, and the asterisk indicates a significant difference from Col-0 using the Mann-Whitney test ($P < 0.01$). B, RNAs from leaves were harvested after infiltration of water or CB (genotypes and times indicated). RT-PCR results with the indicated defense gene-specific primers are shown. The constitutive *EF1α* gene was used as a control. C, Expression profiles of *AtFER1* and *PR1* monitored by RT-PCR. RNAs were extracted from leaves of the indicated mutant genotypes harvested at the given times after the treatment indicated.

mined concentrations of ligand and Fe(III) and pH; for CB, pFe = 14.5 [Tomisić 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; Tomisić 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 *FRO2* genes, encoding the iron transporter IRT1 (Eide et al., 1996) and the ferric chelate reductase

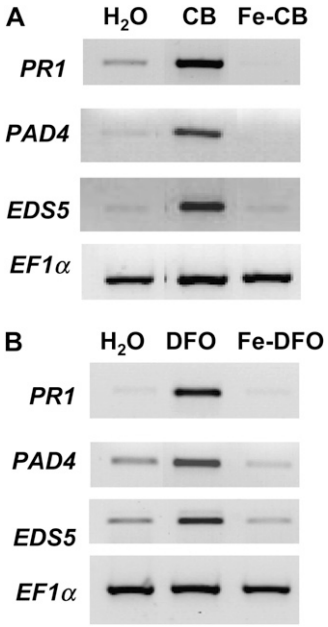

Figure 3. Importance of the chemical state of different siderophores in the activation of the SA pathway. Col-0 leaves were treated with water, CB, Fe-CB, DFO, or Fe-DFO as indicated. RNAs were extracted from leaves harvested 24 h after treatment, and RT-PCR with the indicated defense markers was performed. The constitutive *EF1α* gene was used as a control.

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*.

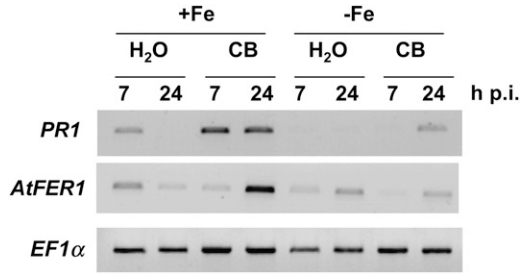

Figure 4. Influence of plant iron nutrition on *PR1* and *AtFER1* gene expression levels following CB treatment. Hydroponically grown plants under 50 μM Fe-EDTA for approximately 6 weeks (+Fe) or under 50 μM Fe-EDTA for 5 weeks and then without iron for 5 d (−Fe) were infiltrated with water or CB (1 mM) as indicated. RT-PCR with the indicated gene-specific primers was performed with RNAs extracted from leaves harvested at the indicated times after treatment. The constitutive *EF1α* gene was used as a control.

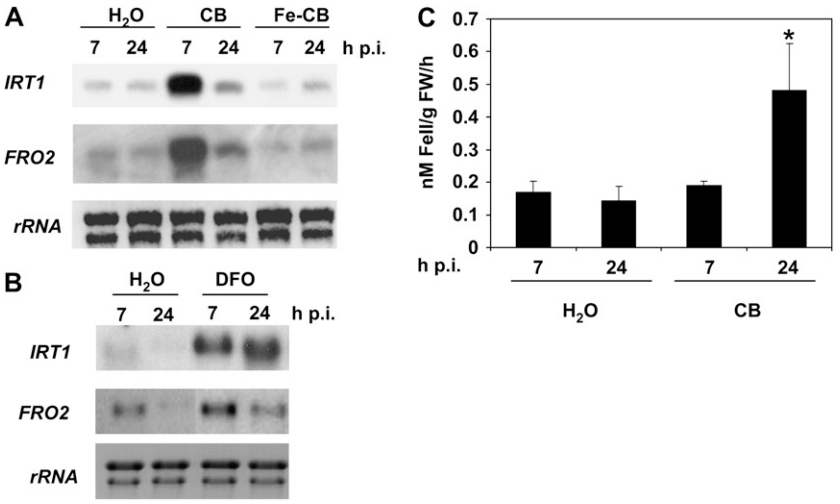

Figure 5. Iron deficiency root response caused by CB or DFO leaf infiltration. Col-0 plants were grown under hydroponic conditions with 50 μM Fe-EDTA. A and B, Leaves were infiltrated with water, CB, or DFO, and then RNAs were extracted from roots harvested at the indicated times after treatment. Expression patterns of *IRT1* and *FRO2* genes were analyzed by northern blots. Ethidium bromide staining is shown as a loading control. C, Ferric chelate reductase activity measured in roots at the indicated times after CB treatment. $n = 6$, error bars indicate SD, and the asterisk indicates a significant difference from the control using the Mann-Whitney test ($P < 0.01$). FW, Fresh weight.

Previous studies using bacterial mutants unable to produce CB or achromobactin 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 *PR1*. 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 *PR1* gene via NPR1. We also show that siderophore-mediated *PR1* up-regulation does not require *PAD4*. *PAD4* encodes a triacyl-glycerol lipase acting upstream

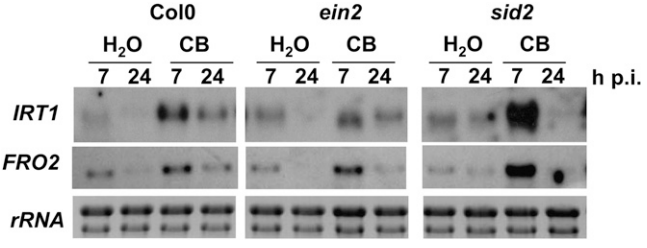

Figure 6. *IRT1* and *FRO2* gene expression in Arabidopsis SA and ET mutants following CB leaf treatment. Plants were grown under hydroponic conditions with 50 μM Fe-EDTA. Expression patterns of *IRT1* and *FRO2* genes were analyzed by northern blots using RNA extracted from roots harvested from ET (*ein2*) and SA (*sid2*) mutants at the indicated times after treatment. Ethidium bromide staining is shown as a loading control.

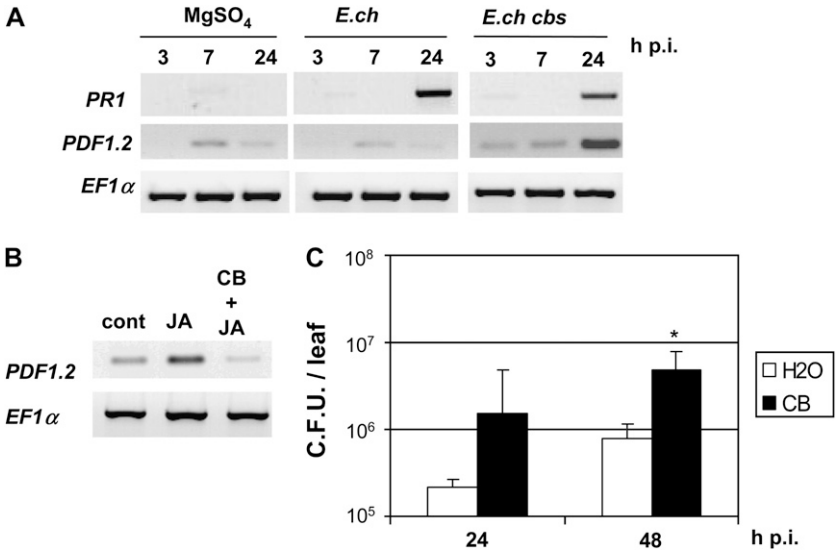

Figure 7. Effects of CB on the expression of *PR1* and *PDF1.2* genes during *E. chrysanthemi* infection. A, Col-0 leaves were infiltrated with 10 mM $MgSO_4$ or 10^7 colony-forming units mL^{-1} bacterial suspension of *E. chrysanthemi* wild type (*E.ch*) or CB negative mutant (*E.ch cbs*). Leaves were 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." $n = 6$, error bars indicate SD, and the asterisk indicates a significant difference from the control using the Mann-Whitney test ($P < 0.05$).

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

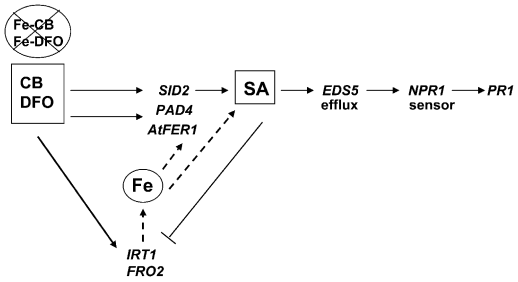

Figure 8. Diagram showing the responses of Arabidopsis to microbial siderophores. Leaf infiltration of iron-free siderophores (CB or DFO) activates the SA-mediated signaling pathway leading to *PR1* up-regulation, the basal defense marker *PAD4*, ferritin accumulation via *AtFER1*, and root iron uptake 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 Fe^{2+}, including Zn^{2+}, Mn^{2+}, and Cd^{2+} (Korshunova 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; Franza 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 mM) and the chelator bathophenanthroline-disulfonic acid (0.3 mM), 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. Kachadourian) were prepared as described by Kachadourian 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 *cbsE* (*cbs-E1*::**Ω**; 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 5×10^7 colony-forming units mL^{-1} made up in 50 mM potassium 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 5×10^7 colony-forming units mL^{-1} in 10 mM $MgSO_4$ (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. (2005). *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 *EF1α*, *PR1*, *PAD4*, *CHIB*, and *EDS5* were described by Fagard et al. (2007). The other gene-specific primers were as follows: *PDF1.2-F* (AT5G44420; 5′-TCATGGCTAAGTTTGCTTCCATCATCACCC-3′) and *PDF1.2-R* (5′-GTAGATTTAACATGGGAC-3′). Equal cDNA amounts were checked by performing different PCR cycles with *EF1α* 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-^{14}C]SA (1 nCi, 54 mCi $mmol^{-1}$; 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 *EF1α* as a constitutively expressed gene.

ACKNOWLEDGMENTS

We thank Floriant Belvert for help with SA quantification. We thank J.-P. Métraux 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.

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