Ferritin accumulation and degradation in different organs of pea (*Pisum sativum*) during development

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Iron concentration and ferritin distribution have been determined in different organs of pea (*Pisum sativum*) during development under conditions of continuous iron supply from hydroponic cultures. No ferritin was detected in total protein extracts from roots or leaves. However, a transient iron accumulation in the roots, which corresponds to an increase in iron uptake, was observed when young fruits started to develop. Ferritin was detectable in total protein extracts of flowers and pods, and it accumulated in seeds. In seeds, the same relative amount of ferritin was detected in cotyledons and in the embryo axis. In cotyledons, ferritin and iron concentration decrease progressively during the first week of germination. Ferritin in the embryo axis was processed, and disappeared, during germination, within the first 4 days of radicle and epicotyl growth. This degradation of ferritin *in vivo* was marked by a shortening of a 28 kDa subunit, giving 26.5 and 25 kDa polypeptides, reminiscent of the radical damage occurring in pea seed ferritin during iron exchange *in vitro* [Laulhere, Laboure & Briat (1989) J. Biol. Chem. **264**, 3629–3635]. Developmental control of iron concentration and ferritin distribution in different organs of pea is discussed.

INTRODUCTION

Living organisms frequently face variations in the concentration of available essential nutrients present in their environment. Uptake of these nutrients and their distribution in different specialized organs during normal development is strictly controlled by a series of homeostatic mechanisms. Among these essential nutrients, iron is of great interest because of its role in important metabolic processes such as oxygen transfer, nitrogen fixation, electron transfer and DNA synthesis (ribonucleotide reduction). However, its tendency to form insoluble salts in aqueous solutions and its potential for toxicity via free-radical formation as a result of redox reactions in the presence of oxygen led to the evolution of specific genetic systems which control iron homeostasis in cells. These systems include iron uptake, transport and storage [for reviews, see Crichton & Charlotiaux-Wauters (1987) and Theil (1987)].

Iron storage is achieved by a class of multimeric (24-mer) proteins called ferritins (Theil, 1987). They are organized in hollow spheres able to accommodate a few thousand iron atoms inside their central cavity and they are present in all living organisms. Ferritins are known to sequester and thus detoxify iron taken up by cells which is not utilized for metabolic requirements. Under conditions of iron need, ferritin-Fe(III) can be released by reduction for cellular use (Bienfait & van den Briel, 1980; Laulhere *et al.*, 1990). Therefore ferritins are key proteins acting as a buffer for iron, protecting cells from a harmful concentration of free iron and regulating their immediate need.

Animal ferritins have been extensively studied, and a wealth of information is now available concerning their structure and function (Crichton & Charlotiaux-Wauters, 1987; Theil, 1987). In plants, most of the information concerning ferritins has been gained from electron-microscopy studies in the 1960s and 1970s (for a review, see Seckbach, 1982). These cytological studies have indicated that plant ferritins were detectable in plastids of many cells, mainly in tissues which were not involved in full photosynthetic activity and throughout the plant life cycle.

Electron-microscopy studies have also shown that, when iron was given to chlorotic bean plants, they accumulated large deposits of ferritins within a few days (Seckbach, 1968). These electron-microscopy observations of plant ferritins have the disadvantage that only the iron cores of well-filled ferritin molecules can be recognized. The non-detectability of dispersed ferritin molecules and those with small iron cores limits the information from these studies. The only quantitative data available about ferritin protein content in plants are restricted to bean leaves (van der Mark *et al.*, 1981, 1982, 1983a,b,c) and soybean cell suspensions (Proudhon *et al.*, 1989). In both systems the environmental effect of iron on the induction of ferritin synthesis has been studied by using immunological methods. Both studies reached the same conclusions, namely that plant ferritins are nuclear-encoded, located in plastids and that the control of iron induction of their synthesis may occur at the transcriptional level.

Knowledge about the ferritin content and the iron concentration in different organs of a plant, supplied continuously with iron (i.e. not iron-loaded after a deficiency period) throughout its life cycle is clearly needed in order to start the study of the developmental regulation of ferritin synthesis in plants. We have recently purified and characterized a pea (*Pisum sativum*) seed ferritin and raised antibodies against it (Laulhere *et al.*, 1988, 1989); we have also investigated the environmental effect of iron on the induction of the synthesis of ferritin in soybean (*Glycine max*) cell suspensions (Proudhon *et al.*, 1989). In the present paper we report how ferritins are distributed in different organs of pea throughout its development, with continuous iron supply during growth. We show that ferritin accumulates during seed formation in the embryo axis and cotyledons in order to store iron and they are processed and disappear during the first week of germination. In our system, under continuous iron supply, no ferritin is detectable in vegetative organs (roots and leaves). From our results we conclude that the synthesis and degradation of ferritin in plants continuously supplied with iron, as well as iron distribution in different organs during their life cycle, is developmentally regulated.

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Fig. 1. Schematic representation of hydroponic cultures of pea supplied with $100 \mu\text{M-Fe}^{3+}\text{-EDTA}$

The nomenclature attributed to different samples is as follows: Em, embryo axis; C, cotyledons; Ep, epicotyls; H, radicles; R, roots; L, leaves; F, flowers; P, pods; S, seeds; the numbers indicate the age (in days) of organs when collected. S42 (1-2-3) are 42-day-old seeds of calibrated sizes of respectively 4, 7 and 9 mm in length; I, imbibition (seeds were allowed to imbibe for 24 h in aerated distilled water); II, seeds were laid in the dark for 6 days on Whatman 3MM filters wetted with distilled water; III, seedlings were in the light for 12 h before being put in hydroponic cultures; IV, hydroponic cultures.

Fig. 2. Iron concentration in different organs of pea harvested at different growth stages during its life cycle (a, in roots; b, in leaves)

The nomenclature attributed to the different samples is explained in Fig. 1. Results are means for triplicate experiments. Bars indicate the highest and the lowest values obtained.

MATERIAL AND METHODS

Plant material

Dried peas (*Pisum sativum*, cv. Douce Provence) were obtained from Vilmorin, Paris, France. Pea plants were grown according to the protocol shown in Fig. 1. Briefly, dry seeds were soaked for 24 h in aerated distilled water. Then seedlings were grown for 7 days in the dark on three layers of filter paper (Whatman 3 MM) regularly wetted with distilled water. The 7-day-old plantlets were exposed to light ($100 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) for 12 h before their transfer on to a liquid medium using a hydroponic culture procedure previously described (Laulhere & Mache, 1979). Conditions of the cultures were 16 h of light ($100 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) at

Fig. 3. Measurement of ^{59}Fe incorporation in the vegetative organs of pea at different times (a, in roots; b, in leaves)

Plants were placed for 3 h with their roots soaking in an aerated solution of $^{59}\text{Fe(III)-EDTA}$. Then roots and leaves were ground and assayed for radioactivity. Results are means for duplicate experiments.

23°C and 8 h of dark at 19°C . Iron was supplied as $100 \mu\text{M-Fe(III)-EDTA}$ to the mineral solution throughout the life cycle of the plants. Different organs were harvested at different times (Fig. 1), frozen in liquid N_2 and stored at -70°C .

Protein extractions and analysis

Pea seed ferritin was purified as described (Laulhere *et al.*, 1989). Total proteins were extracted from 0.2–1 g of selected organs as described by Nechustai & Nelson (1985), except that 0.1% *o*-phenanthroline and 20 mM-EDTA were added to the extraction buffer in order to prevent Fenton reactions leading to possible ferritin degradation during sample preparation (Laulhere *et al.*, 1990). Protein concentrations were determined by the method of Bradford (1976). Protein electrophoresis and immunoblots were performed as previously described (Laulhere *et al.*, 1988).

Iron concentration and uptake measurements

Total iron concentration of the different organs analysed at different time of pea development was determined in triplicate for each of these organs from samples harvested at random from several plants. Tissues were ground in a mortar with a pestle in the presence of liquid nitrogen and 100–200 mg (wet weight) from each sample were mineralized according to Beinert's (1978) procedure. Iron concentration was measured by absorbance of Fe^{2+} -*o*-phenanthroline (0.02%) at 510 nm at pH 6.0 (50 mM-acetic acid/NaOH buffer) using thioglycolic acid (Sigma) as reducing agent. The absorption coefficient used for the Fe^{2+} -*o*-phenanthroline complex was $9780 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Dry weight was estimated by weighing samples before and after desiccation at 100°C .

Iron-uptake experiments were performed as follows. Plants from hydroponic cultures were placed in vials with their roots soaked in a 30 ml of an aerated solution containing $100 \mu\text{M}$ - Fe(III)-EDTA and $20 \mu\text{Ci}$ of ^{59}Fe [Amersham, iron(III) chloride in 0.1 M-HCl; 39 MBq/ml; $0.11 \mu\text{g}$ of Fe/ml]. After 3 h at room temperature, roots were washed with distilled water, then with 20 mM-KCl/5 mM-Na-EDTA until no radioactivity was detect-

able in the washing solution. Roots and leaves were weighed. Incorporation of ^{59}Fe was estimated by measuring ground root and leaf samples counts in liquid-scintillation cocktail (Ready Safe; Beckman). To reveal ^{59}Fe distribution in the whole plant, autoradiography at -70°C on Kodak XAR5 film was performed.

RESULTS

Ferritin is not detectable in vegetative organs

We determined the total iron concentration in roots and leaves taken from plants grown in a hydroponic culture containing stable iron concentration. Representative results obtained with plants from one culture are shown in Fig. 2. In the roots (Fig. 2), starting from $10 \mu\text{mol}$ of iron/g of dry matter in 4-day-old radicles, a maximum of $33 \mu\text{mol}$ of iron/g of dry matter is reached in the roots at day 31, followed by a rapid decrease. This 3-fold increase has been repeatedly observed in different cultures with the maximum varying between day 28 and day 31. This maximum iron concentration in the roots is 5 times higher than in 42-day-old seeds. It is noteworthy that, by this time, young fruits

Fig. 4. Distribution of ferritin in different vegetative organs of pea during development

(a) In the roots: (i) Coomassie Blue R250 staining of an SDS/15%-(w/v)-polyacrylamide gel loaded with $20 \mu\text{g}$ of total protein extracts (H4 to R42). The nomenclature is as in Fig. 1. M, Molecular-mass markers: 94, 67, 43, 30, 20 and 14 kDa. Fe, 250 ng of purified pea seed ferritin. (ii) Immunoblot of a duplicate of (i) probed with antibodies raised against pea seed ferritin. (ii) Immunoblot of proteins from roots ($20 \mu\text{g}/\text{lane}$), including R31. (b) In leaves: (i) same as (a) (i), but with leaf samples; (ii) immunoblot of a duplicate of (i) probed with antibodies raised against pea seed ferritin.

have started to develop, most of the plants have flowered between day 26 and day 28. Iron accumulation in the roots in the early stages of fruit development were also observed in a ^{59}Fe -uptake experiment (Fig. 3). A strong stimulation of iron uptake by the roots is clearly observed at day 32.

In green leaves, iron concentration was almost constant, with a mean value of $8\ \mu\text{mol}$ of iron/g of dry matter (Fig. 2b), except at day 42, for which it increases slightly to $10\ \mu\text{mol}$. Translocation of iron in the leaves has been estimated at different times by determining ^{59}Fe activity in the leaves (Fig. 3b). At day 32, no further iron is transported into the leaves over that which occurred at day 28 or 34. Furthermore, an autoradiogram of a 32-day-old plant which had incorporated ^{59}Fe for 3 h showed clearly that the bulk of ^{59}Fe was concentrated in the roots; a small amount of this labelled iron (about 0.3 %) was translocated uniformly during this time into the upper part of the plant (result not shown). Thus an increase of iron uptake by the roots of pea in hydroponic culture occurs at the beginning of fruit formation. This led to a transitory accumulation of iron in the roots.

Fig. 5. Iron concentrations in different reproductive and storage organs of pea

The nomenclature used is as explained in Fig. 1. Results are means for triplicate experiments. Bars indicate the highest and the lowest values obtained.

In order to determine whether these variations of iron concentration influence the accumulation of ferritin in vegetative organs, we analysed total protein extracts prepared from roots and leaves of different ages for ferritin using antibodies raised against pea seed ferritin (Laulhere *et al.*, 1988). When $20\ \mu\text{g}$ of total protein from roots were analysed, no ferritin was detected (Fig. 4a), even at day 31, which corresponds to the maximum iron concentration in this organ (Fig. 2a). However, when $40\ \mu\text{g}$ of protein was analysed, trace amounts of ferritin were detectable uniformly in all samples (results not shown). In 4-day-old epicotyls (Fig. 4b) ferritin was detectable with a pattern very reminiscent of processed ferritin (Laulhere *et al.*, 1989, 1990; and see below). After leaf greening, no ferritin was detectable in the leaves (Fig. 4b), even when up to $40\ \mu\text{g}$ of protein was loaded on to a gel (results not shown).

Ferritin accumulates in reproductive and storage organs

Iron concentration (Fig. 5) decreased in cotyledons between days 2 and 4 from germination, whereas it increased during seed maturation at day 42. Immunodetection experiments confirmed our previous results (Laulhere *et al.*, 1988) that ferritin is present in flowers as well as in pods and seeds, the ferritin concentration of seeds increasing with maturity (Fig. 6b). These observations indicate that ferritin, in normal plants (i.e. not iron-loaded), accumulates in order to store iron in seeds, since purified pea seed ferritins contain 1800 atoms of iron per molecule (Laulhere *et al.*, 1988). This iron could be stored for future use during seedling growth, as has been proposed by Hyde *et al.* (1963). However, iron release from pea seed ferritin has been shown to promote the processing of ferritin subunits *in vitro* (Laulhere *et al.*, 1990).

Seed ferritin is processed and disappears during germination

Our observations raise the question of the fate of seed ferritins during germination. Fig. 6 shows that ferritin was present in the same amount of cotyledons and in the embryo axis of dry seeds. However, the absolute mass was much higher in cotyledons, since, in a pea seed, the embryo axis represents about 1 % of the weight of a dry seed. Clearly ferritin concentration decreases in cotyledons during the first 7 days of germination (Fig. 6b; compare

Fig. 6. Ferritin distribution in different reproductive and storage organs of pea

(a) Coomassie Blue R250 staining of an SDS/15%-(w/v)-polyacrylamide gel loaded with $20\ \mu\text{g}$ of total protein extracts (Em0 to P42); the nomenclature is defined in Fig. 1; S42 corresponds to protein extracted from a mix of 42-day-old seeds of different sizes. M, molecular-mass markers; Fe, 250 ng of purified pea seed ferritin. (b) Immunoblot of a duplicate of (a) and of total protein extracts from three stages of maturation of 42-day-old seeds (S42-1, S42-2 and S42-3 are 42-day-old seeds of calibrated sizes of respectively 4, 7 and 9 mm in length) probed with antibodies raised against pea seed ferritin.

Fig. 7. Disappearance of ferritin from the embryo axis during the early stages of germination

Protein was loaded at 40 μg /lane. After electrophoresis and electrotransfer on to nitrocellulose filters, samples were probed with antibodies raised against pea seed ferritin. The nomenclature of samples is as described in Fig. 1. Fe, 250 ng of purified pea seed ferritin.

Scheme 1. Hypothetical pathway for the utilization of iron stored in seed ferritin and subsequently used for seedling development

Abbreviations: P, plastid; Fs, ferritin subunit; PE, plastid envelope; T, transport system; C, cytoplasm; Fe(III), ferric iron; Fe(II), ferrous iron; OH $\cdot$, hydroxyl radical.

lanes C1 and C7). In the embryo axis between zero time and day 4, ferritin concentration decreases greatly (Fig. 7; for example, compare lane Ep2 with lane Ep4, and lane H2 with lane H4) and polypeptides of molecular mass lower than the basic 28 kDa pea seed ferritin subunit (Laulhere *et al.*, 1989, 1990) are revealed with time by the antibodies. These observations were true both for the epicotyl and radicle. Ferritin degradation seems to be faster in radicles, since no ferritin was detectable in 4-day-old radicles although it was still detectable in a processed form in

4-day-old epicotyls when 20 μg of total proteins was loaded on to the gel (Figs. 4a, ii, R4 and 4b, ii, Ep4). When 40 μg of protein was loaded on to the gel, traces of ferritin were visible in 4-day-old radicles (Fig. 7). Possibly a faster growth rate of the radicle compared with the epicotyl is responsible.

DISCUSSION

We examined the iron concentration and the ferritin distribution in different organs of pea during development when grown in hydroponic cultures under conditions of continuous iron supply. Ferritin was detectable in reproductive and storage organs, but not in roots and leaves. These observations are in agreement with most of the cytological studies reported (Seckbach, 1982). However, van der Mark *et al.* (1981, 1982) have reported that ferritin containing a low number of iron atoms per molecule can be detected in small amounts in developing primary bean leaves under various light conditions and with constant and changing iron status. This discrepancy could be due either to a difference in the iron supply conditions used in the two studies or to the fact that our antibody raised against pea seed ferritin (which is able to detect 5 ng of pure antigen when diluted 1:500 using the peroxidase method) may be less titrated than the antibody raised against bean seed ferritin used by van der Mark *et al.* (1981, 1982). Nevertheless ferritin clearly accumulates in seeds as an iron-storage compound, each molecule containing a mean value of 1800 atoms of iron (Laulhere *et al.*, 1988).

Marinos (1967) reported that ferritin localized in the plastids of the meristematic dome and leaf primordia of dormant potato (*Solanum tuberosum*) tuber buds disappears when the buds sprout. Hyde *et al.* (1963), who have identified ferritin in ungerminated pea cotyledons, propose that ferritin iron is mobilized for young seedling axis development. Supporting this suggestion, Tiffin *et al.* (1973) have reported a translocation of iron from soybean cotyledons to other parts of the plant, and those authors raised the question of whether a proteinase is involved in releasing iron from the ferritin protein shell and also discussed a mechanism for iron release from the germinating cotyledon. We conclude that the embryo axis has its own iron reserve to start its growth during the early steps of the germination process (Figs. 6 and 7). Clearly, ferritin from the embryo axis disappears in radicles and epicotyls during the first 4 days of germination (Fig. 7), whereas in cotyledons at day 7 it is still present, but at a much lower concentration than at day 1 (Fig. 6). These results are consistent with a proposal by Hyde *et al.* (1963) concerning the utilization of the ferritin iron from seeds for young seedling axis development. At least in the embryo axis, the disappearance of ferritin with time is accompanied by the appearance of ferritin fragments of lower molecular mass than the basic 28 kDa subunit. These shorter polypeptides have the same molecular mass as those observed *in vitro* in response to free-radical damage produced by reduction-mediated iron release (Laulhere *et al.*, 1989, 1990) using purified pea seed ferritin. Possibly specific free-radical cleavage at the N-terminal part of the 28 kDa subunit of pea seed ferritin (Laulhere *et al.*, 1989) is the initial signal for ferritin degradation. It is well known that proteins affected by radical damage are more susceptible to proteinases (Davies, 1987; Davies & Delsignore, 1987; Davies *et al.*, 1987). Therefore, conceivably, proteinases degrade ferritin after free-radical damage occurs, initiated from oxidation of Fe(II) to Fe(III) during metabolic reactions requiring iron during germination. In order to answer the questions posed by Tiffin *et al.* (1973) about the mechanism responsible for iron release during germination, we propose the model given in Scheme 1. During germination a metabolic switch increases the reducing

power of plastids, which contain the ferritin. This allows reduction of the Fe(III) to Fe(II) in the mineral core of ferritin, inducing the release of Fe(II) (Laulhere *et al.*, 1989, 1990). This Fe(II) is either reincorporated into the mineral core of ferritin (Laulhere *et al.*, 1990) or reoxidized by O_2 and chelated by organic acids, among which the principal one could be citrate (Cataldo *et al.*, 1988; van der Mark *et al.*, 1981). This Fe(II)/Fe(III) cycle, in the vicinity of ferritin molecules and in the presence of O_2 , generates free radicals by the Fenton reaction, leading to the degradation of ferritin subunits (Laulhere *et al.*, 1989, 1990) and to the disappearance of the protein. A portion of the Fe(III)-chelates is used by plastids for their functions; another portion could be translocated to other parts of the developing seedlings, as mentioned by Tiffin *et al.* (1973). This model implies the transport of iron through the plastid envelope. The mechanism of such a transport is unknown and deserves further investigation.

At a time corresponding to the early stages of pea fruit development (days 28–32), the iron concentration is 5 times higher in roots, where ferritins are not detectable, than in seeds at day 42, where ferritin is observed. On the basis of our ^{59}Fe -uptake experiments (Fig. 3) and because iron concentrations in leaves are rather constant at that time (Fig. 2), it is more likely that this root iron has been accumulated from the culture medium. Surprisingly, iron accumulation in the root did not lead to ferritin accumulation. The limit of detection in our experiments is 5 ng of ferritin protein. Bean and pea seed ferritins contain an average of 1800 atoms of iron per molecule (Laulhere *et al.*, 1988; van der Mark *et al.*, 1982), as does iron-induced ferritin in bean leaves and in soybean cell suspensions (van der Mark *et al.*, 1982; Lescure *et al.*, 1990). Therefore if ferritin (containing 1800 atoms of iron/molecule) were present in the roots at that time, this ferritin iron would represent less than 0.07% of the iron concentration we detected. Less than 0.2% of the iron determined in roots, when young fruits start to develop, could be stored inside ferritin, even if the ferritin were completely saturated with iron (i.e. about 5000 iron atoms per molecule; Crichton *et al.*, 1978). We have no idea where this root iron is located. It is unlikely that it is located on the root surface, since Branton & Jacobson (1962) have shown that plants grown in the presence of iron had little ^{59}Fe coating the roots, even after 3 h of incubation with ^{59}Fe . Therefore it is plausible that a transitory non-ferritin iron pool exists inside the roots at that particular moment. It has been noted that iron accumulates in the root parenchyma before release to the xylem occurs (Branton & Jacobson, 1962). Citrate could play a role in this storage, since it has been reported to be the main iron transporter in plants (Cataldo *et al.*, 1988). Also, in yeast, a large amount of iron can be stored in the chelated form in small unidentified molecules located in the vacuoles (Raguzzi *et al.*, 1988). Furthermore, soluble iron, not associated with ferritin, can be stored in or around the vessels in leaves (Branton & Jacobson, 1962; van der Mark *et al.*, 1982), this iron being easily accessible for transport from the leaves to the other parts of the plant.

Supplying surplus iron to iron-deficient plants leads to the induction of ferritin synthesis; this environmental induction could be controlled at the transcriptional level (Proudhon *et al.*, 1989; van der Mark *et al.*, 1983c). Developmental control of

ferritin synthesis in plants is very little documented. However, a question which arises from our results is: can the transitory increase of iron concentration in the roots at the beginning of fruit development influence the induction of ferritin synthesis in the fruits? If these two events were directly related, mechanisms controlling ferritin synthesis for iron storage or for iron detoxification could be the same, with iron being the main effector. An alternative would be that apoferritin synthesis in fruit, as well as the iron-uptake system in the roots, are turned on by a co-ordinate developmental signal leading to iron storage in seed ferritins.

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