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Review

Regulation of plant ferritin synthesis: how and why

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Abstract. Plant ferritins are key iron-storage proteins that share important structural and functional similarities with animal ferritins. However, specific features characterize plant ferritins, among which are plastid cellular localization and transcriptional regulation by iron. Ferritin synthesis is developmentally and environmentally controlled, in part through the differential expression of the various members of a small gene family. Furthermore, a strict requirement for plant ferritin synthesis regulation is attested to by alterations of the photosynthetic apparatus and of iron homeostasis in transgenic tobaccos overexpressing these proteins. Plant ferritin gene regulation appears to consist of a complex interplay of transcriptional and posttranscriptional mechanisms, involving cellular relays such as plant hormones, oxidative steps and Ser/Thr phosphatase.

Key words. Ferritin; iron; homeostasis; development; stress; gene regulation.

Introduction

Iron is one of the major metals involved in electron transfer in biological systems, and is therefore essential to life. However, iron physicochemical properties make this element uneasy to use by aerobic living organisms. In aqueous phase, at physiological pH, it tends to precipitate under insoluble forms. Furthermore, its redox cycling participates in activation of reduced forms of oxygen through Fenton chemistry, leading to lipid peroxidation, protein oxidation and DNA mutations, and consequently to cellular damage [1]. Iron homeostasis thus needs to be strictly controlled, and this is achieved by complex iron trafficking networks that involve chelation and reduction processes [2].

A major molecular component required for iron homeostasis control in eukaryotes is the ferritin, a widely distributed class of iron-storage proteins, consisting of a hollow sphere made of 24 subunits and able to accommodate 4500 iron atoms per molecule inside its internal cavity [3, and see below]. Ferritin iron accounts for 92% of the total iron in mature pea embryos [4], evidencing the major role that ferritins play in plant development. Plant ferritin synthesis is also strictly controlled by the iron status of the cells, being accumulated, in the case of iron excess to store and detoxify iron, in a form unable to react with oxygen [5]. The principal aim of this paper is to review our knowledge of the circuits responsible for ferritin synthesis regulation in relation to the biological function that these proteins might play in plants.

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Plant ferritin genes and protein structure

Ferritin gene organisation divergence between animals and plants

Ferritins are coded by small gene families, both in plants and animals. Comparison of ferritin gene organisation between plants and animals has been made possi-

ble by cloning and sequencing two maize ferritin genes [6], one soybean ferritin gene [7] and one *Arabidopsis thaliana* ferritin gene [Van Wuytswinkel and Briat, unpublished observations]. From an evolutionary point of view, plant and animal ferritins arose from a common ancestor and are highly conserved proteins [3]. However, their gene organisation is remarkably different. Although within each kingdom the number and position of introns are strictly conserved in ferritin genes, these parameters are different among plants and animals. Seven introns in higher plant ferritin genes and three in animal ferritin genes are found at different positions. In animal ferritins, intron position has been correlated with the secondary structure domains of the protein [8], suggesting that contemporary animal ferritin gene structure has evolved by exon shuffling. This is not the case for plant ferritin gene structure, since the secondary structure domains of the plant protein have no apparent relationship with the intron/exon boundaries. Also, the three animal ferritin introns are in frame with exon sequences, whereas four out of seven plant ferritin introns are out of frame. The genetic mechanisms involved in iron regulation of ferritin gene expression are different among plants and animals (see below). At a structural level, it is consistent with the absence of an iron responsive element (IRE), responsible for the iron-dependent translational regulation of animal ferritin messenger RNA (mRNA), in all plant ferritin mRNA and genes characterised so far [9]. However, all the members of the plant ferritin complementary DNA (cDNA) and gene family have not yet been fully characterised [10], and it cannot be ruled out that additional structural features remain to be discovered for this class of plant genes.

Plant ferritin protein: an animal-type ferritin in a prokaryotic environment

Plant ferritin protein has been observed in plant plastids [11], and this subcellular localisation has been confirmed biochemically and immunologically [12–14]. A transit peptide, responsible for polypeptide precursor targeting to plastids [15], was characterised at the N-terminal extremity of plant ferritins [16]. Plant ferritins share an amino acid sequence identity varying between 39 and 49% with animal ferritin, but no significative homology with bacterial ferritins [17], including cyanobacteria [18]. Therefore, plant ferritins, although located in the plastids, appear to be more related to animal ferritins than to bacterioferritins. Relics of a nonfunctional bacterioferritin gene are still observed, scattered throughout the chloroplast genome [16]. It is therefore plausible that during the course of evolution, the endosymbiont bacterioferritin was lost and replaced by a nuclear gene product, homologous to contemporary animal ferritin, with, in addition, a specific sequence in its NH2-terminal extremity responsible for plastid subcellular localisation. The seven amino acids required in animal H ferritin for oxidation and rapid uptake of iron inside the mineral core are conserved in plant ferritin subunits [19, 20]. Despite a higher identity of plant ferritins with H-type animal ferritins, the carboxylic residues found at the cavity surface of L-type animal ferritins, and responsible for an efficient iron nucleation and a better stability of the mineral core, are also observed in plant ferritin subunits. An additional plant-specific sequence is observed at the amino terminus of the protein subunit. The first part of this plant-specific amino-terminus sequence encodes a transit peptide responsible for the plastid targeting of plant ferritin precursors, as already mentioned. The second part of this specific plant ferritin domain belongs to the mature ferritin subunit and was named extension peptide [16]. Nearly half of the residues from this extension region assume a helical conformation which folds back onto the surface of the subunit. Free radical-mediated cleavage occurs within this extension peptide during iron exchanges in vitro [21], and possibly in vivo [22], leading to ferritin degradation (see 'Ferritins and plant development' section). Analysis of mutagenesis has confirmed the role of the extension peptide in plant ferritin protein stability [23].

A high helical content of plant ferritin is consistent with the adoption of a four-helix bundle conformation as described in animal ferritins. A nonhelical stretch of 21 residues showed either turn/coil or β-strand predictions in pea seed ferritin [24], and would link the B and C helices via an L-loop characteristic of the animal ferritin subunit fold [3]. A further consequence of the secondary structure as predicted [24] is the conservation of the short C-terminal E-helix, despite the divergent primary structures of plant and animal ferritins in this region [3]. Ferritin subunits assemble in a compact 24-mer arrangement with 4.3.2 symmetry. The resulting packed protein shell contains many intersubunit interactions except that at the four-fold and three-fold symmetry axes, channels of about 3 Å wide are formed [3]. Using the coordinates of the recombinant human H ferritin structure [20], a model of the three-dimensional (3D) structure of pea seed ferritin subunit has been proposed, highlighting the remarkable conservation of the 3D structure of plant as modelled to human ferritins, and the predicted regions of change [24]. The plant-specific N-terminal extension mentioned above would form a compact domain localised on the external surface of the 24-mer. The pea three-fold channels would remain hydrophilic as in animal ferritins, whereas the four-fold channels would be hydrophilic, in contrast with the mammal hydrophobic four-fold channels. Hydrophobic channels at four-fold axes present an energy barrier to unshielded Fe^{2+}ions,

whereas the hydrophilic three-fold channels have been considered as a possible entry or exit port, and a site for Fe(II) oxidation in animal ferritins [25]. The consequences of both four-fold and three-fold channels being hydrophilic, and the effects this would have on iron exchange between plant ferritins and their environment are unknown.

The nucleation and growth of the iron core are active biomineralisation processes involving functions of the protein coat [3, 26], and demonstration of ferroxidase activity associated with the H-type mammal ferritin has highlighted this point [20]. The predicted ferroxidase activity of plant ferritins [24] has been confirmed experimentally either with purified pea seed ferritin [27] or with recombinant pea seed ferritin expressed in *Escherichia coli* [23]. Ferrous iron is the substrate of the ferroxidase activity. It can be formed in vitro by reduction with ascorbate of exogenous ferric chelates and/or of ferric ferritin-iron itself [28]. Depending on the concentration of ascorbate, either overall iron release or uptake by pea seed ferritin may occur. Photoreduction of ferric citrate and/or of ferritin-iron may also be a source of ferrous iron [29]. Iron fluxes in and out of pea ferritin core are influenced by pH, uptake being faster at alkaline pH. Free iron-binding ligands in excess are inhibitory both of iron uptake and release [28].

Animal ferritin and bacterioferritin mineral cores have different chemical and structural properties [3, 26]. Human and horse ferritin mineral cores are made of a single-domain crystal of ferrihydrite, associated with variable amounts of inorganic phosphate. Bacterioferritin cores are hydrous ferric phosphate, with high amounts of phosphate; they exhibit a poor crystallinity and very low magnetic ordering temperatures. These physicochemical differences between animal and bacterial iron cores have been attributed to variations in phosphate concentration between bacteria and animal cells [30]. Plant ferritin proteins are similar to their animal counterpart, but they are localised within plastids, a prokaryotic-type environment. Such a situation influences the composition and structure of plant ferritin cores as determined by Fe:P ratio measurement, and by Mösbauer spectroscopy, electron microscopy and X-ray absorption spectroscopy analysis of native and reconstituted pea seed ferritin mineral cores [27, 31]. Plant ferritin mineral cores have a high phosphate content and are amorphous. In contrast, reconstituted mineral cores using pea seed apoferritin in absence of phosphate were crystalline ferrihydrite. Plant mineral cores are therefore more related to their bacterial counterpart than to their animal one, and this is likely to be due to the high phosphate concentration found within plastids.

Stress and developmental regulation of ferritin synthesis

Ferritins and plant development

Existence of iron under two redox states involves this element in electron transfer reactions occurring in processes like respiration and photosynthesis. Iron is also an essential cofactor of enzymes required for fundamental reactions such as DNA synthesis, or plant-specific pathways like nitrogen fixation or phytohormone synthesis. Therefore, iron starvation is responsible for severe nutritional disorders that profoundly affect the physiology of plants [5]. However, in the presence of oxygen, iron insolubility and toxicity, through free-radical production by reactivity with reduced oxygen intermediates, are also major problems for the use of this element by living organisms [32]. Due to their immobility, plants are highly dependent on their environment and need tight regulation of iron uptake, transport in various organs and storage to ensure optimal development of the plant by preventing both iron starvation and toxicity. Iron storage takes place in the apoplasmic space, between the plasma membrane and the cell wall of plant cells, and perhaps in the vacuole, where low pH and high organic acid concentrations represent optimal conditions for iron deposit [33]. Ferritins are also part of these mechanisms by their capacity to store up to 4500 iron atoms in their cavity in a soluble and bioavailable form [3]. It must be remembered, however, that plant ferritins are localised in plant-specific organelles, the plastids [12, 14]. This localisation and the observation of ferritin at specific plant development stages was first observed by electron microscopy studies, due to electron density of the mineral cores (for review see [11]). These investigations revealed that ferritins accumulate mainly in nongreen plastids, like etioplasts or amyloplasts, whereas a low level of this protein was found in chloroplasts where the photosynthetic process is active. These results have been confirmed by immunodetection of ferritin subunits in protein extracts during the life cycle of pea plants [22]. Ferritins were only detected in roots and leaves of young plantlets, and remained undetectable in the corresponding organs of adult plants at later developmental stages. These data have been further documented by using the determinate nature of maize leaf development [34]. In this plant, the leaf grows from the basal part, creating a cell age gradient from the base to the tip of the leaf. Using immunodetection, high ferritin levels were detected in the young basal section of the leaf and in the tip, the older part of the leaf. On the opposite, a very low level of ferritin was present in the central section of this organ. This part has the higher chlorophyll content, and the detection of the phosphoenolpyruvate carboxylase enzyme in this region, a marker of photosynthetic activity in C4 plants like maize, proves that the photosyn-

thetic process is active in these cells. These results demonstrate the developmental regulation of ferritin synthesis in leaves. Such data are in complete agreement with previous electron microscopy observations showing that ferritins were synthesised in meristematic zones and apices, in leaf primordia, and disappeared during bud development [11, 35]. All these observations suggest that ferritin would be an iron source at early stages of development for the synthesis of iron-containing proteins involved in photosynthesis. This hypothesis is consistent with the fact that ferritins are present in young leaves or etiolated leaves containing nonphotosynthetic etioplasts, and then become undectable in photosynthetic or de-etiolated leaves [11, 22]. The high ferritin concentration in maize leaf tips could be linked to older cell senescence. Ultrastructural studies have clearly established that ferritin accumulates in senescing tissues [11]. Furthermore, in a recent study, a *Brassica napus* complementary DNA (cDNA) corresponding to a messenger RNA (mRNA) encoding a ferritin subunit has been cloned as a senescence-induced mRNA [36]. The neosynthesis of plant ferritins at old stages of development could be linked to iron release during the disorganisation of the photosynthetic apparatus and the requirement to store this metal. The regulatory mechanisms controlling ferritin synthesis during leaf development are still unknown, but it has been established in soybean and maize that there is no direct correlation between the levels of ferritin subunits and mRNA [16, 34]. Ferritin mRNA was detected in mature leaves where ferritin subunits were not detected by Western blot experiments, demonstrating that posttranscriptional controls are involved in the regulation of ferritin synthesis in this organ.

Plants from the legume family specifically develop a symbiosis with some soil bacteria enabling nitrogen fixation. This symbiotic process takes place in specific structures, the nodules, and requires some essential iron-containing proteins [37]. Nitrogenase is directly involved in nitrogen assimilation, and requires low oxygen concentration. This protection against oxygen is achieved by the leghemoglobin, another iron protein. Ferritin has been shown to accumulate at early stages of nodule development, resulting from the interaction between soybean and a *Bradyrhizobium* strain [38]. Ferritin levels decrease with the appearance of nitrogenase and leghemoglobin when the nodules become mature for nitrogen fixation [39]. In contrast, when soybean plants are cultured in the presence of a mutant strain of *Bradyrhizobium* unable to develop functional nodules, ferritins are detected at any stages [38]. These data suggest again that iron is transiently stored in ferritins and used for the accumulation of iron-containing proteins. Ferritin mRNA levels have also been investigated during nodulation. This study reveals that, as for leaf development, no strict correlation between ferritin subunit and mRNA levels is observed [40]. This suggests that a number of posttranscriptional events would occur in the regulation of ferritin level during nodule development.

Thus, these developmental studies underline the role of ferritin as a transient iron buffer for important iron-dependent processes such as photosynthesis and nitrogen fixation. But ferritins are also key proteins in long-term iron storage, as evidenced by seed formation studies. An important amount of iron is stored in pea seeds, and an increase in iron uptake by the roots occurs at early stages of seed development [22]. Iron is also remobilized from vegetative organs to the seed; this iron accounts for 20–30% of the total seed iron content [41, 42]. Iron is probably used during germination for the plantlet development. Immunodetection experiments revealed that ferritin subunits accumulated in seed during their maturation and remained present in dry seeds [22]. This accumulation occurred in the embryo, and no ferritins were detected in the seed coat [4, 22]. The amount of iron stored inside ferritins was estimated to be 92% of the total seed iron content [4], suggesting that this protein is the major form of iron storage in seeds. Whether this iron pool is used only for plastid development or is also transferred to the cytosol remains to be determined.

During germination, ferritins are degraded and iron is used for the growth of the seedling. This process would involved a protein stability control to degrade the protein shell when iron is released from the mineral core. A model has been proposed based on the observation that the immunodetection pattern of pea seedling ferritin is similar to the pattern of ferritin degraded by free radicals during in vitro iron exchanges [22]. When iron release is induced from purified pea seed ferritins by incubation in the presence of ascorbate or light, a progressive degradation of the protein is detected [21, 29]. This degradation is initiated by cleavages at the amino terminus of the ferritin subunit, as demonstrated by microsequence comparison between the mature and the cleaved subunits [21]. The preferential site for cleavage is localized within the α helix of the NH2 extension peptide [24], specific to plant ferritin and important for the stability of the protein as demonstrated by mutagenesis experiments [23]. The pea seed ferritin 3D model suggests that this domain is closed from the three-fold channel, a putative site for iron exchanges between the cavity and the ferritin environment [24]. In vitro inhibition of this degradation, obtained by the presence of free radical scavengers or ferrous iron chelators, indicated that free radical cleavages promoted by free iron occurred [29]. It is known that free-radical-mediated alteration of proteins is a signal for their degradation [43]. These data lead to the hypothesis that a similar mechanism occurs during seedling germination. When

iron is released from the ferritin, free-radical cleavages alter the protein shell, which is then degraded by proteases.

These studies of the developmental regulation of ferritin synthesis indicate that posttranscriptional mechanisms are involved in this control. Some additional control mechanisms remained to be elucidated to explain how ferritins are synthesised at the proper level in particular cells to act as an iron buffer. It is now clear that plant ferritins are encoded by a multigene family [6, 10, 44], which could participate in this coordinated expression by differential regulation of these genes [6]. Indeed, ferritin overexpression under the control of a constitutive promoter in transgenic tobaccos has clearly demonstrated that strict control of ferritin gene expression is required to prevent alteration of the plant physiology by inappropriate ferritin levels (see below).

Iron-dependent regulation of ferritin gene expression

Plant and animal ferritins have evolved from a common ancestor gene as suggested by amino acid sequence comparison of ferritin subunits [17]. However, in the two kingdoms, ferritins are localised in different compartments; plant ferritins are localised in the plastids [12, 14], whereas animal ferritins are cytosolic proteins [3]. This specific compartmentalisation and, as described above, the need to adapt the ferritin content to plant development suggest the involvement of some plant-specific pathways in the regulation of the ferritin subunit and mRNA levels in this organism. Furthermore, regulation of animal and plant ferritin gene expression in response to iron excess is known to be achieved through different mechanisms.

Regulation of ferritin synthesis in animal cells in response to iron occurs mainly at the translational level [45]. A specific structure called the iron regulatory element (IRE) has been identified in the 5′ untranslated region of ferritin mRNAs [46]. When the iron level is low, iron regulatory proteins (IRP1 or -2) bind to this structure and repress the initiation of ferritin mRNA translation. In the presence of iron excess, IRPs are released from the IRE structure, and the translation of ferritin mRNA allows ferritin synthesis in order to store iron. The IRP1 protein corresponds to the animal cytosolic aconitase, the amount of which is not affected by iron [47]. This enzyme contains a labile 4Fe–4S cluster, essential for the sensing of iron. In the presence of iron, the protein has a 4Fe–4S cluster and aconitase activity, whereas the affinity for the IRE is low. When the iron concentration is lower, an apoprotein without an Fe–S cluster corresponds to the active form of IRP1, which lacks aconitase activity but binds with high affinity to the IRE structure and consequently represses ferritin mRNA translation [48, 49]. The IRP2 binding activity is regulated in a different way: the protein is degraded in response to iron overload [50, 51].

A plant cytosolic aconitase gene has been characterised [52], but it is unlikely to participate in translational control of plant ferritin synthesis involving an IRE. Indeed, no IRE sequences are present in the 5′ untranslated region of any known plant ferritin mRNA sequences deduced from cloned cDNA [14, 44, 53, 54] or genes [6, 7]. Furthermore, no IRP activity is detectable in plant cell extracts by RNA gel-shift experiments using animal IRE as a probe [55], or in a translational assay using animal ferritin mRNA and wheat germ extract [56]. However, it cannot be ruled out from these experiments that plant cytosolic apoaconitase could bind to an RNA regulatory sequence other than animal IRE. Nevertheless, all these data suggest that a different mechanism is involved in the regulation of ferritin synthesis by iron in plant and animal cells.

In order to identify these regulatory mechanisms, the effect of iron overload on ferritin gene expression in plants has been investigated. Iron starvation of plants leads to symptoms of chlorosis resulting from chlorophyll deficiency and impaired photosynthesis [5]. Under such conditions, root iron uptake systems are induced in order to enhance iron acquisition and to maintain physiological integrity [2]. Next, addition of excess iron in the culture medium of iron-starved plantlets leads to a large iron influx into the plant [53]. This iron is translocated within 3 h into the leaves to restore the essential photosynthetic process in chloroplasts [57, 58]. During this period of regreening, which takes about 24–48 h, ferritins are used as a safe iron buffer and transiently store the iron required for the synthesis of iron-containing proteins. These conditions have been used as a model to better understand the regulation of ferritin synthesis by iron in plants.

The use of hydroponic growth conditions has allowed strict control of the iron concentration of the liquid culture medium in order to perform both iron starvation and overload [53]. Ferritin mRNAs are already detectable in maize leaf plantlets 3 h after iron resupply. This accumulation reaches its maximum 6 and 24 h, respectively, after treatment in leaves and roots, and then gradually decreases [59]. This increase in ferritin mRNA abundance precedes the accumulation of ferritin subunits, with a maximum detected 24 h after the start of iron treatment. A gradual decrease of ferritin content is observed, consistent with a transient iron buffer function of this protein during the regreening of plants [53]. The increased abundance of ferritin mRNA measured in response to iron overload in plants confirms that IRE-dependent translational control is not the mechanism involved in this regulation. Furthermore, it has been demonstrated that iron resupply to ironstarved soybean cell suspension cultures induces ferritin mRNA accumulation that is controlled at the transcriptional level [14].

Genes responsive to the plant hormone abscisic acid (ABA) are also expressed in response to the iron treatment of iron-starved maize plantlets [59]. This suggests that ABA could be involved in the transduction pathway leading to ferritin synthesis in response to iron. This plant hormone is known to be involved in various stress responses in plants such as water stress or pathogen attack [60]. Indeed, iron overload of maize plantlets leads to a fivefold increase in ABA concentration in roots and leaves, and ferritin mRNA accumulates in response to exogenous ABA treatment [59]. It is interesting to notice that in maize leaves an increase of the endogenous ABA level induced by dessiccation leads to accumulation of ferritin mRNA [6]. This result supports the hypothesis of possible ABA involvement in ferritin gene regulation, but suggests also a role for the ferritins under these stress conditions. The proof that ABA is indeed involved in iron-induced ferritin synthesis came from experiments performed with an ABA-deficient mutant [59]. This maize mutant (*Vp2*) is deficient in carotenoid biosynthesis, which is a precursor for ABA synthesis. Ferritin mRNA accumulation is strongly reduced in this mutant, demonstrating that ABA is involved in a pathway leading to ferritin accumulation in response to iron. However, comparison of ABA and iron treatments revealed that ABA cannot account for the total accumulation of ferritin mRNA measured in response to iron, suggesting activation of an additional pathway. The existence of a second pathway has been clearly demonstrated by investigating the differential regulation of maize ferritin genes during iron resupply to iron-starved maize plantlets [6]. Two subclasses of maize ferritin cDNAs and genes (*ZmFer1* and *-2*) have been characterised [6, 53]. The use of specific probes in Northern blot experiments enabled discrimination of the two classes of transcripts, and revealed that these transcripts accumulate with differential kinetics in response to iron. Furthermore, *ZmFer2* mRNA accumulates in response to both iron and ABA treatments, whereas the *ZmFer1* mRNA level is only increased by iron treatment, revealing that an ABA-independent pathway is required for full expression of ferritin genes (fig. 1).

Regulation of ferritin gene expression under oxidative stress conditions

Iron as a transition metal can react with oxygen to produce reactive oxygen intermediates through the Fenton reaction [1]. Such molecules are highly reactive and can damage most components of the cell. Thus, a high concentration of free iron in aerobic organisms can generate oxidative stress. Indeed, in flooded soils, ferric iron is reduced to the ferrous form, which is stabilised by low pH and anaerobic conditions [61]. This ferrous iron is taken up by the roots, accumulates in plants and induces a physiological disease called 'bronzing' in rice [62]. The leaves of iron-overloaded rice plants present necrotic bronze spots corresponding to damage generated by iron-mediated oxidative stress. A similar phenotype has been reproduced by dipping rice stems or tobacco de-rooted plantlets in an iron solution [63, 64]. In living cells, H_2O_2 is naturally produced by cellular metabolism, and this compound can react with ferrous iron to produce hydroxyl radicals, the most dangerous reactive oxygen species for the cell. Furthermore, recall that oxygen is produced in chloroplasts by the photosynthetic process and that this compartment is highly exposed to oxidative injury. Different mechanisms of reactive oxygen intermediate detoxification have been described in chloroplasts [65]. Another strategy to protect this organelle could be to strictly control the concentration of free iron. Since in plants ferritins are localised in the plastids, they could play an important role in preventing oxidative damage by storing free iron in a safe form. Such a hypothesis is supported by cytological studies demonstrating that an oxidising agent such as ozone induces ferritin accumulation in plants [11]. More recently, it has been shown that the *brz* pea mutant, which accumulates excess iron and

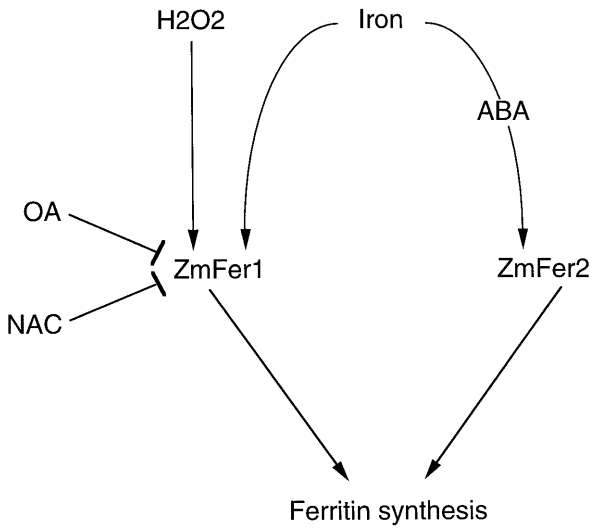

Figure 1. Schematic representation of the pathways involved in maize ferritin gene expression. Expression of two subclasses of maize ferritin genes *(ZmFer1* and *ZmFer2*) is activated by iron. The plant hormone abscisic acid (ABA) is required for the iron-regulated expression of the *ZmFer2* gene, but not for the iron-regulated expression of the *ZmFer1* gene. In addition, expression of the *ZmFer1* gene can be activated by hydrogen peroxide (H_2O_2). Both iron- and H_2O_2-regulated expression of the *ZmFer1* gene are antagonised by an antioxidant (NAC: *N*-acetylcysteine) or a Ser/Thr phosphatase inhibitor (OA: okadaic acid).

presents bronze necrotic spots, contains iron deposits immunocytochemically identified as ferritins [66].

In order to study the regulation of ferritin synthesis under these particular physiological conditions, de-rooted maize plantlets have been used to bypass the root barrier and induce a rapid iron overload of the leaves [67]. Such a treatment leads to rapid accumulation of ferritin subunits and mRNA when compared with the results obtained during iron deficiency recovery from hydroponically grown maize plantlets. Other transition metals such as copper, for example, were not able to induce an increase of ferritin mRNA abundance, which appears to be specific to iron overload [67]. Whether the ABA pathway involved in the regulation of ferritin genes is activated was investigated. A normal ferritin mRNA accumulation was measured in response to iron overload in a *Vp2* ABA-deficient mutant [67]. This result indicates that the activated transduction pathway is ABA-independent, which is consistent with the observation that no *ZmFer2* mRNA, known to accumulate in response to ABA, has been detected under these conditions [68]. *ZmFer1* mRNA is the only ferritin transcript accumulated in response to the incubation of de-rooted maize plantlets in iron-citrate solution [68]. Whether the ABA-independent pathway involved in this physiological system is the same as that inducing *ZmFer1* ferritin mRNA accumulation during iron deficiency recovery remains to be determined.

Since ferritin could be involved in defence mechanisms against iron-mediated oxidative stress, the effect of oxidant and antioxidant molecules on *ZmFer1* ferritin gene expression has been investigated. Antioxidant compounds like glutathione or *N*-acetylcysteine, are able to completely abolish the iron-induced increase abundance of *ZmFer1* ferritin mRNA in de-rooted maize plantlets [67, 68]. In contrast, hydrogen peroxide promotes this transcript accumulation [67, 68]. These results demonstrate that reactive oxygen species would be involved in the regulation of *ZmFer1* gene expression (fig. 1). Similar results have been obtained using *Arabidopsis thaliana* plantlets [44] or maize cell suspension cultures [68], and this system has been used to further investigate the iron-activated ABA-independent pathway that induces *ZmFer1* ferritin transcript accumulation. It has been demonstrated that phosphatase inhibitors such as calyculin A or okadaic acid specifically inhibit iron-induced accumulation of *ZmFer1* ferritin mRNA [68]. Dose-dependent inhibition was observed, demonstrating that a Ser/Thr phosphatase is involved in *ZmFer1* ferritin gene regulation. Since it has been shown in soybean cells that the iron-induced ferritin mRNA accumulation is regulated at the transcriptional level [14], a transient expression assay has been developed to investigate these mechanisms [68]. A 2.2-kb fragment of the *ZmFer1* ferritin gene, containing the promoter, was fused to the *β-glucuronidase* gene and introduced in maize cells by biolistic transformation. An eightfold increase in β-glucuronidase (GUS) activity was observed in response to iron treatment. Furthermore, in this transient expression assay, both okadaic acid and *N*-acetylcysteine inhibit the iron-induced increase in GUS activity. This assay will allow identification of regulatory sequences in the *ZmFer1* gene to better understand how iron, reactive oxygen intermediates and phosphatases interfere in the regulation of this gene.

Ferritin synthesis deregulation in transgenic plants and its consequences

Our knowledge of the role that ferritins play in plant physiology is still very limited, and their function has not been addressed directly. The information available is based on correlating observations obtained from cytology, biochemistry and molecular biology [69]. However, a molecular physiological approach was recently initiated with the aim of deregulating ferritin synthesis by overexpressing them in transgenic plants, either in the plastids (their natural cytological localisation) or in the cytoplasm, to evaluate the consequences of such deregulation on plant development and physiology [70]. Overexpression of this protein was obtained in transgenic tobaccos by placing soybean ferritin cDNA cassettes, either with or without a transit peptide sequence, under the control of the CAMV 35S promoter. Ferritin accumulation in seeds and in 2-day-old seedlings was identical between transformed and control plants. In contrast, ferritin was illegitimally accumulated in leaves of 3-week-old transformed plants compared with control plants [70]. Leaf-overaccumulated ferritin was correctly assembled as 24-mers.

Yellow zones are observed on leaves of transformed plants when grown in vitro on media containing 25 μM Fe(III)-EDTA, as when control plants are grown without iron. Sometimes this phenotype was observed as a spotted mozaic, reminiscent of leaf senescence [71–73], rather than the typical interveinal yellowing syndrome observed during iron deficiency-mediated chlorosis [74]. Consistent with this phenotype, chlorophyll concentrations were decreased by 20% in transgenic tobacco plants, whatever the cellular localisation of the overexpressed ferritin.

The yellow leaf phenotype of transgenic tobacco plants overexpressing ferritin indicated that subcellular alterations occurred, in particular at the chloroplast level. This was confirmed by electron microscopy examination of leaf thin sections (fig. 2). In control A plants, chloroplast structure was normal, with well-defined thylakoid membranes and important grana stacking (fig. 2A). In contrast, when A plants grew under iron-defi-

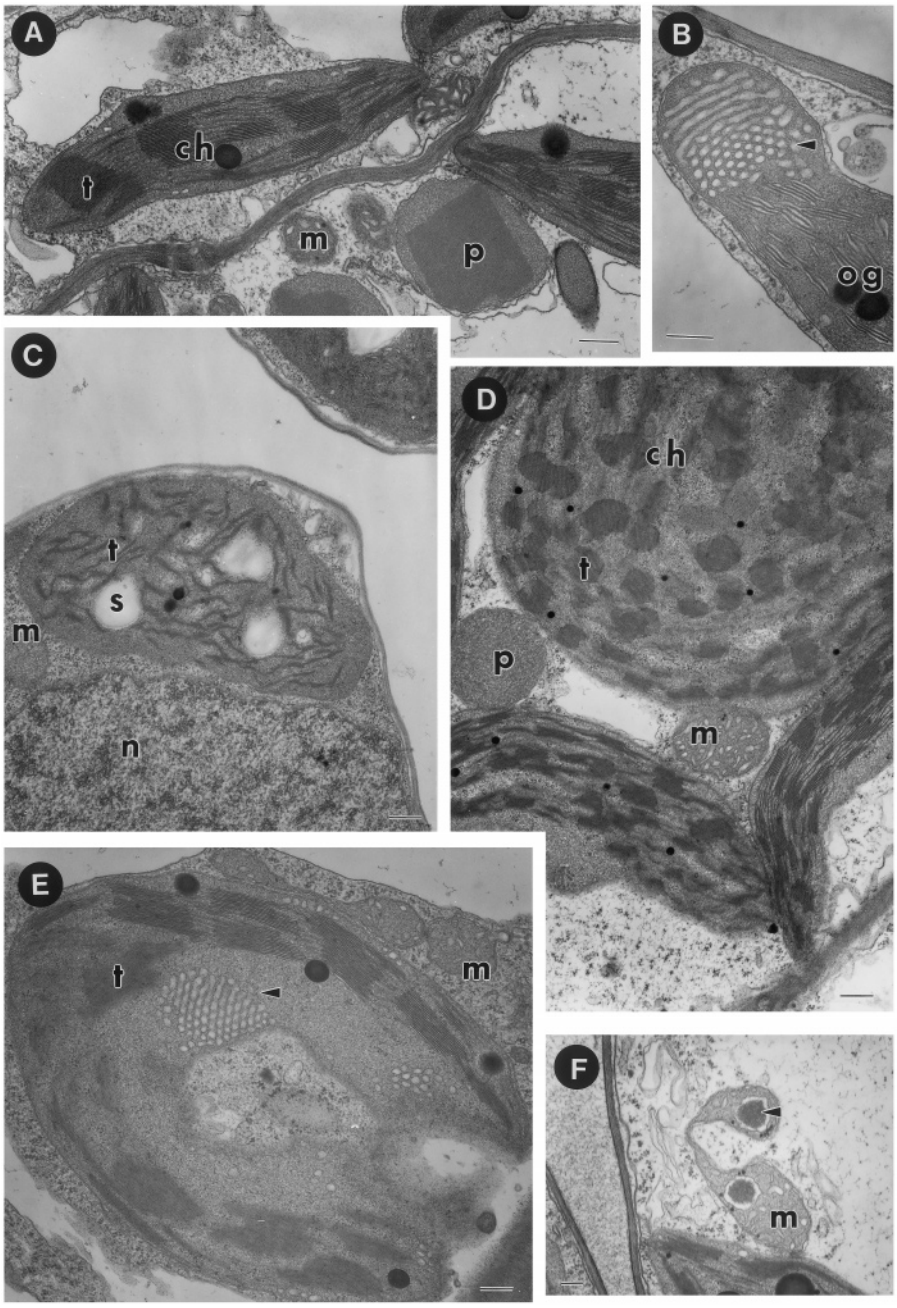

Figure 2. Chloroplast and mitochondria ultrastructure in (*A*) Control A plants grown under iron-sufficient conditions (25 μM Fe(III)-EDTA); (*B* and *C*) control A plants grown under iron-deficient conditions (without iron); (*D*) tobacco P plants overexpressing ferritin in the plastids and grown under iron-sufficient conditions; (*E* and *F*) tobacco C plants overexpressing ferritin in the cytoplasm and grown under iron-sufficient conditions. Leaf thin sections examined by electron microscopy were from 21-day-old plants grown in vitro. ch, chloroplast; n, nucleus; t, thylakoids; s, starch; m, mitochondria; p, peroxisome; og, osmiophilic globuli. Arrowheads indicate unusual vesicles in the chloroplasts (panels *B* and *E*) and abnormal dense particles in mitochondria (*F*). Bars indicate a 250-nm scale.

cient conditions, a chlorosis phenotype was observed, and the chloroplast structure was altered, showing unstacked and diffused thylakoids (fig. 2B, C). Starch accumulation and unusual vesicles (indicated by an arrowhead in fig. 2B), which could have arisen from thylakoid breakdown, were observed in numerous chloroplasts of iron-deficient A plants. In P plants overexpressing ferritin in leaf chloroplasts, and grown under

iron-sufficient conditions (i.e. 25 μM Fe(III)-EDTA), most of the chloroplast thylakoids were diffused, and large areas with very weak electron density were visualised in the stroma (fig. 2D). P plant chloroplast structure was reminiscent of chloroplast structure of iron-deficient control plants (fig. 2C and [74]). Unusual vesicles observed in numerous chloroplasts of control A plants grown under iron-deficient conditions (arrowhead in fig. 2B) were not observed, however, in P plant chloroplasts. On the other hand, in C plants grown under iron-sufficient conditions, and overexpressing ferritin in leaf cell cytoplasm, most of the chloroplast contained these unusual vesicles (fig. 2E). Furthermore, C plant chloroplasts also presented diffused thylakoids, and large areas of weak electron density in the stroma (fig. 2E). In addition, some C plant mitochondria, although still able to divide, contained abnormal very dense electron regions (fig. 2F). Taken together, these observations indicate that accumulation of ferritin in leaves of transgenic tobacco plants grown under iron-sufficient conditions leads to chloroplast alterations similar to those induced by iron deficiency in control plants.

A major consequence of the ferritin accumulation in transgenic tobacco was to increase leaf iron concentration [70, 75] and root ferric reductase activity [70], a key step in iron uptake by dicotyledonous plants [76, 77]. This can be explained by the increased iron storage capacity of the ferritin-transformed tobacco plants in which excessive iron sequestration disturbs metabolism, driving leaf physiology towards an iron-deficient state as mentioned above, and as illustrated by the altered chloroplast substructure (fig. 2). As a consequence, these transgenic plants, sensing an iron deficiency, logically activate their iron uptake systems [2, 32]. Such a situation of increased iron uptake in plants which sense their iron status as deficient, whereas they paradoxically accumulate too much iron, has already been described in the case of the *brz* and *dgl* pea mutants [78, 79] and of the *chloronerva* tomato mutant [80]. With regard to the latter, it is interesting to notice that its leaf phenotype is very reminiscent of the yellow leaf phenotype of the transgenic tobacco overexpressing ferritin.

Iron and oxygen metabolism can interact to promote oxidative stress (see above). Increased iron sequestration in ferritin-transformed tobacco plants could have a negative effect on iron-mediated oxidative stress. Methylviologen, the active molecule of the herbicide paraquat, acts by promoting an oxidative stress in the chloroplast, leading to proteolysis, lipid peroxidation and ultimately to cell death [81]. The toxic effect of methylviologen requires free iron to take place, and can be antagonised by iron chelators such as desferrioxamine [82, 83]. Tobacco transformants overexpressing ferritins are more resistant to methylviologen toxicity, confirming that the transgenic ferritins were functional in vivo—that is able to sequester iron atoms [70]. However, it has been documented in animal cells that ferritin can act either as anti- and prooxidants [84]. Therefore, the increased resistance to paraquat treatment mentioned above could also have arisen, at least in part, from a general activation of plant defence against oxidative stress generated in response to illegitimate accumulation of ferritin in leaves. This point was addressed by measuring various enzyme activities involved in oxygen detoxification in leaf discs of control plants (A1), plants overexpressing ferritin in their cytoplasm (C5) and plants overexpressing ferritin in their plastids (P6). All the enzyme activities measured were indeed increased in tobacco leaves overexpressing ferritin (fig. 3). Catalase activity was 24 and 60% higher in P6 and C5 plant leaves, respectively, than in A1 control plants. Ascorbate peroxidase and gaiacol peroxidase

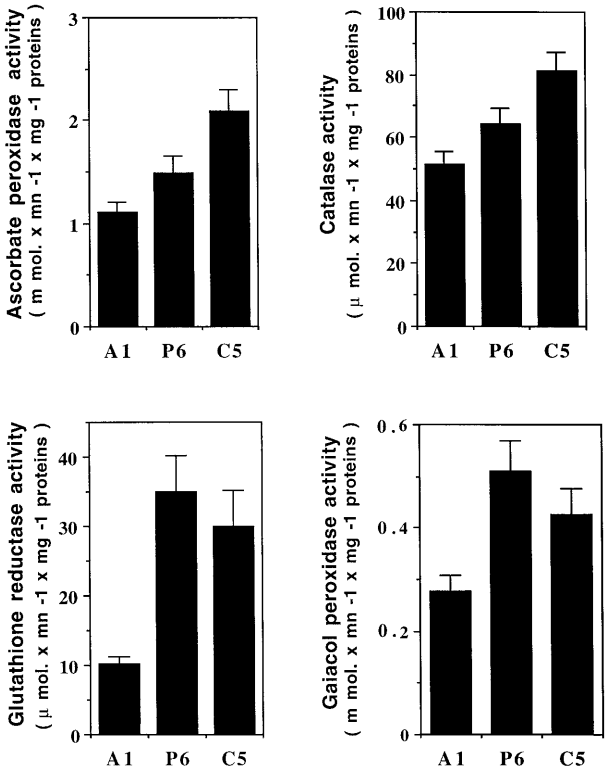

Figure 3. Influence of ferritin overexpression on ascorbate peroxidase, gaiacol peroxidase, catalase and gluthatione reductase activities in tobacco leaves. A1 plants (control), P6 plants (transformants overaccumulating ferritins in their plastids) and C5 plants (transformants overaccumulating ferritins in their cytoplasm) were grown in vitro for 21 days on a medium containing 25 μM Fe(III)-EDTA. Crude extract preparation and enzyme activity measurements were as described [85]. Mean values and standard deviations are calculated from six independent experiments.

activities increased by 34 and 83%, respectively, in P6 plants and by 90 and 53%, respectively in C5 plants. The more dramatic effect concerned glutathione reductase activity, which was increased 3-fold and 2.5-fold in P6 and C5 plants, respectively, compared with A1 plants. Thus, although resistant to methylviologen treatment, transgenic tobacco plants overexpressing ferritin experience oxidative stress. This observation is consistent with the senescence phenotype of ferritin-transformed plants. Strict developmental control of plant ferritin synthesis thus emerges as an important process for normal development of the photosynthetic apparatus.

Conclusions

Plant ferritin synthesis is regulated by iron. This aspect is by far the more documented and implies differential regulation of a small gene family. Also, plant ferritin requires strict control both spatially and temporally. However, our knowledge of the developmental regulation of ferritin synthesis is still very limited. Expression analysis of the various members of the ferritin gene family at different stages of plant development will be necessary to clarify the role that these proteins play in the plant biology.

1 Briat J. F. and Lebrun M. (1999) Plant responses to metal toxicity. C. R. Acad. Sci., in press
2 Briat J. F. and Lobréaux S. (1997) Iron transport and storage in plants. Trends Plant Sci. **2:** 187–193
3 Harrison P. M. and Arosio P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. Biochem. Biophys. Acta **1275:** 161–203
4 Marentes E. and Grusak M. A. (1998) Iron transport and storage within the seed coat and embryo of developing seeds of pea (*Pisum sativum* L.). Soil Science Res. **8:** 367–375
5 Briat J. F., Fobis-Loisy I., Grignon N., Lobréaux S., Pascal N., Savino G. et al. (1995) Cellular and molecular aspects of iron metabolism in plants. Biol. Cell **84:** 69–81
6 Fobis-Loisy I., Loridon K., Lobréaux S., Lebrun M. and Briat J. F. (1995) Structure and differential expression in response to iron and abscisic acid of two maize ferritin genes. Eur. J. Biochem. **231:** 609–619
7 Proudhon D., Wei J., Briat J. F. and Theil E. C. (1996) Ferritin gene organization: differences between plants and animals suggest possible kingdom-specific selective constraints. J. Mol. Evol. **42:** 325–336
8 Harrison P. M., Ford G. C., Smith J. M. A. and White J. L. (1991) The location of exon boundaries in the multimeric iron-storage protein ferritin. Biol. Metals **4:** 95–99
9 Theil E. C. (1994) Iron regulatory elements (IREs): a family of mRNA non-coding sequences. Biochem. J. **304:** 1–11
10 Wicks R. and Entsch B. (1993) Functional genes found for three different plant ferritin subunits in the legume *Vigna unguiculata*. Biochem. Biophys. Res. Commun. **192:** 813–819
11 Seckback J. J. (1982) Ferreting out the secret of plant ferritin – a review. J. Plant Nutr. **5:** 369–394
12 Van der Mark F., Van der Briel W. and Huisman H. G. (1983) Phytoferritin is synthesized in vitro as a high-molecular-weight precursor. Biochem. J. **214:** 943–950
13 Proudhon D., Briat J. F. and Lescure A. M. (1989) Iron induction of ferritin synthesis in soybean cell suspensions. Plant Physiol. **90:** 586–590
14 Lescure A. M., Proudhon D., Pesey H., Ragland M., Theil E. C. and Briat J. F. (1991) Ferritin gene transcription is regulated by iron in soybean cell cultures. Proc. Natl. Acad. Sci. USA **88:** 8222–8226
15 Heijne G. V., Steppuhn J. and Herrmann R. G. (1989) Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. **180:** 535–545
16 Ragland M., Briat J. F., Gagnon J., Laulhère J. P., Massenet O. and Theil E. C. (1990) Evidence for conservation of ferritin sequences among plants and animals and for a transit peptide in soybean. J. Biol. Chem. **265:** 18339–18344
17 Andrews S. C., Arosio P., Bottke W., Briat J. F., von Darl M., Harrison P. M. et al. (1992) Structure, function and evolution of ferritins. J. Inorg. Biochem. **47:** 161–174
18 Laulhère J. P., Labouré A. M., van Wuytswinkel O., Gagnon J. and Briat J. F. (1992) Purification, characterization and function of bacterioferritin from the cyanobacyeria *Synechocystis* PCC 6803. Biochem. J. **281:** 785–793
19 Bauminger E. R., Harrison P. M., Hechel D., Nowik I. and Treffry A. (1991) Mossbauer spectroscopic investigation of structure-function relations in ferritins. Biochem. Biophys. Acta **1118:** 48–58
20 Lawson D. M., Artymiuk P. J., Yewdall S. J., Smith J. M. A., Livingston J. C., Treffry A. et al. (1991) Solving the structure of human H ferritin by genetically engineering intermolecular crystal contacts. Nature **349:** 541–544
21 Laulhère J. P., Labouré A. M. and Briat J. F. (1989) Mechanism of the transition from plant ferritin to phytosiderin. J. Biol. Chem. **264:** 3629–3635
22 Lobréaux S. and Briat J. F. (1991) Ferritin accumulation and degradation in different organs of pea (*Pisum sativum*) during development. Biochem. J. **274:** 601–606
23 Van Wuytswinkel O., Savino G. and Briat J. F. (1995) Purification and characterization of recombinant pea seed ferritins expressed in *Escherichia coli*: influence of amino terminus deletions on protein solubility and *in vitro* core formation. Biochem. J. **305:** 253–261
24 Lobréaux S., Yewdall S., Briat J. F. and Harrison P. M. (1992) Amino-acid sequence and predicted three-dimensional structure of pea seed (*Pisum sativum*) ferritin. Biochem. J. **288:** 931–939
25 Levi S., Luzzago A., Franceschinelli F., Santambrogio P., Cesareni G. and Arosio P. (1989) Mutational analysis of the channel and loop sequences of human ferritin H-chain. Biochem. J. **264:** 381–388
26 Harrison P. M., Artymiuk P. J., Ford G. C., Guest J. R., Hirzman J., Lawson D. M. et al. (1991) Probing structure-function relations in ferritin and bacterioferritin. Adv. Inorg. Chem. **36:** 449–486
27 Wade V. J., Treffry A., Laulhère J. P., Bauminger E. R., Cleton M. I., Mann S. et al. (1993) Structure and composition of ferritin cores from pea seed (*Pisum sativum*). Biochim. Biophys. Acta **1161:** 91–96
28 Laulhère J. P. and Briat J. F. (1993) Iron release and uptake by plant ferritin as affected by pH, reduction and chelation. Biochem. J. **290:** 693–699
29 Laulhère J. P., Labouré A. M and Briat J. F. (1990) Photoreduction and incorporation of iron into ferritins. Biochem. J. **269:** 79–84
30 Mann S., Williams J. M., Treffry A. and Harrison P. M. (1987) Reconstituted and native iron-cores of bacterioferritin and ferritin. J. Mol. Biol. **198:** 405–416
31 Waldo G. S., Wright E., Wang Z. H., Briat J. F., Theil E. C. and Sayers D. E. (1995) Formation of the ferritin iron mineral occurs in plastids: an X-ray absorption spectroscopy study. Plant Physiol. **109:** 797–802
32 Guerinot M. L. and Yi Y. (1994) Iron: nutritious, noxious and not readily available. Plant Physiol. **104:** 815–820
33 Briat J. F. and Lobréaux S. (1998). Iron storage and ferritin in plants. In: Iron Transport and Storage in Microorganism,

Plants and Animals, vol. 25, Metal Ions in Biological Systems, pp. 563–584, Sigel A. and Sigel H. (eds), Marcel Dekker, New York
34 Theil E. C. and Hase T. (1993) Plant and microbial ferritins. In: Iron Chelation in Plants and Soil Microorganisms, pp. 133–156, Barton, L. L. and Hemming B. C. (eds), Academic Press, New York
35 Marinos N. G. (1967) Multifunctionnal plastids in the meristematic region of potato tuber buds. J. Ultrastr. Res. **17:** 91–113
36 Buchanan-Wollaston V. and Ainsworth C. (1997) Leaf senescence in *Brassica napus*: cloning of senescence related genes by substractive hybridisation. Plant Mol. Biol. **33:** 821–834
37 Tang C., Robson A. D. and Dilworth M. J. (1990) The role of iron in nodulation and nitrogen fixation in *Lupinus augustifolius* L. New Phytol. **115:** 61–67
38 Ko M. P., Huang P. Y., Huang J. S. and Baker K. R. (1987) The occurrence of phytoferritin and its relationship to effectiveness of soybean nodules. Plant Physiol. **83:** 299–305
39 Bergersen F. J. (1963) Iron in the developing soybean nodules. Aust. J. Biol. Sci. **16:** 916–919
40 Ragland M. and Theil E. C. (1993) Ferritin (mRNA, protein) and iron concentrations during soybean nodule development. Plant Mol. Biol. **21:** 555–560
41 Hocking P. J. and Pate J. S. (1978) Mobilization of minerals to developing seeds of legumes. Ann. Bot. **41:** 1259–1278
42 Grusak M. A. (1994) Iron transport to developing ovules of *Pisum sativum* seed import characteristics and phloem iron-loading capacity of sources regions. Plant Physiol. **104:** 649–655
43 Davies K. J. A. (1987) Protein damage and degradation by oxygen radicals. J. Biol. Chem. **26:** 9895–9902
44 Gaymard F., Boucherez J. and Briat J. F. (1996) Characterization of a ferritin mRNA from *Arabidopsis thaliana* accumulated in response to iron through an oxidative pathway independent of abscisic acid. Biochem. J. **318:** 67–73
45 Hentze M. W. and Kühn L. C. (1996) Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. Proc. Natl. Acad. Sci. USA **93:** 8175–8182
46 Hentze M. W., Rouault T. A., Caughman S. W., Dancis A., Harford J. B. and Klausner R. D. (1987) A cis-acting element is necessary and sufficient for translational regulation of human ferritin expression in response to iron. Proc. Natl. Acad. Sci. USA **84:** 6730–6734
47 Klausner R. D., Rouault T. A. and Harford J. B. (1993) Regulating the fate of mRNA: the control of cellular iron metabolism. Cell **72:** 19–28
48 Basilion J. P., Kennedy M. C., Beinert H., Massinople C. M., Klausner R. D. and Rouault T. A. (1994) Overexpression of iron-responsive element-binding protein and its analytical characterization as the RNA-binding form, devoid of an iron-sulfur cluster. Arch. Biochem. Biophys. **311:** 517–522
49 Hirling H., Henderson B. R. and Kuhn L. C. (1994) Mutational analysis of the [4Fe-4S]-cluster converting iron regulatory factor from its RNA-binding form to cytoplasmic aconitase. EMBO J. **15:** 453–461
50 Guo B., Yu Y. and Leibold E. A. (1994) Iron regulates cytoplasmic levels of a novel iron-responsive element-binding protein without aconitase activity. J. Biol. Chem. **269:** 24252–24260
51 Iwai K., Klausner R. D. and Rouault T. A (1995) Requirements for iron-regulated degradation of the RNA binding protein, iron regulatory protein 2. EMBO J. **14:** 5350–5357
52 Peyret P., Perez P. and Alric M. (1995) Structure, genomic organization and expression of the *Arabidopsis thaliana* aconitase gene. Plant aconitase shows significant homology with mammalian iron-responsive element-binding protein. J. Biol. Chem. **270:** 8131–8137
53 Lobréaux S., Massenet O. and Briat J. F. (1992) Iron induces ferritin synthesis in maize plantlets. Plant Mol. Biol. **19:** 563–575
54 Spence M. J., Henzl M. T. and Lammers P. J. (1991) The structure of a *Phaseolus vulgaris* cDNA encoding the iron storage protein ferritin. Plant Mol. Biol. **17:** 499–504
55 Rothenberger S., Müllner E. W. and Kühn L. C. (1990) The mRNA-binding protein which controls ferritin and transferrin receptor expression is conserved during evolution. Nucleic Acids Res. **18:** 1175–1179
56 Brown H. P., McQueen S. D., Walden W. E., Patino M. M., Gaffield L., Bielser D. et al. (1989) Requirements for the translational repression of ferritin transcripts in wheat germ extracts by 90 kDa protein from rabbit liver. J. Biol. Chem. **264:** 13383–13386
57 Branton D. and Jacobson L. (1962) Iron transport in pea plants. Plant Physiol. **37:** 539–545
58 Young T. F. and Terry N. (1982) Transport of iron into leaves following iron resupply to iron-stressed sugar beet plants. J. Plant Nutr. **5:** 1273–1283
59 Lobréaux S., Hardy T. and Briat J. F. (1993) Abscisic acid is involved in the iron-induced synthesis of maize ferritin. EMBO J. **12:** 651–657
60 Giraudat J., Parcy F., Bertauche N., Gosti F., Leung J., Morris P. C. et al. (1994) Current advances in abscisic acid action and signalling. Plant Mol. Biol. **26:** 1557–1577
61 Fitter A. H. and Hay R. K. (1989) Environmental physiology of plants, Academic Press, London
62 Ponnamperuma F. N., Bradfield R. and Peech M. (1955) Physiological disease of rice attributable to iron toxicity. Nature **175:** 275
63 Peng X. X. and Yamauchi M. (1993) Ethylene production in rice bronzing leaves induced by ferrous iron. Plant and Soil **149:** 227–234
64 Kampfenkel K., Van Montagu M. and Inze D. (1995) Effects if iron excess on *Nicotiana plumbaginifolia* plants. Implications to oxidative stress. Plant Physiol. **107:** 725–735
65 Halliwell B. (1987) Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. Chem. Phys. Lipids **44:** 327–340
66 Becker R., Manteuffel R., Neumann D. and Scholz G. (1998) Excessive iron accumulation in the pea mutants *dgl* and *brz*: subcellular localization of iron and ferritin. Planta **207:** 217–223
67 Lobréaux S., Thoiron S. and Briat J. F. (1995) Induction of ferritin synthesis in maize leaves by an iron-mediated oxidative stress. Plant J. **8:** 443–449
68 Savino G., Briat J. F. and Lobréaux S. (1997) Inhibition of the iron-induced *ZmFer1* maize ferritin gene expression by antioxidants and Ser/Thr phosphatase inhibitors. J. Biol. Chem. **272:** 33319–33326
69 Briat J. F. (1996) Roles of ferritin in plants. J. Plant Nutr. **19:** 1331–1342
70 Van Wuytswinkel O., Vansuyt G., Grignon N., Fourcroy P. and Briat J. F. (1999) Iron homeostasis alteration by ferritin overexpression in transgenic tobacco. Plant J. **17:** 93–98
71 Bleecker C. K. and Patterson S. E. (1997) Last exit: senescence, abscission, and meristem arrest in *Arabidopsis*. Plant Cell **9:** 1169–1179
72 Buchanan-Wollaston V. (1997) The molecular biology of leaf senescence. J. Exp. Bot. **48:** 181–199
73 Parthier B. (1988) Gerontoplasts: the yellow end in the ontogenesis of chloroplasts. Endocyt. C. Res. **5:** 163–190
74 Thoiron S., Pascal N. and Briat J. F. (1997) Impact of iron deficiency and iron re-supply during the early stages of vegetative development in maize (*Zea mays* L.). Plant Cell Environ. **20:** 1051–1060
75 Goto F., Yoshihara T. and Saiki H. (1998) Iron accumulation in tobacco plants expressing soyabean ferritin gene. Transgenic Res. **7:** 173–180
76 Bienfait H. F. (1985) Regulated redox processes at the plasmalemma of plant root cells and their function in iron uptake. J. Bioenerg. Biomembr. **17:** 73–83
77 Holden M. J., Luster D. G., Chaney R. L. and Buckhout T. J. (1992) Enzymology of ferric chelate reduction at the root plasma membrane. J. Plant Nutr. **15:** 1667–1678
78 Kneen B. E., Larue T. A., Welch R. M. and Weeden N. F. (1990) Pleiotropic effects of *brz*. A mutation in *Pisum sativum*

(L.) cv 'Sparkle' conditioning decreased nodulation and increased iron uptake and leaf necrosis. Plant Physiol. **93:** 717–722
79 Grusak M. A. and Pezeshgi S. (1996) Shoot-to-root signal transmission regulates root Fe(III) reductase activity in the *dgl* mutant of pea. Plant Physiol. **110:** 329–334
80 Ling H. Q., Pich A., Scholz G. and Ganal M. W. (1996) Genetic analysis of two tomato mutants affected in the regulation of iron metabolism. Mol. Gen. Genet. **252:** 87–92
81 Dodge A. (1994) Herbicide action and effects on detoxification processes. In: Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants, pp. 219–236, Foyer C. H. and Mullineaux P. M. (eds), CRC Press, Boca Raton
82 Korbaschi P., Kohen R., Katzhendler J. and Chevion M. (1986) Iron mediates paraquat toxicity in *Escherichia coli*. J. Biol. Chem. **261:** 12472–12476
83 Zer H., Peleg I. and Chevion M. (1994) The protective effect of desferrioxamine on paraquat-treated pea (*Pisum sativum*). Physiol. Plant. **92:** 437–442
84 Cairo G., Tacchini L., Pogliaghi G., Anzon E., Tomasi A. and Bernelli-Zazzera A. (1995) Induction of ferritin synthesis by oxidative stress. Transcriptional and post-transcriptional regulation by expansion of the 'free' iron pool. J. Biol. Chem. **270:** 700–703
85 Cakmak I. and Marschner H. (1992) Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. Physiol. Plant. **98:** 1222–1227