Iron homeostasis and plant immune responses: Recent insights and translational implications

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Iron metabolism and the plant immune system are both critical for plant vigor in natural ecosystems and for reliable agricultural productivity. Mechanistic studies of plant iron homeostasis and plant immunity have traditionally been carried out in isolation from each other; however, our growing understanding of both processes has uncovered significant connections. For example, iron plays a critical role in the generation of reactive oxygen intermediates during immunity and has been recently implicated as a critical factor for immune-initiated cell death via ferroptosis. Moreover, plant iron stress triggers immune activation, suggesting that sensing of iron depletion is a mechanism by which plants recognize a pathogen threat. The iron deficiency response engages hormone signaling sectors that are also utilized for plant immune signaling, providing a probable explanation for iron-immunity cross-talk. Finally, interference with iron acquisition by pathogens might be a critical component of the immune response. Efforts to address the global burden of iron deficiency–related anemia have focused on classical breeding and transgenic approaches to develop crops biofortified for iron content. However, our improved mechanistic understanding of plant iron metabolism suggests that such alterations could promote or impede plant immunity, depending on the nature of the alteration and the virulence strategy of the pathogen. Effects of iron biofortification on disease resistance should be evaluated while developing plants for iron biofortification.

Iron (Fe) is an essential micronutrient for all living organisms, including plants and their associated microbes (1). Iron readily donates and accepts electrons, as it can exist in multiple oxidation states, particularly its ferric (Fe^{3+}) and ferrous forms (Fe^{2+}). Therefore, iron 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 (1). In plants, iron is also essential for chlorophyll and hormone synthesis and photosynthesis. Despite iron's essentiality, iron overload can cause damage in any organism. This is because 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 (Table 1), producing more dangerous reactive oxygen species (ROS), including the highly reactive hydroxide ion (2). These potent oxidizers damage lipids, proteins, and nucleic acids (3, 4). When the damage becomes too severe, the cell cannot be saved and undergoes programmed cell death (5). Thus, balance of iron levels is imperative for all organisms. Accordingly, plants tightly regulate iron uptake, localization, transport, and storage. Exciting recent progress has been achieved in understanding how plants acquire and transport biologically active iron from the soil and respond to iron-deficient environments (6, 7).

Along with the challenge of maintaining nutrient homeostasis, plants also must cope with a wide variety of pathogens and pests. Plants have evolved robust mechanisms for perception of detrimental microbes, which in turn trigger physiological responses to impede infection (8). Recent progress on iron homeostasis has been paralleled by progress in the molecular plant-microbe interaction field on understanding plant pathogen surveillance proteins, immune system signaling, and suppression of immunity by pathogen virulence proteins. These foci have provided huge payoffs in understanding how plants and microbes interact at the molecular level (9). The impact of iron on plant-pathogen interactions has been acknowledged for a considerable span of time but has received limited attention; indeed, iron homeostasis and plant immunity are typically studied in isolation from each other. One goal of this review is to highlight recent studies that connect iron and plant-pathogen interactions. We also discuss the implications of iron-immunity cross-talk on efforts to breed iron-fortified crops. We begin with primers on the regulatory networks that mediate plant immunity and plant iron homeostasis.

Overview of plant immune responses

Plant immune responses are activated when the plant detects signals that are diagnostic of pathogen invasion. For example, plants recognize a variety of pathogen-associated molecular patterns (PAMPs), initiating pattern-triggered immunity (PTI; Fig. 1*C*) (10). PAMPs are epitopes such as 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 (11). PAMPs can be detected in the apoplast by cell-surface receptors (12). Such recognition initiates cytoplasmic protein kinase cascades, Ca^{2+} influx, and rapid production of ROS (13). As discussed below, iron plays a key role in ROS generation. ROS and hormone signals interact with each other to stimulate diverse molecular and cellular responses that strengthen plant

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Table 1
Glossary

Fenton reaction—A catalytic process by which free iron converts hydrogen peroxide to the biologically dangerous hydroxide radical.
Pathogen-associated molecular pattern (PAMP)—Conserved epitopes of plant-pathogenic microbes that are recognized by plants to initiate an immune response.
Pattern-triggered immunity (PTI)—A plant immune response triggered by receptor-mediated perception of PAMPs, typified by production of ROS, cell wall reinforcement, and transcriptional reprogramming.
Pathogen effector—Proteinaceous virulence factor secreted by plant pathogens into host tissues or cells to disrupt immune functioning and accommodate pathogen growth and reproduction.
Effector-triggered immunity (ETI)—A potent plant immune response triggered by perception of intracellular pathogen effectors or their activity; typified by programmed cell death called hypersensitive response (HR) to limit pathogen spread.
Biotrophic pathogen—A plant pathogen that subsists on living host tissue during its entire life cycle.
Necrotrophic pathogen—A plant pathogen that secretes virulence factors to kill host tissues and facilitate its feeding or reproduction.
Hemibiotrophic pathogen—A plant pathogen that employs a biotrophic lifestyle at the start of infection but transitions to a necrotroph to complete its life cycle.
Strategy I iron uptake—Mechanism for plant iron acquisition that relies on rhizosphere acidification and iron reduction, followed by direct import of ferrous iron; utilized by all non-Poaceae (nongrass) plants.
Strategy II iron uptake—Mechanism for plant iron acquisition employed by the Poaceae (grasses); involves secretion of iron-binding phytosiderophores into the rhizosphere followed by uptake of the iron-siderophore complex.
Nutritional immunity—A process, first described in mammals, by which a host organism restricts availability of nutrients following infection to starve a pathogen.
Ferroptosis—Programmed cell death marked by accumulation of iron and loss of antioxidant protections, culminating in a runaway Fenton reaction and lipid peroxidation.
Biofortification—Breeding or genetic engineering efforts designed to improve the nutritional content of edible plant tissues.

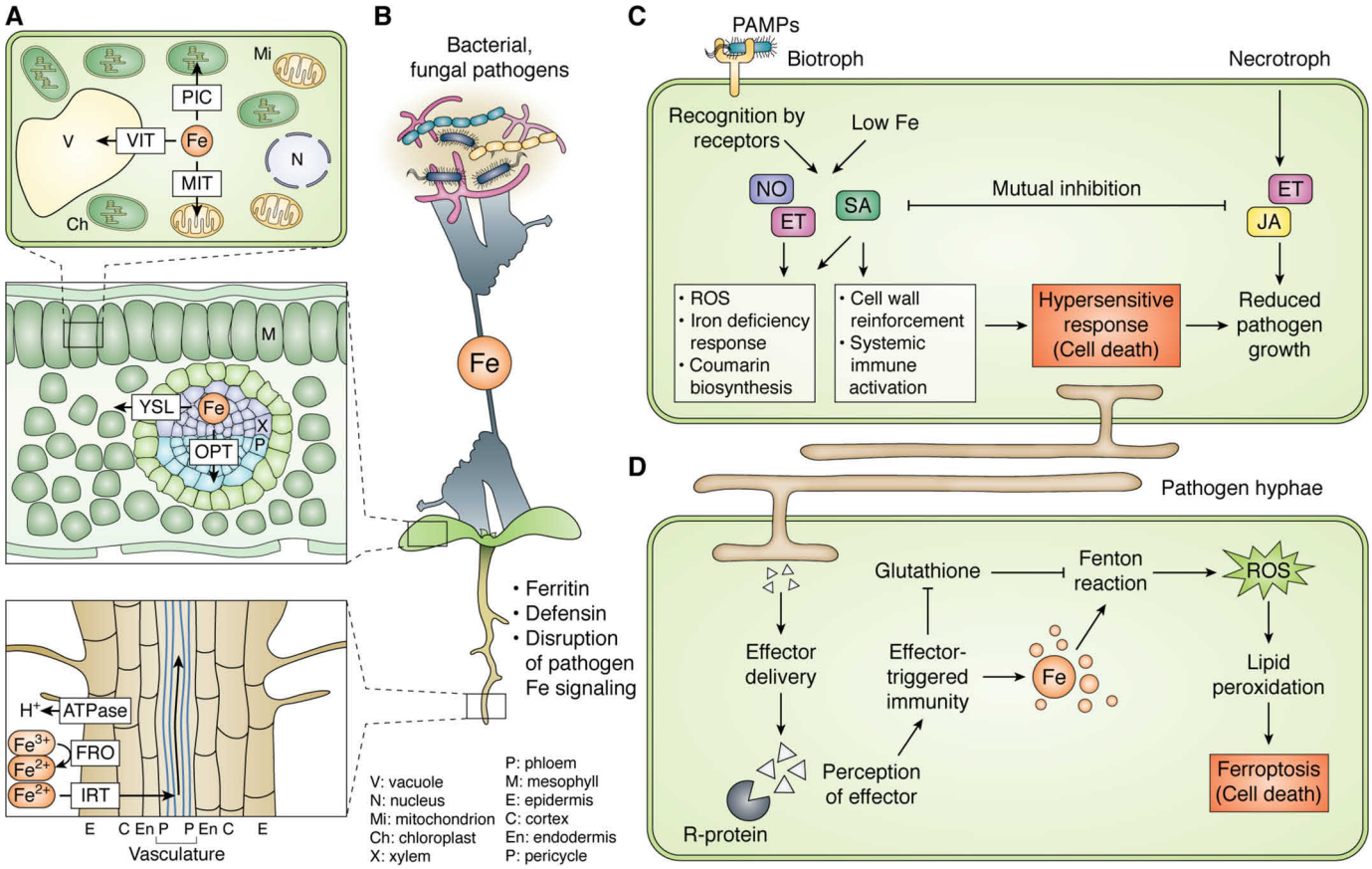

Figure 1. Iron's influence on immunity. *A*, iron uptake, transport, and storage in plants. *Bottom*, Fe is absorbed from the rhizosphere, loaded into the xylem, and transported via the vasculature from the root to the shoot (Strategy I is shown). *Middle*, in the shoot Fe is unloaded from the xylem to the mesophyll cell or the phloem for transport to other sinks. *Top*, inside cells Fe is transported into organelles for assimilation or to the cell wall or stored in vacuoles and ferritin complexes. *B*, tug of war between plants and pathogens for iron. Pathogens use varied mechanisms to scavenge plant iron during infection, whereas plants deploy mechanisms, such as iron-sequestering ferritin and defensins and disruption of Fe signaling, to interfere with pathogen iron scavenging. *C*, activation of immune responses by pathogens and by iron deficiency. PAMPs are recognized by plants to initiate PTI. PTI and iron deficiency initiate similar hormonal responses, and both activate resistance to pathogens. NO and ET buffer SA, modulating the response, and JA/ET act antagonistically to SA. *D*, perception of pathogen effectors triggers ferroptosis as a mechanism of HR cell death. ETI is initiated through R-protein–mediated recognition of effectors secreted by the pathogen. In the rice interaction with *M. oryzae*, the plant recruits iron-dependent cell death (ferroptosis) to trigger the HR and halt pathogen growth. Iron-derived ROS lead to runaway lipid peroxidization, as in mammalian ferroptosis.

cells against pathogen attack (14). PAMP perception leads to reprogramming of thousands of genes, including genes for antimicrobial proteins (*e.g.* iron-sequestering defensins discussed below) and secondary metabolites with antimicrobial activity (13). 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 (15).

All microbial pathogens produce PAMPs and are therefore vulnerable to PTI. Accordingly, pathogen success depends on evasion of detection and/or suppression of PTI signaling (16). Many pathogens disguise themselves by secreting proteins to bind PAMPs, thereby obscuring recognition, leading to PTI (17). 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 (18). Effectors from bacteria, fungi, and oomycetes have been shown to target similar hubs in the host immune signaling network (19). The action of these effectors results in an attenuated immune response called effector-triggered susceptibility (20).

To counter the threat of effector-triggered susceptibility, plants have evolved resistance proteins (R proteins) to detect pathogen effectors and initiate effector-triggered immunity (ETI; Fig. 1*D*) (21). 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 indirectly detect effectors by "guarding" immune hubs that effectors target (21). By perceiving the virulence activities of effectors (*e.g.* proteolytic degradation of an immune signaling protein) 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 (22). 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 the downstream responses are stronger than in PTI (10). Moreover, ETI is often distinguished from PTI by activation of programmed cell death at the site of infection, referred to as the hypersensitive response (HR). A recent study describes a novel role for iron in activation of ETI and will be discussed below (23).

Plant pathogens typically follow one of three lifestyles: biotrophic, hemibiotrophic, or necrotrophic (Fig. 1*C*). Biotrophic pathogens can only extract nutrients from living host cells (24). 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 (25). Hemibiotrophic pathogens begin the infection cycle with an extended period of biotrophy before triggering a necrotrophic program. Plants 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 (26). For example, the phenolic phytohormone salicylic acid (SA) is typically produced in response to biotrophic pathogens and activates the hypersensitive response (27, 28). Contrastingly, immune responses against necrotrophs and herbivores do not involve cell death and are activated by the phytohormones jasmonic acid (JA) and ethylene (ET) (25). The SA and JA/ET pathways antagonize each other to tailor the response to the invading pathogen, so that the plant utilizes its resources most efficiently (Fig. 1*C*) (26, 29). As a general rule, biotrophs are more vulnerable to ETI than are necrotrophs. This is because biotrophs depend on living plant cells for nutrients, whereas necrotrophs purposefully trigger host cell death. Indeed, some necrotrophs activate ETI as a virulence strategy (30). These differing pathogen lifestyles hold important implications for predicting whether crop biofortification could impact disease resistance and will be discussed below.

Overview of plant iron metabolism

Iron is highly abundant in soil, but most iron is bound in oxidized and insoluble ferric forms, which are biologically inactive (31). Consequently, the concentration of free iron in most soils is estimated at 10^{-15} to 10^{-17} M, far below what is required for optimal plant growth, 10^{-9} to 10^{-4} M (32). Moreover, iron is considered an immobile mineral; once it is assimilated in older tissues, there is little movement to younger tissues. As a result, plants constantly live on the edge of iron starvation. To maintain homeostasis, plants use two strategies to increase iron solubility and uptake under low-iron conditions (33–36). Strategy I plants, including all non-Poaceae angiosperms, primarily acquire iron by acidifying the rhizophere via proton ATPases to increase iron solubility and then reducing iron in the soil before direct uptake (7) (Fig. 1*A*). 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 (7, 36). Siderophore is Greek for "iron bearer." Siderophores are used by diverse organisms, including microbial pathogens of animals and plants, to acquire iron and facilitate its uptake (37–39). The primary plant phytosiderophore for Strategy II uptake from the soil is the methionine derivative mugineic acid (40, 41). The Fe-mugineic acid complex is taken into the root by members of yellow stripe-like (YSL) transporter family (42). Strategy I plants also utilize small iron-binding compounds for acquisition, especially the phenolic coumarins (43). The regulatory pathways that control activation of Strategy I uptake genes have been studied intensively and have been reviewed (44). In the following paragraphs, we summarize pathways with connections to immune signaling or potential roles in iron biofortification.

Basic helix-loop-helix (bHLH) transcription factors (TFs) play a key role in regulating iron homeostasis, characterized by heterodimerization between different clades of the bHLH superfamily. Activation of the Strategy I iron uptake response in the outer cells of the root is primarily regulated by the bHLH TF Fe deficiency–induced transcription factor (FIT) (45). FIT heterodimerizes with clade Ib bHLHs, which facilitate FIT stability upon iron deficiency (46). FIT then promotes transcription of iron mobilization genes, including ferric reduction oxidase 2 (*FRO2*), which encodes a protein for reduction of ferric iron in the rhizosphere, and iron-regulated transporter 1 (*IRT1*), which encodes a transporter that delivers reduced ferrous iron into the root epidermis (45, 47) (Fig. 1*A*). Monocots utilize the Strategy II iron uptake mechanism, which occurs through extrusion of mugineic acid family phytosiderophores, such as deoxymugineic acid, via the transporter of mugineic acid 2 (TOM2) (48). Ferric-mugineic acid family phy-tosiderophore chelates are subsequently transported into the root via YSL transporters and reduced for utilization after uptake. Moreover, the response of rice and other monocots to iron

deficiency differs from that of maize by utilizing aspects of both Strategy I and II for iron uptake (49).

After uptake into the root epidermis from the rhizosphere, small molecules facilitate solubility and transport of iron to the root vasculature (7, 50). In *Arabidopsis*, iron is chelated to nicotianamine (similar to the phytosiderophore mugineic acid referenced above) and transferred from the epidermis to the vasculature. It is likely that iron is actively transported into the xylem via the metal efflux protein ferroportin (FPN1) (51) in the vasculature, where another bHLH TF, called POPEYE (PYE), heterodimerizes with homologous TFs that are members of the bHLH clade IVc, including IAA-leucine–resistant 3 (ILR3), to regulate the expression of genes involved in metal ion storage and translocation (52). In these vascular cells, iron is thought to be sensed by BRUTUS (BTS), an iron-binding E3 ligase that facilitates the degradation of ILR3 and other group IVc TFs, resulting in a decrease in iron uptake (53, 54). Adding to this complexity, another bHLH transcriptional regulator in the same clade as PYE, called upstream regulator of IRT1 (URI) (bHLH121), has been recently shown to interact with ILR3 and other bHLH clade IVc transcription factors, forming heterodimers that presumably transcriptionally up-regulate regulate FIT binding partners (55–57). Furthermore, FIT itself appears to be degraded by BTS-like proteins, BTSL1 and BTSL2, in the outer cells of the root (58). Thus, the interplay between BTS proteins and bHLH TF proteins plays an essential role in bridging iron-sensing and transport mechanisms between the outer and inner cell types within the root (59).

After efflux into the xylem, iron is bound to citrate before long distance transport to the shoot (60, 61) (Fig. 1*A*). Once ferric citrate is translocated to the shoot via the xylem, oligopeptide transporter 3 (OPT3) loads iron into phloem companion cells (62, 63), or iron is bound to nicotianamine and loaded into neighboring cells, such as leaf mesophyll cells, by YSL transporters (64–66). In mesophyll cells, various transporters load iron into organelles for storage or metabolism, including vacuolar iron transporter VIT1, which loads iron into the vacuole and is important for iron storage, particularly in developing embryos (67) (Fig. 1*A*). In addition, permease in chloroplasts 1 (PIC1) and mitochondrial Fe uptake transporter (MIT) have been shown to load Fe into chloroplasts (68) and mitochondria, respectively (69). Plastids also contain ferritin, iron storage proteins that are up-regulated in response to ROS detection to bind iron and thereby mitigate damage from the Fenton reaction (70, 71). Ferritin has been implicated in pathogen responses, as discussed below.

Plant pathogens use diverse strategies to steal iron from plants

Bacteria and fungi employ varied strategies for iron acquisition that are analogous to Strategy I and II described above for plants (39, 72, 73). The best-studied mechanism of iron acquisition by pathogens of plants and animals is based on secretion of high-affinity iron-binding siderophores to acquire iron from their hosts, analogous to Strategy II (74, 75). These have been reviewed extensively and insightfully for phytopathogenic bacteria and fungi (38, 76, 77); here, we summarize major themes. First, bacteria produce a large diversity of peptide and small-molecule siderophores. These were first validated as critical virulence factors for *Erwinia* (78) and subsequently studied in several other bacterial genera (76). In pathogenic *Pseudomonas*, *Ralstonia*, and *Erwinia*, the transcriptional regulation of siderophore biosynthesis is mediated by so-called *hrp* (hypersensitive response and pathogenicity) regulatory factors that also control expression of secreted effectors and other virulence factors (79–81). This molecular association places siderophore synthesis under the control of the pathogen virulence program. Conversely, a major regulator of bacterial iron uptake (ferric uptake regulator, *Fur*) also regulates genes for other virulence processes, such as toxin production or cell wall degradation, in several genera of bacterial phytopathogens (76). These regulatory connections between siderophore biosynthesis and other virulence processes indicate the importance of siderophores for bacterial success inside the plant host during infection. Phytopathogenic fungi produce nonribosomal peptide synthases that function as siderophores (82). The nonribosomal peptide synthase family is conserved among ascomycete fungi and has been experimentally validated as important for virulence (83). Contrastingly, experiments in basidiomycetes indicate that siderophores are dispensable for virulence, perhaps because of compensatory systems that are summarized in the following paragraph (84, 85)

Alternate mechanisms for iron acquisition have also been shown to be crucial for bacteria and fungi. For example, reduction and subsequent transport of ferrous iron (akin to Strategy I) has been demonstrated for fungal pathogens. 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 that was first described in yeast (72). Briefly, ferrireductases are active in reducing ferric iron at the cell membrane (86). Next, the reduced iron is loaded into a protein complex of a ferroxidase (FET) and a ferric permease (FTR) (87). In *Saccharomyces*, ScFET3 oxidizes the iron before transport into the yeast by ScFTR1. The purpose of this oxidation step has not been clarified. Contrastingly, the FET/FTR complex has been shown to possess high affinity for iron, allowing fungi to scavenge iron at low concentrations. The purpose of ferrireductase family reductases in plant pathogenic fungi has not been investigated; however, Albarouki *et al.* (88) used a genetic approach to validate the importance of FET-mediated iron uptake in the maize pathogen *Colletotrichum graminicola*. Mutant fungi lacking the iron deficiency–induced FET protein grew as well as WT fungi on iron-sufficient media but exhibited abnormal morphology and reduced virulence on maize. The authors concluded 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 corn smut fungus *Ustilago maydis* utilizes a high-affinity iron uptake system composed of a high-affinity iron permease and an iron multicopper oxidase. Experiments with genetic knockouts demonstrated that this system is important for virulence (84). Fungi also utilize low-affinity iron transport systems, as exemplified in rice false smut, where a probable divalent metal transporter plays a role in virulence (89).

Bacterial pathogens of mammals can "pirate" iron by specifically importing host iron-binding proteins such as transferrin, along with their bound iron (90). Some phytopathogenic bacteria utilize similar strategies. For example, *Petrobacterium* utilizes a "Browninan rachet" to import plant ferritin as an iron source (91). Studies such as these illuminate the complex "tug of war" for iron inside the plant and demonstrate that iron acquisition is central to pathogen success.

Mechanisms through which plants interfere with pathogen iron acquisition

Considering the importance of iron scavenging for microbial fitness and virulence, it stands to reason that hosts might have evolved mechanisms to sequester iron from pathogens during infection, resulting in a tug of war for this essential nutrient (Fig. 1*B*). Indeed, this "nutritional immunity" became apparent in the 1940s for animals, including humans, and is now well-established (92–94). For example, the mammalian hormone hepcidin is deployed to block iron transport and retain intracellular iron pools, particularly in macrophages (95). Concurrently, mammals utilize sidoclarin to bind pathogen siderophores to further limit their capacity for iron assimilation (96). Analogous iron sequestration strategies have not been described in plants, but some lines of evidence indicate that iron restriction is a component of plant immunity. For example, plant genes encoding iron-binding ferritins (*FER*) are upregulated in many plants following infection, including potato tubers during infection by the oomycete *Phytophthora infestans* and in *Arabidopsis* during infection by the bacteria *D. dadantii* (97, 98). *Arabidopsis* deficient in *FER* expression are more susceptible to *D. dadantii*. Interestingly, *FER* gene expression is activated by application of siderophores but is not triggered by *D. dadantii* deficient in siderophore production or by application of iron-bound siderophores. This suggests that *FER* upregulation is triggered by the plant's perception of iron scavenging, and not the siderophore itself. These findings indicate that *Arabidopsis* competes with *D. dadantii* for iron during infection (98). In another study, overexpression of *FER* in *Nicotiana tabaccum* inhibited oxidative damage from virulence activity of necrotrophic fungal pathogens, thereby providing resistance (70). The ectopically expressed *FER* also prevented paraquat-induced ROS damage, suggesting the sequestration of iron to limit the Fenton reaction is effective against some pathogens. Despite these interesting results, little follow-up work has been done, especially with biotrophic pathogens, and the role of *FER* in plant-pathogen interactions warrants further exploration (99).

A second iron-sequestering protein with a known role in pathogen responses was also recently identified in *Arabidopsis* (100): Plant defensin proteins (PDF1.1, 1.2, and 1.3) are immune-regulated and inhibit the growth of fungal plant pathogens *in vitro* (101–104). Like FER, AtPDF1.1 binds iron at high affinity. The *AtPDF1.1* gene is induced in response to pathogen invasion and is secreted into the apoplast. *Arabidopsis* lines in which *PDF1.1* is silenced exhibit enhanced disease caused by the necrotrophic bacterium *Pectobacterium carotovorum*. Conversely, lines overexpressing *PDF1.1* exhibit an iron deficiency response and are more resistant to *P. carotovorum*. The resistance to bacteria can be mitigated by exogenous application of iron, and the overexpression lines also exhibit restriction of iron in the apoplast, consistent with a role in nutritional immunity similar to that proposed for FER. However, *PDF1.1* overexpression lines also exhibit a systemic activation of immune responses that appears to be mediated at least in part through a burst of ethylene production, as discussed further below. Altogether, these data indicate that *PDF1.1* might have a dual role in resistance as an iron sink and as a trigger of ethylene-dependent immunity (100).

Although the above examples suggest that iron restriction is an important immune response in some pathosystems, iron does not appear to be a limiting nutrient in others. For example, iron appears to exist in the apoplast at concentrations greater than 1 μM in bacterially infected leaves, and some bacterial siderophores are dispensable for virulence (105). This enigmatic aspect is further highlighted in a study that used a transcriptomic approach, in combination with plant mutants compromised for immunity, for an unbiased survey of how bacterial gene expression is altered during growth in plants in which PTI or ETI is activated. The bacterial transcriptomic data demonstrated that 69 of 133 previously reported iron-responsive genes were reprogrammed in plants activated for PTI or ETI, compared with transcriptomes from bacteria grown in disease-susceptible plants (106). In general, bacterial genes that are repressed by iron were also repressed by plant immunity, suggesting that a component of plant immunity is to repress genes involved in iron uptake. Repressed genes included a master regulator of iron homeostasis (pyoverdine sigma factor, *pvdS*), and transgenic bacteria overexpressing *pvdS* could partially overcome the growth restriction imposed by activation of plant immunity, thereby demonstrating that reprogramming of iron-uptake genes is a relevant mechanism for suppression of bacterial growth. The mechanism through which the plant manipulates bacterial iron-related genes remains to be established but does not appear to correlate with iron concentration in the apoplast. Further exploration of this and other mechanisms through which plants interfere with pathogen iron acquisition will likely provide important insight into plant immunity along with the mechanisms and physiological relevance of iron scavenging by plant pathogens.

Iron as a tool in plant immunity

Whereas iron sequestration is proven to provide pathogen resistance in mammals and may play a similar role in plants, iron can also be a powerful weapon for the plant when wielded against pathogens. Recruitment of iron to infection 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, and this correlates with increased susceptibility to this hemibiotrophic fungal pathogen (107). Maize recruits ferric iron to the infection site against corn powdery mildew (*Blumeria graminis*), and application of iron chelators decreases resistance to this pathogen (108).

The mechanistic connection between recruitment of iron and a successful immune response can be explained at least in part by iron's capacity to produce ROS. ROS are crucial components of plant immunity with multiple roles: They can act as second messengers, transmitting perception of a pathogen to nearby cells, they can promote oxidative cross-linking of cell wall components, and they can also act as direct weapons against microbes, which lack the capacity to detoxify large quantities of free radicals (109). ROS generation begins quickly after pathogen perception and has been linked to the action of iron-containing transmembrane NADPH oxidases that generate superoxide radicals in the apoplast (109, 110). Superoxide ions act as a signal for further immune events, as precursors for additional reactive oxygen species, and as an apoplastic toxin against microbes (111). The plant cell membrane is impermeable to superoxide, but superoxide can be converted to membrane-permeable hydrogen peroxide (H_2O_2) by superoxide dismutases, some of which contain iron as a cofactor (112).

Recently, an innovative study in rice revealed a new role for iron as a central executioner of HR cell death during ETI, through a mechanism called ferroptosis (23) (Fig. 1*D*). Ferroptosis was first characterized in mammalian systems and is triggered by iron accumulation and concurrent loss of antioxidant protection by GSH (113). The subsequent accumulation of ROS is exacerbated by the iron-dependent Fenton reaction and leads to peroxidation of lipids, which in turn initiates cell death. Dangol *et al.* (23) reported multiple lines of evidence that ferroptosis is the causative mechanism for HR cell death in response to an avirulent isolate of the rice blast pathogen *Magnaporthe oryzae*. First, Fe^{3+} ions and H_2O_2 accumulate in rice leaf sheath cells that are in proximity to *M. oryzae* infection structures, prior to HR cell death. Second, HR cell death is suppressed by treatment with an iron chelator and by chemical inhibitors of lipid peroxidation, GSH transport, and NADPH oxidase activity, all of which rendered the plant susceptible to the otherwise avirulent *M. oryzae* strain. A virulent strain of *M. oryzae* did not induce accumulation of Fe^{3+}, but application of the small molecule erastin, which triggers depletion of the antioxidant GSH and induction of ferroptosis, was sufficient to induce HR-like cell death during infection by the virulent strain. These results are very interesting, because a major unresolved aspect of ETI is how HR cell death is executed following effector perception (27). A recent paper used a similar approach to provide evidence for ferroptotic cell death in *N. benthamiana*, caused by a fast-replicating mutant of tobacco mosaic virus, but it is not clear how cell death caused by the hypervirulent virus relates to HR cell death (114). Thus, it will be of great interest to determine whether ferroptosis is a trigger of HR cell death during ETI in other plant-pathogen interactions.

Regulatory overlap between iron deficiency and pathogen stress

One of the most important aspects of the relationship between iron homeostasis and immunity stems from evidence of regulatory connections between these processes (99, 115). A number of papers provide evidence that imposition of iron stress can also activate immune responses. One of the first clues to this connection came from experiments in which purified siderophores from bacterial phytopathogens were applied to *Arabidopsis* and shown to activate immune responses (116). The siderophore chrysobactin is required for the virulence of *Dickeya dadantii* on *Arabidopsis* and also stimulates immunity. Importantly, chrysobactin loses its immune-triggering capability when it is applied already bound to iron (117). Similarly, Aznar *et al.* showed that iron deficiency responses and immunity are activated in *Arabidopsis* by treatment with deferoxamine, a derivative of a bacterial siderophore, and by a synthetic siderophore, EDDHA (118). Importantly, these siderophores also fail to trigger immunity when applied in iron-bound forms (117). Thus, it seems likely that siderophores trigger plant immunity as a result of their iron-scavenging activity (*i.e.* via perturbation of plant iron levels) rather than molecular recognition of the siderophore itself as a PAMP. As mentioned above, a similar immune-stimulatory effect was linked to iron-scavenging activity of the *Arabidopsis* PDF1.1 protein (100). Finally, strong connections between iron deficiency responses and induced systemic resistance, triggered by beneficial rhizosphere bacteria, have been discovered recently; bacteria that stimulate ISR can also stimulate the iron deficiency response, due in part to regulatory pathways shared between these processes, which encompass ethylene, auxin, nitric oxide, and the MYB72 transcription factor. These microbes might have dual utility as biofertilizers and biopesticides. These findings have recently been reviewed elsewhere (115, 119).

How might cross-talk between iron deficiency response and immunity occur? One obvious connection lies in hormone and small-molecule signaling sectors that are employed by both responses (99, 115) (Fig. 1*C*). Nitric oxide (NO) and ET have been well-established as positive regulators of iron deficiency responses and as activators of immune responses (120, 121) (Fig. 1*C*). The immune response hormone salicylic acid is another potential connection, first noted during the experiments with application of siderophores and explored further in a notable recent paper (122), where Shen *et al.* imposed iron stress on *Arabidopsis* in a hydroponic system and documented that iron deficiency was accompanied by elevated levels of SA and induction of SA-responsive immune gene expression. Several *Arabidopsis* mutants deficient in SA signaling or biosynthesis were less sensitive to iron deficiency (*i.e.* displaying reduced chlorosis and reduced inhibition of root growth). These morphological phenotypes correlated with an increase in soluble Fe in the roots. Finally, induction of several iron homeostasis genes was disrupted in the SA mutants grown under iron-deficient conditions.

At a broad level, these observations indicate that NO, ET, and SA signaling induced by iron deficiency may regulate the expression of a range of genes through which plant immunity is controlled (123) (Fig. 1*C*). Recent molecular experiments have identified regulatory modules involved in iron deficiency response that also act at the intersection of hormone and ROS signaling and immune responses. For example, FIT directly regulates MYB72, a transcription factor that is induced by ET and NO, as well as microbes and iron deficiency (124, 125). In turn, MYB72 regulates systemic immunity and iron acquisition by

controlling biosynthesis of coumarins, which facilitate iron uptake from the soil. These coumarins are bioactive against fungal pathogens, while not impacting plant growth–promoting bacteria, thereby sculpting the microbiome to favor the plant (126). In addition to interacting with clade IV bHLHs and BTS-like, which controls its regulatory capacity and stability, respectively, FIT also interacts with ET signaling transcription factors EIN3 and EIL1, which promotes its stability and contributes to iron acquisition (127). Application of the ROS H_2O_2, has also been shown to stabilize FIT. However, this stabilization requires the presence of the ROS-inducible transcriptional regulator ZAT12, which is required for ROS signaling. Similar to EIN3 and EIL1, ZAT12 interacts with FIT, suggesting that ROS prevents FIT degradation through its interaction with ZAT12. However, loss of ZAT12 function leads to increased accumulation of *FIT* transcript abundance. The repressive effect of ZAT12 on *FIT* transcript abundance, and consequently Fe uptake, indicates a more complex relationship whereby oxidative stress finely tunes FIT abundance, both transcriptionally and posttranscriptionally (128, 129).

In addition to controlling the iron deficiency response by regulating FIT binding partners, ILR3 also directly represses the expression of many genes that encode proteins essential for glucosinolate synthesis in *Arabidopsis* (130). Glucosinolates are secondary metabolites found only in the Brassicaceae family of plants that are produced in response to wounding to protect against herbivorous insects. Consequently, PYE/ILR3 help control wounding response caused by cyst nematode infection, by modulating glucosinolate accumulation under iron deficiency (131, 132).

There is also increasing evidence that glucosinolates play a role in protection against bacterial and fungal pathogens (133), including *Sclerotinia sclerotiorum*, and *Colletotricum* sp. (134, 135). ILR3 also regulates NEET, which transfers Fe-S clusters between proteins in intracellular organelles and the cytosol, playing a critical role in ROS accumulation (136, 137). Finally, ILR3 interacts with the Alfalfa mosaic virus coat protein and positively regulates ROS accumulation and pathogenesis-related protein 1 (PR1) mRNA, SA, and JA accumulation, providing a more direct link between iron homeostasis and disease resistance in plants (137).

BTS (Brutus), the iron-binding E3 ligases that likely post-translationally regulate many of the transcription factors that control iron response, and the rice ortholog, OsHRZ (138), may also play a role in disease response. In *Arabidopsis* BTS interacts with AtVOZ1 and AtVOZ2, which are NAC transcriptional regulators that repress tolerance to abiotic stress conditions yet activate defense response to fungal and bacterial infection (139, 140). Consequently, iron conditions may impact disease response through BTS/HRZ-mediated iron sensing. These connections provide a basis to further explore the mechanisms of cross-talk between iron and immunity and ultimately to understand their adaptive significance. Another critical priority is to extend the evidence for iron-immunity cross-talk from model systems into crops, particularly those for which iron biofortification is being engineered.

Current efforts to improve iron content and bioavailability in crops

Anemia caused by iron deficiency afflicts nearly 1 billion people worldwide, with disproportionate impacts on women and children under the age of 5 (141). This disease is estimated to underpin 5% of all global disability, ranking it ninth among the Global Burden of Disease Project's most pressing issues (142). Iron biofortification of food crops has the potential to help millions avoid anemia (143). 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. Biofortification efforts have the added benefit of increasing overall plant tolerance to alkaline soils, a condition that substantially decreases soil iron bioavailability and is prevalent in over 30% of arable land worldwide. Recent achievements in iron biofortification have been reviewed recently (144, 145). Below, we summarize current progress toward this goal to set the stage for consideration of how linkages between iron and immunity might affect the capacity of biofortified crops to resist diseases.

Modern crop varieties offer decreased genetic diversity compared with that of their wild ancestors. Introgression of alleles from wild ancestors to modern crops has increased iron content, particularly in wheat (146). In addition, 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 and increased bioavailability through breeding (147). Leveraging available germplasm, they have achieved iron content targets in beans, millet, and other crops (148) (Rubyogo, J.-C., and Kasuga, R. (2018) Fighting iron deficiency: new improved high-iron and zinc beans released in Tanzania; http://www.pabra-africa.org/fighting-iron-deficiency-new-improved-high-iron-zinc-beans-released-tanzania/) (Accessed 04/06/20). However, in important staples, including rice, wheat, and maize, breeding efforts have been insufficient to raise iron content to desired levels. For example, the basal iron content in polished rice is typically 2 μg/g, and biofortification targets are typically 14 μg/g (149). Additionally, the use of wild germplasm to achieve biofortification targets may impose yield penalties or affect 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 and bioavailability. Vasconcelos *et al.* (144) organizes these efforts into four categories: increased iron storage in edible tissues, increased iron uptake and translocation, alteration of the iron deficiency response, and combinatorial approaches.

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 3-fold by expression of soybean ferritin under an

endosperm-specific promoter (150). Expression of ferritin under constitutive promoters does not greatly alter the content in the edible tissues (151). Additional approaches have been taken to increase iron bioavailability 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 of the YSL family in rice, soybean, sweet potato, and wheat increases iron content, mobility, and availability (145, 152).

When soybean ferritin was expressed alone in rice, the iron accumulation was not commensurate with the increased storage capacity (153). Expression of VIT genes using endosperm-specific promoters also led to modest increases in seed iron content in rice, wheat, and cassava (145). 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 (154). The most successful examples of this have been accomplished in rice (144). Iron mobility is raised synergistically through both up-regulation of importers like YSL2 and production of iron chaperones through NAS (155). These alterations increase free iron, so when ferritin is expressed in the endosperm, the rice grains store even more iron. Trijatmiko *et al.* (156) use a combinational approach to achieve field-grown rice with 15 μg/g of bioavailable iron, reaching targets for biofortification. When Narayanan *et al.* (157) engineered plants to co-express both *Arabidopsis* IRT1 and FER1 in cassava, they observed up to an 18-fold increase in iron content, yet a 7-fold increase by overexpressing AtVIT1 alone. In other promising studies, genes for nicotianamine synthesis were co-expressed with a combination of FER and YSL, resulting in an up to 4-fold increase in iron seed content (145).

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. Overexpression of IRO2, an iron-related bHLH transcription factor, in rice improved growth on iron-deficient soils, with an incremental increase of iron in rice grains (158). The RNAi-based silencing of IRO2 diminished induction of immune-related genes following iron deficiency (PR1) (138). Overexpression of a mutated version of the *Arabidopsis* transcriptional regulator IDT1 (bHLH34) in tobacco caused constitutive activation of the iron deficiency response, which doubled the iron content (159). Finally, loss of function of the BTS and OsHRZ proteins in *Arabidopsis* and rice, respectively, increased seed iron content and increased tolerance to iron deficiency (160) yet led to increased embyro lethality in *Arabidopsis* (53). Such pleiotropic effects may be a consequence of biofortification efforts that involve small genetic changes in iron uptake and regulatory processes. However, our growing understanding of the regulation of iron metabolism may allow for targeted alterations that maximize effects while minimizing off-target impacts (161, 162). In exactly this context, in the next section we will discuss potential tradeoffs that manipulation of iron will have on disease resistance.

How might biofortification impact plant disease resistance?

Franza and Expert noted in 2012 that the interconnections between iron homeostasis and immunity hold implications for how biofortified crops could respond to pathogens (76). We agree that plants with higher levels of iron might either be more resistant or more susceptible to disease, with the outcome depending on several variables (Fig. 2). The first is the nature of the genetic modification used for iron biofortification. Strategies that are organ-specific (*e.g.* seed-specific overexpression of ferritin) might be less impactful than are strategies based on uptake and transport or on alteration of regulatory pathways that could have systemic effects on iron status. Another key variable is the pathogen; different pathogens infect different organs, employ different iron acquisition strategies, and have differing requirements for iron. Some pathogens are biotrophs or hemibiotrophs that are vulnerable to HR cell death (23); others are necrotrophs that induce cell death, in some cases through ROS generation (70). Plants tailor their response to various pathogens, whether sequestering iron away from the pathogen or concentrating iron at the infection site to produce ROS, consequently initiating HR cell death. Additional factors include iron availability in the soil and the composition of the microbiome, both of which could influence iron status even in crops that are biofortified.

Considering all these variables, we cannot make a single, all-encompassing prediction of whether biofortification will generally help or hurt plant resistance to disease. In some plant-pathogen interactions, iron biofortification could impede disease resistance. Most obviously, increased iron could nullify the plant's iron sequestration mechanisms as was demonstrated for PDF1.1-dependent resistance in the interaction of *Arabidopsis* and *Pectobacterium* (100). Systemically elevated iron could also make plants more susceptible to necrotrophic pathogens that trigger ROS production and cell death. Finally, elevated iron could interfere with mechanisms through which plants utilize iron deficiency as an immune-inducing danger signal (Fig. 2). If such signals are important for disease resistance under field conditions, then elevated iron could be a major detriment to disease.

On the other hand, plant iron biofortification could be neutral in many scenarios or could even improve disease resistance. For example, overexpression of ferritin or other iron-sequestering proteins could mitigate the damage from ROS production triggered by necrotrophic fungi (demonstrated in Ref. 70) or limit availability of iron for pathogens to scavenge as a nutrient. For biotrophs, systemically elevated iron could facilitate ROS generation and initiation of HR cell death, by ferroptosis or other mechanisms that would be effective in resistance (Fig. 2). This effect might be particularly beneficial for crops grown on iron-limited land, for which iron deficiency might hinder effective immunity (*e.g.* see Ref. 107). Excess iron could also nullify

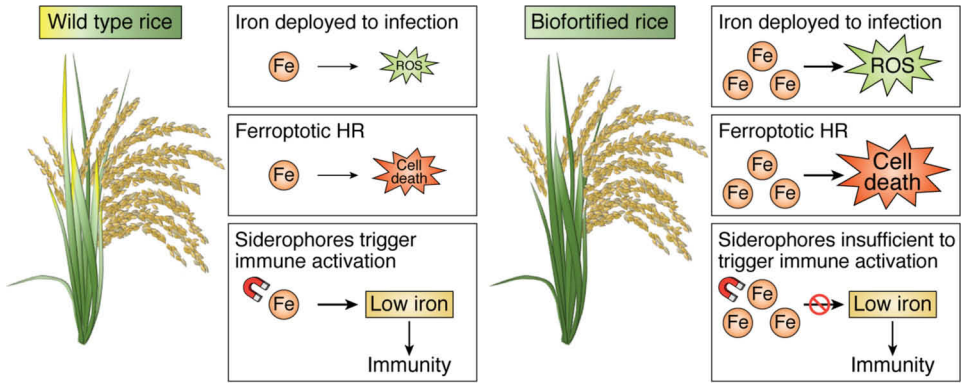

Figure 2. Biofortification of iron will influence plant immunity. In the Poaceae, iron is delivered to infection sites to facilitate the ROS burst. In rice, iron accumulates following ETI, leading to ferroptosis. In all plants, low iron availability, from microbial siderophores or iron-sequestering proteins, triggers immunity and the iron deficiency response. In biofortified plants, especially those with up-regulation of genes related to iron uptake and mobility, these responses may be altered. Additional iron at infection 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.

the benefits that siderophores provide by mitigating oxidative stress (76). Interestingly, exogenous application of iron suppresses disease in turfgrass by mechanisms that are currently unknown but may be due to production of ROS as in maize (163) or repression of iron uptake from the rhizosphere as seen after exogenous application of iron to plant shoots (164). Finally, manipulation of the soil microbiome holds great promise for simultaneously enhancing iron uptake and promoting disease resistance, although the interactions between beneficial microbes and iron-biofortified crops remain to be thoroughly explored (115, 119).

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 been shown to be safe and to provide the iron needed to improve the health of those suffering from anemia (165). However, as we have outlined in this review, biofortification has the potential to influence the interactions plants have with their pathogens. Many important questions remain unanswered about the relationship between iron homeostasis and plant immunity. Through what mechanisms do plants monitor iron and utilize that signal to activate immunity? Is iron sequestration effective against diverse plant pathogens, and can it be engineered into crops? What is the role of iron in the hypersensitive response, and do other plants use ferroptosis to initiate HR cell death? How can the iron scavenging and siderophores of beneficial microbes be leveraged for crop protection?

Using model species, we seek answers to these questions using both breeding and transgenic approaches in the laboratory. In the future, it will be critically important to extend findings from model systems into crops, particularly under field conditions. We also strongly urge the incorporation of disease screening into breeding and assessment of biofortified crops to identify unintended effects on disease resistance or to identify potentially useful enhanced resistance.

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Abbreviations—The abbreviations used are: ROS, reactive oxygen species; PAMP, pathogen-associated molecular pattern; PTI, pattern-triggered immunity; ETI, effector-triggered immunity; R protein, resistance protein; HR, hypersensitive response; EDDHA, ethylenediamine-di(o-hydroxyphenylacetic) acid; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; YSL, yellow stripe-like; bHLH, basic helix-loop-helix; TF, transcription factor; FIT, Fe deficiency–induced transcription factor; NAS, nicotianamine synthase.

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