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REVIEW PAPER

Keep talking: crosstalk between iron and sulfur networks fine-tunes growth and development to promote survival under iron limitation

David G. Mendoza-Cózatl[1,*,†], Arun Gokul[2,†], Mogamat F. Carelse[2], Timothy O. Jobe[3], Terri A. Long[4] and Marshall Keyster[2,5,*]

[1] Division of Plant Sciences, C.S. Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA
[2] Environmental Biotechnology Laboratory, Department of Biotechnology, University of the Western Cape, Bellville, 7535, South Africa
[3] Botanical Institute and Cluster of Excellence on Plant Sciences (CEPLAS), University of Cologne, D-50674 Cologne, Germany
[4] Plant and Microbial Biology, North Carolina State University, Raleigh, NC 27695, USA
[5] DST-NRF Centre of Excellence in Food Security, University of the Western Cape, Bellville, 7530, South Africa

[†] These authors contributed equally to this work.
[*] Correspondence: mendozad@missouri.edu and mkeyster@uwc.ac.za

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Abstract

Plants are capable of synthesizing all the molecules necessary to complete their life cycle from minerals, water, and light. This plasticity, however, comes at a high energetic cost and therefore plants need to regulate their economy and allocate resources accordingly. Iron–sulfur (Fe–S) clusters are at the center of photosynthesis, respiration, amino acid, and DNA metabolism. Fe–S clusters are extraordinary catalysts, but their main components (Fe^{2+} and S^{2-}) are highly reactive and potentially toxic. To prevent toxicity, plants have evolved mechanisms to regulate the uptake, storage, and assimilation of Fe and S. Recent advances have been made in understanding the cellular economy of Fe and S metabolism individually, and growing evidence suggests that there is dynamic crosstalk between Fe and S networks. In this review, we summarize and discuss recent literature on Fe sensing, allocation, use efficiency, and, when pertinent, its relationship to S metabolism. Our future perspectives include a discussion about the open questions and challenges ahead and how the plant nutrition field can come together to approach these questions in a cohesive and more efficient way.

Keywords: Dynamic cell economy, iron sensing, iron use efficiency, nutrient crosstalk, sulfur homeostasis.

Introduction

Plants are among the select group of organisms capable of synthesizing all the molecules needed to complete their life cycle from raw chemical elements (e.g. $Fe^{2+/3+}$, Zn^{2+}, Mn^{2+}, and Cu^{1+}), inorganic compounds (e.g. CO_2, NO_3^-, SO_4^{2-}, NH_4^+, and PO_4^{3-}), water, and light. This includes the synthesis of ≥200 000 metabolites (Dixon and Strack, 2003) such as DNA/RNA precursors, amino acids, lipids, and sugars, in addition to proteins and other macromolecules. Considering that plants are sessile organisms and that they often encounter biotic and abiotic stresses such as fluctuating nutrient availability, drought, and pathogens, the ability of plants to thrive under dynamic environments is a biochemical feat. This plasticity, however, comes with a hefty price tag. Nitrate assimilation, for instance, requires the equivalent of 12 molecules of ATP (~87.6 kcal mol^{-1}) to reduce a molecule of NO_3^- to NH_4^+ and be incorporated into the amide nitrogen of glutamine (Bloom *et al.*, 1992). Sulfate reduction, on the other hand, requires reducing power (8 e^-) and twice as much energy as nitrate reduction to provide the sulfide (S^{2-}) required for cysteine biosynthesis (Hell, 1997). Therefore, plants are under constant pressure to identify and allocate the right amount of resources for metabolic processes such as growth, development, and nutrient assimilation. At the same time, plants also need to determine when to delay or even stop growth and development when resources are scarce (i.e. nutrient limitation) or when resources need to be diverted to support other critical processes such as defense (Katsir *et al.*, 2008; Yang *et al.*, 2012). For decades, understanding the basis of this 'cell economy' has been a major driving force in plant biology and it is at the core of the systems biology field, where the goal is to understand organisms as a whole so we can predict their behavior. In addition, and considering the pressing issues of climate change and food security, this knowledge is essential to develop high-yielding resilient crops capable of producing nutritious food with minimal input of pesticides and fertilizers (Khan *et al.*, 2014; Acosta-Gamboa *et al.*, 2017).

In terms of nutrient availability, and like any other economy, plants also experience abundances and shortages of nutrients, sometimes occurring in a single day or even within hours (i.e. light quality or changes in the rhizosphere composition). Thus, plants have evolved mechanisms to sense resource availability and regulate growth and development accordingly. Plants have also evolved mechanisms to store excess nutrients when possible and decrease their uptake when the storage capacity has been surpassed to prevent the accumulation of toxic levels of nutrients (for reviews, see Mendoza-Cózatl *et al.*, 2011; Jeong *et al.*, 2017). This is particularly important for reactive nutrients such as iron (Fe) and copper (Cu), which are needed in relatively low quantities (micromolar range) but can become toxic at relatively low concentrations (submillimolar range). In recent years, it has become clear that there is an active crosstalk between regulatory networks controlling the uptake and use of nutrients (Forieri *et al.*, 2013; Zuchi *et al.*, 2015; Hantzis *et al.*, 2018). This crosstalk is expected as nutrients are incorporated into molecules of diverse composition, but the molecular and physiological mechanisms driving this crosstalk have only begun to be discovered. Also expected is the extraordinary complexity of nutrient crosstalk in multicellular organisms such as plants, where the crosstalk needs to transcend cellular, tissue, and organ barriers and yet be effective and fast enough to cope with environmental changes that may happen within seconds to minutes. In this review, we will summarize recent advances in the field of Fe homeostasis with emphasis on Fe deficiency responses and their interaction with S metabolism. Recent research demonstrates that the crosstalk between Fe and S is critical to survive during longer periods of low Fe availability (Hantzis *et al.*, 2018). Because of the complexity of the topic, we will mainly focus on data from the reference plant Arabidopsis, but also include other species, particularly crops, when the data are available. We will also discuss current challenges in the Fe and S fields that prevent a seamless integration of data from different sources. Finally, our 'Conclusions and perspectives' section aims to begin a broader discussion that we hope will help pave the way for more cohesive research efforts and to speed up the pace and depth of research on one of the most fundamental fields in plant biology: nutrition.

Cell economy is dynamic

Homeostasis is a term that has been used for almost a century to describe the processes that systems use to maintain conditions necessary for survival (Cannon, 1932). Figure 1A shows the standard diagram used in textbooks to describe how homeostasis only occurs when certain conditions are met (Taiz and Zeiger, 2010); that is, optimal growth and development is only achieved when nutrients are available at certain concentrations (demand≈supply; green zone). Below such levels (yellow zone), the demand for nutrients exceeds the supply and therefore organisms, in this example plants, experience a nutritional deficiency that ultimately limits their growth. Conversely, excess of any nutrient (supply>>demand), regardless of their micro- or macronutrient definition, is toxic to plants and negatively affects plant growth and development (red zone). What is usually less described, and often overlooked, is the fact that the green zone is far from being strictly 'stable'. Optimal growth is certainly achieved over the entire gradient

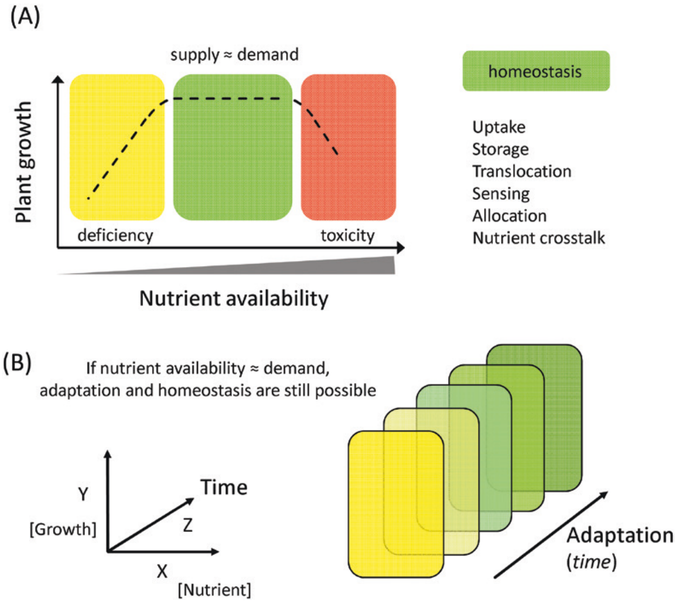

Fig. 1. Cellular homeostasis is based on supply and demand. (A) Nutrient availability is a major determinant for plant growth and development. Optimal growth depends on sufficient availability of nutrients, but, even within the optimal growth space (green), plants need to adapt their nutrient use efficiency and these adaptations may include allocating resources to maximize nutrient uptake (green–yellow border) or activate storage mechanisms to reduce uptake and prevent toxicity (green–red border). (B) Adaptation to environmental changes that include fluctuations in nutrient availability is time dependent (*x*-axis) and plants have evolved different levels of responses in an attempt to maintain or re-gain homeostasis. These may include immediate responses such as re-mobilization of transporters to the plasma membrane, transcriptional activation, nutrient sparing, and re-prioritization of nutrient use.

of the green zone, but the concentration of nutrients can vary greatly within this optimal zone. For instance, plants growing under nutritional conditions near the boundary of the deficiency zone must maximize their nutrient use efficiency to ensure optimal growth and have few opportunities for nutrient storage. On the opposite side of the green zone (right), plants growing under nutritional conditions near the boundary of the toxicity zone may be able to store nutrients for future needs without experiencing any visible toxicity. For Fe, these storage compartments are mainly the cell wall and the vacuole (Lanquar *et al.*, 2005; Ivanov *et al.*, 2014), but not the chloroplast (Reyt *et al.*, 2015). S on the other hand, is primarily stored in the vacuole in the form of sulfate (Kataoka *et al.*, 2004), but can also be stored as S-containing compounds, such as glutathione, sulfolipids, and glucosinolates (Bourgis *et al.*, 1999; Rennenberg and Herschbach, 2014). Hence, the breadth of the green zone depends on the plant's ability to sense nutrient availability and adjust their metabolism accordingly. This adaptation may require adjustments in nutrient uptake, intracellular compartmentalization, changes in source-to-sink relationships, use of stored resources, prioritization of protein synthesis, and nutrient sparing/recycling to optimize nutrient use efficiency (Blaby-Haas and Merchant, 2017; Hantzis *et al.*, 2018). Some of these processes can be activated within seconds/minutes (i.e. phosphorylation or ubiquitination), while others may require minutes to hours (i.e. changes in gene expression and protein translation) and even days (i.e cell and tissue re-programming and/or nutrient sparing). Consequently, plants may experience transitions between deficiency or toxicity zones due to changes in nutrient availability and growth requirements but, as long as the plant's responses are adequate and sufficient, plants may be able to return to their green zone (Fig. 1B). These adjustments may require time, but it is the basis of adaptation. They are dynamic and should be included in any experimental design and discussions related to plant nutrient homeostasis.

The role of iron–sulfur clusters in plant metabolism

At the core, literally, of mitochondrial respiration, photosynthesis, amino acid and purine metabolism, DNA repair, and sulfate and nitrate assimilation are a particular type of cofactors called iron–sulfur (Fe–S) clusters (reviewed by Balk and Pilon, 2011). These are rather simple chemical complexes that are thought to pre-date even life itself, occurring naturally in the early reducing Earth's atmosphere where oxygen levels were low. Due to their delocalized π-electrons, Fe–S clusters are extraordinary catalysts with redox potentials ranging between −600 mV and +400 mV. Therefore, it is not surprising that they were selected early in evolution as prime catalysts and are now essential for plant metabolism. The most common Fe–S clusters found in plants are the 2Fe–2S and 4Fe–4S ferredoxin type clusters, which are coordinated by four Cys residues; the 2Fe–2S Rieske-type clusters coordinated by two Cys and two His residues, and the recently described 2Fe–2S found in NEET proteins, which are coordinated by three Cys residues and one His residue (Nechushtai *et al.*, 2012; Balk and Schaedler, 2014). Fe–S clusters are the major sink for Fe in plants, and proteins containing Fe–S clusters are present in plastids, mitochondria, cytosol, and the nucleus (Balk and Pilon, 2011). Incidentally, Fe^{2+} and S^{2-} are among the most toxic nutrients/intermediates in plant metabolism; thus, plants need to tightly coordinate Fe uptake with S assimilation. In the following sections, we will briefly summarize our current understanding of Fe homeostasis and, when pertinent, its relationship to S metabolism.

Regulation of iron homeostasis

Fe in soils is often found as precipitates that are not readily available for plants. To solubilize Fe, plants have evolved different mechanisms to increase Fe availability. These include reducing Fe^{3+} complexes to Fe^{2+} for uptake (Strategy I) or releasing Fe-binding molecules to the rhizosphere, known as phytosiderophores (Strategy II), to facilitate the uptake of Fe–phytosiderophore complexes into the root system (for a review, see Hindt and Guerinot (2012). Dicotyledonous plants, such as *Arabidopsis thaliana*, rely on Strategy I to mine Fe from the soil, and the main components of this reduction-based system are: a plasma membrane H^+-ATPase (AHA2), that acidifies the rhizosphere to increase Fe solubility, a FERRIC REDUCTASE OXIDASE2 (FRO2), which reduces Fe^{3+} complexes to Fe^{2+}, and an IRON REGULATED TRANSPORTER1 (IRT1), which takes up Fe^{2+} from the rhizosphere into root cells (recently reviewed by Jeong *et al.*, 2017; see Fig. 2). The expression and protein abundance of these three components, known as the Fe regulon, is controlled at both transcriptional and post-transcriptional levels depending on Fe availability (Mendoza-Cózatl *et al.*, 2014; Wild *et al.*, 2016; Dubeaux *et al.*, 2018). In Arabidopsis, transcriptional activation of the Fe regulon during Fe deficiency depends on several transcription factors (TFs) including the FER-LIKE Fe-DEFICIENCY INDUCED TRANSCRIPTION FACTOR [FIT, basic helix–loop–helix bHLH029] and the redundant bHLH038/039 pair (Colangelo

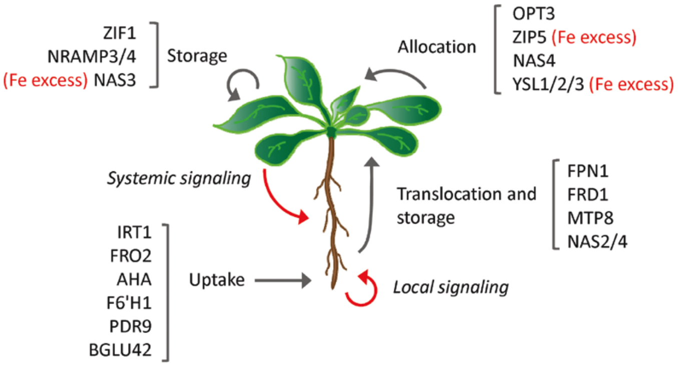

Fig. 2. Overview of Fe uptake and allocation in the reference plant Arabidopsis. Gray arrows represent the four major processes regulating Fe homeostasis in Arabidopsis including uptake, storage, release, solubilization (nicotianamine synthesis), translocation, and re-circulation of Fe through the phloem. Red arrows represent the two major points controlling Fe uptake, a local signaling that controls the localization and abundance of IRT1 at the plasma membrane in root epidermis cells and a systemic signal that controls the Fe uptake machinery at the transcriptional level. For simplicity, only the names of proteins and transporters are shown, and the transcriptional regulation of their corresponding genes is discussed in the text.

and Guerinot, 2004; Yuan *et al.*, 2008). In addition to these bHLH TFs, other TFs such as MYB10 and MYB72 have also been identified as regulators of the Fe deficiency downstream of FIT. MYB10 and MYB72 are required to induce the expression of NICOTIANAMINE SYNTHASE 4 (NAS4), an enzyme that catalyzes the synthesis of nicotianamine (NA), which is an Fe-binding molecule that allows the mobilization of Fe within plant tissues. Failure to induce *NAS4*, as shown in the Arabidopsis mutant *myb10myb72*, affects plant survival under Fe-limiting conditions (Koen *et al.*, 2013; Knuesting *et al.*, 2015). More recently, MYB72 has also been shown to regulate the synthesis of an additional type of Fe-chelating molecules known as coumarins, more specifically scopoletin (Stringlis *et al.*, 2018). Coumarins have antimicrobial properties, and the release of scopoletin into the rhizosphere helped in re-shaping the microbiome for the benefit of the plant. Interestingly, MYB72 was previously found to interact physically with SLIM1 (Van der Ent *et al.*, 2008), one of the very few TFs known to regulate S assimilation, but how the interaction between MYB72 and SLIM1 impacts Fe and/or S homeostasis is currently not known.

An additional transcriptional network, known as the POPEYE (PYE) network, has also been identified and characterized in Arabidopsis (Long *et al.*, 2010). PYE is also a bHLH protein and interacts with additional PYE-like proteins induced during Fe-limiting conditions including bHLH104, bHLH115, and ILR3 (bHLH105) (Long *et al.*, 2010; Zhang *et al.*, 2015). Furthermore, PYE-like proteins interact with BRUTUS (BTS), which is an Fe-binding E3 ligase found in Arabidopsis and rice that is also induced during Fe-limiting conditions (Kobayashi *et al.*, 2013; Selote *et al.*, 2015). In contrast to PYE-like proteins, however, BTS is a negative regulator of the Fe deficiency response and targets PYE-like proteins for degradation, thus dampening the original Fe deficiency response. The structure of BTS is fascinating. Besides being an E3 ligase, BTS also contains three hemerythrin (HHE) domains capable of binding Fe (Selote *et al.*, 2015). It has been suggested that this domain is critical to sense when sufficient Fe levels have been restored after inducing the Fe uptake machinery (Rodríguez-Celma *et al.*, 2019). Once activated, BTS targets positive regulators of the Fe deficiency response to prevent an Fe overload.

Fe acquisition in grasses (Strategy II) is fundamentally different from that in dicots in the sense that Fe complexes, instead of free Fe^{2+}, are taken up from the rhizosphere. This chelating strategy requires the release of Fe-chelating molecules known as phytosiderophores. In rice and maize, the main phytosiderophore is 2-deoxy-mugineic acid (DMA), which is synthesized from NA and released from roots by Transporter Of Mugineic acid 1 (TOM1) (Nozoye *et al.*, 2011). Once Fe^{3+}–DMA complexes are formed, transporters from the YELLOW STRIPE family (ZmYS1 in maize and OsYSL15 in rice) translocate the Fe–phytosiderophore complexes into root cells (Curie *et al.*, 2001; Lee *et al.*, 2009). Similar to Arabidopsis, many of these Fe uptake genes are induced during Fe deficiency and are also transcriptionally regulated by orthologs of the TFs previously discussed. For instance, the BTS ortholog in rice is called Hemerythrin motif-containing Really interesting new gene and Zinc-finger protein 1 (OsHRZ1) (Kobayashi *et al.*, 2013). OsHRZ1 is thought to be a negative regulator of the Fe deficiency response by modulating the activity of OsIRO3 (the rice PYE ortholog), which in turn coordinates the expression of the bHLH TF gene *OsIRO2* and the synthesis of NA. OsIRO2 is also a homolog of bHLH039 and regulates the biosynthesis of DMA and the expression of OsYSL15. In contrast to Arabidopsis, however, rice has two additional transcriptional regulators named Iron Deficiency-responsive *cis*-acting Element binding Factors 1 and 2 (IDEF1 and IDEF2), which also control the expression of *OsIRT1*, *OsIRO2*, and *YSL2*, and the biosynthesis of phytosiderophores (Ogo *et al.*, 2008; Kobayashi *et al.*, 2009, 2014). Notably, DMA and NA share the same S-containing precursors, L-Met and *S*-adenosylmethionine (SAM), offering a first glimpse of how Fe and S metabolism may be connected. As we will discuss later, however, the crosstalk between Fe and S networks may have evolved in a species-specific manner based on the demand for both metabolites and the different environments where plants natively grow. For instance, dicots such as Arabidopsis rely solely on Strategy I for Fe uptake, while maize, a monocot plant, relies mostly on Strategy II (Curie *et al.*, 2001; Vert *et al.*, 2002; Nozoye *et al.*, 2011). Rice, however, and despite being a monocot, seems to have functional components of both strategies and they are thought to be active depending on the growth conditions and Fe availability (i.e. anaerobic paddy soils or aerobic dry soils) (Ishimaru *et al.*, 2006; Ricachenevsky and Sperotto, 2014). Such environmental plasticity suggests that the crosstalk between Fe and S networks may have evolved differently across species and therefore understanding the mechanistic basis of this Fe–S crosstalk may require studies in a species-, tissue-, and environmental-specific conditions.

From the root to the shoot

Once inside the root, Fe needs to be chelated by either organic acids or NA, and subsequently transported to organelles such as the mitochondria and plastids (for Fe–S cluster assembly), the vacuole (for storage), or the xylem parenchyma for subsequent root to shoot translocation (Fig. 2). In Arabidopsis, Fe is thought to be loaded into the xylem by FERROPORTIN 1 (FPN1), where it is once again chelated by either citrate or NA and moved to the aerial part of the plant through the transpiration stream (Morrissey *et al.*, 2009). Allocation of Fe within the shoots is mediated by transporter proteins from different families including the OLIGOPEPTIDE TRANSPORTER 3 (OPT3), which loads Fe into companion cells for source to sink long-distance transport through the phloem (Mendoza-Cózatl *et al.*, 2014; Zhai *et al.*, 2014), and YELLOW STRIPE-LIKE transporters, particularly YSL2, which distributes Fe–NA complexes from the xylem into neighboring cells (DiDonato *et al.*, 2004). In grasses such as rice, Fe allocation between tissues follows a similar path, with two major differences derived from the unique anatomy of grasses and the chelating strategy used to take up Fe^{3+}–DMA from soils. First, while both DMA and NA can bind Fe, Fe^{3+}–DMA in the cytosol of root cells is reduced by ascorbate to form Fe^{2+}, which binds NA to form Fe^{2+}–NA complexes that are then loaded into the xylem stream (von Wiren *et al.*, 1999; Weber *et al.*, 2008). However, because Fe^{2+}–NA complexes are more stable at the pH of the cytosol and the phloem sap (pH 7.2) and less stable at the acidic pH of the xylem sap or vacuolar lumen (pH 5.0), Fe in the xylem sap and vacuole is transferred to and chelated by citrate and DMA, while in the cytosol and phloem sap Fe is predominantly bound to NA and proteins (Mendoza-Cózatl *et al.*, 2008; Ariga *et al.*, 2014). The second major difference between monocots and dicots are the nodes, which is the place where most of the xylem to phloem transfer occurs for long-distance transport of Fe into sink tissues, including young leaves and grains. In rice, these processes are mediated by members of the YSL family such as OsYSL2, OsYSL15, OsYSL16, and OsYSL18 (Aoyama *et al.*, 2009; Lee *et al.*, 2009; Kakei *et al.*, 2012).

While the transcriptional network regulating Fe uptake in roots has been reasonably well described, mostly from work on Arabidopsis seedlings, the transcriptional regulation of Fe deficiency in leaves remains largely unknown. Whole-genome transcriptome profiling of Arabidopsis leaves during Fe deficiency has shown that transporters such as OPT3, ZIF1, a vacuolar NA transporter (Haydon *et al.*, 2012), and the NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN 4 (NRAMP4), which releases Fe from the vacuole (Lanquar *et al.*, 2005), are induced during Fe deficiency (Fig. 2; Schmidt and Buckhout, 2011; Khan *et al.*, 2018). In contrast, the Zrt- and Irt-like Protein 5 [ZIP5; which is also a phloem-localized Fe transporter (Mendoza lab, unpublished)] and YSL1/2/3 are repressed (Klatte *et al.*, 2009), suggesting that limited mobilization of Fe may be a component of adaptation during Fe limitation. In addition, an RNA sequencing (RNA-seq) experiment in Arabidopsis leaves experiencing Fe deficiency for 3 d described a re-programming of plastid metabolism presumably to prevent oxidative stress from reactive molecules such as tetrapyrroles (Rodriguez-Celma *et al.*, 2013). Interestingly, this single point transcriptome experiment also identified a discrete set of genes in leaves that are part of the Fe regulon in roots, including the TFs bHLH038, bHLH039, and bHLH100. Considering that these bHLHs have historically been associated with root-specific responses and that FIT, the master regulator of the Fe regulon, is also considered a root-specific TF, the up-regulation of bHLH038/039/100 in leaves suggests that there may be a leaf-specific Fe regulon. However, the leaf targets of these bHLHs as well as the mechanism regulating their expression in response to changes in Fe availability have yet to be identified.

Fe deficiency responses through time

As described in previous sections, adaptation requires time and, while several major players of the Fe deficiency response have been identified, a dynamic and integrative view of these responses, at the physiological and transcriptional level, is still lacking, particularly when it comes to: (i) leaf responses to Fe deficiency; (ii) the crosstalk between leaves and roots; and (iii) the crosstalk between nutrients such as Fe and S. Time-series experiments and co-expression analyses in Arabidopsis roots over 72 h of Fe deficiency led to the initial identification of BTS and, more recently, the identification of additional candidates that may be part of the gene regulatory network mediating Fe deficiency responses in roots (Koryachko *et al.*, 2015). These types of time-dependent experiments have not been performed in leaves, but recent data from Fe limitation over a period of 7 d (sampling occurring every day), together with previous gene expression data, begin to offer a more complete picture of the different regulatory layers required to adapt and survive under Fe-limiting conditions (Hantzis *et al.*, 2018; Fig. 3). For instance, the leaf ionome of plants transferred from medium containing 10 μM Fe to medium with 10 nM Fe remained virtually unchanged for 2 d, probably due to stored Fe in the cell wall and vacuoles, and only Fe and Mn levels were found to be significantly lower after that. Changes in photosynthetic activity were not detected either until the second day of Fe deficiency. Interestingly, the only mineral that was found to be accumulated at higher levels was S, which is consistent with previous observations showing that sulfate transporters are induced

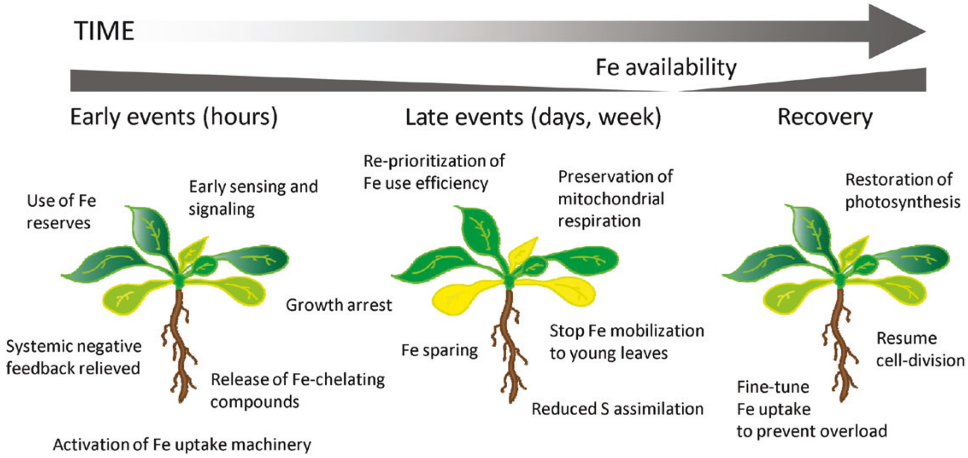

Fig. 3. Dynamic view of Fe deficiency responses in Arabidopsis. During Fe deficiency, Arabidopsis activates different mechanisms to restore Fe levels within the plant, and the magnitude of these responses depends on the severity and duration of the Fe deficiency. Early events include the re-localization of IRT1 to the plasma membrane followed by the release of Fe reserves from the cell wall and the vacuole. If needed, more dramatic events such as growth delay, Fe sparing, and re-prioritization of Fe use are set in motion. All these programmed events are critical for survival and eventual recovery if external Fe once again becomes available (Fe re-supply).

by Fe deficiency (Hantzis *et al.*, 2018). In addition, younger leaves were the first ones to display chlorosis, which is consistent with transcriptional data showing that YSL1/2/3, which mediate the allocation of Fe to sink tissues, are repressed during Fe deficiency (Klatte *et al.*, 2009). Changes after day 2 were somewhat unexpected, but give us some insight into the sophisticated mechanisms that plants have evolved to cope with changes in nutrient availability. As discussed before, the major sink for Fe are Fe–S clusters, and Fe^{2+} and S^{2-} are among the most toxic nutrients. In turn, two key enzymes in the S assimilation pathway, adenosine 5' phosphosulfate reductase (APR) and sulfite reductase (SiR), were strongly repressed (Hantzis *et al.*, 2018). Notably, *SiR* is not typically induced by S deprivation, but it has been found consistently repressed during Fe limitation, suggesting that in the absence of Fe, plants actively prevent the accumulation of S^{2-} (Forieri *et al.*, 2017; Hantzis *et al.*, 2018). Additional chloroplast proteins mediating Fe–S cluster assembly, SUFA and SUFB, were also decreased. However, the down-regulation of Fe–S metabolism seemed to target chloroplasts specifically. In fact, 7 d after Fe deficiency, no significant changes in mitochondrial respiration were detected, suggesting that maintenance of mitochondrial function was prioritized over chloroplast function. Interestingly, this rather dramatic re-programming of photosynthetic tissues allowed plants to fully recover when Fe supply was restored after 7 d of deprivation.

Resilience during Fe limitation requires one more staggered event at the organ level: growth delay. As discussed previously, re-allocation of resources to support processes critical for survival is part of adaptation. Several hormones have been shown to play different roles during Fe limitation (reviewed by Hindt and Guerinot, 2012). However, the underlying molecular mechanisms mediating hormone signaling in response to Fe availability are just being uncovered. For instance, DELLA proteins, which are repressors of gibberellin (GA) signaling and partially control root growth, have been shown to accumulate in the root meristem during Fe deficiency, thus restricting root growth while allowing FIT to become active in epidermal cells within the root differentiation zone (Wild *et al.*, 2016). A similar process in leaves has not been described, but an auxin–cytokinin circuit has been associated with delayed leaf growth due to resource re-allocation to promote stem growth during shade avoidance (Jaillais and Chory, 2010). While reduced root and shoot growth are hallmarks of Fe deficiency, there has to be a distinction between a programmed growth delay that conserves resources and activates uptake mechanisms that may eventually restore nutrient levels (transition between the yellow and green zone in Fig. 1B), and stunted growth due to nutritional deficiencies that cannot be overcome by any homeostatic mechanism (yellow zone; Fig. 1B). Altogether, the data suggest that Fe deficiency responses include a well-defined series of events (summarized in Fig. 3) where, upon sensing Fe limitation, Fe uptake is activated, the release of Fe reserves from the cell wall and vacuole is initiated, the mobilization of Fe to young tissues is paused, and growth is delayed. If these adjustments are insufficient to regain homeostasis, additional steps such as Fe sparing, cessation of sulfate reduction and Fe–S assembly in the chloroplast, and preservation of mitochondrial metabolism are set in motion. At the center of all these processes is Fe sensing, which is critical to fine-tune Fe uptake to prevent an Fe overload and to resume growth once Fe levels have been restored. Fe sensing, however, is perhaps one of the major open questions in the Fe field that certainly deserves immediate, careful, and systematic examination.

Fe sensing and the role of negative feedback loops

Supply and demand models have been successfully used to describe the behavior of metabolic pathways and cellular systems under different scenarios, and one of the major regulatory mechanisms that consistently emerge in these models is the need for negative feedback loops (Hofmeyr and Cornish-Bowden, 2000; Cornish-Bowden and Cardenas, 2001). These demand-driven feedback loops are very effective at fine-tuning biological processes to prevent unnecessary use of resources (i.e. ATP) but also to prevent the accumulation of potentially toxic levels of nutrients or metabolic intermediates (Creissen *et al.*, 1999; Mendoza-Cózatl and Moreno-Sanchez, 2006). Assimilation of both Fe and S in plants is subject to strict negative feedback loops that prevent the accumulation of toxic levels of Fe or S intermediates such as S^{2-}, and disruption of these regulatory loops often leads to aberrant accumulation of Fe or S intermediates with negative impact on plant growth and fitness (Creissen *et al.*, 1999; Hofmeyr and Cornish-Bowden, 2000; Mendoza-Cózatl and Moreno-Sanchez, 2006; Wild *et al.*, 2016; Khan *et al.*, 2018).

More than 15 years ago it was first described that Fe homeostasis in plants had two components: a local sensing system in roots and a systemic shoot to root signaling system that allows leaves to communicate the Fe status of the whole plant to roots and regulate Fe uptake accordingly. The underlying mechanisms regulating the local and systemic regulation have recently been found to be fundamentally different. Local signaling relies on post-translational modifications, while the systemic signaling operates mostly at the transcriptional level. For instance, in roots, the large cytosolic histidine-rich loop in IRT1 is capable of sensing the amount of transition elements available for uptake, including Fe (Wild *et al.*, 2016). Under Fe-sufficient or excess conditions, IRT1 recruits the machinery to trigger its monoubiquitination followed by its removal from the plasma membrane to be held in internal vesicles or to be degraded. Variants of IRT1, where the ubiquitinatable lysine residues (K159 and K174) were mutated to arginines, showed uncontrolled metal uptake leading to plant death (Barberon *et al.*, 2011). Note that this autoregulation does not make IRT1 a *transceptor*. By definition, a transceptor is a transporter protein capable of moving a substrate across a membrane while independently transducing a signal leading to a defined transcriptional output [as shown for the nitrate transceptor NRT1.1 (Ho *et al.*, 2009)]. Since no transcriptional output has been identified as a result of the metal binding to the His loop, describing IRT1 as a transceptor is, if anything, premature. This feedback-mediated autoregulation of IRT1 however, does represent a form of Fe sensing and it is certainly the first line of defense aimed at preventing an overload of Fe or other IRT1 substrates such as Mn, Zn, or the non-essential element Cd.

Systemic signaling, on the other hand, relies on a phloem mobile signal originated in leaves that leads to either the induction of the Fe regulon in roots or its repression. The chemical nature of this mobile signal is under intense scrutiny and has not been unequivocally identified. However, results from the Arabidopsis mutant (*opt3*), in which shoot to root communication of the Fe status is impaired, suggests that (i) proper loading of Fe into the phloem is required to synthesize/stabilize the mobile signal and (ii) the signal is unlikely to be free Fe^{2+}, as *opt3* mutants overaccumulate large amounts of mobile Fe in roots but fail to repress the Fe regulon (Mendoza-Cózatl *et al.*, 2014; Zhai *et al.*, 2014). Moreover, these results confirm previous studies suggesting that leaves dictate the transcriptional behavior of the root system and that the loss of the shoot to root feedback signaling has the capacity to surpass the local autoregulation of IRT1, leading to an overaccumulation of Fe, Zn, and Mn in roots and leaves.

Small peptides have been shown to play important roles in root to shoot and shoot to root communication, and to regulate processes such as nitrogen signaling and nodulation (Imaizumi-Anraku *et al.*, 2010; de Bang *et al.*, 2017). A small family of peptides in Arabidopsis, but ubiquitous within plants, have recently been identified as components necessary to induce the root ferric-chelate reductase FRO2 (Grillet *et al.*, 2018). These 50+ amino acid long peptides, generically called IRON MAN (IMA), are rapidly induced upon Fe deficiency in both leaves and roots, and are predicted to be phloem mobile. Furthermore, an Arabidopsis mutant lacking all eight members of the gene family (*ima8x*) was unable to induce the activity of FRO2 (Grillet *et al.*, 2018). However, grafting experiments between *ima8x* scions and wild-type rootstocks displayed wild-type levels of FRO2 activity, suggesting that while IMA peptides are required for activation of FRO2, shoot to root transport of these peptides is not. Interestingly, these peptides are capable of binding Fe^{2+}, $Cu^{1+/2+}$, Mn^{2+}, and Zn^{2+}, and, when saturated, they precipitate from the aqueous solution. These results led the authors to suggest that the instability of IMAs in the presence of transition elements may be part of a negative feedback system required to regulate Fe uptake (Grillet *et al.*, 2018). This is certainly an exciting hypothesis worth pursuing, and future research will be needed to elucidate the precise mechanism by which IMAs help in regulating Fe homeostasis.

The role of leaves in Fe sensing and, more specifically, that of the leaf vasculature has also recently been explored in greater detail (Khan *et al.*, 2018). Using dynamic imaging of phloem-specific Fe deficiency markers, it was determined that the leaf vasculature is among the first tissues within the plant that respond to Fe deficiency. The transcriptional activation of these phloem-specific markers occurred hours before the up-regulation of the Fe regulon in roots, which in turn suggests that leaves have their own Fe-sensing mechanism, independent of roots. The use of non-destructive dynamic imaging allows the tracking of transcriptional responses in real-time and over long periods of time, offering a very detailed view of the magnitude of these responses. Figure 4 shows a representative trace of the luciferase activity (reporter gene) driven by the OPT3 promoter when plants experience Fe limitation and Fe re-supply (from Khan *et al.*, 2018). It is tempting to suggest that the oscillatory behavior of the reporter gene is the result of circadian regulation, which has been shown to control in part the expression of Fe-regulated genes, including IRT1 (Hong *et al.*, 2013). However, oscillation of OPT3 is evident during Fe-replete conditions, but the magnitude of the oscillations is negligible compared with the changes observed during Fe limitation. Moreover, the repression of the reporter gene when Fe supply was restored occurred in a relatively short period of time (i.e. <2 h) and lacked any sort of oscillatory behavior. Considering that transcriptional responses for Fe deficiency are under tight control by negative feedback loops, the oscillatory behavior of *OPT3* during Fe limitation is more likely to be the result of Fe levels being transiently restored within the vasculature by the release of Fe reserves (e.g. cell wall and vacuoles) and the later activation of Fe uptake. Once Fe levels are restored, even if this is transient, the expression of *OPT3* is repressed until the levels of Fe become limiting again, leading to the up-regulation of *OPT3*. These high-resolution experiments offer another glimpse of the significant negative feedback regulation controlling Fe deficiency responses and the high sensitivity of the Fe sensing system in leaves.

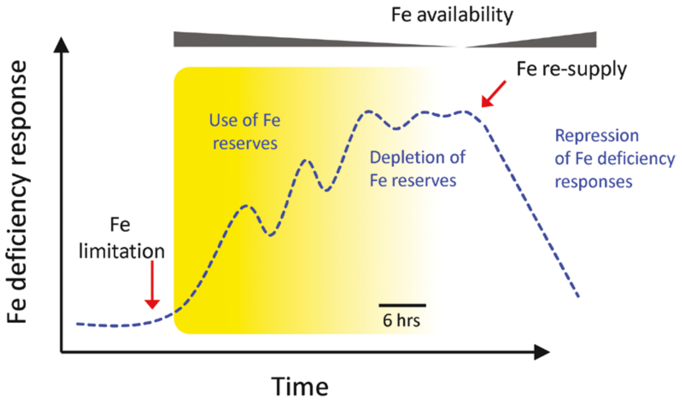

Fig. 4. Iron homeostasis is tightly regulated through negative feedback loops. The dotted line is a representation from experimental data describing the timing of Fe deficiency responses in Arabidopsis leaves. The original data were obtained by tracking the activity of the reporter gene luciferase driven by the Fe-responsive OPT3 promoter (from Khan *et al.*, 2018). In the original experiment, plants were transferred from replete conditions to Fe deficiency conditions and then back to Fe-sufficient conditions at the indicated times (red arrows). The oscillatory behavior of the reporter gene during the initial phase of Fe deficiency is discussed in the text as a function of possible circadian regulation but also as the result of negative feedback loops.

Evidence for an additional negative feedback loop within the Fe homeostatic network came from the characterization of Arabidopsis mutants lacking the bHLH100/101 TFs (Sivitz *et al.*, 2012). Transcriptional profiling of this mutant under Fe limitation showed that Fe deficiency-responsive genes such as *BTS*, *OPT3*, *ORG1*, *bHLH039*, *ZIF1*, and *FRO3* were up-regulated to higher levels in leaves compared with wild-type plants. These results suggest that bHLH100/101 may be necessary to induce negative regulators of the Fe deficiency response in leaves. In this context, the role of BTS—a negative regulator of Fe deficiency responses—becomes relevant again. In the Arabidopsis double mutant *bhlh100/101*, BTS is active and even overexpressed during Fe deficiency conditions, so why are *OPT3, ORG1, bHLH039* and others genes transcribed at higher levels compared with wild type under similar growth conditions? The answer may come from a recent characterization of BTS-like proteins and an additional allele of BTS (*bts-3*) (Hindt *et al.*, 2017). *bts-3* overaccumulates Fe, and other transition elements, in roots, leaves, and seeds due to constitutive activation of the Fe regulon in roots, thus confirming the role of BTS as a negative regulator of the Fe deficiency response. However, the constitutive activation of the Fe regulon in *bts-3* under replete conditions is a minor fraction (a fifth) of the full wild-type response to Fe limitation and, more significantly, *bts-3* was unable to activate the Fe regulon further under Fe deprivation (Hindt *et al.*, 2017). These results suggest that there may be additional negative regulators, on top of the BTS network, regulating root and leaf responses when Fe becomes limiting.

Crosstalk between Fe and S homeostatic networks.

Crosstalk between nutrients is critical for optimal nutrient use efficiency and to prevent the accumulation of toxic intermediates. Over the last decade, there has been increasing evidence suggesting an active crosstalk between Fe and S networks in different plant species. These data include physiological and molecular evidence in dicots and monocots but, most strikingly, as we discussed previously, this Fe–S crosstalk seems to be species specific (Ciaffi *et al.*, 2013; Paolacci *et al.*, 2014; Zuchi *et al.*, 2015; Forieri *et al.*, 2017; Garnica *et al.*, 2018). One example of crosstalk between Fe and S networks is the cessation of sulfite reduction under Fe limitation discussed previously (Hantzis *et al.*, 2018; Fig. 3), but the opposite scenario has also been demonstrated. During S limitation, plants are unable to fully induce their Fe uptake machinery, which includes the Fe regulon in dicots but also the release of phytosiderophores in plants using Strategy II for Fe uptake such as barley, maize, and wheat (Zuchi *et al.*, 2015; Garnica *et al.*, 2018). These observations are further evidence that S and Fe networks are closely connected. While the underlying mechanisms of this crosstalk remain to be identified, transcriptome analyses of Arabidopsis plants experiencing S deficiency showed that the TF ZAT12 is highly induced in roots (Bielecka *et al.*, 2014). ZAT12 has been implicated in several processes, mostly related to stress. Notably, ZAT12 was found to physically interact with FIT, and this interaction negatively affects the induction of the Fe regulon (Le *et al.*, 2016). Certainly, crosstalk between S and Fe networks is likely to exist at additional levels, but the ZAT12–FIT interaction provides a solid clue to further explore how these signaling networks communicate with each other. The interplay between ILR3 and PYE may also play a role in this crosstalk. Glucosinolate (GL) synthesis requires S assimilation and appears to be partially regulated by ILR3, which is transcriptionally elevated in *pye-1* mutants. Therefore, ILR3 and PYE may regulate opposing sides of the GL synthesis under Fe deficiency in order to conserve S for the synthesis of Fe-binding by NA or other processes (Samira *et al.*, 2018). In addition, the previously discussed MYB72–SLIM1 physical interaction is another example of the crosstalk between Fe and S networks, but the consequences of this interaction are at the moment uncertain (Van der Ent *et al.*, 2008). Interestingly, it was recently discovered that the crosstalk between Fe and S networks might be regulated by transcriptional networks independent of those controlling either S assimilation or Fe uptake separately. This evidence was revealed by exploring long-term responses of Arabidopsis to single or combined nutrient deficiency regimes (Forieri *et al.*, 2017). For instance, transcriptome analyses of Arabidopsis roots experiencing Fe limitation for 5 weeks showed a down-regulation of genes mediating S assimilation such as *SULTR1;1*, *APR*, and *SiR* (Forieri *et al.*, 2017). The down-regulation of SiR is particularly interesting as this gene is not affected by S limitation and suggests that a network independent of the S limitation response can also regulate the expression of S assimilation genes during Fe deprivation. In tomato roots, however, repression of *SULTR1* genes by Fe deficiency was not observed, suggesting that the regulation of the Fe–S crosstalk is different across species (Zuchi *et al.*, 2015). Notably, the repression observed in Arabidopsis was eliminated when plants were subjected to dual Fe and S deficiencies. Similarly, during single S limitation conditions, the expression of key regulators of Fe uptake including *FIT*, *bHLH039*, and *bHLH100* was dramatically reduced. Since Fe–S clusters are the major Fe sink in plants, and Fe–S clusters require equimolar concentrations of these two elements, a plausible explanation for these observations is that in the absence of S, plants reduce their Fe uptake capacity to prevent overaccumulation of Fe that cannot be effectively incorporated into Fe–S clusters. This cross-network repression, however, is eliminated when plants experience dual S and Fe deficiencies, suggesting the existence of a *hierarchical regulation* of Fe and S assimilation where the transcriptional regulation controlling the uptake of one element (e.g. Fe) can over-ride the cross-transcriptional regulation imposed by the absence of the other element (e.g. S; Fig. 5). It should be noted that because of the duration of the nutrient deprivation experiments (more than a week), the changes seen probably represent late stages of plant adaptation and that experiments done at early stages (hours, days) may reveal additional cues about the mechanistic basis of the Fe and S crosstalk network. In addition to time, tissue specificity should be considered in future experiments, particularly when exploring sensing mechanisms. For instance, while the FIT network controls Fe uptake at the root level, this network is downstream of the systemic signal derived from shoots (Mendoza-Cózatl *et al.*, 2014; Zhai *et al.*, 2014). As discussed previously, the transcriptional networks regulating Fe deficiency in leaves are just being uncovered, but short-term, time-series experiments in a tissue-specific manner and under single and dual nutrient deprivation conditions are likely to provide more insight into the mechanisms regulating Fe and S networks at the whole-plant level.

Crosstalk between Fe and S networks has also been explored in grasses, particularly in wheat, and additional levels of regulation between these networks have been uncovered (Ciaffi *et al.*, 2013; Zamboni *et al.*, 2017). Data collected over recent years suggest that a major determinant for these differences is the chelating strategy used by grasses for Fe uptake (Strategy II), which requires the synthesis of phytosiderophores derived from S-containing molecules such as L-Met and SAM (i.e. DMA; Fig. 5) (Mori and Nishizawa, 1987; Suzuki *et al.*, 2006). In turn, Fe deficiency in wheat under sufficient levels of S induced the expression of most of the genes of the S assimilatory pathway, suggesting that part of the Fe deficiency

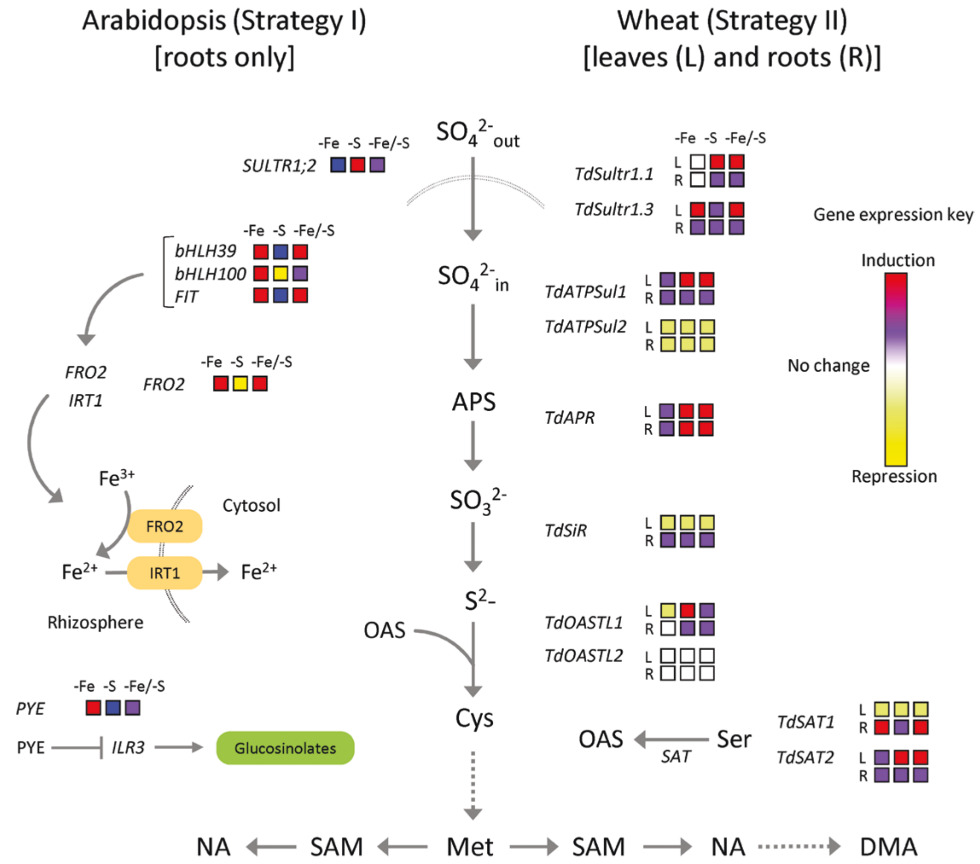

Fig. 5. Crosstalk between iron and sulfur metabolism has distinct signatures during single or dual nutritional deficiencies. The expression of sulfur assimilation and iron uptake genes was measured under single Fe or S deficiencies or combined Fe and S deficiencies (data from Ciaffi *et al.*, 2013; Forieri *et al.*, 2017). The color of the squares represents gene induction or repression compared with plants grown in replete media. The data for Arabidopsis (Strategy I) represents gene expression in roots only, while gene expression in wheat was available for leaves (L) and roots (R). Only the names of the enzymes where gene expression was measured is shown. Dotted lines represent reactions catalyzed by several enzymes. Abbreviations are as follow: *SULTR1;2 or TdSultr1.1*, sulfate transporters; *TdATPSul1*, ATP sulfurylase; *TdAPR*, adenosine 5′-phosphosulfate reductase; *TdSiR*, sulfite reductase; *TdOASTL*, *O*-acetylserine(thiol)lyase; *SAT*, serine acetyltransferase; *FRO2*, ferric chelate reductase; NA, nicotianamine; SAM, *S*-adenosylmethionine; DMA, 2′-deoxymugineic acid.

response in grasses includes the induction of S assimilation to increase the synthesis of phytosiderophores (Ciaffi *et al.*, 2013; Zamboni *et al.*, 2017). Similarly, during S deficiency, grasses often display Fe deficiency symptoms probably derived from their inability to synthesize the S-derived phytosiderophores necessary for Fe uptake (Mori and Nishizawa, 1987). Research in wheat, however, has also shown that the differences between Strategy I and Strategy II plants cannot fully explain the responses observed at the gene expression level and the activity of enzymes within the S assimilation pathways. For instance, the expression of two high-affinity sulfate transporter genes in wheat, Td*Sultr1.1* and Td*Sultr1.3*, was found to be strikingly different during nutrient deprivation experiments. While the induction of Td*Sultr1.1* during S limitation was not affected by Fe availability, the expression of Td*Sultr1.3* was induced by both Fe and S limitation, but the highest expression was found during Fe deficiency at adequate levels of S supply (Fig. 5) (Ciaffi *et al.*, 2013). These results suggest that Fe–S crosstalk in wheat includes additional levels of regulation (transcriptional and post-transcriptional) to fine-tune enzyme activities and the expression of gene isoforms.

Interestingly, separate experiments showed that when S is supplied in excess, wheat accumulates more Fe, and this behavior is also evident when plants experience Fe limitation (Celletti *et al.*, 2016). These results are particularly exciting for wheat production as dual S and Fe scarcity is often found in fields and have a negative impact on yield (Hawkesford *et al.*, 2014). Unfortunately, this increased Fe use efficiency in the presence of excess S was not observed in other grasses such as barley and maize. It was proposed that the rate of phytosiderophore release between species may explain these differences. In support of this hypothesis, the release of phytosiderophores in maize under Fe limitation is moderate compared with barley and wheat (Celletti *et al.*, 2016).

One major theme of this review has been the role that time plays in adaptive responses of plants to changes in nutrient availability (Fig. 1). It is then important to emphasize that the transcriptional responses to Fe, S, or Fe/S deprivation responses, previously described for Arabidopsis (Forieri *et al.*, 2017; Hantzis *et al.*, 2018) and wheat (Ciaffi *et al.*, 2013; Zamboni *et al.*, 2017), represent long-term plant responses, as the deficiency experiments were conducted for >1 week. Transcriptional reprogramming however, happens in phases (Li *et al.*, 2015), and may include rapid/transient changes within minutes/hours to maintain homeostasis. This reprogramming also includes long-term changes (i.e. days/weeks) where plants, based on their nutrient economy, have decided to maintain growth, delay growth, or even trigger an early senescence response (recently reviewed by Sade *et al.*, 2018). In light of the possible transcriptional response scenarios, we should be cautious when jumping to major conclusions related to plant responses to nutrient deficiencies based on single experimental points and consider that plants adapt to changes in their environment and that these changes may be species-, time-, tissue-, and even organelle-specific responses.

Beyond plants—what have we learned about Fe sensing from other systems?

Fe–S clusters are essential components of Fe-sensing mechanisms in organisms ranging from yeast to humans. In the yeast *Saccharomyces cerevisiae*, Fe deficiency responses are regulated by the TFs Aft1 and Aft2 (Rutherford *et al.*, 2001, 2003). Under Fe-sufficient conditions, an Fe–S complex, stabilized by glutaredoxins (GRX3/4) and glutathione, prevents Aft1/Aft2 from moving into the nucleus to activate the Fe uptake machinery. In the absence of Fe–S clusters (i.e. Fe deficiency), Aft1/Aft2 are mobilized to the nucleus where they activate Fe uptake (Pujol-Carrion *et al.*, 2006; Kumánovics *et al.*, 2008). Fe excess in *S. cerevisiae* is also sensed through Fe–S clusters; the bZIP TF YAP5 has two independent Fe–S cluster-binding sites. If these two sites are occupied, YAP5 activates genes mediating Fe detoxification, including the vacuolar Fe transporter *CCC1* (Rietzschel *et al.*, 2015). In mammals, the iron regulatory proteins (IRP1 and IRP2) regulate the stability of mRNAs responsible for iron uptake and homeostasis, and a 4Fe–4S cluster within IRP1 dictates whether it represses or activates Fe uptake (Haile *et al.*, 1992; Walden *et al.*, 2006).

Surprisingly, the role of Fe–S clusters in relation to Fe homeostasis in plants remains vague. Arabidopsis GRX17 is a nuclear-localized glutaredoxin able to bind Fe–S clusters in a similar way to the yeast GRX3/4; however, its function so far has been associated only with the redox signaling necessary for the maintenance of the shoot apical meristem but not in Fe homeostasis (Knuesting *et al.*, 2015). Fe–S cluster assembly in the mitochondria and their translocation to the cytosol are additional processes required for proper Fe sensing in yeast. The mitochondrial ATP-binding cassette Atm1 of *S. cerevisiae* mediates the transfer of Fe–S clusters, or intermediates, from the mitochondrial matrix to the cytosol for proper assembly of cytosolic Fe–S proteins (Kispal *et al.*, 1997). Since Fe–S clusters in the cytosol are necessary for Fe sensing (Outten, 2017), yeast mutants lacking Atm1 overaccumulate Fe due to the constitutive activation of the Fe uptake machinery. The paralog of Atm1 in Arabidopsis is ATM3, and *atm3* mutants display significant chlorosis and reduced rosette size. While the transcriptional status of the Fe regulon in *atm3* has not been explored in detail, IRT1 protein levels were only slightly elevated in *atm3* under Fe-sufficient conditions (Bernard *et al.*, 2009). Based on the presence of several overlapping mechanisms regulating the Fe uptake in Arabidopsis (discussed in previous sections), it would be interesting to explore whether these mild Fe deficiency responses in *atm3* are the result of the significant negative feedback loops that have evolved to prevent an overaccumulation of Fe. Such conservative regulation of Fe homoeostasis would prevent Fe uptake even under Fe-limiting conditions if Fe cannot be properly incorporated into Fe–S clusters, thus preventing cell damage by generation of reactive oxygen species (ROS). Another example of an Fe–S protein that deserves further examination is the Arabidopsis NEET protein (Nechushtai *et al.*, 2012). At-NEET is a 2Fe–2S protein that regulates several plant processes including senescence and ROS metabolism. Interestingly, plants in which NEET expression was decreased by RNAi technology were more sensitive to Fe deficiency and more tolerant to Fe excess (Nechushtai *et al.*, 2012); however, the molecular mechanisms behind these phenotypes remain to be discovered.

Communication between mitochondria and the nucleus, known as mitochondrial retrograde signaling, is another mechanism widely studied in yeast that has recently gained attention in plants and has been proposed to play a role in Fe and S homeostasis (Liu and Butow, 2006; Vigani and Briat, 2015; Vigani *et al.*, 2018). Retrograde signaling is important to adjust metabolism when the function of the mitochondria is impaired either by mutations in respiratory complexes or by nutritional deficiencies. For instance, Arabidopsis mutants with defects in the mitochondrial Complex I show a constitutive activation of genes that regulate Fe uptake and distribution (e.g. *OPT3*, *FRO3*, *BTS*, and *PYE*) (Vigani and Briat, 2015). However, understanding the molecular basis of retrograde signaling in plants is significantly more complex than in yeast, and remains largely obscure. This is due to the additional levels of regulation present in plants (shoot to root communication) plus the presence of plastids, which are also proposed to have a retrograde signaling. One example of this higher complexity is citrate, which is accumulated at higher levels during Fe or S deficiency and has been proposed as a signaling molecule involved in retrograde signaling (Vigani and Briat, 2015; Vigani *et al.*, 2018). In fact, addition of external citrate to Arabidopsis plants results in the induction of *FER1*, which is also induced during Fe excess (Finkemeier *et al.*, 2013). So how can a putative signal of Fe and S deficiency also induce genes that are typically induced during Fe excess? One plausible explanation is that in plants, citrate plays a key role in Fe translocation by keeping Fe soluble in the xylem. External citrate may help to solubilize additional Fe attached to the cell wall, causing the induction of *FER1*. Moreover, aconitase, which is the enzyme that catalyzes the isomerization of citrate into isocitrate in the Krebs cycle, requires an Fe–S cluster as a cofactor. Thus, in the absence of Fe/S availability, some accumulation of citrate may be expected. Finally, in tomato, the accumulation of citrate during Fe and S deficiency happens exclusively in roots, not in shoots (Vigani *et al.*, 2018), and, as we have described throughout this review, shoot signaling plays a major role in dictating the transcriptional responses of roots during Fe deficiency. More work is certainly needed to clarify the mechanisms of retrograde signaling in plants, but a meta-analysis of different transcriptome data from Arabidopsis mutants with diverse mitochondrial dysfunction phenotypes concluded that oxidative stress was probably responsible for the transcriptional reprogramming observed across mutants (Schwarzländer *et al.*, 2012). Therefore, ROS signaling may also be a major player driving the retrograde signaling in plants during Fe, S, or Fe/S deficiency.

Conclusions and perspectives

So, where exactly in the plant is Fe sensed? In several places. How exactly is Fe sensed? In many ways, and most of them are still unclear. However, the progress made in recent years has been outstanding. Novel players have been discovered, old players seem to have additional roles, and new candidate genes hold great potential to move the field forward, so the future certainly looks promising. The recently described role of leaves in Fe sensing is a renewed opportunity to tackle Fe homeostasis from a systems-level perspective and will probably provide a more integrated view of whole-plant responses to changes in Fe availability. Equally exciting is the crosstalk between Fe and S, which remains a fairly open field that is ready to be fully explored. Technologies such as TRAP-seq, DAP-seq, ATAC-seq, proximity labeling, and LAESI-MS (Castro-Guerrero *et al.*, 2016; Kim *et al.*, 2016; Bartlett *et al.*, 2017; Etalo *et al.*, 2018; Sijacic *et al.*, 2018) allow us to track the behavior of genomes, genes, metabolites, and proteins through time at cell-specific resolution. These techniques in combination with bioinformatic tools such as machine learning for the identification of gene and protein regulatory networks will be instrumental to better understand the molecular basis behind the crosstalk between nutrient networks.

Moving forward

A major issue found during the preparation of this review was the enormous difficulty in integrating data sets from different publications. All of them were certainly conducted properly, with well-described conditions and proper controls, but, unless the data came from the same lab, we found that plants were grown in substantially different conditions. For instance, 'replete' Fe conditions ranged from 10 μM to 50 μM (Ivanov *et al.*, 2014; Hindt *et al.*, 2017; Hantzis *et al.*, 2018; Khan *et al.*, 2018), which in turn may impact Fe reserves and the timing/magnitude of Fe deficiency responses. The addition of sucrose to the growth medium was another major variable found across the literature. Understandably, the use of sucrose results in healthier plants for downstream experiments but it will also increase their resilience to stress. Moreover, sucrose will switch the metabolism of plants from autotrophic to mixotrophic and, lastly, a '2-week'-old plant would be in a completely different developmental stage when grown with sucrose compared with plants grown without sucrose as an additional carbon source. These issues are not exclusive to the plant field. The yeast field experienced a similar situation when the regulatory mechanisms controlling glycolysis were being resolved. However, as a good example of what a truly collaborative effort means, different parties came together to agree on what was later described as the *standardized conditions for systems biology* (van Eunen *et al.*, 2010). We, the authors, look forward to beginning similar discussions within the plant field through local seminars, and domestic and international meetings to eventually agree, as a community, on the *standardized conditions for plant systems biology studies*. Without a doubt, this effort will help to advance the pace and depth of research, not only in the Fe and S fields, but all across the field of plant nutrition.

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