The Impact of Iron Deficiency on Plant-Oomycete Interactions

John Henry Herlihy

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John M. McDowell
Guillaume Pilot
Terri A. Long
Aureliano Bombarely Gomez

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ABSTRACT

Plants are sessile organisms adapted to cope with dynamic changes in their environment. Abiotic stresses, such as heat, drought, or nutrient deficiency must be overcome simultaneously with biotic threats such as pathogens and herbivores. Oomycete pathogens represent a significant threat to global food production and natural ecosystems. Novel modes of oomycete disease control could increase crop yield and reduce pesticide application. Overlaps between the plant response to iron deficiency and pathogens have been documented, but the impact of simultaneous imposition of both stresses on the plant have not been studied. Additionally, nothing is known about the impact of iron deficiency on oomycete infection, or mechanisms of oomycete iron uptake. We adapted a hydroponic system to simultaneously impose iron deficiency and monitor pathogen infection. The oomycete pathogens *Hyaloperonospora arabidopsidis*, and *Phytophthora capsici* grew less well on iron-deficient *Arabidopsis thaliana*, at least in part because of observed activation of immunity due to iron stress. We screened *A. thaliana* T-DNA insertion mutants defective in iron metabolism and transport and identified potential mechanisms of *H. arabidopsidis* iron acquisition. We conducted RNA sequencing to understand how *A. thaliana* responds to iron deficiency and root infection of *P. capsici*. 323 genes were differentially upregulated in iron-starved plants over three days, irrespective of pathogen infection, representing a core iron deficiency response. This group of core genes included the primary *A. thaliana* iron uptake pathway and genes for

coumarin biosynthesis. Salicylic acid responsive genes were observed in both treatments consistent with this defense hormone's previously identified role in iron deficiency. Genes related to glucosinolate production – shown to be important in defense against *P. capsici* – were down regulated during infection, potentially due to the activity of virulence effectors. Our work demonstrates crosstalk between the iron deficiency response and plant immunity, and that iron acquisition remains important to the plant even after pathogen invasion. These new insights provide a first step in developing novel resistance strategies to control oomycetes in agronomically important crops.

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GENERAL AUDIENCE ABSTRACT

Oomycetes can cause diseases in plants resulting in loss of crops and requiring application of chemical pesticides. Better understanding of how oomycetes interact with plants will lead to new strategies to control them and more efficient agriculture. In this study, we investigated the role of iron in plant-oomycete interactions, to see what this important metal nutrient might be doing to help or hurt the plants response to infection. We developed a growth system to limit iron to the plant and simultaneously observe oomycete infection. We studied the leaf pathogen *Hyaloperonospora arabidopsidis* or downy mildew, and *Phytophthora capsici*, a root pathogen that infects many types of vegetable crops. In rice, iron restriction hurt the plant's ability to fight off disease, but we observed the opposite: iron limitation caused the plant to be more resistant to both oomycete pathogens. Microscopic observation revealed that the plants ability to fight off downy mildew was not compromised by iron deficiency. Our results suggest that iron limitation triggers an immune response in the plant, which limits pathogen growth. We performed RNA sequencing on iron-deficient roots also infected with the root pathogen. This allowed us to observe how the plant responded to both stresses. The plant balances the response to iron deficiency and infection. Again, we found that iron deficiency triggers immune activation, and observed that iron-deficient plants are more resistant to infection.

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Chapter 1

Iron Biofortification of Crops: Help or Hindrance to Plant Immunity?

John Herlihy[1], Terri A. Long[2], John McDowell[1]

[1]Virginia Tech, School of Plant and Environmental Sciences, Latham Hall (0329), Blacksburg, VA, 24061-0329

[2]North Carolina State University, Department of Plant Biology, 2115 Gardner Hall, Box 7612, Raleigh, NC 27695-7612

Abstract

Iron (Fe) metabolism and immunity are critical for plant vigor and, consequently, human nutrition and health. Our growing understanding of the plant iron deficiency and immune responses has uncovered surprising cross talk between the two. Plants have evolved to detect and react to the presence and activity of pathogens, through a variety of signals, including various pathogen-derived molecules that are secreted during infection. Additionally, sensing of nutrient depletion may represent another mechanism by which plants recognize a pathogen threat: new evidence is emerging that plants may respond to iron depletion with immune activation. Plants can also deploy iron as a weapon of the immune system after a pathogen has been detected to produce reactive oxygen species (ROS) or initiating programmed cell death via ferroptosis. Beyond iron's role in defense, the iron deficiency response and immune response utilize overlapping signal transduction mechanisms, further entangling these two processes. Classical breeding and transgenic approaches have been used to develop crops biofortified for iron content

in order to alleviate the global burden of iron deficiency-related anemia. These alterations impact how biofortified crops perceive iron and, consequently, may substantially impact plant immunity. In this article, we provide overviews of the regulation of plant immunity and iron homeostasis, and summarize evidence for the interconnection of these two networks. We emphasize exciting recent advances and consider whether engineering of for iron biofortification might also affect disease resistance.

Overview of the Plant Immune Response

Signaling components

Plants have evolved robust mechanisms for perception of detrimental microbes, which in turn, trigger physiological responses to impede infection (Cook, Mesarich et al. 2015). One strategy for detecting pathogens occurs through recognition of pathogen-associated molecular patterns (PAMPs), initiating pattern-triggered immunity (PTI)(Figure 1a) (Katagiri and Tsuda 2010). PAMPs are epitopes, often derived from proteins or carbohydrates, exemplified by bacterial flagellin or fungal and oomycete cell wall components. Such epitopes are often evolutionarily conserved, allowing for detection of groups of pathogens (e.g., multiple species) that share the epitope (Boller and He 2009). PAMPs can be detected in the apoplast by cell-surface receptors (Gust and Felix, 2014). Such recognition initiates phosphorylation of cytoplasmic protein kinase cascades, Ca^{2+} influx, and rapid production of reactive oxygen species (ROS) (Macho and Zipfel 2014). ROS generation begins quickly after PAMP perception and is due to the action of iron-containing transmembrane NADPH oxidases that generate

superoxide radicals in the apoplast (Torres, Jones et al. 2006). Superoxide ions act as a signal for further immune events, as precursors for additional reactive oxygen species, and as an apoplastic toxin against microbes (McDowell and Dangl 2000). The plant cell membrane is impermeable to superoxide, but superoxide can be converted to membrane-diffusible hydrogen peroxide by superoxide dismutases, some of which contain iron. Hydrogen peroxide can stimulate additional calcium influx in a signal amplification loop (Marcec, Gilroy et al. 2019). ROS act as second messengers, transmitting perception of a pathogen to nearby cells, but they can also as direct weapons against microbes, which lack the capacity to detoxify large quantities of free radicals (Torres, Jones et al. 2006). Nitric oxide (NO) and ethylene gas (ET) rapidly diffuse through the plant following pathogen perception, alerting local tissues as part of a generalized stress response (Dubois, Van den Broeck et al. 2018). Plants produce NO and ET under many stress conditions, including iron deficiency, to modulate hormone signals, and activate appropriate long-term responses (Frederickson Matika and Loake 2013). Hormonal regulation is shared by plant immunity and the iron deficiency response. In Arabidopsis, the bHLH transcription factor MYB72 is induced by the hormones ET and NO, and also microbes and iron deficiency (García, Suárez et al. 2011, Palmer, Hindt et al. 2013). In turn, MYB72 regulates systemic immunity and iron acquisition (Zamioudis, Korteland et al. 2015). controls biosynthesis of coumarins which facilitate iron uptake from the soil and are bioactive against microbes which sculpts the microbiome to favor the plant (Stringlis, Yu et al. 2018). The integration of immune responses and iron deficiency responses, shows the plant is already coregulating these pathways. Other potential regulatory connections between iron deficiency and immunity have been reviewed and will be further discussed below (Aznar, Chen et al. 2015, Verbon, Trapet et al. 2017). Our goal

in this review is to illustrate how these connections could affect future attempts to utilize crops biofortified for iron

Plants immunity is tailored to pathogens of different lifestyles

Plant pathogens typically follow one of three lifestyles: biotrophic, hemi-biotrophic, or necrotrophic. Biotrophic pathogens can only extract nutrients from living host cells (McDowell 2011). Such pathogens are able to suppress host immunity, extract nutrients, and complete their life cycle without killing host cells. Contrastingly, necrotrophic pathogens kill host cells with toxins and complete their life cycle by feeding from dead or dying plant tissue (Mengiste 2011). Hemi-biotrophic pathogens begin the infection cycle with an extended period of biotrophy before triggering a necrotrophic program. Plant deploy different immune responses against pathogens with these contrasting lifestyles, and plant hormones play key roles in coordinating the immune responses that are most efficient against pathogens with these contrasting lifestyles (Spoel and Dong 2008). For example, the phenolic phytohormone salicylic acid (SA) is typically produced in response to biotrophic pathogens and activates immune responses that typically culminate in programmed cell death (PCD) at the infection site (Glazebrook 2005). The PCD associated with SA in plant immunity is termed the Hypersensitive Response (HR), and restricts pathogen access to plant tissues (Mukhtar, McCormack et al. 2016). Contrastingly, immune responses against necrotrophs and herbivores do not involve cell death and are activated by the phytohormones jasmonic acid (JA) and ethylene (ET) (Mengiste 2011). The SA and JA/ET pathways antagonize and buffer each other to tailor the response to the invading pathogen, so that the plant utilizes its resources most efficiently (Figure 1a) (Spoel and Dong 2008, Hillmer, Tsuda et al. 2017). While

pathogen lifestyle and the corresponding plant response is encompassed by these three strategies, mechanisms of pathogen iron acquisition are more varied and more research is needed to draw conclusions between pathogen lifestyle and their relationship with host iron.

ROS and hormone signals interact with each other to stimulate a plethora of molecular and cellular responses that strengthen plant cells against pathogen attack (Karapetyan and Dong 2018). At the molecular level, PAMP perception, and a buffered SA signal impact expression of thousands of genes, including genes for antimicrobial proteins (e.g., iron-sequestering defensins discussed below) and secondary metabolites with antimicrobial activity (Macho and Zipfel 2014). At the cellular level, pathogens often require access to individual cells or host vasculature, thus the plant produces callose to reinforce cell walls against hydrolases and pathogen secretion systems (Luna, Pastor et al. 2011). The precise roles of these immune responses and their mechanisms of pathogen antagonism are unclear and under investigation, but it is clear that iron plays multiple, important roles (Aznar, Chen et al. 2015, Verbon, Trapet et al. 2017).

Pathogen virulence proteins suppress immunity and facilitate nutrient acquisition

Considering the potency of the plant immune system, pathogen success depends on evasion of detection and suppression of host immune signaling (Nobori, Mine et al. 2018). Many pathogens disguise themselves by secreting proteins to bind PAMPs, thereby obscuring recognition leading to PTI. In a second strategy to interfere with activation of host immunity, pathogens secrete virulence proteins called effectors to inhibit critical regulatory components of host immune signaling (Toruno, Stergiopoulos et al. 2016). Effectors from bacteria, fungi, and

oomycetes have been shown to target similar hubs in the host immune signaling network. The action of these effectors results in attenuated immune response called effector triggered susceptibility (ETS) (Jones and Dangl 2006). Pathogen effectors can also influence other aspects of host physiology, including nutrition (Toruno, Stergiopoulos et al. 2016). For example, bacterial effector mimics a plant transcription factor to upregulate transcription of a sugar transporter (Yang, Sugio et al. 2006). The partitioning of additional sugar to the apoplast promotes pathogen growth and underscores that pathogens require host derived nutrition as well as immune evasion to proliferate. No effectors that manipulate iron has of yet been identified. However, the importance of iron as a nutrient, and the energy intensive mechanisms that pathogens employ to acquire host iron (explained below), would suggest that effector manipulation of host iron metabolism would be a profitable strategy for plant pathogens.

Effector-triggered immunity

To counter the threat of ETS, plants have evolved resistance proteins (R proteins) to detect the effects of pathogen effectors (Kourelis and van der Hoorn 2018). Some R proteins bind directly to the cognate effector, similar to direct binding of PAMP ligands by pattern recognition receptors. However, it is more common for R proteins to "guard" immune hubs targeted by pathogen effectors (Kourelis and van der Hoorn 2018). By perceiving the virulence activities of effectors, and disruption of immune signaling, rather than the effectors themselves, a single R protein can protect the plant from multiple pathogens that have converged to target the same protein complex (Van Der Biezen and Jones 1998).

ETI and PTI activate many of the same signaling pathways and defense responses. However, ETI is typically faster, its signaling is more resistant to pathogen interference, and immunity is stronger than PTI (Katagiri and Tsuda 2010). The exact molecular events that differentiate PTI and ETI are under investigation. Respiratory burst oxidase homolog (RBOH) derived ROS accumulate at high levels during ETI, which in some interactions is critical for the aforementioned HR PCD to halt the spread of pathogens (Torres, Dangl et al. 2002). The HR is particularly effective against biotrophic pathogens that require living host tissue. However, as most pathogens are biotrophic in the early stages of infection, and initiation of HR to limit pathogen growth is often the best defense for a plant (Mukhtar, McCormack et al. 2016). Recent work on synthetic R proteins and molecular mechanisms of R protein and HR activation offer exciting glimpses into how plant immunity may be improved (Michelmore, Coaker et al. 2017). Moreover, new studies, discussed below, provide a direct role for iron in activation of ETI.

Overview of Plant Iron Metabolism

Iron is an essential micronutrient for all living organisms including plants and their associated microbes (Camprubi, Jordan et al. 2017). Because iron can exist in multiple oxidation states and a wide redox potential range, Fe can readily donate and accept electrons. Therefore, Fe cofactors such as heme and Fe-sulfur clusters function in all primary metabolic processes including respiration, DNA synthesis and repair, and cell proliferation and differentiation (Camprubi, Jordan et al. 2017). In plants Fe is also essential for chlorophyll and hormone synthesis, and photosynthesis among other processes. Iron is highly abundant in soil, but most

iron is bound in oxidized and insoluble ferric forms (Fe^{3+}), which are biologically inactive (Chen and Barak 1982).

Iron's potent electron chemistry also makes it dangerous when it is in physiological excess. Iron acts as a catalyst with hydrogen peroxide through the Fenton reaction, producing more dangerous ROS including the highly reactive hydroxide ion (Winterbourn 1995). These potent oxidizers damage lipids, proteins, and nucleic acids (Becana, Moran et al. 1998, Pinto, Souza et al. 2016). When the damage becomes too grievous, the cell cannot be saved and undergoes PCD (Tsai and Huang 2006). Thus, iron overload can cause damage in any organism. To balance iron levels, plants tightly regulate iron uptake, localization, transport and storage (Kobayashi and Nishizawa 2012, Xing, Wang et al. 2015).

When a plant perceives iron deficiency, iron uptake is increased through one of two strategies (Marschner, Römheld et al. 1986). Strategy I plants, including all non-Poaceae angiosperms, primarily acquire iron through reduction of iron in the soil and direct uptake (Kobayashi, Nozoye et al. 2019). The Strategy II plants (including Poaceae such as maize and rice) instead secrete phytosiderophores to bind ferric iron in the rhizosphere for transport back into the root (Marschner, Römheld et al. 1986, Kobayashi, Nozoye et al. 2019). Siderophore is Greek for iron bearer. These molecules are used diverse organisms, including microbial pathogens, to acquire iron and facilitate its uptake (Andrews, Robinson et al. 2003, Haas, Eisendle et al. 2008, Niehus, Picot et al. 2017). Despite differences in mechanisms of Strategy I and II plants, there remains considerable overlap between iron uptake in all plants. Uptake via

assimilation by iron-binding compounds is still important for iron acquisition in Strategy I plants, especially the phenolic coumarins (Tsai and Schmidt 2017).

The regulatory pathways that control activation of Strategy I uptake genes have been studied intensively in Arabidopsis, and we will highlight pathways and genes that are discussed later in the article due to their entanglement with immune signaling or potential role in iron biofortification. Activation of the Strategy I iron deficiency response is primarily regulated by the basic helix-loop-helix (bHLH) transcription factor (TF) FIT (Bauer, Ling et al. 2007). FIT heterodimerizes with other bHLHs upon iron deficiency and promote transcription of Fe mobilization genes including *FRO2,* which encodes a protein for reduction of ferric iron in the rhizosphere and *IRT1*, which encodes a transporter of reduced ferrous iron into the root (Connolly, Campbell et al. 2003, Bauer, Ling et al. 2007). A number of WRKY and MYB transcription factors respond to both iron deficiency and immune signaling, which might allow the plant to integrate these two stresses (Urzica, Casero et al. 2012, Zamioudis, Korteland et al. 2015, Yan, Li et al. 2016). The bHLH TF PYE heterodimerizes with a different group of TFs to regulate iron deficiency response such as lateral root formation (Long, Tsukagoshi et al. 2010). It also interacts with the bHLH ILR3 to coordinate iron deficiency response with wounding and nematode infection (Rampey, Woodward et al. 2006, Samira, Li et al. 2018). The primary plant phytosiderophore for strategy II uptake from the soil is the methionine derivative mugineic acid (Takemoto, Nomoto et al. 1978, Higuchi, Suzuki et al. 1999). The Fe-mugineic acid complex is taken into the root by members of yellow stripe like (YSL) transporter family (Curie, Panaviene et al. 2001).

Small molecules facilitate solubility and transport of iron in plants (Tiffin 1970, Kobayashi, Nozoye et al. 2019). In Arabidopsis, once Fe is taken from the rhizosphere into the outer cells of the root it is chelated to nicotianamine (similar to the methionine-derived mugineic acid), transferred to the vasculature and effluxed into the xylem via FPN1 (Morrissey, Baxter et al. 2009). Once in the xylem Fe is bound to citrate for long distance transport to the shoot (Bienfait, van den Briel et al. 1985, Rogers and Guerinot 2002). YSL family proteins unload Fe-nicotianamine from the vasculature and are expressed in many other tissues (Curie, Cassin et al. 2008). Various transporters load iron into organelles in leaf mesophyll cells for storage or metabolism, including NRAMP3 and 4 which load iron into the vacuole, which is important for iron storage, particularly in developing embryos (Lanquar, Lelièvre et al. 2005, Bastow, Garcia de la Torre et al. 2018). Ferritins are iron storage proteins found in plastids, and are upregulated in response to ROS detection to bind iron and thereby mitigate damage from the Fenton reaction (Deák, Horváth et al. 1999, Briat, Duc et al. 2010).

Just as we saw with ET and NO, SA and JA regulate the iron deficiency response, further connecting it with biotic stress responses. ET, as mentioned above, promotes immune activation, ROS production, coumarin biosynthesis, but also modifies root architecture and promotes increased iron uptake from the soil (Pitts, Cernac et al. 1998). In Arabidopsis, SA and JA display a similar antagonistic effect in regulation of iron deficiency as to immune responses (Maurer, Müller et al. 2011, Shen, Yang et al. 2016). Iron deficiency stimulates production of SA and vice versa (Shen, Yang et al. 2016). However, JA downregulates expression of FIT, suppressing the iron deficiency response (Cui, Chen et al. 2018). The shared regulatory pathways of the plant immune system and iron deficiency response alludes to iron's role in immunity.

Plant pathogens use iron uptake strategies analogous to plant system I and system II

As stated above, plant pathogens are dependent on iron from the plant during infection. Microbial pathogens (bacteria and fungi) employ varied strategies for iron acquisition that are analogous to Strategy 1 and 2 described above for plants (Andrews, Robinson et al. 2003, Philpott 2006, Sandy and Butler 2009). For example, many plant pathogens secrete high affinity, iron-binding siderophores to acquire iron from their immediate environment, analogous to Strategy II (Neilands 1995, Khan, Singh et al. 2018). Fungi express nonribosomal peptide synthases (NPS) to produce siderophores (Carroll and Moore 2018). In the plant pathogenic ascomycete fungi, the NPS family is conserved and has been experimentally validated as important for virulence (Oide, Moeder et al. 2006). Bacteria produce a large diversity of peptide and small molecule siderophores, which in some cases have been validated as critical virulence factors. In pathogenic *Pseudomonas*, *Ralstonia*, and *Erwinia* the transcriptional regulation of siderophore biosynthesis is mediated by so-called hrp regulatory factors that also control expression of effectors (Occhialini, Cunnac et al. 2005, Zhao, Blumer et al. 2005, Lan, Deng et al. 2006). This molecular association would put siderophore synthesis under the control of the pathogen virulence program, not an iron deficiency program, and indicates the importance of siderophores for success inside the host during infection. One of the most interesting recent studies on this topic showed that suppression of iron deficiency-related gene expression in bacteria growing in the apoplast, is a component of PTI and ETI (Nobori, Velasquez et al. 2018). This global suppression is likely due to specific suppression of one or more regulators of iron homeostasis by the plant host, and the physiological relevance of this interplay was demonstrated

by experiments in which transgenic bacteria overexpressing a regulator of iron acquisition could partially overcome growth restriction imposed by artificial induction of PTI. The mechanism underpinning this manipulation of bacterial physiology remains to be determined and will be a very interesting area of future study.

While siderophore-mediated uptake has received the most experimental attention from plant pathology researchers, reduction, and subsequent transport of ferrous iron (akin to Strategy I) has been shown to be crucial for some pathogens, especially fungi. Just as in the rhizosphere, iron inside the plant is more soluble and available when reduced. Fungal reductive iron assimilation is a three part process best described in yeast (Philpott 2006). Briefly, FRE family, iron reductases are active in reducing ferric iron at cell membrane (Dancis, Roman et al. 1992). Next the reduced iron is loaded in to a protein complex of FET and FTR (Askwith, Eide et al. 1994). ScFET3 oxidizes the iron before transport into the yeast by ScFTR1. The role of this oxidation step is unknown, but the FET/FTR complex exhibits high affinity, allowing fungi to scavenge iron at low concentrations. The role of FRE family reductases in plant pathogenic fungi has not been reported, however, Albarouki et al. demonstrated the importance of FET-mediated iron uptake in the maize pathogen *Colletotrichum graminicola* (Albarouki and Deising 2013). Mutant fungi, lacking the iron deficiency-induced FET protein, grew as wild type fungi on iron sufficient media. However, during infection on maize, those mutants exhibited abnormal morphology and reduced virulence. The authors conclude that fungal reductive iron assimilation is important in supplying the pathogen with this critical nutrient *in planta*, and removing potentially dangerous free iron from the environment. The expression of iron transporters during infection has also been implicated in pathogen virulence (Eichhorn, Lessing et al. 2006). In rice

smut, a transporter plays a role in virulence, indicating apoplastic iron availability is important for pathogen success (Zheng, Ding et al. 2016). Studies such as these illuminate the tug-of-war for iron inside the plant, and confirm that nutrient acquisition is central to pathogen success.

Pathogens compete with the plant and beneficial endophytes for iron

The competition for iron involves more than plants and their pathogens: This competition also shapes and is shaped by communities of beneficial microbes that live as non-pathogenic endophytes in plant tissues or occupy the soil in close proximity to plant organs (Pii, Borruso et al. 2016). These microbes also produce siderophores, which allows them to compete for iron which is often a limiting mineral nutrient (Scher and Baker 1982, Gómez Expósito, de Bruijn et al. 2017). A number of studies have demonstrated that siderophore-producing beneficial microbes inhibit plant pathogen growth and provide improved disease outcomes. For example, in a screen for bacteria that antagonize the rice blast fungus (*Magnaporthe oryzae*), those with high copy numbers of siderophore genes, and siderophore production under iron stress performed best at pathogen suppression (Zeng, Xu et al. 2018). Moreover, purified bacterial siderophores are sufficient to antagonize growth of pathogenic Fusarium (Scher and Baker 1982). Both siderophore-producing bacteria, and purified siderophore inhibit Fusarium growth *in vitro*. Treatment of soil with siderophore-producing bacteria, also reduced the virulence of Fusarium *in planta (Adesina, Lembke et al. 2007).* The mechanism of pathogen suppression was confirmed to be competition for iron through application of iron-bound siderophore. Bound to iron, the microbial siderophores where unable to sequester more iron, and consequently showed no antagonism toward Fusarium. Additional examples, summarized in recent reviews underscore

the importance of siderophores from beneficial microbes, and their potential roles in agricultural settings (Saha, Sarkar et al. 2016).

Microbial siderophores trigger plant immunity

Microbial siderophores help plant pathogens establish virulence and allow beneficial microbes to outcompete plant pathogens for iron. However abundant evidence shows that siderophores are also potent triggers for plant immunity (Figure 1a) (Aznar, Chen et al. 2014). Activation of plant immunity by siderophores is likely to occur by one of two paths. The first would be analogous to PTI, in which direct recognition of pathogen siderophores triggers immunity. The second would act through iron acquisition, in which siderophore iron scavenging initiates an iron deficiency response. The coregulation of plant immunity and the iron deficiency response would then prompt the plant to trigger immunity based on perception of that iron scavenging (Verbon, Trapet et al. 2017).

Unlike in PTI, no receptors of immune-activating siderophores have been identified. Treatment with siderophores in a diverse group of plants triggers immunity and provides resistance to pathogens in distal tissues. In eucalyptus, growth of the bacterial pathogen *Ralstonia solanacearum* was inhibited by application of siderophore-producing Pseudomonads (Ran, Li et al. 2005). Root application of the Pseudomonas or their purified siderophore reduced the pathogen's growth in shoot tissues. This systemic immune activation was perturbed when the siderophore genes were knocked out in the Pseudomonad. Without the siderophore, these bacteria lost their capability to provoke an immune reaction from plants and inhibit pathogen

growth. Similar observations were seen in other models. The suppression of Fusarium infection in tomato is diminished when triggering bacteria are mutated to inhibit siderophore biosynthesis. Additionally, microbial and synthetic siderophores both trigger immunity. Aznar et al. showed that deferoxamine, a derivative of a bacterial siderophore, and ethylenediamine-di(o-hydroxyphenylacetic) acid (EDDHA), a synthetic siderophore both induced iron deficiency and immune related gene expression (Aznar, Patrit et al. 2015). The plant has no evolutionary history with the synthetic siderophore, and presumably no receptor for its recognition.

Further supporting this hypothesis, siderophores fail to trigger immunity when their iron scavenging ability is compromised. EDDHA does not trigger does not trigger immunity when applied in its iron-bound form (Scher and Baker 1982). The siderophore chrysobactin is required for the virulence of *Dickeya dadantii* on Arabidopsis, and also stimulates immunity. It too loses its immune-triggering capability when it is applied bound to iron (Dellagi, Segond et al. 2009). The authors conclude that the triggering of plant immunity relates to perturbation of plant iron levels not molecular recognition of the siderophore itself. The absence of a siderophore receptor, and the diversity of immune-triggering siderophores, including synthetic molecules, suggests that iron depletion, rather than perception of siderophores, is the mechanism by which siderophores activate plant defense responses and confer immunity to pathogens.

Further research is required to understand the exact mechanisms of plant immune activation by siderophores. While it is possible that certain siderophores are recognized as PAMPs, plants would require many unique receptors for the numerous molecular forms of siderophores. The detection of iron scavenging is a robust mechanism for plants to detect a universal pathogen

activity. The overlaps in the plant iron deficiency response and immune activation provide a simple way to achieve this monitoring. Research into the plant perception of iron and the shared signaling mechanisms of these pathways will help us better understand the exact role of siderophores in immune activation.

Mechanisms through which plants restrict iron availability

Host iron sequestration is a potent tool to prevent pathogens from acquiring this crucial nutrient. Iron availability is correlated to virulence of some pathogens (Zanette, Bitencourt et al. 2015), so mammals have evolved mechanisms to restrict iron and prevent pathogen uptake (Skaar 2010). Specifically, the mammalian hormone hepcidin is deployed to block iron transport, and retain intracellular iron pools, particularly in macrophages (Stefanova, Raychev et al. 2017). Concurrently, mammals utilize sidoclarin to bind pathogen siderophores to further limit their capacity for iron assimilation (Nelson, Barasch et al. 2005). Similarly, plants are thought to have evolved mechanisms to sequester iron during infection in order to limit pathogen growth. However, definitive support has not yet emerged for this mechanism. Plant ferritins (FER) may play just such a role. FER is upregulated in many plants following infection, including potato tubers during late blight infection and Arabidopsis upon perception of *D. dadantii* (Mata, Lamattina et al. 2001, Dellagi, Rigault et al. 2005). Arabidopsis deficient in FER expression are more susceptible to *D. dadantii*. The expression of FER in this pathosystem relies on the presence of iron scavenging from pathogen siderophores. Bacteria deficient in siderophore production, or application of iron-bound siderophore are insufficient to activate FER expression, indicating that the pathogen and host are in competition for iron (Dellagi, Rigault et

al. 2005). Overexpression of FER behind a 35S promoter in *Nicotiana tobacum* provides resistance to fungal pathogens (Deák, Horváth et al. 1999). A concurrent study on a similar tobacco transformant reported an iron deficiency response in these plants (Van Wuytswinkel, Vansuyt et al. 1999). Despite these interesting results little follow up work has been done on these studies. The results are intriguing, as FER overexpression systemically, or in specific tissues is a common tool for iron biofortification (Vasconcelos, Gruissem et al. 2017).

Iron restriction may play another role: activating a systemic signal that triggers both iron deficiency response and immunity. This mechanism would mirror immune stimulation by microbial siderophores. Recent work demonstrates that plant defensins, a class of proteins long known to play a role in pathogen defense, likely act through iron restriction (Hsiao, Cheng et al. 2017). Plant defensins (PDF) are immune-regulated proteins that provide resistance to pathogens (Kaur, Velivelli et al. 2018). The family of PDF1 (1.1, 1.2, and 1.3) share high sequence similarity across the Brassicaceae, and inhibit the growth of fungal plant pathogens and yeast *in vitro* (Terras, Eggermont et al. 1995, Thomma, Cammue et al. 2002, Sels, Delauré et al. 2007). Like FER, Arabidopsis PDF1.1 appears to sequester iron to restrict pathogen growth. Hsiao et al. in 2017 demonstrated that AtPDF1.1 binds iron at high affinity, and is expressed in response to pathogen invasion or ROS. PDF1.1's iron binding activity stimulates plant immunity, similarly to microbial siderophores. The subsequent iron deficiency response and accompanying ET burst following PDF1.1 secretion activate immunity throughout the plant. Arabidopsis overexpressing PDF1.1 is more resistant to *Pectobacterium carotovorum*, and this resistance can be mitigated by exogenous application of iron. The role of pathogen starvation in this process is unclear, as isolating the growth inhibition from iron deficiency versus immune

stimulation is challenging. Future experiments in which key signaling components of plant immunity have been compromised may reveal the individual contributions of immune activation and pathogen starvation.

Iron as a tool in plant immunity

While iron sequestration is proven to provide pathogen resistance in mammals, and may pay a similar role in plants, it can also be a powerful weapon when wielded against pathogens. Iron has the ability to produce dangerous ROS through the Fenton reaction (Winterbourn 1995). Delivery of iron to infections sites, to exploit its redox chemistry is a critical immune response for many plants, particularly the Poaceae. Iron-deficient maize is unable to produce ROS at Colletotrichum infection sites that is required for defense (Ye, Albarouki et al. 2014). This correlates with susceptibility to this hemibiotrophic fungal pathogen. If the plant is wounded before infection, the pathogen can proceed more quickly to a necrotrophic stage and virulence conferred by iron deficiency is masked (Albarouki, Schafferer et al. 2014). Maize also uses this strategy against the biotrophic pathogen corn powdery mildew (*Blumeria graminis*) (Liu, Greenshields et al. 2007). In this interaction the plant recruits ferric iron to the infection site where it plays a role in ROS production. Application of iron chelators inhibits this effect, and decreases resistance to the pathogen (Liu, Greenshields et al. 2007). The iron-derived ROS provides protection from pathogens through its cytotoxic effect, as well as amplifying immune signaling as discussed above (Torres, Jones et al. 2006). While the above example suggest this mechanism is effective against pathogens in biotrophic life stages, insufficient examples exist to generalize the efficacy of iron-derived ROS against any particular pathogen.

A recent study in rice by Dangol et al. puts iron in a central role in execution of HR cell death during ETI (Dangol, Chen et al. 2019). The inspiration from this study came from studies in mammalian systems that discovered a mechanism of cell death called ferroptosis (Stockwell 2018). This process is triggered by iron accumulation, and concurrent loss of glutathione antioxidant protection. The subsequent accumulation of ROS is exacerbated by the Fenton reaction and leads to peroxidation of lipids, which in turn initiates cell death. In rice, PTI from fungal elicitors is insufficient to initiate ferroptosis, but ETI from recognition of avirulent *Magnaporthe oryzae* is sufficient, indicating that ferroptosis may important for HR. Upon initiation of ETI-induced HR, iron and hydrogen peroxide are recruited to infected cells causing a run-away Fenton reaction. As in animals, cell death in rice can be triggered by exogenous inhibition of the glutathione pathway to accelerate the ferroptotic cascade. This is sufficient to trigger HR in response to otherwise virulent isolates of *M. oryzae*. Conversely, chelation of iron or addition of antioxidants prevents the HR, even in the avirulent interaction. ETI, culminating in HR, remains one of the most potent weapons against plant pathogens. A major unresolved aspect of ETI is how immunity is activated following effector perception, so it will be of great interest to generalize whether ferroptosis is a trigger of cell death during ETI in other plants.

Current Efforts to Biofortify Crops for Iron

Anemia caused by iron deficiency afflicts nearly one billion people worldwide, with disproportionate impacts on women and children under five (Kassebaum 2016). This disease is estimated to underpin five percent of all global disability, ranking it ninth among the Global

Burden of Disease Project's most pressing issues (Kassebaum, Jasrasaria et al. 2014). Iron biofortification of food crops has the potential to help millions avoid anemia (Murgia, Arosio et al. 2012). By definition, biofortified crops contain more of a desired nutrient in edible plant tissues. Plant breeders and genetic engineers are altering the genes of agriculturally important varieties to produce food with higher iron content. Below we summarize current progress towards this goal, to set the stage for consideration of how linkages between iron homeostasis and immunity might affect biofortified crops' capacity to resist diseases.

Some important crops exhibit natural, intraspecific variation in iron content, allowing breeders to attempt iron biofortification through conventional breeding. The Consultative Group for International Agricultural Research (CGIAR) has been a global leader in biofortification through breeding (Brooks and Johnson-Beebout 2012). Leveraging available germplasm, they have achieved iron content targets in beans, millet, and others (Garcia-Oliveira, Chander et al. 2018, Rubyogo and Kasuga 2018). However, in important staples including rice, breeding efforts have been insufficient to raise iron content to desired levels. For example, the basal iron content in polished rice is typically 2 g/kg, and biofortification targets are typically 14 g/kg (Bhullar and Gruissem 2013). Additionally, the use of wild germplasm to achieve biofortification targets may impose yield penalties or effect agronomic traits to preclude industrial scale adoption.

To overcome difficulties presented by biofortification through breeding, some researchers have turned to genetic engineering. With a greater understanding of plant iron metabolism, alterations in a few key genes hold the potential to greatly increase iron content. Vasconcelos et

al. 2017 organizes these efforts into 4 categories: increased iron storage in edible tissues, increased iron uptake and translocation, alteration of the iron deficiency response, and combinatorial approaches (Vasconcelos, Gruissem et al. 2017).

Overexpression of iron storage proteins has been attempted to create iron sinks in edible tissues for human consumption. For example, edible iron content in rice grains was increased as much as three-fold by expression of soybean ferritin under an endosperm-specific promoter (Goto, Yoshihara et al. 1999). Expression of ferritin under constitutive promoters does not greatly alter the content in the edible tissues (Drakakaki, Christou et al. 2000). Additional approaches have been taken to increase iron availability throughout the plant through enhancement of iron uptake or iron mobilization from the roots. When the overall abundance and mobility of iron is increased, more can be transported to edible tissues. YSL2 is important for iron uptake from the soil in Strategy II plants. Nicotianamine synthase (NAS) produces nicotianamine which acts an iron chelator and enhances transport. Overexpression of NAS or genes the YSL family in rice, increases iron mobility and availability (Masuda, Usuda et al. 2009). Rice grains of these plants typically show double the iron content, but some transformants produce grains with 19 g/kg iron in greenhouse conditions (Ishimaru, Masuda et al. 2010).

When soybean ferritin was expressed alone in rice the iron accumulation was not commensurate with the increased storage capacity (Qu, Yoshihara et al. 2005). This would suggest that general increase of iron storage in the grain is not sufficient to deliver iron to the edible tissue. Combinatorial approaches that combine increased iron mobility and iron storage

have been attempted, to further increase iron content (Wirth, Poletti et al. 2009). The most successful examples of this have been accomplished in rice (Vasconcelos, Gruissem et al. 2017). Iron mobility is raised synergistically through upregulation of both importers like YSL2, and production of iron chaperones through NAS (Masuda, Ishimaru et al. 2012). These alterations increase free iron, so when ferritin is expressed in the endosperm, the rice grains store even more iron. Trijatmiko et al. use a combinational approach to achieve field grown rice with 15 g/kg of bioavailable iron, tantalizingly close to targets for biofortification (Trijatmiko, Dueñas et al. 2016).

Additional promising approaches could be possible through manipulation of the regulatory components that exert major effects on the plant's iron deficiency response. The iron deficiency response promotes additional iron uptake from the soil and mediates partitioning of iron in the plant. Overexpressed IRO2, an iron-related bHLH transcription factor, in rice improved growth on iron deficient soils, with an incremental increase of iron in rice grains (Ogo, Itai et al. 2011). The RNAi-based silencing of IRO2 diminished induction of immune related genes following iron deficiency (PR1) (Ogo, Nakanishi Itai et al. 2007). Biofortification with small genetic changes to regulatory process is alluring, but also carries the most risk of pleiotropic effects. Our growing understanding of the regulation of iron metabolism may allow for targeted alterations that maximize effects while minimizing off-target impacts (Urzica, Casero et al. 2012, Wu and Ling 2019). In exactly this context, in the next section we will discuss potential tradeoffs that manipulation of iron will have on disease resistance.

How will biofortification impact plant disease outcomes?

As described above, iron homeostasis and immunity are greatly interconnected, and alterations in plant iron levels for biofortification could impact how plants respond to pathogens. Moreover, different pathogens employ different iron acquisition strategies; including siderophore production or expression of transporters. On top of that, plants tailor their response to various pathogens, whether wielding iron as a weapon to produce ROS and to initiate ferroptosis, or sequestering iron. Due to the specific mechanisms by which plants detect and fight off various pathogens, we cannot make a single, all-encompassing prediction of whether biofortification will generally help or hurt plant resistance to disease. Rather, the downstream implications of these specific alterations will depend on the nature of the alteration(s), the plant, the pathogen and specific conditions in which the infection occurs. The following sections we discuss examples in which increases in plant iron could potentially aid or inhibit plant disease resistance.

In some plant pathogen interactions, iron biofortification could impede disease resistance (Figure 2). Iron deficiency can be a potent immune activator whether originating from microbial siderophores, or host iron sequestration. Increased iron availability and mobility could very well inhibit this process, which could manifest as increased susceptibility. Similarly, iron sequestration is important for defense against pathogens, in certain plants. Increased iron could nullify the plant's sequestration efforts as we saw in the interaction of Arabidopsis and Pectobacterium. If iron levels are increased in the plant through biofortification, resistance strategies like Arabidopsis expression of PDF1.1 may be impossible.

On a more optimistic note, evidence from some studies indicate that plant iron biofortification could improve disease resistance. For example, exogenous application of iron suppresses disease in turfgrass (McCall, Ervin et al. 2017). The mechanism of this disease suppression is unknown, but may be due to production of ROS as in maize (Liu, Greenshields et al. 2007). Many plants divert iron to the site of infection to generate ROS. In rice, sufficient iron is required for resistance to fungal pathogens. The knockdown of the plasma membrane iron importer OsNRAMP6 improves fungal resistance, demonstrating that the partitioning, not just the presence of iron is critical for its role in disease suppression (Peris-Peris, Serra-Cardona et al. 2017). This could be related with iron's importance in ferroptosis, as import of iron to infected cells is critical for this response (Dangol, Chen et al. 2019). In plants capable of ferroptosis, if cellular iron levels are increased, the threshold for programmed cell death may be decreased. In these cases, biofortification efforts in which plant iron content is increased, plant disease resistance would improve.

Conclusions and Future Directions

Iron deficiency related anemia in humans is a global problem that demands interventions including the release of biofortified crops to help those in need. Staple crops biofortified for iron though breeding or genetic engineering have the potential to improve the lives of millions. Already many biofortified crops have shown to be safe, and to provide the iron they need to improve the health of those suffering from anemia. However, as we have outlined in this article, biofortification has the potential to influence the interactions plants have with their pathogens. In a production setting, biofortified crops will contain more iron, and be less able to detect iron

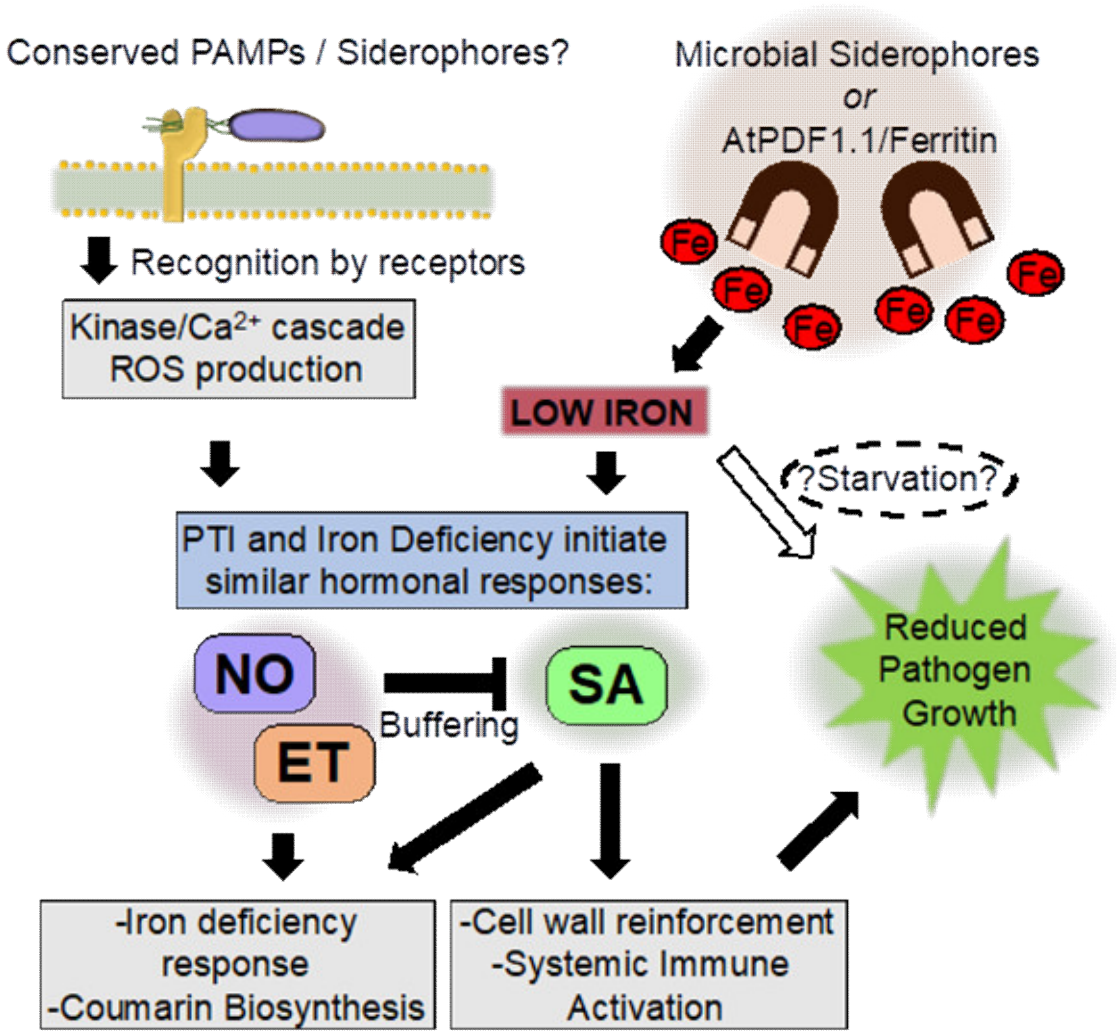
depletion associated with pathogen acquisition. If a plant uses iron sequestration to signal the immune system or control pathogen invasion, a biofortified variety will be less likely to succeed in that strategy, as observed in iron application studies of Pectobacterium-infected Arabidopsis. For some plants however, iron biofortification will help immunity; making ferroptosis easier and facilitating the production of more ROS to combat pathogens.

When it comes to plant iron metabolism and immunity, important questions remain unanswered. Through what mechanisms do plants detect iron and utilize that signal to activate immunity? Is iron sequestration effective against pathogens, and can it be engineered into new crops? What is the role of iron in the hypersensitive response, and do other plants use ferroptosis? How can the iron scavenging, and siderophores of beneficial microbes be leveraged for crop protection? As research on biofortification of iron, as well as other nutrients continues, emphasis should be placed on the role of iron in plant health.

Both plant and human health depend on iron and its role in plant immunity. Despite the lingering questions, our current understanding of connections between plant immunity and iron metabolism, point to the importance of these responses to each other. Coregulation of the responses underscores how they have evolved together. Depending on the pathosystem, iron sequestration, or deployment as a weapon is important for plant resistance. Either way, alterations made for iron biofortification could impact the plant immune response. Critically, if iron sensing is an alarm for pathogen invasion, biofortification efforts will inhibit plant mechanisms of pathogen detection. While, it is possible to predict the influence of additional iron in some specific plant-pathogen interactions, it is impossible to generalize the impact of

biofortification on plant disease resistance. Researchers, breeders, and growers must have this relationship in mind, as small alterations in either with have large impacts on both. It will be important for future work into iron biofortified crops, to test for enhanced susceptibility to pathogens.

Figures



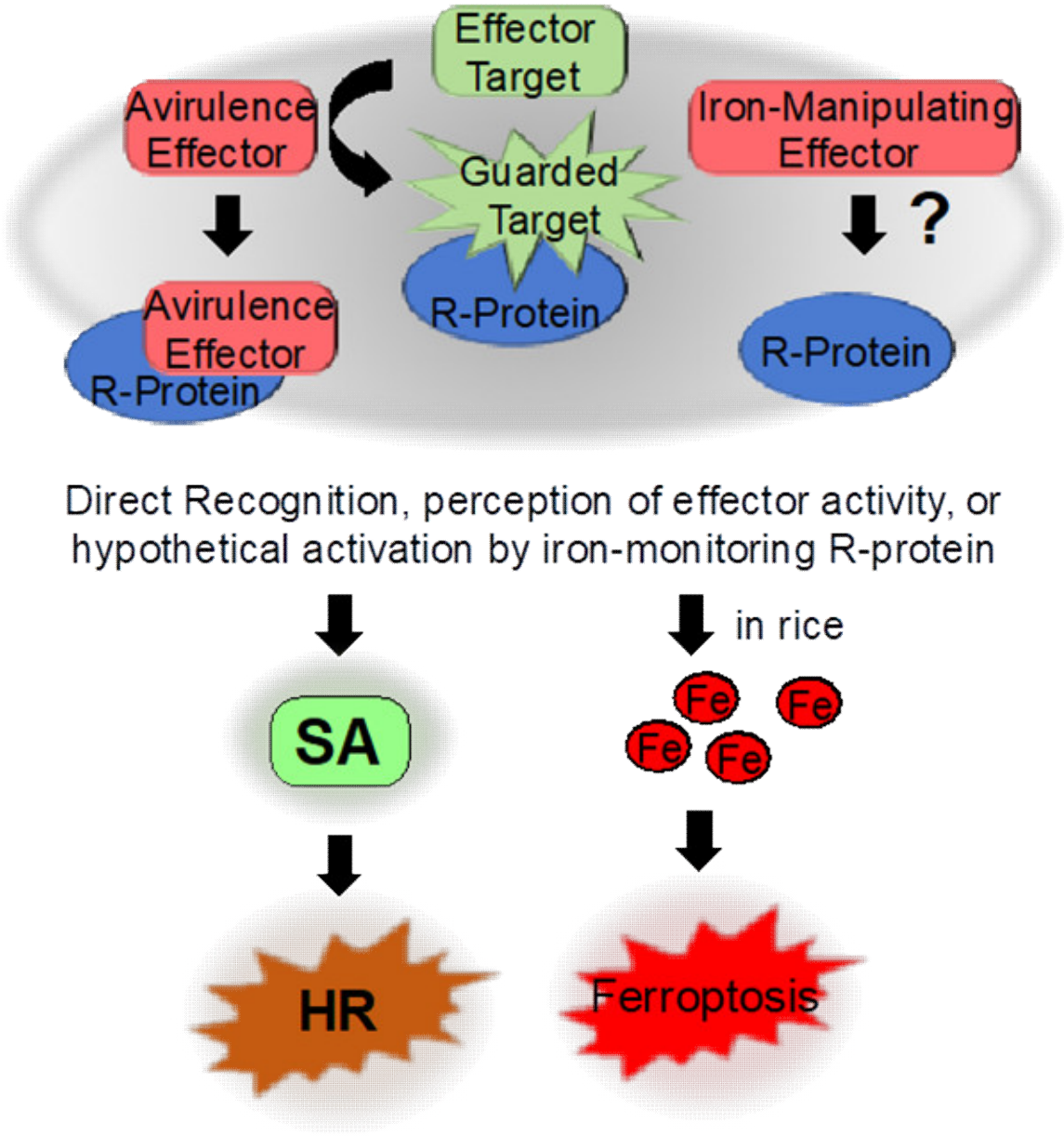


Figure 1: Iron's influence on PTI and ETI. (a) Pathogen associated molecular patterns (PAMPs) are recognized by plants to initiate pattern-triggered immunity (PTI). Siderophores may be recognized as well, but no receptors have been identified. Iron binding by microbial siderophores, or plant iron-sequestering proteins reduces iron availability in the phyllosphere. It is unknown whether iron starvation contributes to observed reductions in pathogen growth. PTI

and iron deficiency initiate similar hormonal responses, and both contribute to resistance to pathogens. Gaseous hormones nitrous oxide (NO) and ethylene (ET) buffer salicylic acid (SA), modulating the response. (b) Effector-triggered immunity (ETI) is initiated following recognition of avirulence effectors, or their actions, by plant resistance proteins (R-proteins). It is possible that pathogen effectors manipulate host iron metabolism, though this has not been described in plants. Upon activation, the R-protein signals for a strong immune response. SA, unbuffered by other hormones, leads to the hypersensitive response (HR): cell death to halt pathogen growth. In the rice interaction with *Magnaporthe oryzae*, the plant recruits iron to facilitate the HR. Iron-derived ROS lead to runaway lipid peroxidation, as in mammalian ferroptosis.

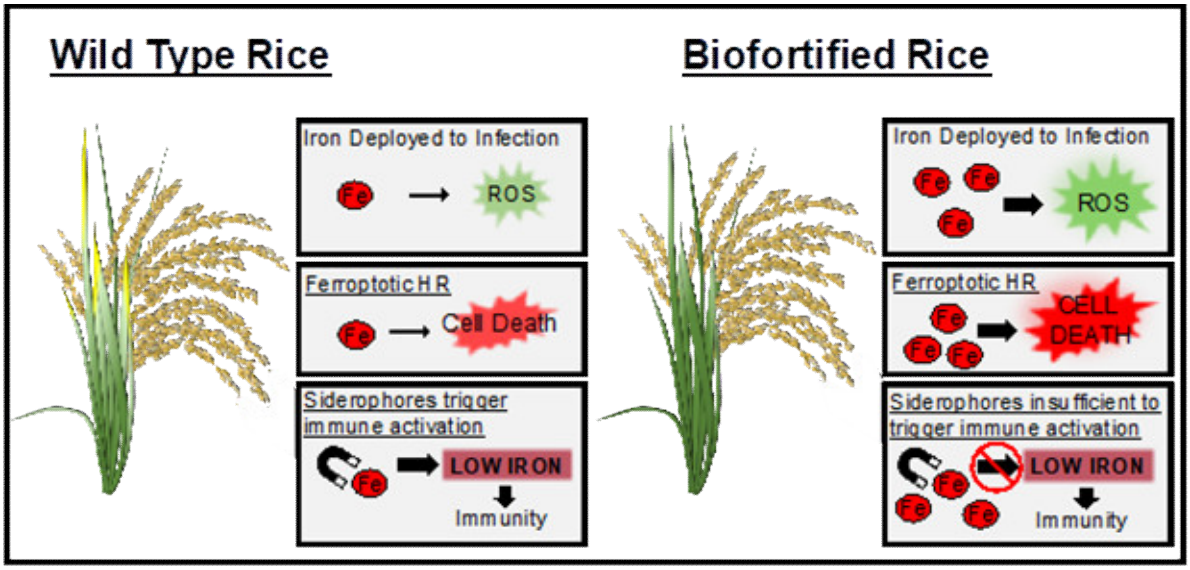


Figure 2: Biofortification of iron will influence plant immunity. In the Poaceae, iron is delivered to infection sites to facilitate the ROS burst. In rice, iron accumulates following ETI,

leading to ferroptosis. In all plants, low iron availability, from microbial siderophores, or iron-sequestering proteins, triggers immunity and the iron-deficiency response. In biofortified plants, especially those with upregualtion of genes related to iron uptake and mobility, these responses may be altered. Additional iron at infections sites, could produce more ROS and a stronger ferroptotic HR. This might slow pathogen growth. However, additional iron may limit the plant's capacity to use low iron status to trigger immunity, and promote pathogen growth.

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Chapter 2

Arabidopsis thaliana Iron Homeostasis Affects Oomycete Parasitism

John Herlihy[1], John McDowell[1]

[1]Virginia Tech, School of Plant and Environmental Sciences, Latham Hall (0329), Blacksburg, VA, 24061-0329

Abstract

Recent studies have put iron at the center of plant-pathogen relationships. For example, iron-dependent ferroptosis in rice has been reported as a key mechanism underpinning the hypersensitive response (HR) to the rice blast fungus. It is unknown how iron deficiency impacts plant-oomycete interactions. We developed three methods to restrict iron to Arabidopsis and study the impact on the infection of the model oomycete Hyaloperonospora arabidopsidis (Hpa). We adapted a hydroponic system to limit iron uptake, and in iron-starved plants observed a reduction in avirulent Hpa growth. We saw no disruption of avirulence effector-mediated HR. Similarly, we directly applied iron chelator to plants and observed similar reduction in pathogen growth, accompanied by additional cell death. These results suggest that the Arabidopsis immune system is robust to iron deficiency and likely does not deploy ferroptosis against Hpa. We infected T-DNA insertion mutants, deficient in key iron metabolism genes and again saw reduction in pathogen growth. Quantification of defense marker gene expression indicated that iron limitation induces SA-responsive immunity in plants and is responsible for some of the observed pathogen growth reduction. Some mutants tested demonstrated characteristics of

susceptibility genes, providing putative routes by which oomycetes acquire iron from their hosts. While iron does not seem to play role in Hpa-induced HR in Arabidopsis, iron deficiency provokes an immune response and in turn reduces growth of an oomycete pathogen.

Introduction

Iron plays an oversized role in plant-pathogen interactions. Despite iron's low bioavailability, it is required by all organisms for its critical function in electron transport, and as an essential cofactor in enzymes (Camprubi, Jordan et al. 2017). Plants also require iron for biosynthesis of chlorophyll (Larbi, Abadía et al. 2006, Andresen, Peiter et al. 2018), while plant pathogens must acquire all of their iron from their host during growth inside the host (Howard 1999, Johnson 2008). However, too much iron damages organisms, as the same electron chemistry that makes the metal indispensable to life, also generates dangerous free radicals via the Fenton reaction (Winterbourn 1995). The many roles of iron put it at the center of plant-pathogen interactions, and a better understanding of iron's role will help provide better resistance strategies for control of plant disease.

Model oomycetes allow for investigation of iron in plant-pathogen interactions

The oomycetes are a family of fungus-like pathogens that inflict high losses on potato, tomato, grapes, brassicas, and others (Kamoun, Furzer et al. 2015). Despite their global impact, oomycete pathogens are poorly understood compared to bacterial and fungal pathogens. Researchers often use *Hyaloperonospora arabidopsidis* (*Hpa*) as a model oomycete to gain a

better understanding of these important organisms and develop strategies for resistance in the field (McDowell 2014). Due to its obligate biotroph lifestyle, *Hpa* cannot grow or complete its life cycle apart from its host *Arabidopsis thaliana* (At) (Baxter, Tripathy et al. 2010). Plant pathogens, including *Hpa*, need to overcome accomplish two tasks to establish a successful infection: suppress host immune functions (Stassen and Van den Ackerveken 2011), and acquire nutrients from the host plant (Fatima and Senthil-Kumar 2015, Cox, Meng et al. 2017, Sonawala, Dinkeloo et al. 2018). Pathogens, including *Hpa*, deliver virulence proteins called effector proteins to plant cells to disrupt critical signaling components of host immunity (Nobori, Mine et al. 2018). It is not known whether any oomycete effectors alter host metabolism for better access to sugar, iron, or other resources.

To initiate infection, airborne-*Hpa* spores must contact Arabidopsis leaves (McDowell 2014). Then the pathogen produces an appresorial penetration structure to gain access to the leaf tissues. Pathogen hyphae grow throughout the plant, secreting cell wall-degrading enzymes and other virulence proteins to facilitate infection and neutralize host defenses (Baxter, Tripathy et al. 2010). Projections from the hyphae, called haustoria, invaginate the host cell membranes and play a role in effector delivery (Mims, Richardson et al. 2004, Van den Ackerveken 2017). In fungal pathogens, homologous haustorial structures are important for nutrient uptake and delivery of additional virulence proteins (Presti and Kahmann 2017, De Jong and van den Ackerveken 2018). However, in *Hpa* and other oomycetes, the role of the haustoria for acquisition of iron and other nutrients is unknown.

Plant iron metabolism

Iron uptake for all organisms is tightly controlled, as iron is critically important, but dangerous in excess. Plants rely on two strategies for iron uptake: I. reductive iron assimilation, and II. phytosiderophore-mediated assimilation (Marschner, Römheld et al. 1986, Kobayashi, Nozoye et al. 2019). Strategy I plants include all the non-Poaceae angiosperms, and primarily utilize reductive iron assimilation. Here, we summarize Strategy I with emphasis on Arabidopsis genes/proteins that are important for this manuscript (Figure 1 a): Upon perception of low iron, the basic helix-loop-helix (bHLH) transcription factor FIT heterodimerizes with other bHLHs and activates transcription of genes responsible for iron uptake and translocation in the root (Bauer, Ling et al. 2007). Following FIT activation, the rhizosphere is acidified to solubilize iron, and *FRO2* is expressed for reduction of ferric iron to its more soluble and bioactive ferrous form (Connolly, Campbell et al. 2003). IRT1 is the primary transporter in Arabidopsis for uptake from the soil (Vert, Grotz et al. 2002). Plants lacking this critical transporter exhibit symptoms of iron deficiency and accumulate less iron than wild type plants. Despite the importance of reductive assimilation, Strategy I plants also employ phytosiderophores for iron solubilization and uptake. The most important siderophore in Arabidopsis is courmarin and its molecular isoforms. These phenolic compounds are secreted, bind iron, and are reassimilated as an iron complex (Sisó-Terraza, Luis-Villarroya et al. 2016). Coumarins are also antimicrobial, playing a role in sculpting the rhizosphere microbiome (Sardari, Nishibe et al. 2000, Stringlis, Yu et al. 2018). Iron is stored in the root apoplast (Bienfait, van den Briel et al. 1985). Other iron-binding molecules, such as citrate and the methionine derivative nicotianamine (NA), are important for iron solubilization and transport inside the plant (Higuchi, Suzuki et al. 1999, Schuler, Rellán-Álvarez et al. 2012). The iron transporter FRD3 loads citrate-bound iron into the xylem for

mobilization to the shoot (Rogers and Guerinot 2002). *frd3* mutants display symptoms of iron deficiency and accumulate iron in the roots but not the shoots, due to lack of transport capacity (Durrett, Gassmann et al. 2007). The family of yellow-stripe-like (YSL) transporters, are important in all plants moving NA-bound iron (Curie, Cassin et al. 2008). In the mesophyll, excess iron is stored in the vacuole and plastid. NRAMP3 and -4, as well as the VTL-family transporters redundantly perform a role in iron storage, loading iron into the vacuole (Gollhofer, Timofeev et al. 2014, Bastow, Garcia de la Torre et al. 2018). In the plastid, iron is stored in ferritin (FER). Iron is used in protein cofactors like Fe-S complexes and heme (Bernard, Netz et al. 2013).

The plant immune system

Once a plant's physical barriers have been breached, the plant relies on detection of pathogens to coordinate an immune response (Couto and Zipfel 2016). The first immune mechanism is PAMP-triggered immunity (PTI), which is activated by recognition of conserved, pathogen-associated molecular patterns. These so-called PAMPs are motifs on important pathogen proteins or structures that are not easily modified or dispensed of (Boller and He 2009, Katagiri and Tsuda 2010). Plant genomes encode dozens or hundreds of cell-surface receptors to detect PAMPs (Sun, Li et al. 2013). Upon recognition of these motifs, and subsequent signaling by kinases, the plant initiates PTI, typified by reinforcement of the cell wall, production of defense-associated hormones, and induction of defense responses throughout the plant (Luna, Pastor et al. 2011, Macho and Zipfel 2014).

PTI also generates reactive oxygen species (ROS). A major source of ROS for PTI in Arabidopsis is the respiratory burst oxidase homolog D (RBOHD), which utilizes iron-bound heme for production of superoxide following pathogen perception (Torres, Dangl et al. 2002). Subsequently, superoxide dismutase (SOD) converts superoxide to hydrogen peroxide that can be transported into plant cells. Pathogen-induced ROS can damage growing pathogens which, compared to larger plants, lack the reductive capacity to detoxify dangerous free radicals (McDowell and Dangl 2000). ROS can also facilitate cell wall reinforcement via oxidative cross-linking (Kärkönen and Kuchitsu 2015). Finally, ROS act as cellular signals that contribute to local transcription of defense-associated genes, including those with antimicrobial activity such as defensins and pathogenesis-related (PR) proteins (Torres, Jones et al. 2006).

Perception of pathogen virulence proteins initiates effector-triggered immunity (ETI). Plants have evolved resistance proteins (R-proteins), enabling gene-for-gene resistance against specific pathogen effectors (Kourelis and van der Hoorn 2018). Both the pathogen effector and host R-protein are required for this immune response. R-proteins monitor important signaling pathways for pathogen manipulation by effectors. Upon detection, R-proteins activate immune responses that overlap with PTI responses, but are typically stronger and faster. Moreover, ETI often culminates in genetically-programmed host cell death, often termed as the hypersensitive response (HR).

Growing understanding of iron's role in immunity

Resistance to pathogens requires a multilayered plant immune system tightly associated with iron (Verbon, Trapet et al. 2017). In addition to its role as a cofactor for ROS-producing enzymes, iron catalyzes the Fenton Reaction in the presence of hydrogen peroxide, leading to production of hydroxyl radicals. These ROS are among the most dangerous, reacting with all biological molecules, and causing lethal lipid peroxidation (Skorzynska-Polit 2007). Plants mitigate the potential for the Fenton Reaction by expressing ferritins to sequester iron (Ravet, Touraine et al. 2009). In the Poaceae, iron is released into the apoplast after pathogen perception, and is important for successful immunity. In maize and wheat iron accumulates at infection sites (Liu, Greenshields et al. 2007). Ye et al. in 2014 showed that maize resistance to *Colletotrichum graminicola* required adequate iron (Ye, Albarouki et al. 2014). The normally avirulent pathogen could overcome the host when iron was chelated, and the plant was unable to deploy the metal to the infection site. The end result of this iron accumulation is generation of ROS via the Fenton reaction. A similar study shows that the subsequent depletion of cytosolic iron levels induces additional immune responses (Albarouki, Schafferer et al. 2014). Comparatively little is known about the role of iron in pathogen resistance in dicots, and nothing is known about how iron affects resistance to oomycete pathogens.

Plants may use iron itself to monitor for pathogens (Aznar, Chen et al. 2014). Iron acquisition by pathogens and other microbes often provokes systemic immune activation in plants. When pathogen siderophores are applied to plants and sequester iron, distal tissues are more resistant to pathogens (Aznar and Dellagi 2015). This effect would suggest siderophores are acting as PAMPs, and are recognized by plant receptors (Aznar, Chen et al. 2015). However, no receptors have been identified, and when certain siderophores are applied already bound to iron, they do

not stimulate an immune response (Kieu, Aznar et al. 2012). Additionally, some synthetic siderophores also trigger an immune response, and plants are unlikely to have evolved a receptor for a synthetic molecule (Aznar, Chen et al. 2014). These observations indicate that iron availability, not siderophore recognition, is the signal for iron-related immune activation. However, the mechanisms by which perception of iron depletion triggers an immune response are unknown.

Unlike maize and wheat, some plants sequester iron away from the infection site following pathogen perception. Potato expresses *FER* following challenge by the oomycete *Phytophthora infestans* (Mata, Lamattina et al. 2001). The same response is seen in Arabidopsis after infection by the bacteria *Dickeya dadantii* (Dellagi, Rigault et al. 2005). The authors show loss of the *FER* results in enhanced susceptibility to the bacteria, presumably from loss of the iron sequestration mechanism. Hsiao et al. 2017 show the plant defensin PDF1.1 binds iron and is involved in its sequestration following pathogen attack (Hsiao, Cheng et al. 2017). They show that the sequestration provides the same iron depletion as pathogen siderophores, further triggering plant immunity. These findings further support the hypothesis that iron depletion initiates plant immunity. Such sequestration might also deny iron to growing pathogens, but this remains to be substantiated.

In rice, a recent report shows that iron plays in the HR to avirulent pathogens via ferroptosis (Dangol, Chen et al. 2019). Following perception of avirulent fungal pathogen *Magnaporthe oryzae*, rice also accumulated iron inside infected cells. The oxidative damage from the Fenton reaction lead to ferroptotic HR to contain pathogen spread. Fungal PAMPs did not trigger the

response, and antioxidants and iron chelators were sufficient to prevent ferroptosis. This mechanism has not been reported in other species and it remains to be determined whether ferroptosis is a central mechanism underpinning ETI.

This new understanding of HR rice could be useful in developing stronger resistance to plant pathogens, but the mechanism has yet to be generalized to other plants. In this study, we sought to understand how Arabidopsis might utilize iron in defense against *Hpa*. We tested how iron restriction impacted Arabidopsis resistance to avirulent *Hpa*. Unlike ferroptosis-related defense in rice, we observed that iron restriction increased resistance, rather that lowered it. Additionally, we did not observe any alteration in the Arabidopsis defense response to *Hpa* following iron restriction. We propose that ETI against *Hpa* is robust to iron depletion, and that ferroptosis does not play a critical role in the immune response of Arabidopsis to *Hpa*.

Materials and Methods

Plant and Pathogen Growth Conditions

Arabidopsis was grown on Sungro Professional Growing Mixture, in 8h of light at 22˚C and 16h of dark at 20˚C. *Hpa* isolates Emwa1 and Emco5 were propagated on WS-0 Arabidopsis, and all studies, and T-DNA insertion mutant screens were done in a Columbia-0 background. *Hpa* propagation was carried out weekly, by collecting sporulating leaf tissue, dislodging spores in sterile water, diluting to 50,000 spores mL^{-1} and applying to plants by Preval sprayer

(McDowell, Hoff et al. 2011). Iron restriction was introduced with 3 mM Deferoxamine mesylate salt (Sigma Aldrich), applied with a Preval sprayer at 0.375 mL $inch^{-1}$.

Hydroponic Growth Conditions

Columbia-0 Arabidopsis was grown in a hydroponic system adapted from Pratelli et al. 2016 (Pratelli, Boyd et al. 2016). J2 media was prepared with 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1 mM NH_4NO_3, 1 mM $CaSO_4$, 50 µM NaFeEDTA, 30 µM H_3BO_3, 5 µM $MnCl_2$, 1 µM $CuCl_2$, 1 µM $ZnCl_2$, 100 nM $Mo_7O_{24}(NH_4)_6$ (adapted from (Lejay, Tillard et al. 1999)). J2 media with 0.6% w/v agar was cast into pipette tip racks (Olympus brand, Gennesse Scientific, USA) sealed with 3 inch HD Clear packaging tape (Duck Brand, USA). After casting, the tape was removed, excess agar was removed with a razor, and the rack was placed in a square petri dish (120 mm x 120 mm Greiner Bio, USA).

Seeds were sterilized in 70% ethanol in water, with 3% w/v sodium dichloroisocyaburiate, for 20 minutes, washed three times with 100% ethanol, then allowed to dry in a sterile laminar flow hood. Seeds were suspended in sterile water and single seeds were pipetted onto each well of the hydroponic tray. Fifteen mL of J2 medium was added to each Petri plate. The plants were grown in the Petri plate for 2 weeks in an incubator (100 µE, 10h light, 22ºC). At this stage plant the racks were transferred to tip boxes containing 350 mL J2 medium. For iron deficiency treatments, plants were instead transferred to boxes containing J2-Fe media, in which NaFeEDTA was removed and replaced with 300 µM of the iron chelator FerroZine (Acros Organics, USA).

Cytology

For Trypan Blue staining, tissue samples were collected and washed in water, then stained in trypan blue solution made from equal parts water, phenol, lactic acid, and glycerol, and 0.05% w/v Trypan Blue, for 5 minutes at 90° C, then 30 minutes at room temperature. Samples were destained in 2.5:1 chloral hydrate: water solution for three days, changed every 24 hours.

For Diaminobenzidine staining to visualize ROS, plant shoots were detached from roots and hypocotyls were placed in DAB solution, consisting of 0.1% w/v 3,3'-Diaminobenzidine (Sigma-Aldrich) and 0.08% v/v concentrated hydrochloric acid, for 6 hours in the dark. Samples were destained with a solution of 3:1:1 ethanol: lactic acid: glycerol for 24 hours (Thordal-Christensen, Zhang et al. 1997).

For Perls' iron staining plants were stained in Perls' solution, 4% w/v KFeCN and 4% v/v concentrated HCl, for 15 minutes under vacuum, then 30 minute at room temperature and pressure (Meguro, Asano et al. 2003). Samples were washed three times in water, then incubated in methanol with 0.3% v/v H_2O_2 and 10 mM NaN_3 for 30 minutes at room temperature. Samples were washed in 0.1 M phosphate buffer pH 7.4, then intensified with 3,3'-Diaminobenzidine, in the same phosphate buffer with 0.025% w/v DAB, 0.005% w/v $CoCl_2$ and 0.005% v/v H_2O_2 for 10 minutes, before washing with water.

Pathogen Quantification

Avirulent *Hpa* (Emwa1) growth was quantified by counting sporangiophore on infected cotyledons at 7 dpi. Virulent *Hpa* (Emco5) growth was quantified by Taqman PCR (Primers and probes found in Table 1)(Su'udi, Kim et al. 2013, Babu and Sharma 2015, Haudenshield, Song et al. 2017). A multiplex qPCR reaction of single copy pathogen (*Hpa* actin) and plant (*Actin2*) genes provided a relative quantification of pathogen growth. Plant shoots were collected six days post infection. Genomic DNA was extracted using a Qiagen Biosprint 15 DNA Plant kit, and quantified by nanodrop. Ten nanograms of DNA was tested with Applied Biosystem Multiplex SYBR Green Master Mix on an Applied Biosystems qPCR 7500 thermocycler. Delta C_T transformation provided relative growth of *Hpa* as compared to control or other time points.

Iron Quantification by ICP-MS

Plant tissues were ground and dissolved in CEM MARS Xpress microwave digester in concentrated nitric acid (Trace Metal Grade, Fisher Scientific) at 200° C for 30 minutes. Analysis was performed on a Thermo iCAP RQ Inductively coupled plasma mass spectrometer in a 2% nitric acid in water matrix.

Expression Quantification by Quantitative PCR

RNA was extracted using Qiagen RNeasy Plant spin column kit, and quantified by nanodrop. cDNA was synthesized with Invitrogen SuperScript IV and diluted fifty-fold for use in qPCR. PCR was performed with Applied Biosystems Taqman Multiplex Master Mix on an Applied

Biosystems qPCR 7500 thermocycler, according to the manufacturer's instructions and default settings (Primers in Table 1). Delta-delta C_T transformation, compared to *Actin2*, was used to normalize transcript abundance (Han, Yang et al. 2013).

Results

Optimization of a hydroponic system to induce iron deficiency in Arabidopsis

To investigate the role of iron in the immune response of Arabidopsis we adapted a hydroponic growth system to restrict iron availability to the plant. This system allowed for control over the plant growth media and access to the roots for tissue collection and analysis. We cast complete media agar into 96-well trays and sewed sterile *Arabidopsis thaliana* ecotype Columbia-0 seeds on the wells, before placing the trays in plates containing additional media. After two weeks, we transferred the trays to boxes containing complete media or iron-deficient media containing 300 μM of the iron chelator ferrozine. The chelator prevented the Arabidopsis from accessing any remaining iron in the media and has been used previously to accelerate the onset of the plant iron deficiency response (Long, Tsukagoshi et al. 2010). A schematic of the hydroponic system is shown in Figure 1 b.

To validate that the plants grown in the hydroponic system initialize an iron deficiency response, we tested iron-deprived plants, compared to controls, with several assays for the iron deficiency response. The first assay was to visually monitor for yellowing of the leaves (chlorosis) that follows iron deficiency, due to reduction of chlorophyll production. At three days

after treatment, iron chlorosis in the foliar tissues was not yet evident (Figure 2 a and b) but became severe by seven days after (Figure 2 c and d).

The second assay tested for transcriptional activation of two widely used marker genes for iron deficiency responses (Figure 2 e). In the roots, transcript abundance from the iron transporter gene *IRT1* increased linearly over the first three days and plateaued by day three, reaching 23-fold increase in expression, and stabilizing at a minimum of 20-fold increase over untreated plants for the subsequent days. Also in the roots, transcription of *FRO2*, the rhizosphere iron reductase, demonstrated a similar pattern of induction following chelator treatment. The timing of activation of these iron deficiency response marker genes is similar to previous reports (Mukherjee, Campbell et al. 2006, Long, Tsukagoshi et al. 2010).

In the third assay, we used inductively-coupled plasma mass spectrometry (ICP-MS) to directly test whether total iron is reduced in chelator-treated plants (Figure 2 f). Tissue from hydroponically grown plants were separated into roots and shoots. The shoot tissues of iron-replete plants contained 155 ng mg^{-1} dry weight of iron, while shoots of plants grown on iron-deficient media contained 97 ng mg^{-1} dry weight iron. Root samples from the multiple replicates were pooled to obtain sufficient material for testing. The roots of plants grown in the hydroponics contained 2553 ng mg^{-1} dry weight iron, while roots of plants grown in iron-deficient media contained only 39% of that, at 987 ng mg^{-1} dry weight. Values for shoot and root iron are similar to reports using similar growth and iron depletion conditions (Vert, Grotz et al. 2002, Vert, Barberon et al. 2009). Altogether, these experiments validated that our hydroponic system induced an iron-deficiency response in roots and shoots, with comparable timing and

magnitude to previously reported systems. Moreover, as shown below, Arabidopsis plants grown in the hydroponic system can support colonization by virulent *Hpa* and can mount a resistance response against avirulent *Hpa*, indistinguishable from soil-grown plants. Therefore, the hydroponic system can be coupled with *Hpa* infections to test the interplay between iron status and immunity.

Iron deprivation does not compromise resistance to avirulent *Hyaloperonospora arabidopsidis*

After validating that the hydroponic system could induce an iron deficiency response in Arabidopsis, we tested whether ETI against *Hpa* is compromised in iron-deficient plants, using the *Hpa* isolate Emwa1. This isolate is recognized by the *RPP4* resistance gene in Arabidopsis Columbia-0, which encodes an NLR protein with an N-terminal TIR domain (van der Biezen, Freddie et al. 2002). This resistance is relatively weak, compared to other Arabidopsis *RPP* genes: The hypersensitive response is not initiated immediately upon pathogen penetration; rather, it is induced two to three days after inoculation. This delay enables limited hyphal growth and a low level of asexual sporulation (Figure 3 a). The hypersensitive response is manifested as "trailing necrosis" in which hyphae are surrounded by dead cells (Figure 3 b and c). These attributes enable detection of both enhanced susceptibility and enhanced resistance phenotypes (e.g., Figure 3).

We infected hydroponically grown plants with spores of Emwa1 at three days after chelator treatment and quantified growth by counting sporangiophores emerging from cotyledons at seven days post-infection (Figure 3 a). We observed that iron deficiency did not compromise

resistance in Columbia-0 to Emwa1; indeed, iron-deficient plants produced significantly fewer sporangiophores than control plants (p-value 0.005), with an average of 1.4 sporangiophore per cotyledon rather than 3.2 on plants grown in iron-replete media, showing a 45 percent reduction. Cytological assays showed that lack of iron did not greatly alter the infection. In trypan blue-stained plants, the pathogen did not exhibit altered morphology, and a normal plant response of trailing necrosis was apparent (Figure 3 b and c), indicating that iron deficiency does not inhibit the progression of the HR. Diaminobenzidine staining for ROS production showed that the ROS burst following pathogen invasion was not suppressed in iron-starved plants (Figure 3 d and e). Perls' staining identified iron accumulation in iron-replete samples. These sites of iron accumulation were not visible in iron-starved plants (Figure 3 f and g). Iron concentration was visible by Perls' staining in chloroplasts and guard cells of both treatment types, corresponding sites of iron storage in the leaves. Altogether, these assays indicate that the iron starvation response we initiated by chelator treatment did not alter the ability for plants to recognize Emwa1 or inhibit *RPP4*-mediated resistance to the pathogen. The lack of iron accumulation in infected iron-starved leaves did not impact resistance to the avirulent pathogen.

While hydroponic growth in chelated media induced iron deficiency in Arabidopsis, some iron remained available to the plant, as seen in the ICP-MS analysis and with Perls' staining. The chelator deferoxamine has been previously shown to suppress the HR in rice resulting from recognition of avirulent *Magnaporthe oryzae*. This was interpreted as evidence for ferroptotic cell death in the rice-*Magnaporthe* interaction. We utilized a similar approach to more acutely restrict iron availability to plant and pathogen. Soil-grown plants were infected with *Hpa* Emwa1 and then 24 hours later sprayed with the iron chelator deferoxamine or mock-treated with water.

Interestingly, foliar application of DFO reduced pathogen growth similar to chelation through hydroponic media (Figure 4 a). *Hpa* in DFO-treated plants showed only 31 percent of the sporulation (p-value 0.037).

Just as with iron-starved plants grown in hydroponics, cytological examination of DFO-treated plants revealed no qualitative difference in infection progression, or ROS production, compared to mock-treated plants. However, trypan blue staining of DFO-treated plants, infected with *Hpa* isolate Emwa1 at 6 dpi, showed enhanced trailing necrosis (Figure 4 b and c). We observed no indications that iron restriction by chelation interrupted the hypersensitive response. In fact, lesions were more prominent in DFO-treated plants compared to those seen in controls. Morphology of *Hpa* hyphae and haustoria were not altered in DFO-treated samples. DAB staining for detection of ROS showed more ROS production at the infection site of DFO-treated plants, just as trypan blue staining demonstrated more plant cell death (Figure 4 d and e). Unlike in the hydroponic study, Perls' staining did not show altered iron distribution in infected plants at three days post infection. In both treatments, the stain with DAB intensification revealed accumulation of iron in localized regions, which appear to be infection sites (Figure 4f and g). We observed canonical signs of *RPP4*-mediated resistance – trailing necrosis, ROS production, and low sporulation - in iron-starved plants in both treatments. We did see potential evidence of iron accumulation following infection indicative of ferroptosis by Perls' stain, but the presence of HR in trypan stains suggests that this accumulation is not important for *RPP4*-mediated immunity.

Mutations in Arabidopsis iron metabolism alter *Hpa* growth

To complement the experiments with iron chelators, we undertook a genetic approach in which we measured growth of avirulent and virulent *Hpa* isolates on T-DNA insertion mutants for genes related to iron metabolism and transport. We selected 23 genes with established roles in plant iron uptake, transport, metabolism, and/or homeostasis, as summarized in the introduction and in Table 2. Several of these genes had been previously shown to affect plant responses to bacterial pathogens, but effects on oomycetes have not been tested to our knowledge. Other genes such as those related to Fe-S complex, or heme biosynthesis were screened as susceptibility genes, for their potential role in creation of pathogen-exploitable pools of iron. All mutations arose from T-DNA insertion into Columbia-0 background, which was included as a baseline control (Krysan, Young et al. 1999). The ecotype WS lacks *RPP4*-mediated resistance to *Hpa* isolate Emwa1 and serves as control for efficient infection and a standard of comparison for disease susceptibility. The *eds1* knockout mutant is deficient in immune responses, including basal resistance to virulent *Hpa*, and acts as a second control for efficient infection and a standard of comparison for enhanced disease susceptibility.

Growth of avirulent Emwa1 was quantified by counting the number of sporangiophores produced on cotyledons at 7 dpi (Figure 5 b). Susceptible ecotype WS and the susceptible *eds1* mutant demonstrated predicted increases of pathogen growth. Eleven-day old plants of seven mutants showed reduced colonization of avirulent pathogen (*opt3*, *bHLH100*, *bHLH101*, *vtl2*, *frd3-1* and *-3*, and *nramp3*). Other mutants show non-significant resistance to the avirulent pathogen. The only mutant to show increased growth of avirulent pathogen was *irt1*, although this enhanced susceptibility was not significant.

The growth of virulent isolate Emco5 was measures by quantification of pathogen DNA by quantitative PCR at 6 dpi (Figure 5 a). Again, ecotype WS and the *eds1* mutant demonstrated expected increases in susceptibility. This assay only identified four mutants with pathogen growth reduction phenotypes (*fro3*, *frd3-1* and *-3*, and *cog0354*). A third allele of *frd3* showed non-significant pathogen growth reduction, but mutants of this gene were the only to demonstrate decreased growth of both virulent and avirulent pathogen. A second allele of *cog0354* showed a non-significant increase in pathogen growth. Three mutants supported significant increases in growth of virulent pathogen (*fc1*, *hcf101*, and *irt1*). Only *irt1* supported increased growth of both virulent and avirulent *Hpa*.

We followed up the initial experiments on soil-grown plants with tests of select mutants in the hydroponic system, in order to observe the compounding effect of iron starvation and genetic manipulation of host iron metabolism (Figure 6 a). The *frd3* and *fro3* mutants were selected for their putative role as S-genes for pathogen iron acquisition. The *irt1* mutant was selected as the only mutant to demonstrate susceptibility in both screens. The *irt1* and *fit* mutants did not present pathogen growth different than wild type Columbia-0 in the hydroponic system. However, the *frd3* mutant showed an additive effect, in which growth on iron-deficient media resulted in additional reduction of pathogen growth beyond the reduction seen in the mutant alone. The *fro3* mutant was the only genotype that did not exhibit significantly altered pathogen growth on the iron-deficient plants.

It was unclear whether pathogen growth reduction associated with iron deficiency was due to pathogen iron starvation or activation of plant immunity. We tested expression levels of *PR-1* in iron-depleted plants to observe whether the iron deficiency response also stimulated plant immunity. Arabidopsis plants were grown in the hydroponic system, transferred to boxes, and treated with replete media or iron-deficient media for five days. These plants responding to iron-deficiency, all demonstrated significant upregulation of *PR-1*, except *frd3* mutants, which even under iron sufficient conditions is inhibited in root-to-shoot iron transport and demonstrates symptoms of iron deficiency (Figure 6 b). *PR-1* expression levels were significantly elevated in *frd3* mutant plants as well as all plants treated with the chelated media. However, *PR-1* induction did not reach levels observed during avirulent *Hpa* infection, which was used as a positive control of immune activation.

We also tested the impact of DFO treatment on Emwa-infected *frd3* and *irt1* plants. We applied 3 mM DFO to leaves 24 hours after infection and like in Columbia-0, Emwa1 grew less well in both *irt1* and *frd3* mutants. We observed the largest effect in *irt1* plants, where DFO treatment obscured the increase of pathogen growth seen in the mock-treated plants (Figure 7 a). Trypan blue staining of DFO-treated mutant plants was similar to what we observed in Columbia-0 (Figure 7 b, c, d, and e). In both *frd3* and *irt1* mutants DFO treatment increased the abundance of necrotic lesions, suggesting a stronger immune reaction.

We also tested DFO-treated plants for immune induction by comparing *PR-1* expression levels (Figure 7 n). Arabidopsis plants where treated as before. We infected with avirulent pathogen or mock-infected with water. Then half of each treatment group was treated with 3 mM

DFO or mock-treated 24 hours post-infection. Soil grown *frd3* plants exhibited some nonsignificant induction of *PR-1*, but not to the level of hydroponically-grown plants. DFO treatment significantly increased *PR-1* expression even in immune compromised *eds1* plants. As expected, avirulent *Hpa* infection induced *PR-1* expression in Columbia-0 and *frd3* plants. The *PR-1* induction from iron deficiency and pathogen infection was not additive, as plants that received both treatments demonstrated similar *PR-1* induction as the plants treated with *Hpa* alone.

Discussion

Iron plays an important role in defense against bacterial and fungal pathogens in rice, maize, and other grasses. Iron accumulating at infection sites generates ROS to damage pathogens and to promote the HR via ferroptosis. However, the role of iron in the immune responses of dicot plants is less well-understood, and we know very little about how iron contributes to immunity against oomycetes. The potential role of ferroptosis in immunity against *Hpa* is particularly intriguing. Considering the importance of iron accumulation for ferroptototic cell death in the rice-*M. oryzae* interaction, we reasoned that restriction of iron in the Arabidopsis-*Hpa* interaction could weaken or completely suppress ETI. Therefore, we restricted iron to Arabidopsis plants by three mechanisms. We imposed iron restriction by limiting iron in the growth media, chelating iron through siderophore application, and by a genetic approach with mutants that disrupt iron uptake, transport, or metabolism.

Our initial experiments utilized a hydroponic system through which we could reduce iron in the plant and induce an iron deficiency response. This system proved to be effective for inducing the iron deficiency response in Arabidopsis and supported the completion of the *Hpa* life cycle under time frames similar to those observed on soil-grown plants. We transferred healthy plants from complete media to iron-deficient media to minimize the impact of chronic iron deficiency and reduce pleiotropic effects and growth stunting that might confound our results. Three days of growth on iron-deficient media only begun to cause chlorosis, which by seven days was severe (Figure 2 a and b). The treatment did not fully deplete iron in the plants as observed by ICP-MS, but the reduction was significant and comparable to similar studies (Figure 2 b and f). The treatment also induced expression of marker genes of iron deficiency to similar levels as other studies, over a similar time frame following imposition of iron deficiency (Figure 2 e).

With our system, we made direct comparisons of iron-starved plants to healthy plants as soon as the starvation response manifested to achieve our goal of identifying the impacts of host iron deficiency response on immunity and pathogen growth. We reasoned that if iron availability were critical to the *RPP4*-mediated resistance and ferroptosis was integral to RPP4-dependent ETI, then we should see several phenotypes in iron-deficient plants: 1. Enhanced susceptibility to avirulent *Hpa*. 2. Accumulation of iron around infection sites prior to the initiation of the hypersensitive cell death. 3. Inhibition of ROS production and HR cell death. With regard to (1) we did not observe this: iron-deficient plants grown in our hydroponic system did not exhibit decreased resistance to *Hpa* Emwa1. Rather, the plants displayed slightly reduced levels of the background sporulation that occurs during *RPP4*-dependent ETI. With we regard to (2) we did observe accumulation of iron in infected samples that was diminished in iron-deficient plants

(Figure 3 d and e). With regard to (3), the trypan blue staining in iron-deficient plants revealed no qualitative differences in the trailing necrosis in iron-deprived, hydroponically-grown plants. Unlike the report from the rice-*M. oryzae* interaction, iron restriction by chelator in hydroponic media, or direct application, does not limit HR in response to an avirulent pathogen. Additionally, we observed no changes in production or ROS at the infection site in DAB staining. This indicates that neither the HR or the oxidative burst is impacted by iron restriction in the hydroponic system. These results do not support the hypothesis that ferroptosis is an important aspect of *RPP4*-dependent ETI, moreover, they suggest that iron is not a critical limiter for *Hpa*-induced ROS production.

One caveat to the above interpretations is that ICP-MS still detects residual iron in the leaves grown under iron-deficient hydroponic conditions. Therefore, we cannot rule out those reserves playing a role in defense. With this caveat in mind, we employed an independent method of iron restriction. As mentioned previously, the iron chelator DFO has been used in several previous studies to reveal roles of iron in diverse pathosystems. Therefore, we directly applied DFO to infected, soil-grown, Arabidopsis leaves to more acutely restrict iron availability. We employed a similar method as Dangol et al. in which ferroptotic HR was inhibited by 3 mM DFO. Importantly, we applied DFO at a time point after the pathogen has penetrated to the mesophyll to avoid direct effects of the chelator (i.e., toxicity) on pathogen infection. Our results indicated that plant immunity was enhanced rather than inhibited by treatment with the chelator. Pathogen sporulation was reduced rather than enhanced on DFO-treated plants. Trypan blue staining showed that DFO application did not inhibit cell death but, if anything, potentiated more cell death. DFO treatment did stimulate production of additional ROS, perhaps due to the immune

stimulation associated with DFO treatment. In rice, this treatment is sufficient to disrupt ROS production. However, seeing no changes in two iron restriction treatments strengthens the case that iron is not critical for this process in Arabidopsis.

We observed contrasting results in experiments to detect iron accumulation by Perls' statin. Iron-starved plants grown in hydroponics did not show iron accumulation at infections sites at 6 dpi. However, DFO-treated plants showed staining indicative of iron accumulation at infection site, at 3 dpi. Importantly, these samples were taken at 3 dpi (concurrent with the peak of *RPP4*-mediated immunity), rather than those from hydroponically-grown plants taken at 6 dpi. The sampling at 6 dpi may have been too late to detect iron accumulation associated with programmed cell death. Another explanation for the staining could be ROS at the stained sites, which also can be visualized with DAB. Further testing with Perls', without DAB intensification may resolve this issue. Despite this conflicting result, the presence of HR following two methods of iron restriction indicate that iron accumulation/ferroptosis are not necessary for *RPP4*-dependent ETI.

As a third approach to elucidate the role of iron in the plant's interaction with *Hpa*, we screened a large collection of Arabidopsis T-DNA insertion mutants in genes for iron uptake, transport, regulation, and utilization. This approach has been used previously for necrotrophic bacterial and fungal pathogens of Arabidopsis, but has not been applied to biotrophic pathogens or to oomycetes. We infected the mutants with avirulent *Hpa* to identify iron related genes that may play a role in resistance to *Hpa* or *RPP4*-triggered immunity. We also screened mutants with virulent isolate Emco5. Emco5 overcomes host resistance, so these tests would be more

likely to reveal small differences in pathogen growth due to nutritional resistance resulting from loss of access to iron. Any host gene that encodes a protein that facilitates pathogen colonization is called a susceptibility gene (S-gene) (Lapin and Van den Ackerveken 2013). S-genes are typically identified in reverse genetic screens in which gene knockdowns lead to additional pathogen virulence. Other S-genes are found by identification of the targets of pathogen effectors. S-genes include negative regulators of immunity that the pathogen may activate to suppress host defenses. For example, *Hpa* induces expression of *DMR6* in haustoriated cells to reduce host immune responses (Van Damme, Huibers et al. 2008). The sugar transporters discussed above are also S-genes. Without those genes present, *Xanthamonas* does not have access to optimal resources for growth. This nutritional resistance is the target of many studies hoping to reduce the impact of disease on agriculture. If we observed decreased pathogen growth in one of the mutants, it might indicate that *Hpa* requires an Arabidopsis S-genes to acquire iron. Manipulation of S-genes to starve plant pathogens promises durable resistance as pests struggle to find new sources of nutrition.

None of the iron utilization mutants (those involved in Fe-S complex assembly, or Heme biosynthesis) tested showed decreases in pathogen growth that would indicate their role in supplying iron to the pathogen. However, other mutants did show resistance that would indicate a potential role as an S-gene, facilitating pathogen iron acquisition. For example, two transporter mutants showed enhanced resistance: *OPT3* is responsible for fine-tuning iron localization in the plant. The *vtl2* mutant also showed enhanced resistance. This transporter stores iron in the vacuole and is downregulated during iron deficiency. The action of these transporters could be important for *Hpa* iron acquisition, or could have a negative impact on Arabidopsis immunity.

The transcription factors bHLH100 and -101 promote transcription of genes important in iron mobilization and uptake, which could impact the availability of iron to *Hpa*.

The only mutant to show enhanced resistance to both virulent and avirulent *Hpa* was *frd3*, which loads iron-bound citrate into the xylem in the root for long-distance transport. The FRD3 transporter is the primary mechanism for iron transport to the shoot, so *frd3* mutants could mimic iron restriction caused by iron-deficient media in the hydroponic system or chelator application to the shoots. When *frd3* is grown on the hydroponic system with iron-deficient media the phenotype is exacerbated. The combination of iron restriction from the media and compromised root-to-shoot iron transport further reduces pathogen growth. The *frd3* mutant was also the only genotype tested that demonstrated *PR-1* activation in iron sufficient hydroponic conditions, and iron-deficient plants showed additional *PR-1* expression (Figure 8 a). This strengthens the claim that iron deficiency restricts pathogen growth at least in part via activation of the immune system, and suggests that transport might be a process through which immune activation is mediated.

The *fro3* mutation induced significant reductions in growth of virulent *Hpa*, and therefore offers promise as a potential S-gene. *Hpa* is known to lack reductases of nitrate and sulfate, preferring to assimilate reduced metabolites from its host (Baxter, Tripathy et al. 2010). The iron reductase FRO3 could serve to provide a pool of reduced iron to the pathogen, and thereby free *Hpa* from the necessity of spending energy on its own iron reduction. FRO3, unlike FRO2 that works in the rhizosphere, is found in all plant tissues, and may be exploited by *Hpa* growing through the mesophyll. Interestingly, the iron status of the host does not affect pathogen growth

on this mutant when grown in hydroponics. If immune activation by host iron deficiency is the predominant reason for enhanced resistance, we would expect increasing resistance as iron is restricted, such as in *frd3*, in which we see an additive effect of mutant and iron restriction. Instead, we observe that additional restriction of iron to *fro3* mutants does not impact pathogen growth. This could indicate that the iron starvation of the pathogen may play a larger role in this growth reduction than in other mutants. Additional tests of this mutant with a virulent isolate may allow for observation of stronger phenotypes, without the influence of a strong immune response.

The *irt1* mutant was the only mutant to exhibit any enhanced susceptibility. This phenotype is similar to that reported by Aznar et al. 2014 who demonstrated increased growth of the necrotrophic bacteria *D. dadantii* on *irt1*. This was a surprising observation, because iron reduction in the shoot, previously associated with *irt1*, would suggest that it would behave similarly to *frd3* in pathogen growth assay. Previous reports support the enhanced susceptibility phenotype of the *irt1* mutant, but additional studies will be needed to uncover the mechanisms underlying this result.

Conclusions

In contrast to the aforementioned studies in grass species, our results suggest that the immune response against *Hpa* in Arabidopsis defense is robust under conditions of iron deficiency. We undertook three approaches to restrict iron availability, and none of them reduced the efficiency of *RPP4*-dependent ETI, with the possible exception of *irt1* mutation. Thus, our results

demonstrate that iron does not play the same role in promoting defense as observed in other plants, particularly monocots. Rather, our results demonstrate that host iron status can influence Arabidopsis-*Hpa* interactions in the opposite manner. Rather than perturb immunity, iron restriction appears to reduce the pathogen's ability to grow and complete its life cycle. While we observed activation of plant immunity, indicated by increased expression of *PR-1*, it is still unclear whether the inhibition of pathogen growth is solely due to immune stimulation of the iron deficiency response, or in combination with pathogen iron starvation.

Rather than support the hypothesis that iron is critical for immunity against this oomycete pathogen, our results suggest that the reduction of iron renders the plant less susceptible. Iron reduction may impact the Arabidopsis-*Hpa* interaction in two, non-exclusive ways. The first is that the plant iron deficiency response stimulates immunity as documented in other organisms. The second is that reduced iron availability impedes the pathogen's ability to scavenge this critical nutrient from its host. The first hypothesis is supported by activation of the defense marker gene *PR-1*, which is evident in both hydroponically-grown and DFO-treated plants. The activation in DFO-treated plants is similar to similar observations from Aznar et al. 2014. They observed that iron restriction from application of DFO or a similar synthetic siderophore activated immunity. Importantly, application of iron-bound siderophore did not prompt an immune response, ruling out the possibility that the siderophore itself was perceived as an elicitor. This mechanism for immune activation could benefit plants, because scavenging for iron is a critical goal for all pathogens, and detection of that action would provide the plant with additional surveillance capacity beyond canonical pathogen detection machinery. It is even conceivable that iron-associated proteins could be guarded by NLR proteins to detect pathogens'

attempts to perturb host iron homeostasis. The iron deficiency response triggers immune activation, and perturbation of iron sensing would disrupt a plant's capacity to initiate that defense response. Few iron sensors have been described in plants. The E3 ligase BTS senses iron and influences expression of both iron metabolism and defense-related genes, making it a promising candidate for future study (Selote, Samira et al. 2015). The second hypothesis, in which reduced iron availability starves the pathogen, is quite plausible but difficult to assess with our results. Future experiments with virulent pathogens will be designed with this goal in mind.

We believe that there are three questions for the future to further disentangle the role of immune activation and pathogen starvation in this interaction. The first is identifying the mechanism of low iron perception that triggers immune activation. Could known iron sensors be informing the immune systems response in low iron conditions? Certain pathogen siderophores could act as PAMPs, but no receptors have been identified. Secondly, how is pathogen growth impacted when the influence of the immune response is mitigated? Repeating the experiments in this study with virulent *Hpa* as well as immune-compromised plants will remove the pathogen reduction that is associated with a strong resistance to the pathogen, and isolate the impact of iron restriction on *Hpa* growth. To better understand the direct effect of iron starvation on the pathogen we could also assay the oomycete's iron metabolism. Quantitative PCR of *Hpa* genes associated with iron acquisition would help decipher if pathogen in these plants are indeed suffering iron deficiency themselves.

The third question is, what is the role of S-genes for *Hpa* iron acquisition? Further study of *frd3*, *fro3*, additional mutants of S-gene candidates will clarify the importance of host

metabolism in *Hpa* iron uptake. As with the second question, study of these mutant plants with virulent pathogen may reduce the impact of plant immunity. If we see large reduction in growth, without corresponding immune activation, the case for nutritional resistance will be strengthened. As above, quantification of pathogen gene expression related to iron metabolism in these mutants will help resolve whether enhanced resistance, or nutritional resistance plays a larger role in the growth reduction we observe.

Our findings have the potential to improve plant health. Iron accumulation in rice leads to ferroptosis and cell death. We developed a hydroponic system to limit plant iron, and study plant-pathogen interactions under those conditions. We found that in the Arabidopsis-*Hpa* interaction, iron deficiency leads to resistance, but is not essential for ROS production in response to *Hpa*. We identified potential S-genes, which could be manipulated to stymie *Hpa*'s access to iron. This new understanding of iron's role in the plant immune system will help breeders and molecular biologist develop more resistant crops.

Figures

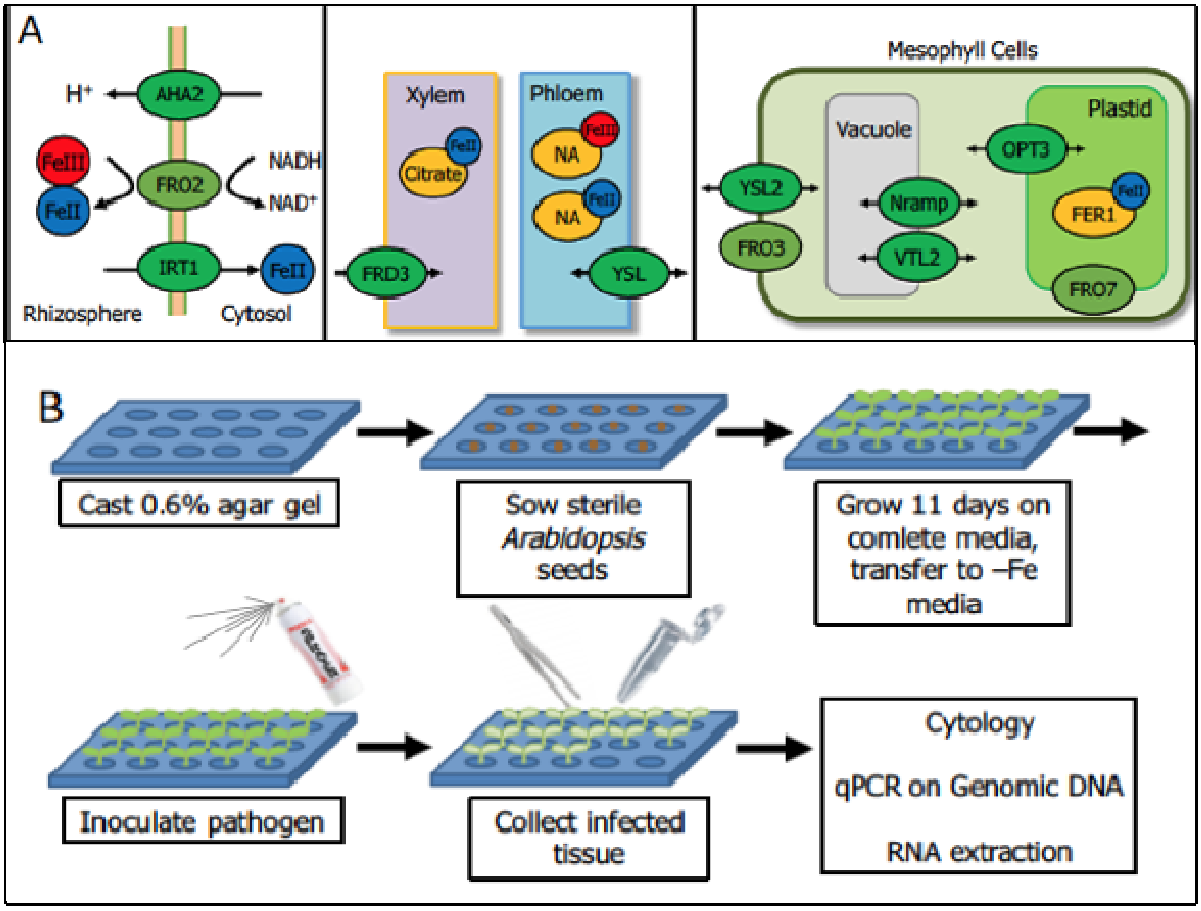

Figure 1: Figure a depicts general iron uptake, transport and storage of Arabidopsis pertinent to this study. The left panel shows iron uptake from the rhizosphere. The proton ATPase AHA acidifies the soil and the reductase FRO2 reduces iron to make it more soluble, and then the membrane transporter IRT1 imports the iron. The middle panel depicts long-range iron transport. FRD3 loads iron-bound citrate to the xylem, and additional transporters including YSL transfer nicotianamine-bound iron between and out of the vasculature. The right panel shows the mesophyll. Iron is loaded by YSL transporters and additional FRO paralogs exist to catalyze reduction of iron. Iron is stored in the vacuole by import from numerous transporters, and in the chloroplast in ferritin complexes. Figure b depicts the hydroponic system used for this study. Complete media agar was cast into 96-well trays. Sterile seeds were sewn onto the tray, one per

well. Trays were placed in square plates with additional media. After 11 days, trays were transferred to boxes with 300 mL complete media, or iron-deficient (-Fe) media, which contains 300 uM of the iron chelator Ferrozine.

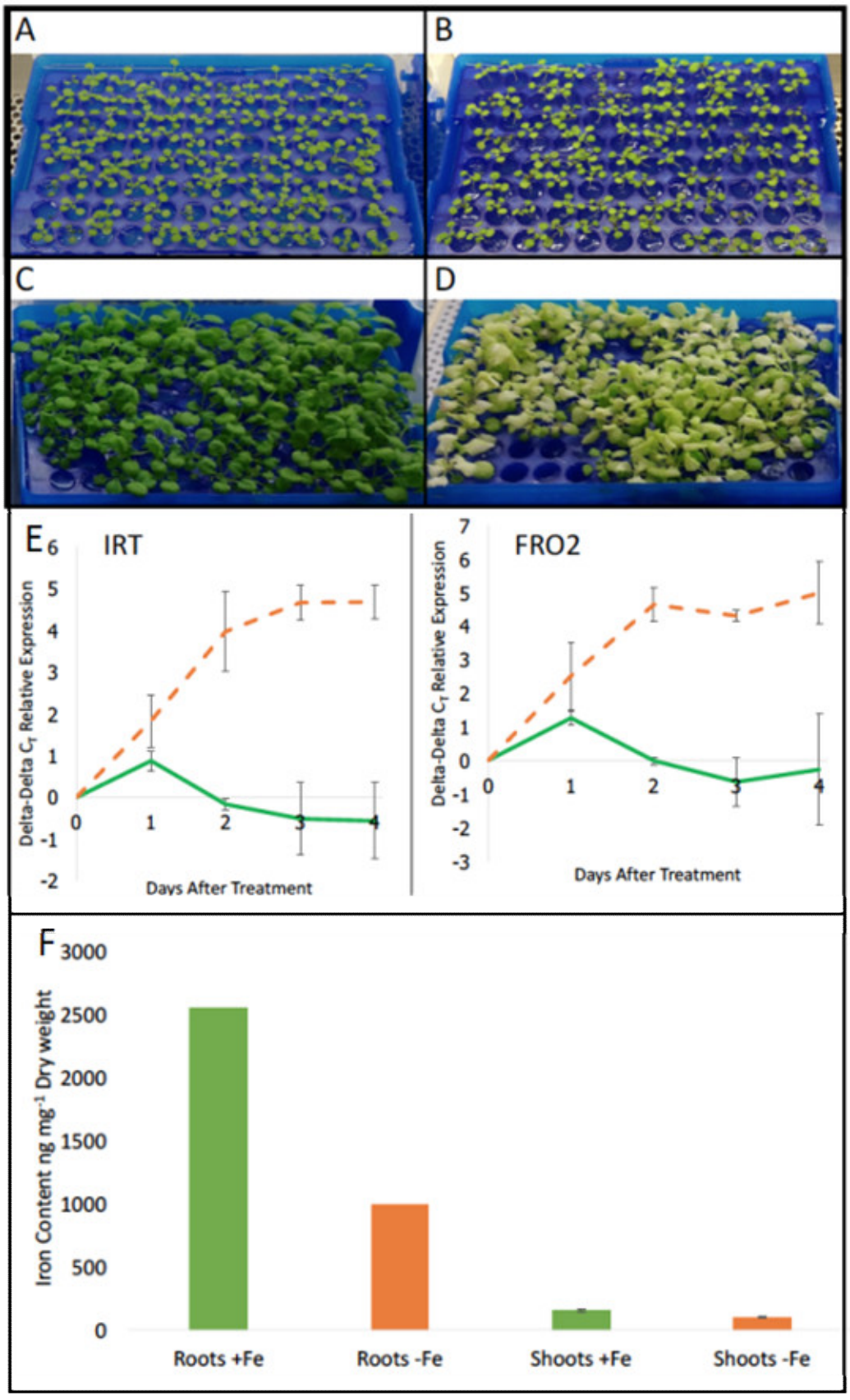
A
B
C
D
E
IRT
FRO2
Delta-Delta C_T Relative Expression
Days After Treatment
F
Iron Content ng mg^{-1} Dry weight
Roots +Fe
Roots -Fe
Shoots +Fe
Shoots -Fe

Figure 2: Hydroponic system restricts iron availability to Arabidopsis. (a, b) Arabidopsis seedlings grown in the hydroponic system, three days after transfer to boxes containing complete media (a) or -Fe media (b). Chlorosis of the leaves is beginning in -Fe plants. By seven days after transfer (c, d), chlorosis of -Fe plants is severe (d) compared to plants on complete media (c). (e) Quantitative PCR measuring gene expression of marker genes induced by iron deficiency. Time in days is measured from transfer to boxes containing complete (Green solid line) or -Fe media (orange dashed line). Expression compared to reference gene Actin2, and error bars represent standard deviation between 3 biological replicates. (f) Inductively coupled plasma mass spectrometry quantifying iron in shoots and roots or hydroponically grown Arabidopsis. Error bars depict standard deviation between three biological replicates. Root samples were pooled to obtain sufficient sample for experimentation.

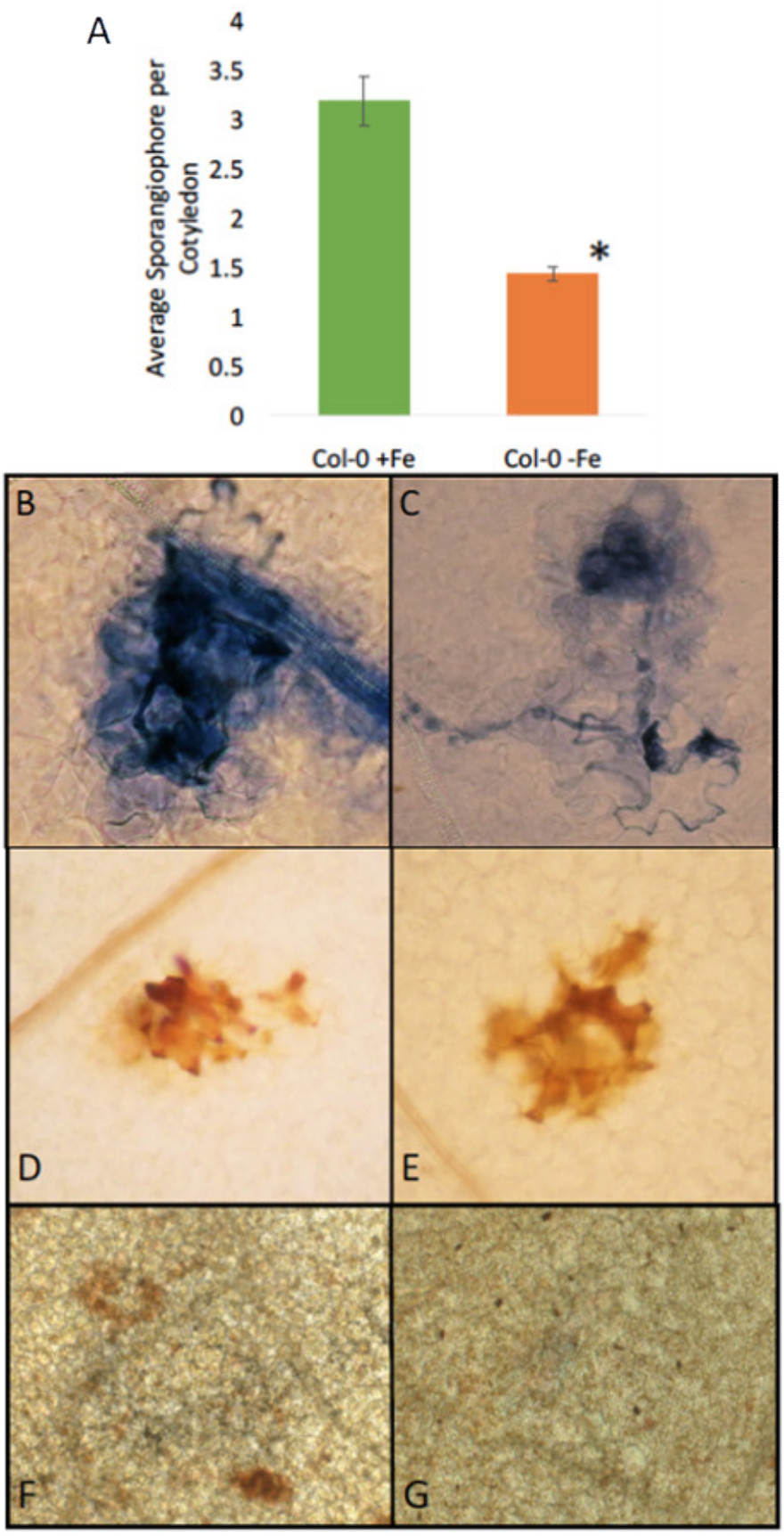
A
Average Sporangiophore per Cotyledon
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Col-0 +Fe
Col-0 -Fe
B
C
D
E
F
G

Figure 3: Pathogen response on iron deprived conditions. (a) Reproduction of the avirulent pathogen Hpa isolate Emwa1 on hydroponically grown Arabidopsis. Plants were grown on complete media for 11 days, then transferred to boxes with complete media or -Fe media for three days before infection with Hpa isolate Emwa1. Pathogen growth was quantified by counting sporangiophores on cotyledons at seven days post-infection. Error bars represent standard deviation of three biological replicates, and asterisk indicates significant difference (p-value < 0.05). (b, c) Trypan Blue stained cotyledons at six dpi in iron-replete plants (b) or iron-deficient plant (c), to highlight pathogen morphology and cell death associated with the HR to the pathogen. (d, e) Perls' stain with diaminobenzidine intensification at 6 dpi to highlight iron in infected cotyledons from iron-replete (d) or deficient (e) plants. (f, g) Diaminobenzidine staining to highlight ROS production during Hpa infection in cotyledons at 3 dpi of iron-replete (f) or deficient (g) plants.

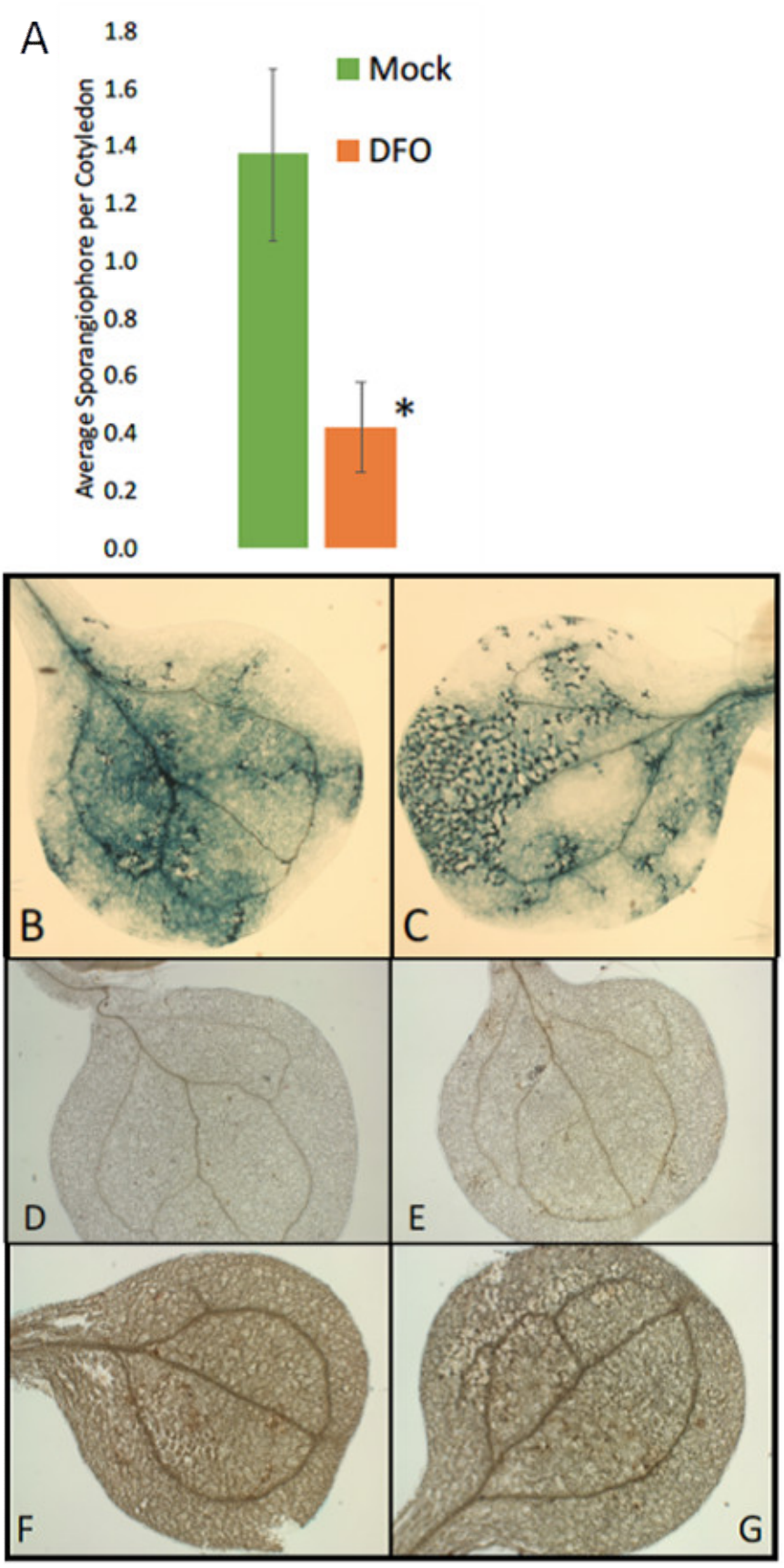
A
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Average Sporangiophore per Cotyledon
Mock
DFO
*
B
C
D
E
F
G

Figure 4: Pathogen Response following DFO Treatment. (a) Growth of the avirulent pathogen Hpa isolate Emwa 1 on soil-grown Arabidopsis treated with iron chelator DFO. Eleven day old plants were infected with Hpa, then at 24 hpi treated with 3 mM DFO or water. Pathogen was quantified as above, by counting reproductive structures on cotyledons at 7 dpi. Asterisk indicates significant difference (p-value < 0.05). (b, c) Trypan Blue stained cotyledons at six dpi in iron-replete plants (b) or iron-deficient plant (c), to highlight pathogen morphology and cell death associated with the HR to the pathogen. (d, e) Perls' stain with diaminobenzidine intensification to highlight iron in infected cotyledons at 3 dpi of iron-replete (d) or deficient (e) plants. (f, g) Diaminobenzidine staining to highlight ROS production during Hpa infection at 3 dpi in cotyledons of iron-replete (f) or deficient (g) plants.

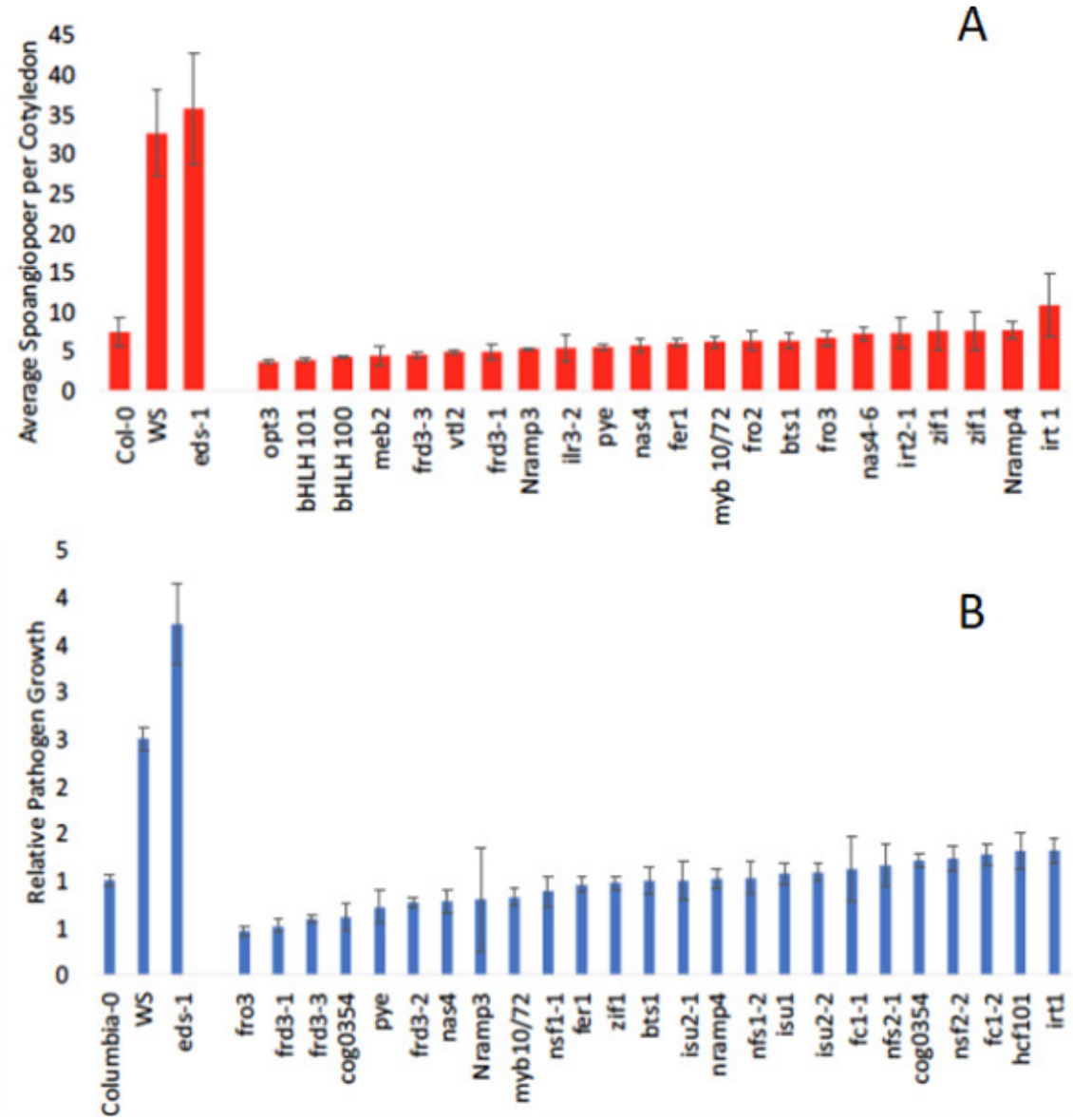

Figure 5: Hpa growth on KO mutants. Quantification of pathogen growth on Arabidopsis T-DNA insertion mutants in genes related to iron uptake, transport, metabolism, and regulation. See Table 2 and the introduction for descriptions of genes. (a) Growth of avirulent Hpa isolate Emwa1 was quantified as above by counting sporangiophores on cotyledons at 7 dpi. (b) Relative growth of virulent Hpa isolate Emco5 was quantified by comparison of PCR amplification of host and pathogen genes, normalized to growth on Columbia-0.

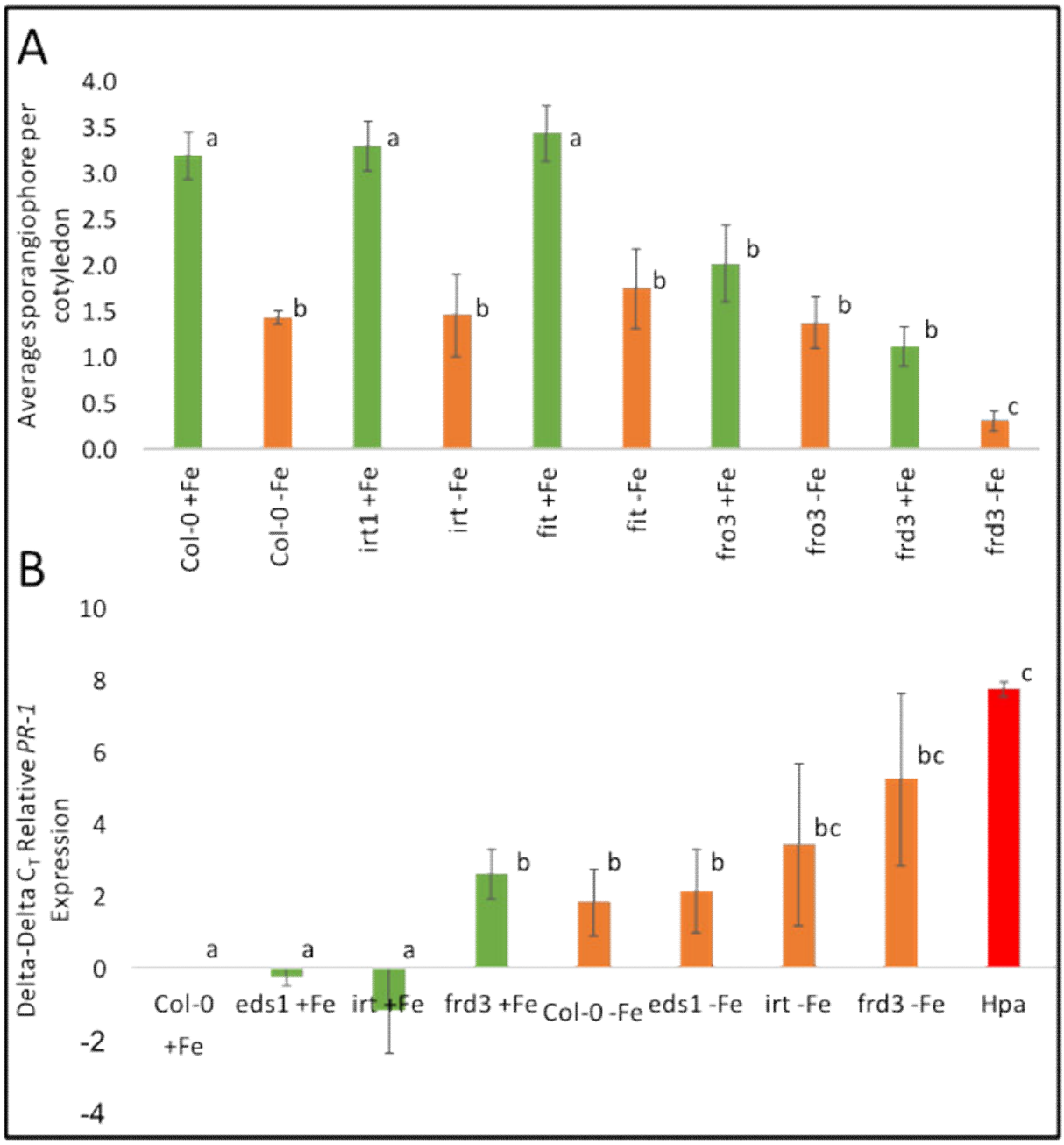

Figure 6: Select mutants on hydroponics. (a) Growth of avirulent Hpa isolate Emwa1 on iron-starved plants grown in the hydroponic system. Arabidopsis was grown as above on iron-replete or deficient media. Pathogen growth was quantified as above. Mutants/ treatments with

different letters represent significant difference (p-value < 0.05). (b) Expression levels of Arabidopsis immune marker gene PR-1 compared to Arabidopsis Actin2 on mutants grown in the hydroponic system. The sample “Hpa” was isolated from soil-grown, infected Arabidopsis Columbia-0. Mutants/ treatments with different letters represent significant difference (p-value < 0.05).

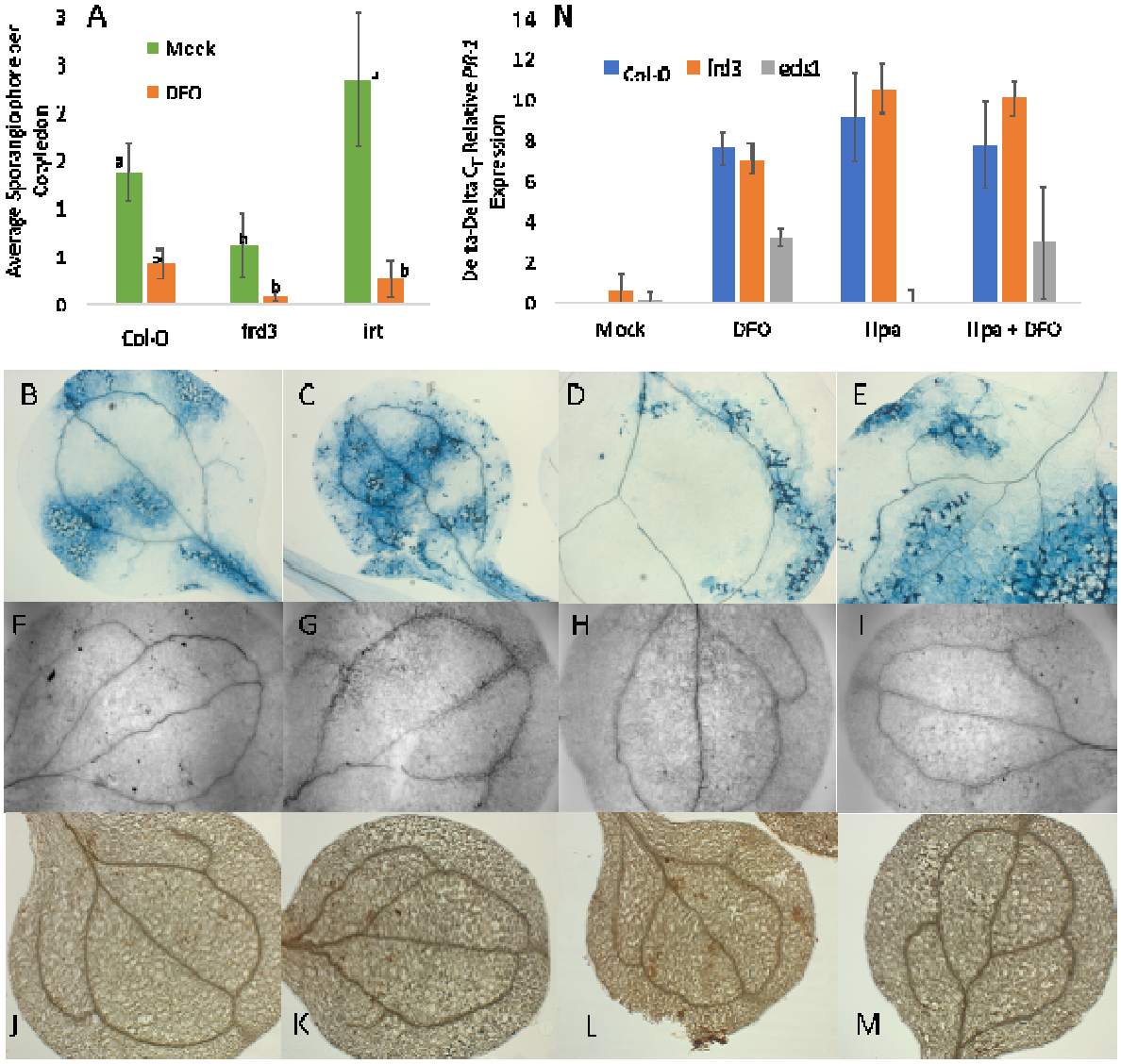

Figure 7: Pathogen response on DFO-treated mutant plants. (a) Growth of avirulent Hpa isolate Emwa1 on mutant plants treated with DFO as above. Mutants/ treatments with different letters represent significant difference (p-value < 0.05). (b, c, d, e) Trypan blue staining to observe pathogen morphology and host HR on iron-replete frd3 (b) irt1 (d) or -deficient frd3 (c) and irt1 (e) plants. (f, g, h, i) Diaminobenzidine staining to highlight ROS production during Hpa infection at 3 dpi in cotyledons of iron-replete frd3 (f) and irt1 (h) or deficient frd3 (e) and irt1 (i) plants. (j, k, l, m) Perls' stain with diaminobenzidine intensification to highlight iron in infected cotyledons at 3 dpi of iron-replete frd3 (f) and irt1 (l) or –deficient frd3 (g) and irt1 (m) plants. (n) Expression levels of immune marker gene PR-1 compared to Actin2 on mutants grown in the hydroponic system, compared to soil-grown, infected Columbia-0.

Arabidopsis Actin2 for Taqman F	5' - ATCACAGCACTTGCACC – 3'
Arabidopsis Actin2 for Taqman R	5' – GGGAAGCAAGAATGGAAC – 3'
Arabidopsis Probe	5' – VIC-AGGTCGTTGCACCACCTGAAAGG-MGB-NFQ – 3'
Hpa Actin for Taqman F	5' – CGCACACTGTACCCATTTAT – 3'
Hpa Actin for Taqman R	5' – CATCATGTAGTCGGTCAAGT – 3'
Hpa Probe	6FAM-CGCGATTGTGCGTTTGGATCT-MGB-NFQ
RT – qPCR Actin2 control F	5' – AATCACAGCACTTGCACCA – 3'
RT – qPCR Actin2 control R	5' – GAGGGAAGCAAGAATGGAAC – 3'
RT – qPCR FRO2 F	5' – GCG ACTTGTAGTGCGGCTATG – 3'
RT – qPCR FRO2 R	5' – CGTTCGACGGATTCTTG – 3'
RT – qPCR IRT1 F	5' – CGTGCGTCAACAAAGCTAAAGC – 3'
RT – qPCR IRT1 R	5' – CGGAGGCG1AAGACTTAATGATA – 3'
RT – qPCR PR-1 F	5' – GGTTCCACCATTGTTACACCT – 3'
RT – qPCR PR-1 R	5' – GAACACGTGCAATGGAGTTT – 3'

Table 1: Primers and probes used in this study

Genotype	Description	Virulent Pathogen Growth	Avirulent Pathogen Growth
Columbia 0	Background	WT control	WT control
WS	Susceptible Ecotype	ES	ES
eds1	Defense Signaling	ES	ES
irt1	Iron import - 1° Uptake	ES (n.s.)	ES
irt2	Iron import	NC	Not screened
fro2	Iron reductase - Rhizosphere	NC	Not screened
fro3	Iron reductase	NC	RC
nas4	Nicotianamine biosynthesis	NC	NC
zif1	Iron transport	NC	NC
frd3	Iron transport – Citrate xylem loader	RC	RC
nramp3	Iron transport - Defense in monocots	NC	NC
nramp4	Iron transport - Defense in monocots	NC	NC
ferritin1	Iron storage in chloroplast	NC	NC
opt3	Plastid transport	RC	Not screened
vtl2	VIT-Like Iron transport	ER	Not Screened
bts1	Iron regulation – E3 ligase	NC	NC
pye	BTS regulated	NC	RC
ilr3	Iron and Auxin regulation	NC	Not screened
myb10 x myb72	Iron and defense regulation	NC	NC
bHLH100	Iron regulation – PYE binding	RC	Not screened
bHLH101	Iron regulation – PYE binding	RC	Not screened
cog0354	Fe-S Cluster Assembly	Not screened	NC
isu1	Fe-S Cluster Assembly	Not screened	NC
isu2	Fe-S Cluster Assembly	Not screened	NC
hcf101	Fe-S Cluster Assembly	Not screened	NC
fc1	Heme biosynthesis	Not screened	NC

Table 2: Summary of infection phenotypes of virulent and avirulent Hpa on iron-related T-DNA insertion mutants of Arabidopsis. Virulent pathogen Emco5 quantified by Taqman PCR. Avirulent Emwa1 quantified by sporangiophore counting on cotyledons. NC – No change. ES – Enhanced susceptibility. RC – Reduced colonization. All ES or RC denotations reflect significance from Columbia-0 control after three biological replicates, except *irt1* mutant which demonstrates nonsignificant enhanced susceptibility.

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Chapter 3

Transcriptome Profiling Reveals a Core Iron Deficiency Response in *Arabidopsis thaliana* that is not Altered by *Phytophthora capsici* Infection

John Herlihy[1], John McDowell[1]

[1]Virginia Tech, School of Plant and Environmental Sciences, Latham Hall (0329), Blacksburg, VA, 24061-0329

Abstract

Plants are sessile organisms adapted to cope with dynamic changes in their environment. Abiotic stresses, such as heat, cold, drought, salinity, or nutrient deficiency, must be overcome simultaneously with biotic threats such as pathogens and herbivores. Overlaps between the plant response to iron deficiency and pathogens have been documented, but the impact of both stresses on the plant transcriptome has not been studied. We adapted a hydroponic system to simultaneously impose iron deficiency and monitor pathogen infection in the roots. The oomycete pathogen *Phytophthora capsici* grew less well on iron-starved *Arabidopsis thaliana*. We conducted RNA sequencing to understand how Arabidopsis responds to these two stresses. 323 genes were differentially upregulated in iron-starved plants over three days, irrespective of pathogen infection, representing a core iron deficiency response. This group of core genes included the primary Arabidopsis iron uptake pathway and genes for coumarin biosynthesis. As the infection progressed, other genes associated with iron deficiency, including lateral root growth were down regulated. Salicylic acid responsive genes were observed in both treatments

consistent with the defense hormone's previously identified role in iron deficiency. Genes related to glucosinolate production – shown to be important in defense against *P. capsici* – were down regulated during infection, potentially due to the activity of virulence effectors. Our work demonstrates further crosstalk between the iron deficiency response and plant immunity, and that iron acquisition remains important to the plant even after pathogen invasion.

Introduction

Phytophthora capsici: model Oomycete

Oomycetes are filamentous organisms originally classified as fungi, until genetic analysis confirmed them to be heterokonts more closely related to algae (Förster, Coffey et al. 1990). The genus *Phytophthora* contains some of the most devastating plant pathogens, including the causative agent of late blight (*P. infestans*) and *P. ramorum*, another broad-host-range pathogen and causative agent of sudden oak death. The oomycete pathogen *Phytophthora capsici* causes disease on over three hundred plant species, infecting shoots, fruits, and roots (Granke, Quesada-Ocampo et al. 2012). Study of *P. capsici* is critical to develop resistance, understand the biology of the destructive genus *Phytophthora*, and to decipher the underpinnings of its broad host range (Lamour, Stam et al. 2012).

P. capsici biflagellated-zoospores are chemotatically-attracted to potential hosts (Khew and Zentmyer 1973, Adorada, Biles et al. 2000). Spores encyst at the plant epidermis and produce appressorial penetration structures to invade host tissues. Once inside the plant, *P. capsici* hyphae grow in the apoplastic space and feed off living host tissues as a biotroph. To facilitate

this initial biotrophic stage of disease, *P. capsici* produces membrane-bound invaginations of host cells, called haustoria. Homologous structures in plant-pathogenic fungi are responsible for nutrient uptake, though this role has not been verified in the oomycetes (De Jong and van den Ackerveken 2018). The haustoria are sites of metabolic activity and production of virulence factors to suppress host immunity. The most important of these are virulence proteins called effectors. Effector proteins, including the oomycete-specific RxLR effectors, are critical for suppression of host immunity and establishment of disease (Anderson, Deb et al. 2015). In Arabidopsis, following one to two days of biotrophic growth, *P. capsici* transitions to necrotrophy (Attard, Gourgues et al. 2010). The onset of necrotrophy is faster in other hosts (Muthuswamy, Balakrishnan et al. 2018). Additional effectors enable the transition to necrotrophy, where the pathogen kills host cells and lives off the dead tissue. *P. capsici* produces asexual reproductive sporangiophores, each containing dozens of zoospores. Upon release, spores travel plant-to-plant completing the life cycle.

P. capsici is an excellent model system for study of *Phytophthora*. It grows well in lab conditions, and some *P. capsici* isolates are virulent on Arabidopsis (Wang, Bouwmeester et al. 2013). Wang et al. 2013, leveraged the genetic tools available in Arabidopsis, including T-DNA insertion mutants, to demonstrate *P. capsici* is an effective pathogen even on this non-host species. Despite *P. capsici*'s efficacy, plant immunity provides some protection. For example, SA-induced defenses and glucosinolates play a role in slowing disease progression. When genes for biosynthesis of these compounds are knocked down, *P. capsici* infection accelerates.

Previous studies have documented the plant transcriptome during *P. capsici* infection. Jupe et al. used microarray to study *P. capsici* infection on detached tomato leaves (Jupe, Stam et al. 2013). The authors of the study conclude that the plant undergoes two shifts in transcriptional response to infection, the first upon initial invasion of *P. capsici*, then again following the onset of pathogen necrotrophy. The first transition is accompanied by downregulation of normal metabolic processes, and the second includes induction of genes related to biotic stress and cell survival, potentially to counteract the pathogen's necrotrophic program. That analysis also reveals downregulation of tomato immune receptors and signaling components, presumably targeted *by P. capsici* virulence effectors to stifle immune activation. Using proteomics, Mahadevan et al. observed similar *P. capsici*-induced changes in *Piper nigrum* (Mahadevan, Krishnan et al. 2016). As in Jupe et al. 2013, the study in pepper observed a rapid downregulation of metabolism genes, and accompanying induction of stress responsive genes. The authors also observed the downregulation of glucosinolate biosynthesis genes, perhaps again the action of *P. capsici* virulence effectors. Importantly, both of these studies were conducted on detached leaves. While both reveal important insights into the behavior of *P. capsici*-infected plants, no study has been done on infected roots, nor in the model plant Arabidopsis.

Iron and Immunity

During biotrophic life stages, plant pathogens require complete nutrition from their host. Iron is required by all organisms, and its low bioavailability imposes limitations on growth, including that of pathogens. Plant pathogenic bacteria and fungi produce iron-binding compounds called

siderophores in order to acquire this essential nutrient (Haas, Eisendle et al. 2008). It is unknown how *P. capsici* and other pathogenic oomycetes assimilate iron.

Arabidopsis thaliana responds similarly to iron deficiency and pathogen invasion (Aznar and Dellagi 2015, Verbon, Trapet et al. 2017). Both stresses induce production of the phytohormones salicylic acid (SA) and ethylene (ET) (Shen, Yang et al. 2016, Dubois, Van den Broeck et al. 2018). SA is commonly associated with plant defense against pathogens, and its role in iron deficiency implies iron metabolism is important during pathogen infection. Other regulators, including transcription factors (TF), act alongside or downstream of these hormones. The TF MYB72 is regulated by ET and iron deficiency, is induced by microbes in the rhizosphere, and in turn MYB72 controls transcription of genes involved in systemic immunity and iron deficiency (Zamioudis, Korteland et al. 2015, Stringlis, Yu et al. 2018). This coregulation is indicative of iron's importance in plant-pathogen interactions.

In this study, we sought to investigate how the duel stresses of iron deficiency and *P. capsici* infection impacted Arabidopsis, using transcriptomics for an unbiased and comprehensive view of the plant's responses. We leveraged a hydroponic system (Chapter 2) to control nutrient level in the media and gain accesses to infected roots. This system allowed us to understand the interplay between immunity and iron deficiency in Arabidopsis and reach the surprising conclusion that both responses remain robust under conditions in which the two stresses are imposed concurrently.

Materials and Methods

Hydroponic Growth Conditions

Columbia-0 Arabidopsis was grown in a hydroponic system adapted from Pratelli et al. 2016 (Pratelli, Boyd et al. 2016). J2 media was prepared with 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1 mM NH_4NO_3, 1 mM $CaSO_4$, 50 µM NaFeEDTA, 30 µM H_3BO_3, 5 µM $MnCl_2$, 1 µM $CuCl_2$, 1 µM $ZnCl_2$, 100 nM $Mo_7O_{24}(NH_4)_6$ (adapted from (Lejay, Tillard et al. 1999)). J2 media with 0.6% w/v agar was cast into pipette tip racks (Olympus brand, Gennesse Scientific, USA) sealed with 3 inch HD Clear packaging tape (Duck Brand, USA). After casting, the tape was removed, excess agar was removed with a razor, and the rack was placed in a square petri dish (120 mm x 120 mm Greiner Bio, USA).

Seeds were surface sterilized in 70% ethanol in water, with 3% w/v sodium dichloroisocyaburiate, for 20 minutes, washed three times with 100% ethanol, then allowed to dry in a sterile laminar flow hood. Seeds were suspended in sterile water, and single seeds were pipetted onto each well of the hydroponic tray. Fifteen mL of J2 medium was added to each Petri plate. The plants were grown in the Petri plate for 2 weeks in an incubator (100 µE, 10h light, 22^{o}C). Plant racks were then transferred to tip boxes containing 350 mL J2 medium. For iron deficiency treatments, plants were instead transferred to boxes containing J2-Fe media, in which NaFeEDTA was removed and replaced with 300 µM of the iron chelator FerroZine (Acros Organics, USA).

Pathogen Infection

Ten days before infection *Phytophthora capsici*, isolate LT263 was transferred to 5% v/v clarified V8, 1.5% w/v agar, 0.3% w/v ß-sitosterol plates. Plates were grown at 28^{o}C in the dark to induce sporangiophore production. Two-week-old, hydroponically-grown plants were transferred to tip boxes, containing J2 or J2-Fe media, three days before infection. The ten-day-old *P. capsici* plates were flooded with sterile water, at room temperature to release swimming zoospores from sporangiophores. Spore suspension was collected from plates and concentration was measured with a haemocytometer. The suspension was adjusted to 20,000 spores mL^{-1} with additional water. Twenty mL of zoospore suspension was added to square Petri plates, and hydroponic trays were removed from boxes and placed into the plates. Plant roots were exposed to the spore suspension for 18 hours, then returned to the tip box, or in the case of soil comparisons, transferred to pots containing Sungro Professional Growing Mixture.

Trypan Blue Staining

Tissue samples were collected and washed in water, then stained in trypan blue solution made from equal parts water, phenol, lactic acid, and glycerol, and 0.05% w/v Trypan Blue, for 30 minutes at room temperature. Samples were destained in 2.5:1 chloral hydrate: water solution for three days, changed every 24 hours.

Pathogen Quantification

P. capsici growth was quantified by Taqman PCR (Primers and probes found in Table 1)(Su'udi, Kim et al. 2013, Babu and Sharma 2015, Haudenshield, Song et al. 2017). A multiplex qPCR reaction of single copy pathogen (*P. capsici*-specific ITS) and plant (Actin2) genes provided a relative quantification of pathogen growth. Genomic DNA was extracted using a Qiagen Biosprint 15 DNA Plant kit, and quantified by nanodrop. Ten nanograms of DNA was tested with Applied Biosystem Multiplex SYBR Green Master Mix on an Applied Biosystems qPCR 7500 thermocycler. Delta C_T transformation provided relative growth of *P. capsici* as compared to control or other time points.

Expression Quantification by Quantitative PCR

RNA was extracted using Qiagen RNeasy Plant spin column kit, and quantified by nanodrop. cDNA was synthesized with Invitrogen SuperScript IV and diluted fifty-fold for use in qPCR. PCR was performed with Applied Biosystems Taqman Multiplex Master Mix on an Applied Biosystems qPCR 7500 thermocycler, according to the manufacturer's instructions and default settings (Primers in Table 1). Delta-delta C_T transformation, compared to *Actin2*, was used to normalize transcript abundance (Han, Yang et al. 2013).

RNA Sequencing and Analysis

RNA was extracted as above, samples were sent to the North Carolina State University Bioinformatics Research Center for library preparation, sequencing, and analysis. Samples were sequenced on five lanes of an Illumina HiSeq 2500. Single-end reads were trimmed and adapted

using Trimmomatic v0.38 (Bolger, Lohse et al. 2014). Trimmed reads were mapped onto a chimeric Arabidopsis (TAIR10) and *P. capsici* genome using HiSAT2.0 (Cui, Herlihy et al. 2019, Kim, Paggi et al. 2019). Mapped reads for each organism were parsed, so two BAM files were prepared for each sample. BAM files were catalogued using Stringtie v1.3.5 and publicly available annotations (Pertea, Pertea et al. 2015). Differential expression analysis was performed using Cuffdiff v2.2.1 (Trapnell, Roberts et al. 2012). Differentially expressed genes were entered into BioVenn to identify commonly up or down-regulated genes (Hulsen, de Vlieg et al. 2008). Expression levels of 323 genes differentially upregulated in iron-deficient samples at 0 dpi and 1 and 2 dpi infected was used as an input for Genevestigator to identify other publically available data sets with similar expression of those 323 genes (Hruz, Laule et al. 2008).

Results

Hydroponic growth conditions for *Phytophthora capsici* infection of Arabidopsis roots

In order to better understand how iron deficiency and the Arabidopsis immune response to *Phytophthora capsici* impacted one another, we developed a procedure to profile the transcriptome during both stresses. We adapted a hydroponic growth system, described in Chapter 2, in which we could modulate iron availability and observe pathogen infection on the roots (Pratelli, Boyd et al. 2016). Plants were grown as in Chapter 2: Arabidopsis seeds were sown onto agar cast in the wells of a pipette tip rack. The racks, with seeds, were placed in complete media for two weeks, then transferred to boxes containing complete media or iron-deficient media. After three days in iron-deficient media, the plants developed symptoms of iron

deficiency, including expression of iron deficiency marker genes and reduced iron in the roots as measured by inductively coupled plasma mass spectrometry (Chapter 2). At three days after imposition of iron stress, both iron-deficient plants and iron-replete plants were inoculated with 20,000 spores mL^{-1} of *Phytophthora capsici* isolate LT263 zoospores. After 18 hours in the inoculation solution, plants were returned to the hydroponic boxes with iron-replete or -deficient media corresponding to their previous treatments.

Validation of the hydroponic system for *P. capsici* infection assays

We demonstrated in Chapter 2 that hydroponically grown plants can support the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) colonization of leaves. To confirm that *P. capsici* infection of roots proceeded similarly in hydroponic and soil-grown conditions, we compared the morphology of pathogen infections in both growing conditions. Following pathogen inoculation as described above we returned some plants to hydroponic boxes and transplanted others into soil. At 2 and 6 dpi we collected roots and stained with trypan blue to observe the pathogen (Figure 1). In all observed roots, pathogen hyphae grew and branched through the root cortex and produced haustoria. Colonization was heavy in hydroponically-grown plants and in soil-grown plants at both time points. The pathogen produced extraradical hyphae external to the root in both hydroponic and soil conditions. Pathogen hyphae and haustoria did not exhibit differences in morphology between hydroponic and soil conditions at 2 or 6 dpi. We did not observe pathogen sporangiophores in any condition during this trial. These data demonstrate that *P. capsici* colonization of roots is not compromised by the hydroponic system.

Phytophthora capsici growth is reduced on iron-deficient Arabidopsis

Our next experiment was to test whether *P. capsici* colonization of roots was impacted by iron deficiency (Figure 2). We collected root tissue from three replicates of infected tissues grown in iron-replete or iron-deficient media, and extracted DNA for pathogen quantification by Taqman PCR. We compared total pathogen DNA to plant DNA to estimate pathogen growth (Figure 2). We normalized infections levels in iron-replete plants at 4 dpi to 1, and we observed that the pathogen growth in iron-deficient plants was only 0.76 of the growth in iron-replete plants at the same time. By 7 dpi, the pathogen had grown to an average of 2.13 in iron-replete samples, but grew less, only 1.46, in iron-deficient samples. The pathogen also grew more slowly iron-deficient conditions, exhibiting an average increase in relative biomass of 192%, compared with the average 213% increase observed in iron-replete samples. These results are similar to iron-deficiency-induced growth reduction of *Hpa*, seen in Chapter 2, and could result from commensurate responses in the plant and/or oomycete pathogens.

Expression of iron-deficiency associated marker genes are not altered by infection

As a first test for alteration in the Arabidopsis iron deficiency response during *P. capsici* infection, we monitored expression levels of marker genes for the iron deficiency response. RNA was extracted from roots of hydroponically grown plants grown in iron-replete or -deficient media, before and after inoculation with *P. capsici* or mock infection. We quantified transcript abundance from FRO2 and IRT1, two marker genes for the primary iron uptake response in

Arabidopsis (Figure 3) (Vert, Grotz et al. 2002, Connolly, Campbell et al. 2003). FRO2 expression increases in the first three days of iron deficiency treatment as we observed in Chapter 2, peaking between a four and five-fold induction, and plateauing for the next four days. *P. capsici* infection does not significantly alter the induction of FRO2 in iron-replete or -deficient samples. IRT shows a similar pattern to FRO2 expression. These data provided the first indication that the kinetics and magnitude of the iron deficiency response are not affected by *P. capsici* infection.

Transcriptome sequencing of oomycete-infected, iron-starved Arabidopsis

To further understand how the Arabidopsis iron deficiency response and immune response influence each other we leveraged our hydroponic system to profile gene expression during both stresses, imposed separately and together. Plants were supplemented with complete media, or subjected to iron deficiency treatment as described above, and then were inoculated with 20,000 spores mL^{-1} or mock-infected. Three time points (0, 1, 2 dpi) were selected to encompass the early stages of infection. The early timepoints would allow for expression profiling before the necrotrophic phase of *P. capsici* infection, at which point infected cells would be dead and no longer produce transcripts for analysis. The 0 dpi time point was collected immediately before infection. There are two treatments at time 0 and four each at 1 and 2 dpi, for 10 total samples, each with three independent biological replicates (Figure 4). Each of the ten samples has a code with the three pertinent identifiers. The first letter is M or P: M indicating uninfected samples given mock treatment, and P for pathogen-treated samples. The next code is +Fe or –Fe denoting the iron status of the sample. The last code is the dpi, 0, 1, or 2. For example, P+Fe1, indicates

the pathogen infected, iron-replete sample at 1 dpi. Following RNA extraction, samples were sent to the North Carolina State University Bioinformatics Research Center for library preparation, sequencing, and analysis.

Seven pairwise comparisons were made to compare the magnitude of differential gene expression across various conditions (Figure 5). All genes differentially expressed across all three biological replicates with p-value less than 0.05 were identified. The lists of differentially expressed genes were compared amongst various conditions, and genes shared between, or unique to, various conditions were delineated. These comparisons comprised the first step towards functional analyses that are described in the next sections.

The highest numbers of differentially expressed genes in any comparison were observed between M+Fe0 and M-Fe0. This comparison illustrates the impact of iron deficiency in our system before *P. capsici* infection. 2229 genes were expressed more highly in the iron-replete sample and 1910 genes expressed more highly in the iron-deficient sample. The second comparison was between M-Fe0 and P-Fe1, and the third was between M-Fe0 and P-Fe2. Both of these comparisons were examined for insight into how the iron deficiency response changed over time following infection by *P. capsici*. The M-Fe0 condition showed higher expression of 401 genes compared to P-Fe1 and 270 genes compared to P-Fe2. M-Fe0 exhibited lower expression of 1165 and 1566 genes compared to P-Fe1 and P-Fe2, respectively. Therefore, gene induction predominated, compared to gene repression, following pathogen infection.

The fourth and fifth comparisons were between P+Fe1 and P-Fe1, and P+Fe2 and P-Fe2, with the goal of observing how iron deficiency impacted the plant's response to *P. capsici* infection. At 1 dpi 1239 genes were expressed more highly and 853 genes exhibited lower expression in iron-replete samples compared to iron-deficient samples. At 2 dpi there were 721 expressed more highly and 837 with lower expression in iron-replete samples.

The final comparisons were of P+Fe1 to P+Fe2, and P-Fe1 to P-Fe2. These comparisons would help us understand how the plant response to infection changed over time in both iron conditions. Unfortunately, these comparisons did not pass quality control. The six replicates, comprising the two timepoints of each comparison did not cluster on a multi-dimensional scaling plot. Additionally, when plotted on a dendrogram, the six replicates of each comparison did not cluster by time. These methods for understanding similarity between replicates should differentiate treatments if there are significant biological differences. Our results could be indicative of high variation between replicates or biological similarity in gene expression at each timepoint. For these reasons, comparisons of P+Fe1 to P+Fe2, and P-Fe1 to P-Fe2 were not included in the analyses described below.

The core iron deficiency response of Arabidopsis is maintained during infection

To confirm that our experimental conditions induced an iron deficiency response comparable to previously reported studies on iron deficiency, we compared iron deficiency-induced genes that we identified to those listed in publicly available data sets. First, we identified genes more highly expressed in iron-starved plants in each comparison of iron-replete to iron-deficient

conditions: M+Fe0 to M-Fe0, P+Fe1 to P-Fe1, and P+Fe2 to P-Fe2. These comparisons also provided insight into the impact of pathogen infection on the iron deficiency response. A total of 2401 genes were more highly expressed in iron-deficient samples in one or more of the three time points, over the course of infection (Figure 6). We then designated a core iron deficiency response of 323 genes that were more highly expressed in all treatments, representing 13.4% of the total collection of iron deficiency-induced genes. These genes comprise those upregulated across iron-deficient samples independently of pathogen infection. We tested that gene list for statistical overrepresentation of biological functions of their protein products using PANTHER (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org) (Mi, Muruganujan et al. 2018). Overrepresented gene ontology categories with Bonferroni corrected p-value less than 0.05 were included. Fifty gene ontology categories were enriched in that list, including categories previously associated with the iron deficiency response, such as metal ion transport and response to iron starvation (Appendix 1: Table 1). Upregulated genes in this collection included known iron-responsive genes, such as marker genes *FRO2* and *IRT1*, which reduce iron in the rhizosphere and transport it into the root, respectively (Figure 2) as well as *NAS2* and *bHLH100* (Figure 7). *NAS4* catalyzes the biosynthesis of nicotianamine, and iron-binding compound important for iron transport throughout the plant (Higuchi, Suzuki et al. 1999). The TF bHLH100 does not regulate *FRO2* and *IRT1* directly, but its activity is still important for full activation of the iron-deficiency response (Sivitz, Hermand et al. 2012). Also included is *CYP82C4*, which catalyzes the production of fraxrtin, a coumarin derivative important in iron acquisition (Rajniak, Giehl et al. 2018). Figure 7 shows that *bHLH100* and *CYP82C4* show the highest level of induction, and that expression levels continue to rise following *P. capsici* infection. Expression of *NAS4*, *IRT1*, and *FRO2* roughly decreases twofold over the two days of

infection, from their peak expression levels at 0 dpi, but remain significantly upregulated compared with iron-replete samples. Altogether, these results indicate that the iron deficiency response is robust under conditions of pathogen infection.

Based on previous reports of coregulation between iron deficiency and SA immune responses, as described above (Shen, Yang et al. 2016), we examined the core set of 323 iron deficiency response genes for evidence of SA signaling. Accordingly, SA signaling genes are differentially expressed under our conditions of iron stress. Such genes included *PBS3*, as well as SA-responsive genes encoding transcription factors: *WRKY38* and *WRKY62*. Figure 7 shows that *PBS3* expression is already induced over 32-fold by 0 dpi, and increases almost another fourfold after two days of *P. capsici* infection. The expression level of *WRKY38* and *-62* is stable from 0 to 1 dpi, and increases twofold by the second day of infection. Response to hypoxia was also overrepresented in the set of 323 genes, likely due to hydroponic growth conditions.

The list of core iron deficiency-responsive genes was also compared to publically available transcriptome data sets through Genevestigator. This tool returns publically-available differential expression comparisons, in which gene expression of a query is similarly perturbed. The ten data sets that included the most similar expression of those 323 genes, included seven iron deficiency sets (Figure 8). One of those ten most similar responses was from SA treatment. Altogether, these data confirm that an iron deficiency response was induced under our experimental conditions, and suggest that the SA signaling sector was also activated by iron deficiency, independently of pathogen infection.

To gain additional insight into the relationship between the iron deficiency response and pathogen infection, we examined genes that were upregulated in uninfected, iron-deficient plants (M-Fe0) but were not upregulated in iron-deficient, infected plants (P-Fe1 and P-Fe2). These genes represent the response to iron deficiency in the absence of *P. capsici* infection. This group of 1178 genes comprised 49.1% of all genes upregulated across the three iron deficiency comparisons (Figure 6). Analysis for statistically overrepresented gene ontology categories in this group revealed 126 GO terms (Appendix 1: Table 2). These included auxin biosynthesis (*NIT2*), sulfur starvation (*LSU1 -2* and *-3*) and glucosinolate biosynthesis (*MYB34*, *MAMA1*). Glucosinolate production has previously been shown to correspond to resistance to *P. capsici*. Response to hypoxia was also enriched, likely due to the hydroponic conditions. Interestingly, induced systemic resistance, and responses to biotic stress like cell wall thickening, were also enriched at this time. Collectively, these categories represent iron-deficiency-induced genes that are suppressed over the first two days of *P. capcisi* infection. This pathogen-dependent suppression may be a result of the plant focusing its resources to cope with the arrival of *P. capsici*, or the effect of the pathogen itself, suppressing immunity with virulence factors to facilitate growth.

We then examined 175 genes that showed higher expression in iron-deficient samples at 0 and 1 dpi but were not present in the core group of 323 genes. In other words, these genes are upregulated at day 0 and 1 but not at day 2 as the infection progresses. These genes showed statistical overrepresentation of 17 GO terms, including cytokinesis and mitosis (Appendix 1: Table 3). These results suggest that root elongation and growth associated with iron deficiency is

diminished as the infection progresses. Collectively, these categories represent facets of the iron deficiency response that are suppressed over the second day of *P. capsici* infection.

To further understand how *P. capsici* impacts the iron deficiency response we performed similar analyses on genes differentially expressed in infected and mock-infected, iron-deficient samples (M-Fe1 vs P-Fe1, and M-Fe2 and P-Fe2). This analysis used genes up- (Figure 9 a) or down-regulated (Figure 9 b) in infected, iron-deficient samples compared to mock-infected, iron-deficient samples, in both days of the infection. Genes up regulated over both days represent processes prioritized by the plant, and may give insight to pathways that take precedence when both stresses are imposed simultaneously. Surprisingly, only 73 genes were upregulated under these conditions on both days. This group was enriched in 19 GO terms, including callose deposition, hypoxia and response to bacteria (Appendix 1: Table 4). A similarly small number of genes were down regulated in these conditions (123 total). These 123 genes were enriched in 13 GO terms, all but one of which related to glucosinolate biosynthesis (Appendix 1: Table 5). This apparent suppression of glucosinolate biosynthesis could be greatly beneficial to the pathogen, as glucosinolate production has been linked to defense against *P. capsici*.

Discussion

Nothing is known about the interplay between iron homeostasis and infection by oomycetes. As a first step towards addressing this knowledge gap, we developed a hydroponic system to enable restriction of iron while simultaneously observing pathogen growth. The growth of the *P. capsici* was similar in the hydroponic system was indistinguishable from growth in soil-grown

plants (Figure 1). Morphology of the pathogen, over the first seven days of infection, including hyphal growth and haustoria formation, was similar in both hydroponic and soil-grown plants. We did observe enrichment of marker genes of hypoxia in some of our data sets, but this was not consistent across all hydroponic treatments, and did not adversely impact pathogen growth compared to soil. Thus, the hydroponic conditions supported normal progression of *P. capsici* colonization of roots, as observed for *Hpa* in leaves (Chapter 2).

Similar to our observations with *Hpa* on leaves in Chapter 2, iron-starved Arabidopsis roots are more resistant to *P. capsici* (Figure 2). *P. capsici* exhibits less growth at four dpi and grows more slowly over the seven-day period tested. Previous reports have demonstrated that the iron deficiency response induces SA signaling and other defense responses in Arabidopsis, and it is possible that the observed reduction in pathogen growth is a result of enhanced resistance of the host. Indeed, our transcription profiling reveals consistent upregulation of SA-related signaling genes in iron-deficient samples, across all three days. However, iron starvation could also impact the pathogen, and we cannot rule out that iron deficiency impacts *P. capsici* growth. Future study of pathogen transcripts in our study will provide better understanding the reduced pathogen growth in iron limiting conditions. A decrease in expression of pathogen proliferation and virulence genes, and concurrent increase in expression of iron scavenging and utilization, would indicate that the iron starvation itself is reducing pathogen growth. A third cause of pathogen growth reduction could be toxicity of the iron chelator itself. Increased expression of detoxification enzymes in the pathogen would indicate this is a reason for decreased growth in the iron-deficient samples. Analysis of the pathogen transcriptome is underway and may provide answers to these questions.

The hydroponic system was also able to induce the iron deficiency response. We demonstrated the reduction of iron availability by ICP-MS and chlorosis of the leaves in Chapter 2. Our initial tests with quantitative PCR demonstrated upregulation of iron deficiency associated marker genes *IRT1* and *FRO2* (Figure 3). The induction of the marker genes is consistent with previous reports (Mukherjee, Campbell et al. 2006, Long, Tsukagoshi et al. 2010). We hypothesized that *P. capsici* infection would alter the iron deficiency response. However, our results show that a portion of the transcriptional changes associated with iron deficiency in Arabidopsis remain unchanged following infection. Figure 3 shows that gene expression of *FRO2* and *IRT1* remain constant following infection. The transcriptome results confirmed that 323 core iron deficiency genes remained upregulated from day 0 uninfected samples through the first two days of *P. capsici* infection. These results demonstrate that response to *P. capsici* does not substantially preclude the iron deficiency response. In other words, the plant maintains the iron response under conditions of pathogen infection.

The composition of the core 323 genes further illuminates the connection between iron deficiency and immunity in Arabidopsis. Figure 7 shows that this group contains central players in both responses. Coumarins additionally play a role in sculpting the rhizosphere microbiome (Stringlis, De Jonge et al. 2019). Induction of coumarin biosynthesis following application of oomycete-derived elicitors improved resistance to *P. palmivora* on rubber (Dutsadee and Nunta 2008). The core iron-deficiency response contains SA-related genes as well. Among the core genes is *PBS3*, responsible for the last step of 90% of pathogen-induced SA (Rekhter, Lüdke et al. 2019). The expression of *PBS3* is strongly induced at 0 dpi, following three days of iron

starvation, and continues to rise following *P. capsici* infection. Its inclusion in the core iron deficiency response genes indicates that it is more highly expressed in the iron-deficient sample at each time point. *WRKY38* and *WRKY62* are both SA-responsive TFs on the list, indicative of the downstream impacts of *PBS3* activation (Figure 7). The presence of both iron and SA-related genes in the core iron deficiency response demonstrates the close relationship these two responses share.

Despite the connections between immunity and iron deficiency, we do observe some major differences between the profile of genes upregulated in M-Fe0 and the subsequently infected samples. At day 0 we observed upregulation of a large number of genes that were not induced at later time points (Figure 6). We interpret this as the generalized stress response following three days of iron deficiency. We see upregulation of genes with functions coregulated with iron deficiency (Appendix 1: Table 2). These include genes previously associated with iron scavenging, including courmarin biosynthesis, and auxin to promote root growth and increase surface area for nutrient assimilation. This gene set also includes genes with functions tangentially related to iron deficiency but known to be upregulated under low iron stress: the sulfur deficiency response, glucosinolate biosynthesis, and defense against pathogens; as has been seen in previous studies (Forieri, Sticht et al. 2017). The coregulation of these stress responses is well documented, including previous demonstrations that these processes are regulated by stress and immune hormones including SA and ET. We also observed upregulation of genes related to callose deposition, regulation of immune responses, and wounding; markers of SA and ET signaling. Whether the evolutionary function of this regulation is convenient use of available hormone signaling cascades, or a mechanism to detect nutrient scavenging by

pathogens is unknown. However, these responses could account for the reduction of *P. capsici* growth on iron-deficient Arabidopsis compared to iron-replete controls.

Despite the reduced pathogen growth in iron-deficient plants, *P. capsici* is able to establish an infection. This could be due to the pathogen's ability to suppress defense responses in its host. Glucosinolates have been shown to play a major role in defense to *P. capsici* in Arabidopsis (Wang, Bouwmeester et al. 2013). Arabidopsis deficient in indole glucosinolate biosynthesis are susceptible to avirulent *P. capsici,* underscoring the importance of indole glucosinolates for resistance to *P. capsici*. Despite the induction of glucosinolate biosynthesis genes in M-Fe0, there is consistent downregulation of these genes in infected plants (Appendix 1: Table 5). Presumably *P. capsici* is able to wield its virulence proteins to suppress transcription of these genes and disrupt the defense response initiated by iron deficiency. Our study supports the centrality of indole glucosinolates in the interaction. Further effectoromic study of *P. capsici* should investigate the pathogens ability to suppress glucosinolate production in the plant.

Conclusions

In this study, we show the utility of a hydroponic system for the simultaneous observation of nutrient stress and *P. capsici* infection on Arabidopsis roots. We used transcriptomics to show that a core iron deficiency response is consistent throughout the first two days of infection despite pathogen progression. This core response is smaller in scale than the large induction of genes seen at day 0, and does not involve some of the tangentially associated pathways typically seen in other studies. We see that root elongation and the sulfur deficiency response, including

glucosinolate metabolism are not among those in the core response. However, further study is needed to show whether the downregulation of these genes during pathogen infection is due to a focusing of the plant response once *P. capsici* is detected, or a result of pathogen virulence activity. Our results reinforce the relationship between the iron deficiency response and the immune response, showing the activation of defense hormone signaling, and resistance to *P. capsici* during iron deficiency. Plant growth-promoting bacteria often produce siderophores that trigger plant the iron deficiency response and systemic immunity. This in turn confers resistance to some fungal and bacterial pathogens. A similar approach may be useful to control *P. capsici*, as the siderophore ferrozine is effective in reducing the growth of this oomycete pathogen in our system. Future use of our system will aid in the understanding of how plants cope with multiple stress. The media and infection procedure could be modified to study other nutrient stresses in combination with root pathogen infection.

Further analysis of our duel transcriptome will yield new and exciting results. The sequencing was able to capture both plant and pathogen transcriptomes, despite the fact that we only analyzed the reads mapped to the Arabidopsis genome for this study. Our analysis here focused on the impact of pathogen infection on the iron deficiency response. Differentially expressed genes in key comparisons were used to answer this question. Future work will be able to look at changes in gene expression over different comparisons to address other questions; particularly, how coexpression of genes across different treatments may cluster together. This will provide insight into the regulation of the iron deficiency response. The regulation of the core response might be different from the wider response seen at day 0. Other comparisons may also explain the dramatic drop in glucosinolate biosynthesis as the infection progress.

Analysis of the pathogen transcripts will also provide insight in oomycete iron metabolism. Compared with fungal and bacterial pathogens, little is known about iron acquisition in oomycetes. It is unknown if oomycetes produce siderophores, or utilize other mechanisms of iron uptake. Study of the transcriptome of pathogenic *P. capsici* during iron deficiency, may reveal how the organism responds to this stress. Our study also included samples of axenically grown *P. capsici* on iron-deficient and iron-replete media. Comparison of these transcriptomes will help us understand how oomycetes acquire iron during saprophytic and pathogenic life stages.

Figures

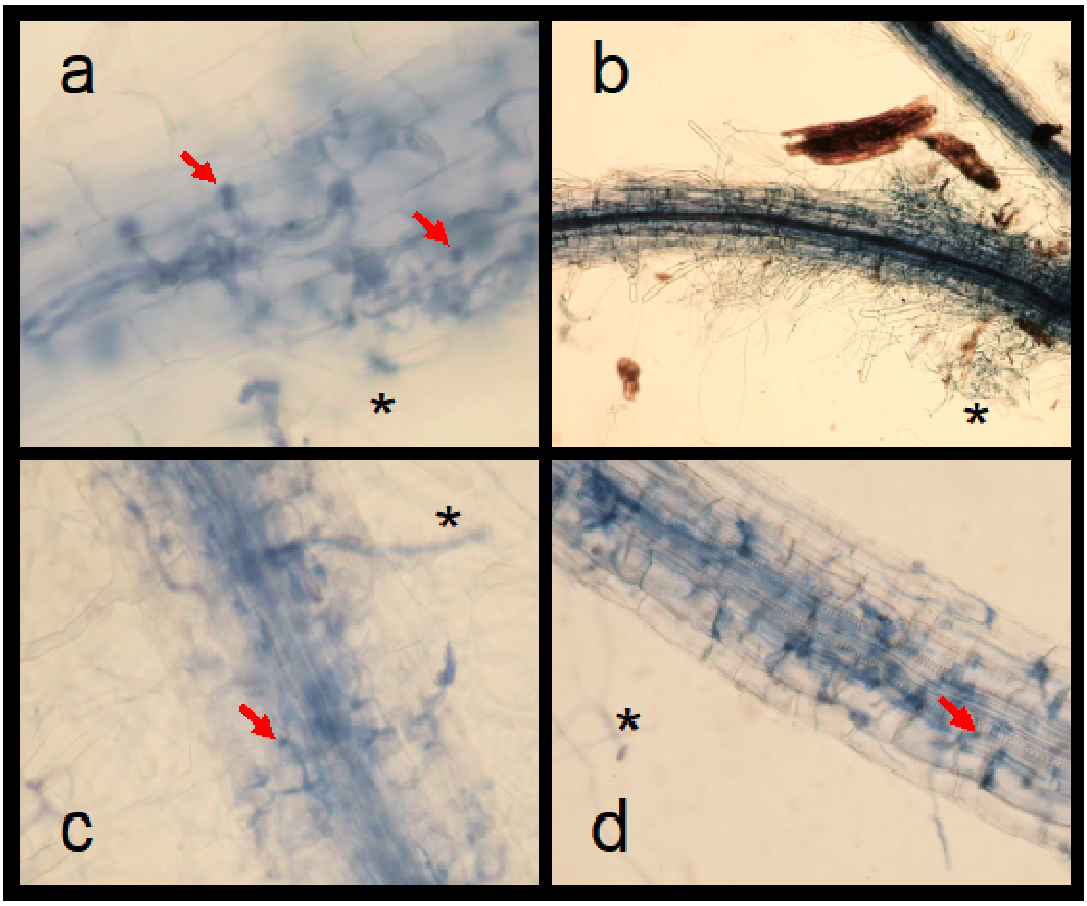

Figure 1: Trypan blue stains of *P. capsici*-infected Arabidopsis roots grown in soil or hydroponics. Arabidopsis was grown on agar for 2 weeks then inoculated with 20,000 spores mL^{-1} of *P. capsici*, and transferred to hydroponic media (a, c) or soil (b, d). Samples were collected at 2 dpi (a, b) and 6 dpi (c, d). Red arrows denote pathogen haustoria, and asterisks denote extraradical hyphae growing outside the root. Images are representative examples of three independent replicates.

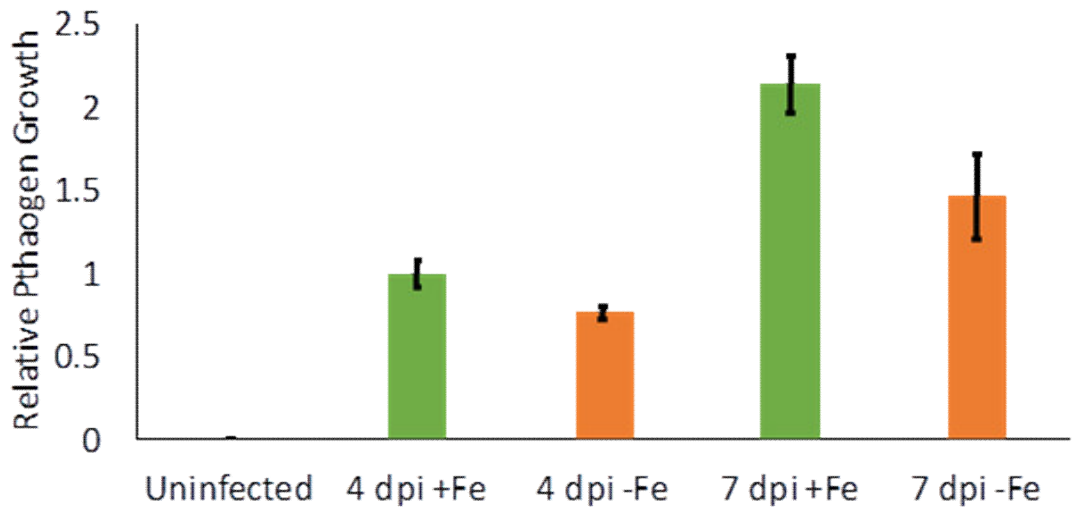

Figure 2: Quantification of *P. capsici* on hydroponically-grown *Arabidopsis thaliana* by quantitative PCR. Arabidopsis was grown for two weeks on complete media then transferred to iron replete or deficient media for three days. Then roots were infected with 20,000 spores mL^{-1} of P. capsici. After 4 and 7 days tissue was collected for DNA extraction and PCR. Error bars represent standard deviation of three biological replicates.

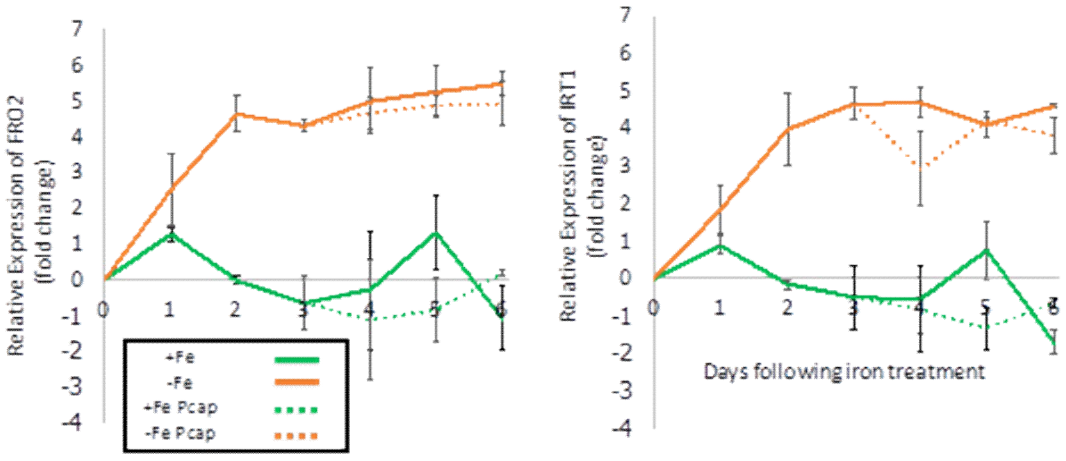

Figure 3: Expression of *FRO2* and *IRT1*, marker genes of the Arabidopsis iron deficiency response, during growth in iron replete or iron deficient hydroponics, and subsequent infection with *P. capsici*. Plants were grown in complete media for 2 weeks, before transfer to iron deficient (orange lines) or complete media (green lines). After three days, plants were inoculated with 20,000 spores mL^{-1} of *P. capsici* (dashed lines), or mock infected (solid lines). Tissue was collected for RNA extraction and cDNA synthesis. Error bars represent standard deviation of three biological replicates.

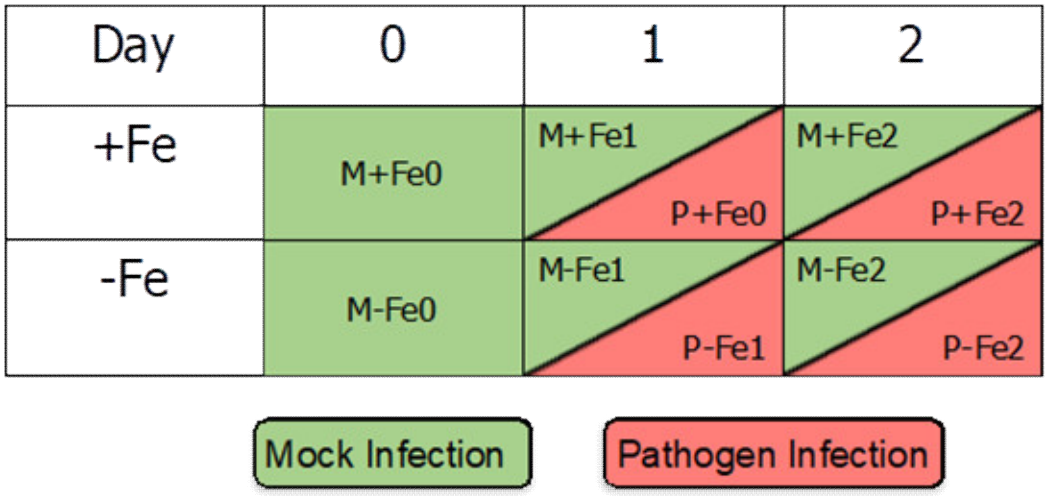

Figure 4: The ten treatments used for transcriptome analysis in this study. Each treatment was assigned a three part code. M or P to denote the infection status, M for mock infected, or P for pathogen infected with 20,000 spores mL^{-1} of P. capsici. +Fe or –Fe for iron-replete or iron-starved. All plants were grown for two weeks on iron-replete media then transferred to iron-replete or iron-deficient media with 300 µM of the iron chelator FerroZine for three days prior to infection. The third part of the code is the days post infection, 0, 1, or 2. 0 dpi samples were collected immediately prior to infection.

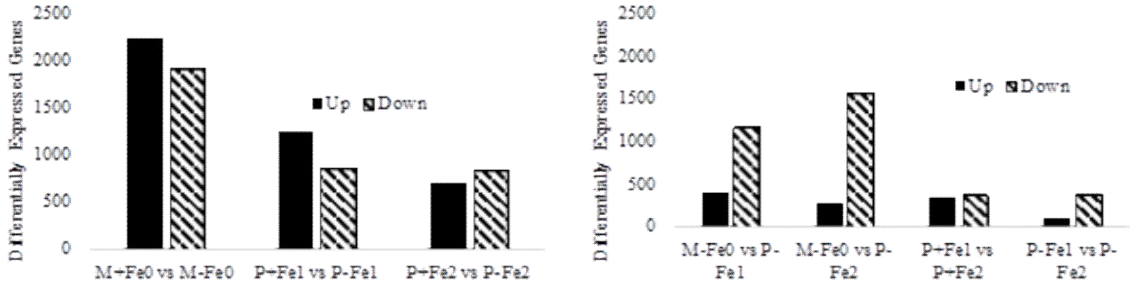

Figure 5: Differentially expressed genes across seven comparisons to understand how iron deficiency and response to pathogen infection influence one another. DEGs were identified by Cuffdiff v2.2.1 with a p-value less than 0.05. Solid bars represent higher expression in the first data set, while dashed bars represent lower expression in the first set. Comparisons of expression levels from iron replete and iron deficient samples (a) revealed how the response to *P. capsici* infection was impacted by iron deficiency, and comparisons of these differentially expressed genes provided insight into the plants response to both stresses over time. Comparisons of the infection across multiple time points (b) revealed which responses were conserved throughout infection, during iron deficiency.

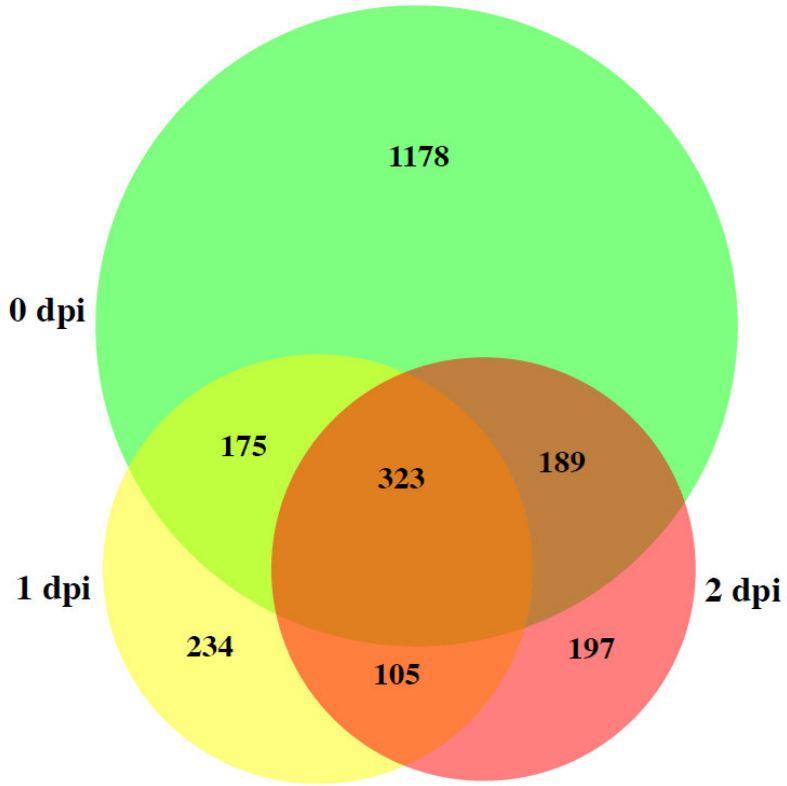

Figure 6: Proportional Venn Diagram showing DEGs up regulated iron deficient samples of the course of *P. capsici* infection. Genes more highly expressed in iron deficient plants than iron replete plants from 0, 1, and 2 dpi infected samples. (BioVenn web tool).

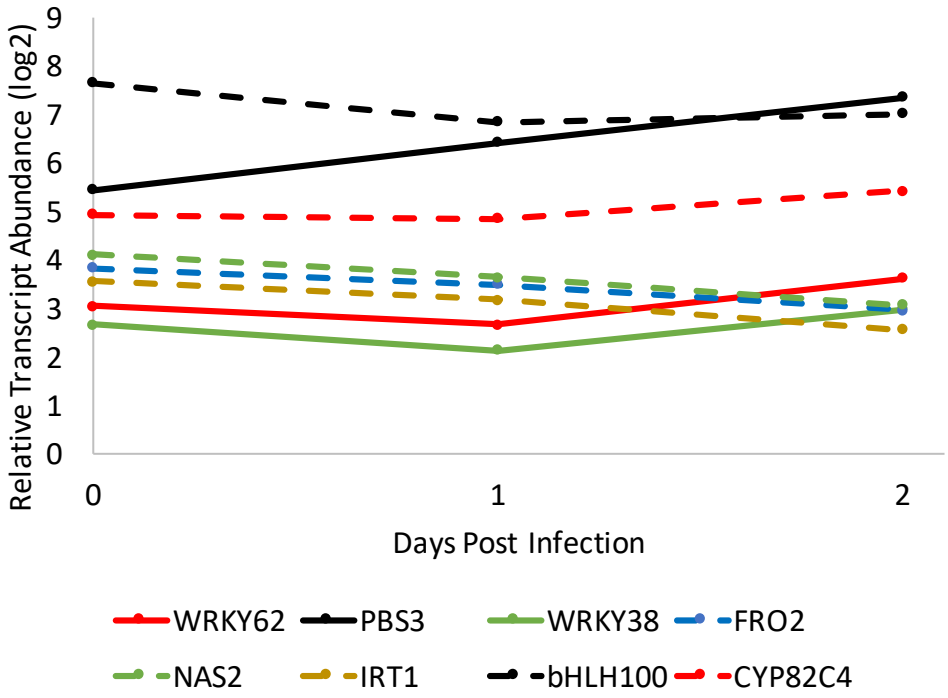

Figure 7: Relative transcript abundance of selected genes in the core iron deficiency response. Transcript levels normalized to that of iron-replete samples at day 0 then transformed to $\log_2$ scale. Genes selected from the 323 core genes that showed upregulation in iron-deficient samples irrespective of pathogen infection. Solid lines are salicylic acid-related, and dashed lines are iron-related.

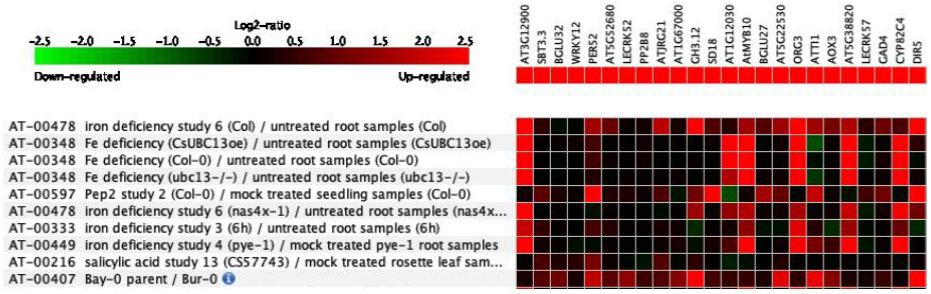

Figure 8: Treatments that induce similar expression patterns of 323 core iron deficiency genes as our hydroponic system. Expression levels of 323 core iron deficiency genes in 0 dpi

used as input in Genevestigator, to identify perturbations that induce similar gene activity. Genes along the top are the most highly upregulated in M-Fe0 vs M+Fe0. Comparisons along the left are those with the most similar pattern of differential expression. Red indicates upregualtion in the comparison, green represents down regulation.

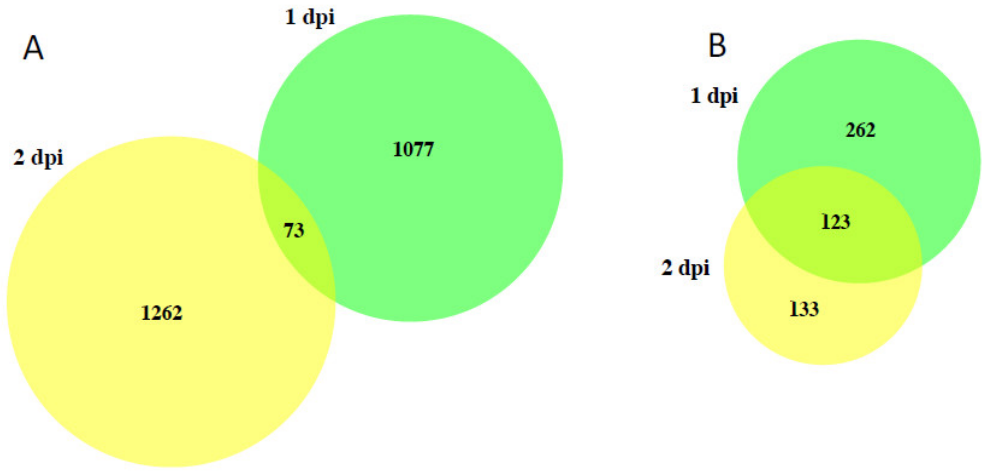

Figure 9: Proportional Venn Diagram of genes expressed at higher levels (a) or lower levels (b) in infected, iron-deficient samples, than mock infected, iron-deficient samples. (BioVenn web tool).

Arabidopsis Actin2 for Taqman F	5' - ATCACAGCACTTGCACC – 3'
Arabidopsis Actin2 for Taqman R	5' – GGGAAGCAAGAATGGAAC – 3'
Arabidopsis Probe	5' – VIC-AGGTCGTTGCACCACCTGAAAGG-MGB-NFQ – 3'
Pcap ITS for Taqman F	5' – TTTAGTTGGGGGTCTTGTACC – 3'

Pcap ITS for Taqman R	5’ – CTAGTTAAAGCAGAGACTTTCGT – 3’
Pcap Probe	5’ – 6FAM-CGGACCGAAGTCCAAACATTCGC-MGB-NFQ – 3’
RT – qPCR *Actin2* control F	5’ – AATCACAGCACTTGCACCA – 3’
RT – qPCR *Actin2* control R	5’ – GAGGGAAGCAAGAATGGAAC – 3’
RT – qPCR *FRO2* F	5’ – GCG ACTTGTAGTGCGGCTATG – 3’
RT – qPCR *FRO2* R	5’ – CGTTCGACGGATTCTTG – 3’
RT – qPCR *IRT1* F	5’ – CGTGCGTCAACAAAGCTAAAGC – 3’
RT – qPCR *IRT1* R	5’ – CGGAGGCG1AAGACTTAATGATA – 3’

Table 1: Primers and probes used in this study

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Appendix A

Tables of Gene Ontology Categories Overrepresented in Comparisons of Differentially Expressed Genes in Chapter 3

GO biological process complete	Total genes in At (27581)	Input	Expected	Fold Enrichment	Bonferroni corrected P-value
cellular response to iron ion starvation (GO:0010106)	6	4	0.07	58.19	8.86E-03
phloem transport (GO:0010233)	21	5	0.24	20.78	2.70E-02
vascular transport (GO:0010232)	21	5	0.24	20.78	2.70E-02
cellular iron ion homeostasis (GO:0006879)	42	8	0.48	16.63	2.43E-04
cellular transition metal ion homeostasis (GO:0046916)	71	9	0.81	11.06	8.66E-04
iron ion homeostasis (GO:0055072)	97	12	1.11	10.8	1.17E-05
transition metal ion homeostasis (GO:0055076)	141	14	1.62	8.67	8.18E-06
cellular metal ion homeostasis (GO:0006875)	143	13	1.64	7.93	7.96E-05
transition metal ion transport	116	10	1.33	7.52	5.03E-03

(GO:0000041)					
cellular cation homeostasis (GO:0030003)	171	13	1.96	6.64	5.56E-04
cellular ion homeostasis (GO:0006873)	181	13	2.07	6.27	1.02E-03
metal ion homeostasis (GO:0055065)	228	16	2.61	6.13	6.20E-05
cellular response to hypoxia (GO:0071456)	234	15	2.68	5.59	5.18E-04
cellular response to oxygen levels (GO:0071453)	236	15	2.7	5.55	5.75E-04
cellular response to decreased oxygen levels (GO:0036294)	236	15	2.7	5.55	5.75E-04
response to salicylic acid (GO:0009751)	206	13	2.36	5.51	4.03E-03
cellular chemical homeostasis (GO:0055082)	207	13	2.37	5.48	4.24E-03
response to hypoxia (GO:0001666)	259	16	2.97	5.39	3.25E-04
response to starvation (GO:0042594)	179	11	2.05	5.36	3.29E-02
response to decreased oxygen levels (GO:0036293)	263	16	3.01	5.31	3.96E-04
response to oxygen levels (GO:0070482)	264	16	3.02	5.29	4.16E-04

cation homeostasis (GO:0055080)	280	16	3.21	4.99	8.81E-04
inorganic ion homeostasis (GO:0098771)	294	16	3.37	4.75	1.63E-03
metal ion transport (GO:0030001)	285	15	3.27	4.59	5.45E-03
response to organic cyclic compound (GO:0014070)	337	17	3.86	4.4	1.97E-03
ion homeostasis (GO:0050801)	326	16	3.74	4.28	5.89E-03
response to antibiotic (GO:0046677)	286	14	3.28	4.27	2.63E-02
cellular homeostasis (GO:0019725)	313	15	3.59	4.18	1.61E-02
chemical homeostasis (GO:0048878)	439	19	5.03	3.78	3.96E-03
drug metabolic process (GO:0017144)	404	17	4.63	3.67	2.03E-02
response to bacterium (GO:0009617)	478	18	5.48	3.29	4.64E-02
homeostatic process (GO:0042592)	583	21	6.68	3.14	1.77E-02
ion transport (GO:0006811)	675	24	7.73	3.1	4.77E-03
oxoacid metabolic process (GO:0043436)	952	30	10.91	2.75	2.69E-03
carboxylic acid metabolic process (GO:0019752)	828	26	9.49	2.74	1.56E-02
organic acid metabolic process (GO:0006082)	959	30	10.99	2.73	3.11E-03
response to acid chemical	1127	33	12.91	2.56	3.61E-03

(GO:0001101)					
response to external biotic stimulus (GO:0043207)	1023	29	11.72	2.47	4.33E-02
response to other organism (GO:0051707)	1023	29	11.72	2.47	4.33E-02
response to biotic stimulus (GO:0009607)	1024	29	11.73	2.47	4.37E-02
response to external stimulus (GO:0009605)	1426	40	16.34	2.45	8.27E-04
regulation of biological quality (GO:0065008)	1157	32	13.26	2.41	1.93E-02
response to oxygen-containing compound (GO:1901700)	1507	41	17.27	2.37	1.83E-03
response to endogenous stimulus (GO:0009719)	1381	37	15.82	2.34	9.23E-03
response to hormone (GO:0009725)	1363	36	15.62	2.31	1.54E-02
small molecule metabolic process (GO:0044281)	1408	37	16.13	2.29	1.17E-02
response to chemical (GO:0042221)	2666	67	30.54	2.19	3.80E-06
response to stress (GO:0006950)	3088	74	35.38	2.09	2.75E-06
response to stimulus (GO:0050896)	5347	101	61.26	1.65	3.89E-04
Unclassified (UNCLASSIFIED)	6709	86	76.87	1.12	0.00E+00

Table 1: GO categories enriched in core iron deficiency genes upregulated in all iron deficient samples independent of infection. Gene list analyzed using PANTHER web tool (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org). Total genes reflects the number of Arabidopsis genes of that category. Input are the genes provided to the service from our analysis. Expected are the number of genes in a category proportional to the total genes in that category encoded in Arabidopsis and the genes provided. Only biological function GO categories with Bonferroni corrected P-values less than 0.05 included.

GO biological process complete	Total genes in At (27581)	Input	Expected	Fold Enrichment	Bonferroni corrected P-value
indoleacetic acid metabolic process (GO:0009683)	12	7	0.51	13.77	1.62E-02
S-glycoside biosynthetic process (GO:0016144)	39	19	1.65	11.5	1.85E-09
glucosinolate biosynthetic process (GO:0019761)	39	19	1.65	11.5	1.85E-09
glycosinolate biosynthetic process (GO:0019758)	39	19	1.65	11.5	1.85E-09
toxin biosynthetic process (GO:0009403)	21	10	0.89	11.24	6.61E-04
defense response by callose deposition (GO:0052542)	19	8	0.8	9.94	2.22E-02

indolalkylamine metabolic process (GO:0006586)	29	11	1.23	8.96	1.02E-03
tryptophan metabolic process (GO:0006568)	29	11	1.23	8.96	1.02E-03
auxin biosynthetic process (GO:0009851)	34	12	1.44	8.33	5.70E-04
indole-containing compound biosynthetic process (GO:0042435)	49	16	2.08	7.71	1.36E-05
secondary metabolite biosynthetic process (GO:0044550)	148	48	6.27	7.66	8.95E-21
response to insect (GO:0009625)	32	10	1.36	7.38	1.46E-02
glycosyl compound biosynthetic process (GO:1901659)	63	19	2.67	7.12	1.53E-06
toxin metabolic process (GO:0009404)	71	21	3.01	6.98	2.58E-07
auxin metabolic process (GO:0009850)	61	18	2.58	6.97	5.79E-06
indole-containing compound metabolic process (GO:0042430)	71	20	3.01	6.65	1.48E-06
aromatic amino acid family metabolic process (GO:0009072)	76	20	3.22	6.21	4.04E-06
aromatic amino acid family biosynthetic process (GO:0009073)	57	15	2.41	6.21	4.49E-04
cellular response to oxygen levels (GO:0071453)	236	59	9.99	5.9	4.27E-21

lignin biosynthetic process (GO:0009809)	44	11	1.86	5.9	3.03E-02
cellular response to decreased oxygen levels (GO:0036294)	236	59	9.99	5.9	4.27E-21
cellular response to hypoxia (GO:0071456)	234	58	9.91	5.85	1.53E-20
phenylpropanoid biosynthetic process (GO:0009699)	85	21	3.6	5.83	4.17E-06
S-glycoside metabolic process (GO:0016143)	112	27	4.74	5.69	3.20E-08
glucosinolate metabolic process (GO:0019760)	112	27	4.74	5.69	3.20E-08
glycosinolate metabolic process (GO:0019757)	112	27	4.74	5.69	3.20E-08
response to chitin (GO:0010200)	138	32	5.84	5.48	1.00E-09
response to decreased oxygen levels (GO:0036293)	263	60	11.14	5.39	9.57E-20
sulfur compound biosynthetic process (GO:0044272)	136	31	5.76	5.38	3.44E-09
response to hypoxia (GO:0001666)	259	59	10.97	5.38	2.37E-19
response to oxygen levels (GO:0070482)	264	60	11.18	5.37	1.13E-19
lignin metabolic process (GO:0009808)	66	15	2.79	5.37	2.25E-03

secondary metabolic process (GO:0019748)	318	72	13.47	5.35	5.57E-24
phenylpropanoid metabolic process (GO:0009698)	115	25	4.87	5.13	1.20E-06
detoxification (GO:0098754)	100	21	4.23	4.96	4.98E-05
response to wounding (GO:0009611)	208	39	8.81	4.43	1.03E-09
glycosyl compound metabolic process (GO:1901657)	170	31	7.2	4.31	4.85E-07
response to toxic substance (GO:0009636)	187	33	7.92	4.17	2.69E-07
sulfur compound metabolic process (GO:0006790)	357	58	15.12	3.84	5.99E-13
regulation of innate immune response (GO:0045088)	127	20	5.38	3.72	6.18E-03
response to organonitrogen compound (GO:0010243)	243	38	10.29	3.69	2.44E-07
response to karrikin (GO:0080167)	128	20	5.42	3.69	6.87E-03
cellular amino acid biosynthetic process (GO:0008652)	186	29	7.88	3.68	4.30E-05
hormone biosynthetic process (GO:0042446)	170	26	7.2	3.61	3.30E-04
regulation of immune response (GO:0050776)	131	20	5.55	3.61	9.41E-03

response to nitrogen compound (GO:1901698)	292	43	12.37	3.48	8.07E-08
alpha-amino acid metabolic process (GO:1901605)	232	34	9.82	3.46	1.04E-05
hormone metabolic process (GO:0042445)	244	35	10.33	3.39	1.01E-05
regulation of response to external stimulus (GO:0032101)	190	27	8.05	3.36	7.12E-04
alpha-amino acid biosynthetic process (GO:1901607)	163	23	6.9	3.33	5.98E-03
regulation of immune system process (GO:0002682)	142	20	6.01	3.33	2.76E-02
regulation of response to biotic stimulus (GO:0002831)	185	26	7.83	3.32	1.43E-03
regulation of defense response (GO:0031347)	260	36	11.01	3.27	1.40E-05
response to jasmonic acid (GO:0009753)	219	30	9.27	3.23	3.22E-04
response to drug (GO:0042493)	501	66	21.22	3.11	4.87E-11
response to ethylene (GO:0009723)	206	27	8.72	3.1	2.95E-03
cellular amino acid metabolic process (GO:0006520)	335	43	14.19	3.03	3.77E-06
response to water (GO:0009415)	348	42	14.74	2.85	2.96E-05

regulation of hormone levels (GO:0010817)	377	45	15.97	2.82	1.21E-05
immune response (GO:0006955)	305	36	12.92	2.79	5.36E-04
immune system process (GO:0002376)	373	44	15.8	2.79	2.47E-05
response to water deprivation (GO:0009414)	340	40	14.4	2.78	1.24E-04
innate immune response (GO:0045087)	301	35	12.75	2.75	1.09E-03
cellular response to chemical stimulus (GO:0070887)	1129	130	47.81	2.72	2.46E-19
organic acid biosynthetic process (GO:0016053)	442	50	18.72	2.67	1.18E-05
carboxylic acid biosynthetic process (GO:0046394)	442	50	18.72	2.67	1.18E-05
response to bacterium (GO:0009617)	478	53	20.24	2.62	6.07E-06
response to external biotic stimulus (GO:0043207)	1023	112	43.32	2.59	1.33E-14
response to other organism (GO:0051707)	1023	112	43.32	2.59	1.33E-14
response to biotic stimulus (GO:0009607)	1024	112	43.36	2.58	1.37E-14
response to fungus (GO:0009620)	321	35	13.59	2.57	5.17E-03
oxoacid metabolic process (GO:0043436)	952	103	40.32	2.55	5.69E-13

organic acid metabolic process (GO:0006082)	959	103	40.61	2.54	7.81E-13
response to chemical (GO:0042221)	2666	279	112.9	2.47	9.56E-40
response to oxidative stress (GO:0006979)	393	41	16.64	2.46	2.29E-03
regulation of response to stress (GO:0080134)	375	39	15.88	2.46	4.75E-03
defense response to bacterium (GO:0042742)	395	41	16.73	2.45	2.44E-03
monocarboxylic acid metabolic process (GO:0032787)	424	43	17.96	2.39	1.87E-03
defense response (GO:0006952)	1009	102	42.73	2.39	3.73E-11
response to inorganic substance (GO:0010035)	892	90	37.77	2.38	1.94E-09
response to oxygen-containing compound (GO:1901700)	1507	149	63.82	2.33	9.66E-17
defense response to other organism (GO:0098542)	765	74	32.4	2.28	1.19E-06
response to acid chemical (GO:0001101)	1127	109	47.73	2.28	7.51E-11
carboxylic acid metabolic process (GO:0019752)	828	80	35.06	2.28	2.77E-07
response to external stimulus	1426	137	60.39	2.27	3.44E-14

(GO:0009605)					
response to osmotic stress (GO:0006970)	545	52	23.08	2.25	7.87E-04
organic cyclic compound biosynthetic process (GO:1901362)	768	73	32.52	2.24	3.59E-06
cellular response to stress (GO:0033554)	1142	108	48.36	2.23	3.65E-10
aromatic compound biosynthetic process (GO:0019438)	679	64	28.75	2.23	6.75E-05
response to stress (GO:0006950)	3088	285	130.77	2.18	2.12E-31
response to metal ion (GO:0010038)	457	42	19.35	2.17	2.64E-02
response to salt stress (GO:0009651)	469	43	19.86	2.17	2.09E-02
response to organic substance (GO:0010033)	1716	154	72.67	2.12	8.20E-14
response to abiotic stimulus (GO:0009628)	2067	183	87.53	2.09	2.06E-16
regulation of response to stimulus (GO:0048583)	692	60	29.3	2.05	2.13E-03
small molecule metabolic process (GO:0044281)	1408	122	59.63	2.05	2.27E-09
small molecule biosynthetic process (GO:0044283)	602	52	25.49	2.04	1.30E-02
multi-organism process (GO:0051704)	1497	129	63.39	2.03	8.32E-10

cellular response to endogenous stimulus (GO:0071495)	644	55	27.27	2.02	1.01E-02
cellular response to hormone stimulus (GO:0032870)	625	53	26.47	2	1.89E-02
response to endogenous stimulus (GO:0009719)	1381	116	58.48	1.98	6.42E-08
response to hormone (GO:0009725)	1363	114	57.72	1.98	1.24E-07
response to stimulus (GO:0050896)	5347	442	226.43	1.95	1.06E-42
cellular response to stimulus (GO:0051716)	2427	199	102.78	1.94	1.11E-14
oxidation-reduction process (GO:0055114)	977	77	41.37	1.86	1.83E-03
regulation of biological quality (GO:0065008)	1157	84	49	1.71	1.26E-02
cell communication (GO:0007154)	1565	112	66.27	1.69	5.62E-04
signal transduction (GO:0007165)	1305	90	55.26	1.63	4.05E-02
biosynthetic process (GO:0009058)	2454	151	103.92	1.45	1.99E-02
biological regulation (GO:0065007)	5664	321	239.86	1.34	8.61E-05
regulation of biological process (GO:0050789)	4958	273	209.96	1.3	1.56E-02
cellular process (GO:0009987)	10105	515	427.93	1.2	8.70E-04
biological_process (GO:0008150)	20872	956	883.89	1.08	2.19E-03
Unclassified (UNCLASSIFIED)	6709	212	284.11	0.75	0.00E+00

macromolecule metabolic process (GO:0043170)	4716	125	199.71	0.63	5.18E-06
cellular component organization (GO:0016043)	2510	64	106.29	0.6	2.23E-02
cellular component organization or biogenesis (GO:0071840)	2830	66	119.84	0.55	1.49E-04
nucleobase-containing compound metabolic process (GO:0006139)	1980	43	83.85	0.51	2.30E-03
cellular component biogenesis (GO:0044085)	1170	21	49.55	0.42	2.28E-02
organelle organization (GO:0006996)	1616	29	68.43	0.42	2.91E-04
RNA metabolic process (GO:0016070)	1235	19	52.3	0.36	6.39E-04
nucleic acid metabolic process (GO:0090304)	1639	24	69.41	0.35	1.01E-06
macromolecule biosynthetic process (GO:0009059)	1065	15	45.1	0.33	1.04E-03
gene expression (GO:0010467)	1410	17	59.71	0.28	3.87E-07
cellular macromolecule biosynthetic process (GO:0034645)	1021	12	43.24	0.28	1.37E-04
vesicle-mediated transport (GO:0016192)	431	2	18.25	0.11	1.68E-02

Table 2: GO categories overrepresented in genes upregulated iron deficient plants at day 0 but in iron deficient infected samples. Gene list analyzed using PANTHER web tool (Protein Analysis

Through Evolutionary Relationships, http://pantherdb.org). Total genes reflects the number of Arabidopsis genes of that category. Input are the genes provided to the service from our analysis. Expected are the number of genes in a category proportional to the total genes in that category encoded in Arabidopsis and the genes provided. Only biological function GO categories with Bonferroni corrected P-values less than 0.05 included.

GO biological process complete	Total genes in At (27581)	Input	Expected	Fold Enrichment	Bonferroni corrected P-value
cytokinesis by cell plate formation (GO:0000911)	61	7	0.37	18.73	5.36E-04
mitotic cell cycle phase transition (GO:0044772)	45	5	0.28	18.13	3.69E-02
cell cycle phase transition (GO:0044770)	46	5	0.28	17.74	4.08E-02
microtubule-based movement (GO:0007018)	65	6	0.4	15.06	1.33E-02
cytokinesis (GO:0000910)	96	8	0.59	13.6	6.93E-04
regulation of protein kinase activity (GO:0045859)	121	7	0.74	9.44	3.85E-02
regulation of kinase activity (GO:0043549)	122	7	0.75	9.36	4.05E-02
regulation of protein phosphorylation	141	8	0.86	9.26	1.06E-02

(GO:0001932)					
cell division (GO:0051301)	297	16	1.82	8.79	2.78E-07
regulation of phosphorylation (GO:0042325)	149	8	0.91	8.76	1.56E-02
mitotic cell cycle process (GO:1903047)	183	9	1.12	8.03	8.00E-03
regulation of cell cycle (GO:0051726)	280	13	1.72	7.58	9.05E-05
cell cycle process (GO:0022402)	397	18	2.43	7.4	2.63E-07
microtubule-based process (GO:0007017)	199	9	1.22	7.38	1.54E-02
mitotic cell cycle (GO:0000278)	230	10	1.41	7.1	6.43E-03
cell cycle (GO:0007049)	492	19	3.01	6.3	1.04E-06
Unclassified (UNCLASSIFIED)	6709	36	41.11	0.88	0.00E+00

Table 3: GO categories overrepresented in genes upregulated iron deficient plants at day 0 and in iron-deficient infected sample at day 1, but not in iron-deficient infected samples at day 2. Gene list analyzed using PANTHER web tool (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org). Total genes reflects the number of Arabidopsis genes of that category. Input are the genes provided to the service from our analysis. Expected are the number of genes in a category proportional to the total genes in that category encoded in Arabidopsis and the genes provided. Only biological function GO categories with Bonferroni corrected P-values less than 0.05 included.

GO biological process complete	Total genes in At (27581)	Input	Expected	Fold Enrichment	Bonferroni corrected P-value
defense response by callose deposition in cell wall (GO:0052544)	16	3	0.04	69.88	4.76E-02
secondary metabolite biosynthetic process (GO:0044550)	148	7	0.4	17.63	5.49E-04
response to hypoxia (GO:0001666)	259	8	0.69	11.51	1.63E-03
response to decreased oxygen levels (GO:0036293)	263	8	0.71	11.34	1.82E-03
response to oxygen levels (GO:0070482)	264	8	0.71	11.29	1.87E-03
cellular response to hypoxia (GO:0071456)	234	7	0.63	11.15	1.06E-02
cellular response to oxygen levels (GO:0071453)	236	7	0.63	11.06	1.12E-02
cellular response to decreased oxygen levels (GO:0036294)	236	7	0.63	11.06	1.12E-02
secondary metabolic process (GO:0019748)	318	9	0.85	10.55	6.28E-04
response to bacterium (GO:0009617)	478	10	1.28	7.8	1.93E-03
cellular response to chemical	1129	18	3.03	5.94	2.56E-06

stimulus (GO:0070887)					
cellular response to stress (GO:0033554)	1142	16	3.06	5.22	1.51E-04
response to oxygen-containing compound (GO:1901700)	1507	16	4.04	3.96	5.89E-03
response to chemical (GO:0042221)	2666	26	7.15	3.63	7.62E-06
cellular response to stimulus (GO:0051716)	2427	23	6.51	3.53	1.46E-04
response to stress (GO:0006950)	3088	27	8.29	3.26	3.49E-05
response to abiotic stimulus (GO:0009628)	2067	18	5.55	3.25	1.94E-02
response to stimulus (GO:0050896)	5347	37	14.35	2.58	9.95E-06
Unclassified (UNCLASSIFIED)	6709	9	18	0.5	0.00E+00

Table 4: GO categories overrepresented in genes upregulated iron deficient infected plants compared to iron-deficient, mock-infected plants over both days of infection. Gene list analyzed using PANTHER web tool (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org). Total genes reflects the number of Arabidopsis genes of that category. Input are the genes provided to the service from our analysis. Expected are the number of genes in a category proportional to the total genes in that category encoded in Arabidopsis and the genes provided. Only biological function GO categories with Bonferroni corrected P-values less than 0.05 included.

GO biological process complete	Total genes in At (27581)	Input	Expected	Fold Enrichment	Bonferroni corrected P-value
S-glycoside biosynthetic process (GO:0016144)	39	8	0.16	49.2	5.23E-08
glucosinolate biosynthetic process (GO:0019761)	39	8	0.16	49.2	5.23E-08
glycosinolate biosynthetic process (GO:0019758)	39	8	0.16	49.2	5.23E-08
glycosyl compound biosynthetic process (GO:1901659)	63	8	0.26	30.46	1.63E-06
S-glycoside metabolic process (GO:0016143)	112	8	0.47	17.13	1.09E-04
glucosinolate metabolic process (GO:0019760)	112	8	0.47	17.13	1.09E-04
glycosinolate metabolic process (GO:0019757)	112	8	0.47	17.13	1.09E-04

sulfur compound biosynthetic process (GO:0044272)	136	9	0.57	15.87	2.79E-05
secondary metabolite biosynthetic process (GO:0044550)	148	8	0.62	12.96	8.28E-04
glycosyl compound metabolic process (GO:1901657)	170	8	0.71	11.29	2.26E-03
secondary metabolic process (GO:0019748)	318	10	1.33	7.54	3.24E-03
sulfur compound metabolic process (GO:0006790)	357	11	1.49	7.39	1.12E-03
Unclassified (UNCLASSIFIED)	6709	26	27.97	0.93	0.00E+00

Table 5: GO categories overrepresented in genes downregulated iron deficient infected plants compared to iron-deficient, mock-infected plants over both days of infection. Gene list analyzed using PANTHER web tool (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org). Total genes reflects the number of Arabidopsis genes of that category. Input are the genes provided to the service from our analysis. Expected are the number of genes in a category proportional to the total genes in that category encoded in Arabidopsis and the genes provided. Only biological function GO categories with Bonferroni corrected P-values less than 0.05 included.