Short communication

Alterations of iron distribution in *Arabidopsis* tissues infected by *Dickeya dadantii*AUDE AZNAR^{1,2}, ORIANE PATRIT³, ADELINE BERGER⁴ AND ALIA DELLAGI^{1,*}¹AgroParisTech, Institut Jean-Pierre Bourgin, UMR 1318, ERL CNRS 3559, Saclay Plant Sciences, RD10, F-78026 Versailles, France²Sorbonne Universités, UPMC, Université Paris 06, UFR927, F-75005 Paris, France³AgroParisTech, 16 rue Claude Bernard, 75231 Paris cedex 05, France⁴INRA, Institut Jean-Pierre Bourgin, UMR 1318, ERL CNRS 3559, Saclay Plant Sciences, RD10, F-78026 Versailles, France**SUMMARY**

Dickeya dadantii is a plant-pathogenic enterobacterium responsible for plant soft rot disease in a wide range of hosts, including the model plant *Arabidopsis thaliana*. Iron distribution in infected *A. thaliana* was investigated at the cellular scale using the Perls'-diaminobenzidine-H₂O₂ (PDH) method. Iron visualization during infection reveals a loss of iron from cellular compartments and plant cell walls. During symptom progression, two distinct zones are clearly visible: a macerated zone displaying weak iron content and a healthy zone displaying strong iron content. Immunolabelling of cell wall methylated pectin shows that pectin degradation is correlated with iron release from cell walls, indicating a strong relationship between cell wall integrity and iron in plant tissues. Using a *D. dadantii* lipopolysaccharide antibody, we show that bacteria are restricted to the infected tissue, and that they accumulate iron *in planta*. In conclusion, weak iron content is strictly correlated with bacterial cell localization in the infected tissues, indicating a crucial role of this element during the interaction. This is the first report of iron localization at the cellular level during a plant-microbe interaction and shows that PDH is a method of choice in this type of investigation.

Keywords: *Arabidopsis*, *Dickeya dadantii*, histochemical staining, infection, iron, Perls'-DAB-H₂O₂.

INTRODUCTION

Iron is an essential nutrient for fundamental metabolic processes in living organisms. Indeed, owing to its capacity to undergo reversible changes in its oxidation state, this metal is involved in an important number of electron transfer reactions. Cellular metabolism uses iron-containing cofactors consisting of Fe-S clusters or haem to transfer electrons during photosynthesis, respiration, nitrogen fixation, DNA synthesis and the degradation of reactive oxygen species (ROS). Although iron is the fourth most

abundant element on Earth, it is poorly bioavailable because of the low solubility of ferric oxyhydroxides prevailing in aerobic soils. Under reductive conditions, iron is in the ferrous form (Fe²⁺) and is more soluble (Lindsay and Schwab, 1982). However, Fe²⁺ can catalyse the Fenton reaction in the presence of hydrogen peroxide (H₂O₂), thus leading to the formation of hydroxyl radicals, resulting in protein denaturation, DNA breaks and lipid peroxidation (Pierre and Fontecave, 1999). The absolute requirement of iron and its potential toxicity have constrained living organisms to develop the tight control of iron uptake and storage.

Plants acquire iron from the soil through the roots. Two strategies of iron uptake have been described (Briat *et al.*, 2007; Kim and Guerinot, 2007): strategy I, which is based on the reduction of iron, followed by its transport via a membrane transporter of the Iron-Regulated Transporter 1 (IRT1) family; and strategy II, which is found only in Poaceae and employs a chelation-based strategy involving the extrusion of phytosiderophores that chelate iron in the rhizosphere, followed by the import of iron-phytosiderophore complexes by the roots. Iron distribution to different organs and cellular compartments is mediated by divalent metal chelators, such as nicotianamine or citrate, which are allowed to cross membranes via transporters from the YSL (yellow-stripe like) family or the protein FERRIC REDUCTASE DEFECTIVE 3 (FRD3) (Morrissey and Guerinot, 2009). Iron is mainly stored in the ferritin shell of mesophyll cell chloroplasts (Briat *et al.*, 2010). The *Arabidopsis* genome contains four ferritin coding genes *AtFER1–AtFER4*. Ravet *et al.* (2009) showed that they mainly play antioxidant functions in *Arabidopsis*. Iron can also be stored in mitochondria (Nouet *et al.*, 2011) or in the vacuoles of mature embryo endodermal cells (Kim *et al.*, 2006; Lanquar *et al.*, 2005). Important amounts of iron were recently detected in the nucleolus (Roschztardt *et al.*, 2011). The role and exact status of this iron are not yet known.

Dickeya dadantii 3937 (formerly named *Erwinia chrysanthemi* 3937) is an enterobacterium that causes soft rot on economically important crops, including potatoes and chicory, and on ornamentals, such as the genus *Saintpaulia* (Toth *et al.*, 2003). Bacterial cells invade the intercellular spaces of parenchymatous tissues and secrete large amounts of plant cell wall-degrading enzymes (CWDEs), leading to tissue disorganization and maceration symptoms (Grimault *et al.*, 1997; Murdoch *et al.*, 1999). Under

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iron-limiting conditions, most microbes synthesize and secrete low-molecular-weight compounds, called siderophores, designated to scavenge iron and deliver it to the bacterial cell. *Dickeya dadantii* synthesizes two siderophores: achromobactin (Munzinger *et al.*, 2000) and chrysobactin (Persmark *et al.*, 1989). Both siderophores are required for the systemic progression of maceration symptoms on the hosts (Dellagi *et al.*, 2005; Enard *et al.*, 1988). Leaf treatment with chrysobactin provokes perturbations of iron distribution which lead to the activation of immune responses, indicating that siderophores produced during infection disturb iron homeostasis and defence (Dellagi *et al.*, 2009). Limiting the iron supply to *Arabidopsis* plants results in reduced maceration symptoms (Nam Phuong *et al.*, 2012). Thus, *D. dadantii* bacterial cells require the presence of physiological amounts of iron to invade plant tissues (Franza and Expert, 2013). Interestingly, the AtFER1 ferritin protein has been shown to accumulate during *Arabidopsis* infection by *D. dadantii*, and an *atfer1* mutant is more susceptible to infection (Dellagi *et al.*, 2005). The role of AtFER1 in this context was attributed to its iron scavenging activity, which could deprive invading bacteria of iron. In addition, iron transport across the vacuolar membrane via the proteins AtNRAMP3 and AtNRAMP4 is involved in the amplification of oxidative stress, thus contributing to plant defence (Segond *et al.*, 2009). These data show that iron is a key modulator of the plant's susceptibility to the pathogenic bacterium *D. dadantii*.

To further investigate the role of iron during this interaction, we studied the localization of iron during disease progression in the leaf. We used a newly developed histochemical staining method based on Prussian blue staining combined with diaminobenzidine (DAB) amplification. The method, named Perls'-DAB-H₂O₂ (PDH), allows the precipitation of non-haem iron with the Perls' reagent, followed by intensification owing to DAB oxidation by H₂O₂. This method is specific for iron staining because the staining of other metals is very poor (Roschztardt *et al.*, 2009, 2010).

In this work, we visualized alterations of iron distribution in the infected tissue at the cellular level. This method allowed us to determine precisely the localization of iron in infected tissues and to compare this localization with the presence of bacteria, the level of cell wall degradation and ferritin accumulation. This novel iron staining method will provide a crucial contribution to a better understanding of the mechanisms underlying plant-microbe interactions known to be influenced by the iron status of the host and/or the microbe.

RESULTS

Characterization of physiological patterns of iron distribution in *A. thaliana* healthy leaf tissues

In order to check that PDH staining (Roschztardt *et al.*, 2010) allowed the localization of iron in *A. thaliana* tissues under our

conditions, we first performed observations on healthy leaf tissues (Fig. S1, see Supporting Information). Cell walls and plastids were strongly stained. The iron staining was also particularly strong in the nucleus, where it seemed to concentrate in a spherical structure, assumed to be the nucleolus. To check this, we stained consecutive sections with PDH and with 4',6-diamidino-2-phenylindole (DAPI). We selected two consecutive sections, the first stained with PDH and the second stained with DAPI, because PDH staining could not be combined on the same section with DAPI. DAPI strongly stained the nuclei, but not the nucleoli, thus allowing us to identify the nucleolus. The strong iron spot visible with PDH in the nucleus co-localizes with the nucleolus visible in DAPI staining. These data are in agreement with previous reports on the presence of large amounts of iron in the nucleolus of pea cells (Roschztardt *et al.*, 2011). In some of the *A. thaliana* mesophyll cells, dark stained spots could be observed in the plastids after PDH staining. To check whether these spots correspond to ferritin, we used consecutive sections, one of which was stained with PDH and the other hybridized with the anti-AtFER1 antibody (Dellagi *et al.*, 2005). AtFER1 antibody hybridizes to the plastids and co-localizes with the dark spots visible in the PDH-stained section.

During the PDH procedure, intensification is performed using DAB tetrahydrochloride, which can detect peroxidase activity on living tissues (Roschztardt *et al.*, 2009). To inhibit peroxidase activity (in particular, when using living tissues), incubation of samples in a methanol solution containing 0.01 M NaN₃ and 0.3% (v/v) H₂O₂ for 1 h can be performed before intensification. Similar results were obtained with or without pretreatment with methanol-H₂O₂-NaN₃ (data not shown). In addition, incubation of fixed tissues in the DAB solution did not allow staining, indicating the absence of background peroxidase activity in our samples.

Thus, the PDH staining method used under our conditions is a relevant method to study iron distribution at the microscopic level, because we were able to see iron at sites described in the literature.

Modification of iron distribution during tissue infection by *D. dadantii*

To investigate the cellular distribution of iron during the infection progress of *D. dadantii* cells in *A. thaliana* leaf tissues, leaves were inoculated by a method published previously which allows the progressive development of symptoms (see Experimental procedures). Samples were selected so as to contain both healthy and macerated tissues and fixed as indicated in Experimental procedures (Fig. 1A). Following PDH staining, two distinct zones with different intensities of PDH staining were observed (Fig. 1B). Iron staining was weaker in the tissues in the vicinity of the infection hole. This zone corresponds to tissue which is starting to be disorganized. In this zone, iron could hardly be detected in the cell

Fig. 1 (A) Description of the experimental procedure used to analyse iron localization during symptom progression. HZ, healthy zone; LE, leading edge of maceration; MZ, macerated zone. Iron localization in healthy and infected zones by Perls'-diaminobenzidine (DAB)-H₂O₂ (PDH) staining. (B) Three photographs mounted side by side spanning the MZ and HZ. PDH staining is reduced in cell walls, plastids and the nuclei of MZ cells. Scale bar: 200 μ m. (C, D) Enlargements of the LE zone. A clear difference in staining is observed at LE. (E, F) Enlargements of the MZ zone. Small strongly stained spots correspond to bacteria (b). Scale bar: (C, E) 50 μ m; (D, F) 20 μ m.

walls, chloroplasts or nuclei. Further away from the infection hole, the staining of iron was strong and evenly distributed in cell walls and plastids. A clear-cut boundary separated the strongly stained zone and the weakly stained zone. We conclude from these observations that the tissue contained two distinct zones: a macerated zone (MZ) that was weakly stained with PDH, and a healthy zone (HZ) that was strongly stained with PDH. The shift of PDH staining between HZ and MZ was not progressive, but sudden. Indeed, at the leading edge of infection (LE), a clear distinction between healthy cells and dying cells was observed (Fig. 1B,C). Staining of cell walls and plastids in MZ was much reduced, indicating a loss of iron. However, some regions of cell walls remained stained, indicating that iron was more strongly bound in some parts of the cell wall. Interestingly, neither nuclei nor nucleoli were visible in

the MZ. Rod-shaped structures with very strong PDH staining were visible in the intercellular spaces of the MZ (Fig. 1E,F). These elements were presumed to be bacteria (see confirmation below). Taken together, these data indicate that, during its progression in leaf tissues, *D. dadantii* causes loss of iron from dedicated cellular compartments and cell walls. Bacterial cells contain a high iron concentration during the infection process.

Comparative localization of iron, bacteria and intact cell walls during the infection process

We investigated whether the loss of iron from the cell walls coincided with cell wall degradation. Pectin demethylation is the first step of cell wall degradation by *D. dadantii* pectinases

Fig. 2 Localization of iron, bacteria and intact cell walls in an infected *Arabidopsis* leaf. (A, C) Perls'-diaminobenzidine (DAB)-H₂O₂ (PDH) staining. (B, D) Anti-methylated pectin immunolabelling using JIM7 antibody indicates intact cell walls. Staining is reduced in MZ because of bacterial cell wall-degrading enzyme (CWDE) activity. (E) Anti-*Dickeya dadantii* lipopolysaccharide immunolabelling. Bacteria are visible in MZ. (C-E) represent the same tissues. Consecutive sections were used in order to be able to co-localize iron, intact cell walls and bacterial cells. Scale bars: (A, B) 50 μ m; (C-E) for LE, 50 μ m; for MZ, 20 μ m. b, bacteria; cw, cell wall; HZ, healthy zone; LE, leading edge of maceration; MZ, macerated zone.

(HugouvieuxCottePattat *et al.*, 1996; Reverchon and Nasser, 2013). Thus, we monitored the level of cell wall integrity in the leaf using an antibody raised against highly methylated pectin, JIM7 (Knox *et al.*, 1990; Vandenbosch *et al.*, 1989). For this purpose, we performed JIM7 immunolabelling and PDH staining on consecutive semi-thin sections of the same samples. Figure 2 shows that, in healthy tissues, JIM7 immunolabelling is evenly distributed along cell walls, as in uninfected samples (Fig. S2B, see Supporting Information). This observation indicates that cell walls are undamaged in HZ. In the colonized tissues of MZ, JIM7 immunolabelling is much reduced. Cell wall integrity is thus strongly affected in this zone. However, some small portions remain labelled with JIM7 antibody, indicating that cell wall degradation is not complete,

which is reminiscent of that observed with PDH staining (Fig. 2C,D). These data indicate that iron is strongly associated with cell walls in *Arabidopsis* leaves and that, during bacterial progression in the leaf, cell wall-associated iron is released.

To investigate the localization of bacteria in the different zones, we used an antibody raised against a specific *D. dadantii* envelope component, the lipopolysaccharide (LPS). Immunolocalization of *D. dadantii* bacterial cells indicates that they are essentially restricted to the intercellular spaces of MZ (Figs 2 and S3, see Supporting Information). LPS labelling is visible around the bacterial cells (Fig. 2E). These data confirm that the rod-shaped structures stained by PDH are *D. dadantii* cells and that bacterial cells accumulate iron in the infected tissue.

Immunolocalization of ferritin during the infection process

Iron mobilization in the infected tissues is likely to provoke perturbations of iron homeostatic genes. In response to *D. dadantii* infection, the ferritin AtFER1 protein accumulates and is involved in the limitation of infection (Dellagi *et al.*, 2005). To investigate the tissue localization of AtFER1 during infection, we performed immunolabelling with the anti-AtFER1 antibody (Dellagi *et al.*, 2005). In control leaves (Fig. 3), AtFER1 was present in some plastids but not in all cells. In the infected sample, AtFER1 was not detected in MZ or LE. HZ could be subdivided into two subzones according to their ferritin content: HZ1 and HZ2 (Figs 3 and 4). In HZ2, close to LE, no signal was detected with the anti-AtFER1 antibody. The cell morphology in HZ2, including plastids and cell walls, was similar to that of HZ1. In HZ1, which corresponds to cells further away from the maceration front, AtFER1 proteins accumulated in the chloroplasts to higher levels than in controls. The AtFER1 protein accumulated ahead of the bacterial progression.

DISCUSSION

We have investigated the localization of iron at the cellular level in tissues of *Arabidopsis* leaves during infection by the bacterial pathogen *D. dadantii*.

During progression of the bacterial pathogen in the leaf tissues of *Arabidopsis*, iron is depleted from cell walls and from cellular compartments that normally contain large amounts of iron, such as plastids. This depletion raises the question of the fate of iron during infection. One possibility is that it is absorbed by bacterial cells. As observed in macerated tissues, bacteria are strongly stained by PDH, indicating that they are loaded with iron. Thus, they probably benefit from the release of large amounts of this element for growth. However, strong oxidative stress is also probably caused by the presence of free iron in the intercellular spaces. A strong oxidative stress occurs in *Arabidopsis* leaves in response to *D. dadantii* (Fagard *et al.*, 2007). This oxidative stress is mainly mediated by the NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD). Free iron could contribute, together

Fig. 3 Ferritin localization in an infected and non-infected *Arabidopsis* leaf. Sections of infected tissue were immunolabelled using an anti-AtFER1 antibody. Two different HZs were identified. In HZ1, ferritin was abundantly present (indicated by arrows). In HZ2 (close to LE), no ferritin could be detected. Scale bars: 50 μ m. Control, non-infected leaf; HZ, healthy zone; LE, leading edge of maceration; MZ, macerated zone.

Element	Healthy Zone 1	Healthy Zone 2	Macerated Zone 1
Iron	+	+	-
AtFer1	+	-	-
Intact cell wall	+	+	-
Bacteria	-	-	+

Fig. 4 Comparative table of iron, ferritin and bacterial localization, as well as cell wall integrity, in *Arabidopsis* leaf tissues infected by *Dickeya dadantii*. +, element is present; –, element is absent. Scale bar: 200 μ m.

with this enzyme, to the amplification of ROS accumulation. Interestingly, in Fagard *et al.* (2007), the authors showed that superoxide anions form a ring surrounding MZ and that this site of superoxide anion accumulation coincides with the LE of maceration. Consistently, the accumulation of ROS in response to *D. dadantii* is drastically reduced in iron-starved plants (Nam Phuong *et al.*, 2012). In addition, iron transport across the vacuolar membrane via the proteins AtNRAMP3 and AtNRAMP4 is involved in the amplification of oxidative stress, thus contributing to plant defence (Segond *et al.*, 2009). Together, these data suggest a role of iron in ROS accumulation during infection. In maize plants grown under adequate iron nutritional status, the establishment of the hemibiotrophic pathogen *Colletotrichum graminicola* was retarded when compared with that in iron-starved plants (Ye *et al.*, 2014). The authors observed that the retarding effect of iron on *C. graminicola* development coincided temporally and spatially with the recruitment of iron to infection sites and the local accumulation of H_2O_2 . Similarly, in barley, infection by the fungal pathogen *Blumeria graminis* triggered iron accumulation in the epidermal cells attacked by the fungal appressorium. This iron accumulation provoked a strong oxidative stress which led to callose deposition, thus preventing further infection (Liu *et al.*, 2007). Together, these data show that modifications of iron localization can be observed during plant–microbe interactions, and that they can provoke oxidative stress, thus contributing to immunity.

Iron release from dying cells can also provoke the transfer of this element to healthy tissues, thus explaining the accumulation of the ferritin AtFER1 behind the LE. These data confirm previous observations of ferritin accumulation around MZ at a macroscopic level using transgenic plants expressing a β -glucuronidase (GUS) fusion under the control of the AtFER1 promoter (Dellagi *et al.*, 2005). The accumulation of AtFER1 ahead of the LE could reflect an iron

buffering effect to protect cells from oxidative damage. This pattern of AtFER1 accumulation can also constitute a barrier for scavenging iron to prevent its acquisition by invading bacteria. The absence of AtFER1 accumulation in HZ2 cells indicates lower iron content in these cells. This could result from bacterial progression in the tissues and the diffusion of small molecules, such as siderophores, ahead of bacterial cells. Indeed, early response to siderophores is the repression of AtFER1 expression (Aznar *et al.*, 2014).

Our data show that cells displaying pectin degradation match well cells losing cell wall-associated iron. This shows that the cell wall structure is an important site for iron storage. It is well known that phenolic compounds present in the cell walls chelate large amounts of iron (Marschner *et al.*, 1986). More data are available about iron in the root apoplast than in the leaf apoplast (Longnecker and Welch, 1990; Maier and Cattani, 1995; Nikolic and Romheld, 2007). However, the exact role and localization of iron in the apoplast is not well understood. Our data show that a co-localization exists between intact cell walls and the presence of iron. This is particularly obvious in MZ, where some cell walls are partially degraded and where the intact segments (labelled with JIM7) co-localize with strong PDH-stained segments (Fig. 4). Recently, Hassan *et al.* (2013) identified a pectate lyase, named PelN, from *D. dadantii* which has the unusual characteristic of requiring Fe^{2+} as a co-factor for its enzymatic activity, and no other cation (among the cations tested). A *pelN*-deficient mutant showed reduced virulence on chicory leaves, indicating the contribution of this enzyme to the pathogenicity process. These results are in agreement with the presence of large amounts of iron in the plant cell wall. They suggest a possible evolutionary adaptation of PelN to the presence of this element in the close environment of its substrate.

In summary, we show that the study of iron localization at the cellular level provides valuable information concerning the role of

iron during pathogen attack. Our work highlights the potential of the powerful technique of *in situ* iron staining for the study of plant–microbe interactions at the cellular level. This method is highly specific and easy to set up.

EXPERIMENTAL PROCEDURES

Plant growth, bacterial culture and inoculation conditions

Arabidopsis Col-0 seeds were sown in non-sterile soil and stratified by incubation for 2 days at 4 °C. Five-week-old plants were heavily watered and covered with transparent plastic 16 h before inoculation. The cover was kept in place throughout the assay to maintain high moisture conditions. Plants were subjected to an 8-h light and 16-h dark cycle at 19 °C.

The wild-type strain *D. dadantii* 3937 (*E. chrysanthemi* 3937) was grown in Luria–Bertani medium (Miller, 1972). For plant inoculations, a small hole was made with a needle within the leaf, and 5 µL of a bacterial suspension at a density of 5×10^8 colony forming units/mL, made up in 50 mM potassium phosphate buffer (pH 7), was spotted.

Preparation of tissues for light and electron microscopy

For light microscopy, small fragments of leaves were sampled. For the infected leaves, the samples were chosen when the MZ reached stage 2 according to the stages described by Nam Phuong *et al.* (2012). This stage corresponds to an MZ spanning a circular area around the inoculation hole of about 3 mm in diameter, which occurs about 24–48 h post-inoculation (Fig. 1A). Samples to be fixed were collected at the boundary between the MZ and non-macerated zone. Samples were fixed for 3 h in a solution consisting of 4% paraformaldehyde and 1% glutaraldehyde in 0.05 M piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 7.3, under intermittent vacuum. They were washed four times in the same buffer and were dehydrated in a graded ethanol series before embedding in LR White resin (Electron Microscopy Sciences, Hatfield, PA.). For light microscopy, transverse semi-thin (0.5 µm) sections were cut with a diamond knife (Diatome histo, Bienne, Switzerland).

Immunolocalization of bacteria, methyl-esterified homogalacturonans and ferritin

For the immunolabelling of cell walls, we used the monoclonal JIM7 antibody, which specifically recognizes methyl-esterified homogalacturonans (Knox *et al.*, 1990). For the immunolabelling of bacteria, we used the anti-*D. dadantii* LPS polyclonal antibody (Fagard *et al.*, 2007). For the immunolabelling of AtFER1, we used an anti-AtFER1 antibody (Dellagi *et al.*, 2005) (see Table S1, Supporting Information, for experimental details).

Consecutive semi-thin sections were positioned in an ordered manner on a multiwell slide. They were incubated in TBSB-T buffer (0.05 M Tris-HCl buffer, pH 7.4, containing 0.9% NaCl, 0.1% bovine serum albumin and 0.01% Tween 20) for 10 min, and then saturated with serum 1 (see Table S1) diluted 1:30 in TBSB-T. They were washed (four times for 10 min), and then incubated overnight at 4 °C with the antibody (serum 2) raised

against LPS, methyl-esterified homogalacturonans or AtFER1, diluted as indicated in Table S1. After four washes for 10 min with TBSB (TBSB-T without Tween), the sections were incubated at room temperature with the labelled antibody [serum 3: anti-rat in rabbit immunoglobulin (IgG) or anti-rabbit in goat IgG, see Table S1] labelled with fluorescein isothiocyanate (FITC) diluted 1:300 in TBSB in the dark. The slides were washed six times for 10 min each with TBSB, rinsed with distilled water and mounted in one drop of Vectashield medium. Controls for antibody specificity consisted of sections for which the primary antibody (serum 2) was replaced by TBSB buffer and all the rest was unchanged (Fig. S2). Fluorescence was observed with a Zeiss microscope excitation filter for wavelengths of 450–490 nm and barrier filter for a wavelength of 520 nm. The slides were then observed with a Zeiss Axiophot light microscope (Zeiss, Oberkochen, Germany).

Histochemical staining of Fe with the PDH procedure

Sections were deposited on glass slides that were incubated for 45 min at room temperature in Perls' stain solution [4% (v/v) HCl and 4% (w/v) K-ferrocyanide]. The slides were then washed twice for 5 min with distilled water and twice with 0.1 M sodium phosphate buffer, pH 7.4. For the intensification reaction, the slides were incubated for 30 min in the dark in a 0.1 M sodium phosphate buffer (pH 7.4) solution containing 0.025% (w/v) DAB tetrahydrochloride (Sigma, Saint Louis, USA), 0.005% (v/v) H₂O₂ and 0.005% (w/v) CoCl₂. The sections were washed three times with distilled water to stop the reaction.

ACKNOWLEDGEMENTS

The imaging studies were performed with the help of the cytology/imaging platform of the Plant Observatory—http://www.observatoire-vegetal.inra.fr/observatoire-vegetal_eng.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Characterization of iron localization sites in Arabidopsis leaves. (A, B) Perls'-diaminobenzidine (DAB)—H₂O₂ (PDH) staining of healthy leaf tissue. Scale bar: (A) 50 µm; (B) 20 µm. (C) Co-localization of nucleolus by 4',6-diamidino-2-phenylindole (DAPI) staining (arrow in left panel) and by PDH staining (arrow in right panel). Scale bar: 20 µm. (D) Co-localization of iron in plastids by anti-AtFer1 immunolabelling (arrows in left panel) and by PDH staining (arrows in right panel). Scale bar: 20 µm. cw, cell wall; nuc, nucleolus; pl, plastid; v, vascular tissue; a, b, c, d, e, ferritin spots.

Fig. S2 Specificity of antibodies used for immunolabelling. (A, B) Anti-methylated pectin immunolabelling with (B) and without (A) JIM7 antibody (serum 2). Cell walls are visible only when serum 2 is used. (C, D) Anti-AtFER1 immunolabelling with (D) and without (C) anti-AtFER1 antibody (serum 2). AtFER1 proteins are visible in plastids only when serum 2 is used (arrows). (E, F) Anti-*Dickeya dadantii* lipopolysaccharide (LPS) immunolabelling with (F) and without (E) anti-LPS antibody (serum 2). Bacteria are visible only when serum 2 is used (arrows). Scale bars: 50 µm.

Fig. S3 Localization of intact cell walls and bacteria in an infected Arabidopsis leaf. (A) Anti-methylated pectin immunolabelling using JIM7 antibody indicates intact cell walls. Staining is reduced in the macerated zone (MZ) because of bacterial cell wall-degrading enzyme (CWDE) activity. (B) Anti-*Dickeya dadantii* lipopolysaccharide immunolabelling. Bacteria are visible in MZ. (A) and (B) represent the same tissue. Consecutive sections were used in order to be able to co-localize intact cell walls and bacterial cells. Scale bars: 50 µm. b, bacteria; cw, cell wall; HZ, healthy zone; LE, leading edge of maceration; MZ, macerated zone.

Table S1 Description of the different sera used in this study. Serum 1 is used to saturate the unspecific sites recognized by the sera of the species from which serum 3 is derived. Serum 2 is the specific serum recognizing the epitope to be labelled. Serum 3 recognizes serum 2 and is linked to fluorescein isothiocyanate (FITC).