Iron-Requiring Enzymes in the Spotlight of Oxygen

Abstract
Iron (Fe) is a cofactor required for a variety of essential redox reactions in plant metabolism. Thus, plants have developed a complex network of interacting pathways to withstand Fe deficiency, including metabolic reprogramming. This opinion aims at revisiting such reprogramming by focusing on: (i) the functional relationships of Fe-requiring enzymes (FeREs) with respect to oxygen; and (ii) the progression of FeREs engagement, occurring under Fe deficiency stress. In particular, we consideredsuch progression ofFeREsengagement asstrainresponsesofincreasing severity during the stress phases of alarm, resistance, and exhaustion. This approachcancontributetoreconcilethevarietyofexperimentalresultsobtainedso far from different plant species and/or different Fe supplies.

Highlights
Iron is an essential cofactor for a variety of redox reactions in plant metabolismand Fe-requiring enzymes (FeRE) catalyse reactions that also involve oxygen, as a reagent/product of the reaction itself or as an entry/end point of a metabolic pathway.
The large amount of data collected by omics technologies revealed that the complex responses of a plant facing iron deficiency involve several metabolic pathways.
Although FeRE are differentially affected in Fe-deficient plants, a clear overview of their involvement during Fe deficiency responses is still lacking.
Fe-oxygen relationship might represent a new point of view to revisit metabolic reprogramming occurring under
Fe deficiency.

Iron-Oxygen Partnership
Iron (Fe) is a ubiquitous element on earth and the evolution of iron biochemistry is heavily intertwined with earth's geochemistry. The central role of iron (Fe) chemistry in biological systems was established shortly after life appeared approximately 3.5 billion years ago, but changed with the oxygenation of the Earth's atmosphere [1] because Fe chemistry and biochemistry are heavily influenced by the presence of oxygen [1,2]. In plants, Fe-requiring enzymes (hereafter referred as FeREs) catalyse reactions that also involve oxygen, where oxygen is a reagent and/or product of the reaction itself or it represents the entry and/or end point of the metabolic pathway in which the enzyme takes part. Except for a few examples, which do not carry out redox activity (i.e., aconitaseand purple acid phosphatase), FeREs display redox functions [2] and can be grouped into six categories listed in the next section. In this opinion article we propose to describe the progress of the engagement of the various FeRE categories taking place during the iron deficiency stress phases of alarm, resistance, and exhaustion, and occurring along with the 'economy of Fe use'.

FeRE Categories
By considering their type of interaction with oxygen as well as their biochemical or metabolic functions, FeREs could be grouped into the following six categories (also explained in more detail below): (i) dioxygenases, that catalyse reactions where both atoms of molecular oxygen

are incorporated into substrates; (ii) monooxygenases, that use molecular oxygen in hydroxylation reactions; (iii) enzymes that scavenge reactive oxygen species (ROS); (iv) enzymes that take part in the oxygen-dependent electron transport chains, such as the photosynthetic electron transport (PET) and the respiratory electron transport (RET); (v) enzymes dependent on molybdenum cofactor; (vi) enzymes involved in the DNA metabolism and repair.

Dioxygenases

Dioxygenases represent a wide class of enzymes, which catalyse reactions involving both atoms of molecular oxygen incorporated into one or more substrates; these enzymes use a variety of reaction mechanisms. A large subclass of dioxygenases is Fe dependent, and in particular, the 2-oxoglutarate/Fe(II)-dependent dioxygenases (20-ODDs), which take part in various oxidative reactions, such as hydroxylation, halogenation, desaturation, epimerization, cyclization, and demethylation [3–5]. Due to their widespread distribution in many metabolic pathways, the effects of Fe deficiency may be due to the 20-ODDs requirements for Fe on almost all metabolic pathways ([6], Box 1).
ThedependenceofODDsonFe(II)suggeststhattheyalsomightactasFesensorswhentheirKmis close to the physiological concentration of free, redox-active Fe ions forming the so-called labile iron pool (LIP) [7]. This hypothesis, although not experimentally confirmed, encourages a functional
screening of this wide class of dioxygenases based on their biochemical parameters (Km for Fe (II)) [5,7,8]. A stochastic simulation of activity of some 20-ODDs shows that dynamically changed availability of exogenous Fe can result in a differential and flexible modulation of 20-ODDs activities [5].

Monooxygenases

The monooxygenases catalyse a reaction in which one atom of oxygen appears in the product while the other one is reduced to water [9], among which is the Fe-heme cytochrome P450 (CYP450) protein superfamily, present in all organisms including plants. CYP450s use NADPH as a reducing molecule and share a common catalytic centre, a heme with Fe coordinated to thiolate of a conserved cysteine. CYP450s contribute to the homeostasis of phytohormones and signalling molecules by controlling their biosynthesis and catabolism. They are also involved in the biosynthesis of pigments; volatiles; antioxidants and defence compounds, including phenolics and their conjugates; flavonoids; coumarins; lignans; and glucosinolates [10–14].
The cytochrome CYP82C4 (At4g31940) is one of the genes highly expressed in Fe-deficient Arabidopsis thaliana roots through a FIT-dependent pathway [15]; its expression highly correlates with the expression of genes involved in the strategy I early Fe-deficiency response

Box 1. Involvement of 20-ODDs in the Metabolic Responses Induced by Fe Deficiency
2-Oxoglutarate/Fe(II)-dependent dioxygenases (20-ODDs) are required for the biosynthesis and catabolism of the plant hormones gibberellins (GAs) (i.e., GA oxidases, [4–6]), the biosynthesis of ethylene (i.e., 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, [5,6]), and for the catabolism of auxin (the irreversible inactivation of IAA to its oxindole
derivative) [5,6]. GAs, ethylene, and IAA are important plant growth regulators; they are also involved in the modulation of plant responses to Fe deficiency [5]. Furthermore, some 20-ODDs (e.g., prolyl 4-hydroxylase P4H) carry out posttranslation modifications (PTMs). The P4H genes are highly expressed in A. thaliana roots and their expression is modulated by different stresses [7]. The impact of Fe deficiency on the catalytic activity of 20-

ODDs would therefore explain the involvement of such pathways in the metabolic responses induced by Fe-deficiency.
20-ODDs enzymes are also involved in the biosynthesis of secondary metabolites, such as flavonoids, benzylisoquinone alkaloid, glucosinolate, tropane alkaloid, monoterpene indole alkaloids, benzoxazinoid, coumarin, and mugineic acid [6].
Coumarin and mugineic acid are directly involved in the Fe deficiency-induced responses; coumarins are derived from phenylpropanoid metabolism and are involved in plant defence but also as reducing molecules assisting Fe uptake [42].
F60H1 is a 20-ODD oxidase, involved in scopoletin biosynthesis [50]. The complete biosynthetic pathway of redox-active metabolites in response to Fe deficiency, deriving from phenylpropanoid metabolism, has been elucidated in A. thaliana: two hydroxylases act in cascade for the synthesis of fraxetin and of the newly identified molecule termed sideretin (5,7,8-trihydroxy-6-methoxycoumarin), from the less oxidized coumarin scopoletin, (i.e., the 20-ODD scopoletin 8-hydroxylase S8H and the cytochrome P450 CYP82C4 [17,18]). These small molecules, fraxetin and sideretin, are secreted from roots through the ABC-type PDR9 transporter [18,51]. These small molecules efficiently mobilize and reduce Fe(III) and
their biosynthesis and secretion may constitute quite a widespread component of the strategy I for Fe-uptake, though this has not been fully appreciated up to now [18]. Notably, At3g12900, coding for 20-ODD scopoletin 8-hydroxylase S8H, is strongly upregulated during early Fe deficiency (6 hours) [16].
Graminaceous plants synthesize mugineic acid-related compounds (named siderophores) through the methionine
pathway and secrete them from roots into the soil to assist Fe uptake. 20-ODDs IDS2, and IDS3 are part of the methionine pathway, being responsible for the oxidation of 20-deoxymugineic acid into 3-epi-hydroxy-deoxy mugineic
acid and mugineic acid, respectively [52].

[16,17]. Indeed, it encodes an enzyme responsible for the last biosynthetic step of sideretin, from the less oxidized fraxetin [18] (Box 1).

Peroxidases and Catalase

Plants cells are exposed to oxygen, and they continuously produce the so-called reactive oxygen species (ROS), i.e., partially reduced or excited forms of oxygen (hydrogen peroxide H2O2, singlet oxygen1O2, ionsuperoxideO2 ⁻,hydroxylradical ⁻OH).InthepresenceofFe(II)andFe(III),theHaber- Weiss reaction can produce the hydroxyl radical ⁻OH from the less reactive H2O2 and O2 ⁻ [19–21].
ROS exert beneficial effects when in a physiological concentration range; however, outside that range, ROS become cytostatic or cytotoxic [19]. Tight control of their concentration is achieved by antioxidant molecules, such as ascorbic acid (ASA), glutathione (GSH), and ROS scavenging enzymes, among which are peroxidases and catalase. Peroxidases contain Fe-heme and they catalyse the oxidation of several molecules by reducing H2O2 to H2O [22]. Catalases contain Fe- heme and dismutate two molecules of H2O2 into water and oxygen. Fe availability therefore influences ROS levels, as well as the activities of ROS scavenging enzymes together with that of enzymes associated with ROS-scavenging pathways [23]. A link between the control of Fe availability, the Fe deficiency responses, and the antioxidant machinery exists; as an example, the zinc-finger protein ZAT12 is required for cytosolic ascorbate peroxidase 1 (cAPX1) expression and ROS signalling in A. thaliana [24,25]. The transcription factor FIT is a central regulator of the Fe-deficiency response and it interactswithZAT12, which acts as a negative regulator of Fe uptake [26].

FeREs in the Oxygen-Dependent Electron Transport Chains

The establishment, in aerobic autotrophic cells, of electron transport chains for oxygenic photosynthesis and respiration (hereafter referred as PET and RET, respectively), leading to

oxygen evolution and consumption, results in a high demand for Fe. Both PET and RET provide energy for cells by transferring electrons across membranes (thylakoids for PET and inner membranes for RET); they play a central role in the primary metabolism. The FeREs belonging to this group, thus taking part in PET or RET chains, possess Fe as either Fe-S clusters or heme groups. PET heavily relies on Fe; each photosystem I (PSI) unit contains a total of 14 Fe atoms [27], each PSII complex (dimer) contains 4 Fe atoms and each functional cytb6f complex necessitates 12 Fe atoms, for a total of at least 30 Fe atoms per linear ETC functional unit [28–30]. RET needs at least 44 Fe atoms per functional unit [31,32]. The impact of low Fe availability on PET and RET has been extensively studied during the last decade (Box 2).

Moco-Dependent FeREs

Moco-enzymes require the molybdopterin cofactor (Moco) [33]; the plant Moco-dependent FeREs are nitrate reductase (NR), xanthine dehydrogenase (XDH), and aldehyde oxidase (AO). These three enzymes have key roles in nitrogen assimilation, purine catabolism, and synthesis of abscisic acid (ABA), respectively [34]. A mutual impact of Mo and Fe homeostasis has been observed in cucumber plants [35]. The enzymatic activities of the various Moco-dependent FeREs are differentially affected under Fe starvation, in either roots or leaves. Such modulation might depend on several factors, such as the relative content of Fe and Mo in the tissues. Indeed, the strong induction of NR activity in Fe- deficient roots might suggest that NR is heavily modulated by Mo levels (Mo strongly accumulates in Fe-deficient roots) and that root NR has priority of Fe use, under Fe deficiency [35].

In addition, Moco biosynthesis is dependent on Fe, as one of its precursors (cPMP) is catalysed by a GTP 30,8-cyclase (CNX2 protein) which contains two Fe-S clusters [36]; cPMP content strongly increases in Fe-deficient roots and leaves [35]. As already observed for NR in roots, FeREs-CNX2 might represent another example of priority of Fe use occurring under Fe deficiency.

FeREs Involved in the DNA Metabolism

FeREs involved in DNA replication and repair do not require oxygen for their catalytic activity; they consist in DNA polymerases, DNA helicases, and DNA primases.

In plants, information concerning the effect of Fe deficiency on DNA stability, replication, and repair is still scarce. UBC13 A/B and RGLG1/2 proteins, which are involved in the differentiation of root epidermal cells, are suggested as nodes in the DNA damage repair mechanisms; UBC13 downregulation under Fe deficiency would prioritize DNA metabolisms [37]. Also, a higher concentration of Fe has been recently observed in the plant nucleolus [38], which represents an important nuclear site for several DNA-repair related proteins [39]. Such nuclear Fe sites might be crucial in controlling the induction of DNA replication and repair mechanisms under low Fe growing conditions [37].

Progression of the Engagement of FeREs during Fe Deficiency: Some Physiological Aspects

Iron deficiency-induced responses in plants are often associated with increased oxygen consumption rates in root tissues [40,41], while both oxygen consumption and evolution decreased in Fe deficient leaves [35]. However, evidence about the impact of Fe deficiency on the dynamics of subcellular oxygen metabolism during the progression of Fe deficiency is still scarce.

The Fe-oxygen partnership allows us to redefine the responses of plants to Fe deficiency in terms of its impact on the various FeREs categories. Recalling the definition of ‘stress’ in the

Levitt as well as in the GAS theories can support this approach (Box 3). We therefore propose to

Box 2. Changes Induced in the Photosynthetic or Respiratory Electron Transport Chains (PET and RET) by Fe Deficiency

PET

The effect of Fe starvation on the light phase of photosynthesis has been analysed in various unicellular organisms [53].

The documented ultrastructural changes observed in chloroplasts under Fe starvation are associated with changes in the composition of the various components of the electron transport chain; for example, a dramatic reduction of thylakoid grana in favour of unstacked thylakoid membranes is observed in barley (Hordeum vulgare), together with an

increase of non-photochemical quenching (NPQ) and a remodelling of the major light-harvesting chlorophyll a/b-binding protein HvLhb1 [54]. Such changes would be aimed at optimizing energy transfer between the photosystems [55].

Fe deficiency affects the stability of the LHC1 subunits more than the PSI core in rice deficient plants [56]. A. thaliana seedlings show a severe reduction of the protein components of the electron transport chain, such as PSI (PsaA), PSII (D1 and the inner antenna protein CP43), as well as a subunit of cytochrome b559 binding heme (PsbE), whereas no alterations in the amounts of the peripheral antenna proteins LHCII were observed [57]. The marginal effects of Fe deprivation on the LHC proteins have been recently confirmed in A. thaliana [46]; in this study, however, the cytb6f appears the most affected PET component, unlike PSI and PSII. These authors also found that the chloroplastic machinery of Fe-S clusters' biosynthesis was strongly affected, in particular the SUFA and SUFB components.

RET

Approximately 30 mitochondrial proteins are differentially expressed under Fe deficiency. Among them, overexpression of external alternative ND DHs proteins as well as proteins related to the TCA cycle were identified. In particular, accumulation of protein (e.g., isovaleryl-CoA dehydrogenase, IVDH) belonging to the branched-chain amino acid

catabolism were identified [35]. This pathway catabolises some amino acids belonging to the aspartate family (e.g., lysine, threonine, methionine). Under energy-limited conditions (like those which occur under Fe deficiency) such an aa family can be degraded in mitochondria providing electrons to the IVDH enzyme and in turn to the electron transfer flavoprotein system, which is a further alternative pathway to transfer electrons to the ubiquinone pool, bypassing complex I and II (the complexes most affected by Fe deficiency) and further providing precursors to TCA [58]. Therefore, under Fe deficiency, cells might induce different alternative pathways to bypass the Fe-requiring complexes of the respiratory chain.

describe the progress of the engagement of the various FeREs categories, occurring under Fe deficiency, with the following stress phases (see below), as reported in Figure 1.

Alarm Phase

If available Fe diminishes within the cell, the homeostatic control of Fe content acts at first on the mobilization of Fe from Fe stores (ferritin and/or vacuolar Fe), to restore Fe availability and to fully satisfy Fe demand. The alarm phase might be triggered when Fe homeostasis fails to efficiently provide Fe to the various FeREs; in such a case, cells start to perceive Fe deficiency. Fe deficiency- induced responses are characterized by the activation of Fe uptake systems [42]. At this stage, total cellular Fe content starts to diminish and LIP can also be altered. It is likely that FeREs are still not affected at the enzyme level in the availability of their Fe-cofactors; however, their expression might already bec hanging if they represent key nodes of the early Fe-deficiency response pathway (Box 1) both in roots [16] and in shoots [43]. The dioxygenases with their Km for Fe close to the physiological concentration of the LIP might be already altered [3,5]. An early engagement of DNA replication and repair-related FeREs would also occur in this phase since it has been suggestedthat DNA metabolism is prioritized under Fe deficiency in order to maintain genome stability and therefore to secure essential housekeeping functions [37]. Also, a

modulation of some ROS- scavenging enzymes might occur; a rise in H2O2 a few hours after exposure to Fe deficiency has been observed in roots of an Fe-deficiency tolerant apple genotype but not in an Fe-deficiency sensitive one [44]. Such early rise in ROS might be a relevant part of this alarm phase.

Resistance Phase

This phase follows the alarm phase, and it occurs when plants' growth starts to be impaired due to Fe deficiency. During this stage, the total Fe content in plant tissues is significantly decreased, leading to impaired distribution of total Fe within cells/plants. A program for Fe economy which occurs under Fe deficiency and targets specific FeREs has been recently described in Chlamy-domonas reinhardtii [45] and in Arabidopsis thaliana [46]. The preferential use of Fe (defined as 'priority of Fe use' [35,45,46]) by some metabolic pathways, at the expense of other pathways, might represent the hallmark of this stress phase. Such strategy of Fe economy would allow a simultaneous modulation of all FeREs, resulting in a decreased content of some of them; indeed, the rates of both PET and RET decrease under Fe deficiency (Box 2), with direct consequences in

Box 3. Definition of Stress in the Biological Systems

The theory of Levitt [59], applied in seed science [60], defined the concept of physical stress as a force (stress) able to deform a body; this deformation (strain) is reversible at first (elastic phase) but, upon intensification of the applied force, that deformation can become irreversible (plastic phase). Such mechanical concepts of stress and strain can be translated into biological systems; the various different environmental factors would represent the 'stress, whereas the changes in the metabolic processes of an organism would constitute the 'strain', whether reversible (elastic phase) or irreversible (plastic phase).

It is possible to define the modulation of the FeREs groups (i.e., the strain in this system, on the extent of perceived Fe deficiency, or the applied stress). The reversible damage that can be repaired by cells constitutes the strain in the elastic range whereas the irreversible damage constitutes the strain in the plastic range and is associated with a failure of repair mechanisms, reaching the breaking point with plant death [60].

Although the concept by Levitt easily explains the relationship between stress and strain, it does not take into account other relevant factors in biological systems, such as duration of the stress and the activation of repair mechanisms (when the strain is still in the elastic range).

A further definition of stress, closely related to a biological system, is based on the general adaptation syndrome (GAS) theory, suggested by the endocrinologist Hans Selye and recently revisited in plant biology [60]. According to this theory, three phases of the link between stress/strain can be described: alarm (stress is perceived), resistance (stress protection mechanisms are activated), and exhaustion (failure to withstand stress).

oxygen metabolism [40,41]. The Fe economy strategy suggests that Fe deficiency firstly affects PET and then RET [45,46], leading to impairment of carbon metabolism and thereby a carbon/ nitrogen metabolisms imbalance [47]. The altered content of other nutrients, such as Mo, might contribute to the modulation of the Moco-dependent FeREs (e.g., NR) [35].

However, an increase in activity of other FeREs, such as some dioxygenases, can be observed during the resistance phase, when their roles support Fe uptake strategies (Box 1). In this case, they would indeed benefit from the Fe economy strategy for their demand of Fe and/or Fe cofactors.

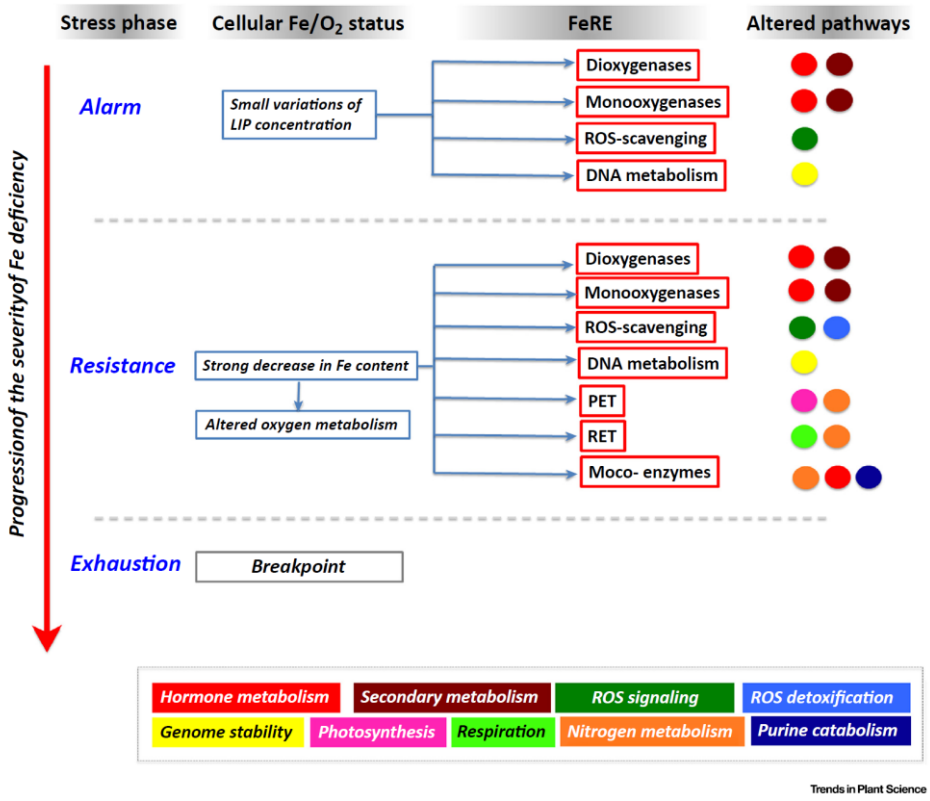

Figure 1. Engagement of Fe-Requiring Enzyme (FeREs) during the Progression of Fe Deficiency. The proposed progression of engagement of various FeREs categories (down- or upregulation) under the various stress phases (alarm, resistance, exhaustion) of Fe deficiency is reported, together with changes in the cellular Fe status and in oxygen metabolism. Breakpoint of the cell occurs in the exhaustion phase, when the severity of Fe deficiency causes irreversible impairment of FeREs, thus leading to cell death. Circles of various colours correspond to the main metabolic pathways affected by the changes in the corresponding FeREs categories. Fe, iron; FeRE, Fe-requiring enzyme; LIP, labile iron pool; PET, photosynthetic electron transport; RET, respiratory electron transport; ROS, reactive oxygen species.

A redox imbalance would be established in the cell leading to a regulation of ROS detoxifying processes [44,48]. During this stage, the ROS scavenging enzymes are engaged in order to avoid oxidative damage [44]. However, the activity of FeREs involved in such reactions (APX, CAT, peroxidases and Fe-superoxide dismutases) decreases in some Fe-deficient plants [48], while it increases in others, that are Fe-efficient genotypes [44]. Hence, the priority of Fe use of the FeREs-ROS scavenging enzymes might influence the genotype susceptibility to Fe deficiency.

An enzymatic and transcriptional regulation (up- or downregulation) of FeREs proteins might occur at this stage. Under Fe shortage, the processes related to DNA replication and repair might be maintained thanks to the 'priority of Fe use', in order to maintain essential housekeeping functions [37].

Exhaustion Phase

Severe or prolonged Fe deficiency leads to growth retardation, stasis, and death [49]. This is likely because many FeREs may display an irreversible deformation, that is, failure of PET and

RET caused by damage and disassembly of their components accompanied by clear ultrastructural changes of chloroplast and mitochondrial membranes (Box 2). Such changes would limit energy provision to the cells and thereby the plant growth. Such conditions would negatively affect the activity of other FeREs subgroups.
Such a proposed progression unmasks several open questions; a more refined scheme of FeREs engagement during Fe deficiency stress phases therefore necessitates further experimental evidences (see Outstanding Questions).

Concluding Remarks

Thanks also to the widespread access to omics technologies, a large amount of data is continuously being acquired on the impact of Fe deficiency in plants [23]. These results reveal that the complex responses of a plant facing Fe nutritional disorder involve several metabolic pathways [31], orchestrated by the 'priority of Fe use' strategy, which implies that an efficient recycling of Fe-cofactors might take place, possibly along with their metabolic channelling.
The classification of FeREs according to their relationship with oxygen, as proposed in this opinion, allows us to consider the different phases of strain responses of the plants under Fe deficiency stress (alarm, resistance, exhaustion) in terms of functional responses of the FeREs groups occurring under Fe economy.
This approach might be useful for better analysing the results obtained in different research groups, which have adopted a variety of experimental plans. Indeed, a given nutritional stress applied to different plant species might modify their proteomes and metabolomes, not only because of the species-specificity of the stress response, but also because it may trigger different strains (i.e., the stress phases described in this article) in the plants analysed (see Outstanding Questions).

Outstanding Questions

Is the affinity of FeREs for Fe (such as in 20-ODDs) responsible for their differential engagement in the Fe deficiency responses? Are FeREs involved in the Fe sensing?
Which reactive oxygen species (ROS) act as signals for Fe deficiency during the alarm phase and which ROS-scavenging FeREs contribute in the changing of ROS levels during such phase?
Do small changes of the LIP contribute in Fe-deficiency signal occurring in the alarm phase and which FeREs contribute in the changing of LIP during such phase?
Are there any FeREs which can be used as species-independent markers of the different phases?
Which is the balance between catabolism and de novo biosynthesis of Fe cofactors, versus their reutilisation to essential FeREs, during the resistance phase of Fe deficiency? Is there a sort of metabolic 'channelling' of such Fe cofactors to favour their use by essential FeREs?

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