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The role of ferritin in developing primary bean leaves under various light conditions

F. van der Mark, T. de Lange, and H.F. Bienfait
Department of Plant Physiology, University of Amsterdam, Kruislaan 318, NL-1098 SM – Amsterdam, The Netherlands

Abstract. Ferritin and ferritin-iron in the primary leaves of *Phaseolus vulgaris* L. were determined during growth in the dark, in the light, and during de-etiolation. The ratio ferritin protein/total protein appeared to be rather constant. In dark-grown leaves maximally 50% of the total extractable iron was found to be present in ferritin. This percentage was lower in de-etiolating and light-grown leaves. In ten-day-old green leaves no ferritin-iron could be measured. The translocation of iron from cotyledons to the developing plant appears to be related to the need for iron in the leaves. These results suggest that ferritin acts as a buffer molecule for iron in plants.

Key words: Etiolation – Ferritin – Greening – Iron-metabolism – *Phaseolus*.

Introduction

Iron is present in the active centre of many electron carriers in photosystems I and II and is therefore essential for the process of photosynthesis. The synthesis of chlorophyll is also dependent on the presence of iron; iron deficiency causes chlorosis in plants. Although its availability is necessary for the development of the photosynthesizing apparatus, soluble iron may have harmful effects on the cell. Ferrous ions, by oxidation with oxygen, give rise to superoxide, O_2^-. Ferric ions should be chelated in order to be soluble and to prevent binding to, and inactivation of, proteins.

The iron-storage protein ferritin makes an intracellular iron reserve possible. Ferritin is a hollow shell with a capacity for 4500 iron atoms and it is known as the major iron storage protein in the mammalian system (Munro and Linder 1978). The protein is also found in plants, certain fungi (David and Easterbrook 1971), and a bacterium (Stiefel and Watt 1979). The presence of ferritin in plants was first noticed by Hyde et al. (1963). They suggested that iron is stored in a nontoxic form as a phyto-ferritin and that this iron may subsequently be used to form iron-containing components of the photosynthetic apparatus. In her study of chloroplast development in leaves and hypocotyls in *Phaseolus vulgaris,* Whatley (1977) found small ferritin accumulations in the plastids of primary leaves in the very early period of development. A disadvantage of these electron-microscope studies is that only highly-filled ferritin can be detected. Our approach was to use quantitative immunological determinations for ferritin protein and ferritin-iron in the leaf and the cotyledons of *Phaseolus vulgaris*.

Materials and methods

Plant material. Seeds of *Phaseolus vulgaris* L. cv. Prelude, obtained from Sluis B.V., Enkhuizen, The Netherlands, were used for the isolation of bean ferritin. Seedlings were grown on Perlite containing low levels of endogenous iron and nutrient medium without iron and with $Ca(NO_3)_2$ instead of $CaSO_4$, but otherwise as described by Sluiters-Scholten et al. (1973). A light regime of 16 h light and 8 h dark was used. During de-etiolation plants were continuously illuminated. The light intensity in all experiments was 8400 lx (Philips TL 33).

Isolation of bean ferritin. Ferritin was isolated by the procedure described by Crichton et al. (1978) with the following modifications. After each ultracentrifugation step only the brown-colored part of the pellet was resuspended. After two cycles of low and high speed centrifugation steps, the solution was adjusted to 0.01% Triton-X 100 (Merck) and was sonicated 3 times for 10 s.

The preparation was recentrifugated and a clear brown pellet was obtained. After DEAE-cellulose chromatography (Whatman DE-52) a pure ferritin fraction was obtained. The isolated protein was judged to be homogeneous on the basis of polyacrylamide gel electrophoresis under denaturating and non-denaturating conditions.

The protein was stored in 60% glycerol (Merck) at −20° C. Under these conditions the protein is stable for at least six months. Protein was measured by the Lowry method or the assay according to Bradford (1976).

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Iron was determined with bathophenantroline sulfonate (Sigma) after reduction (Zuyderhoudt et al. 1978). The iron content of the isolated bean ferritin was 1900 atoms Fe per molecule.

Partial purification of leaf ferritin. Leaf ferritin was partially purified by 50% ammonium sulfate precipitation of a leaf extract. The pellet was resuspended and after dialysis put onto a DEAE-52 (Whatman) column that was eluted with a linear gradient of 0–0.5 M NaCl in 50 mM Tris-HCl, pH 7.5. Ferritin-containing fractions were pooled; about 25% of the protein was ferritin.

Preparation of antibodies. Antisera were obtained from rabbits injected twice with about 100 µg isolated bean ferritin. An immunoglobulin fraction was prepared by 50% ammonium sulphate precipitation and DEAE-cellulose fractionation.

Crossed and tandem-crossed electrophoresis were performed according to Laurell (1965), immunodiffusion according to Mancini (1965).

Quantitative immunoelectrophoresis (Laurell 1966) was performed in 1% agarose M.E. (Marine Colloids Inc.) in Tris-barbital-sodium barbital buffer, pH 8.8, with 0.4 mM calcium lactate and 0.5% Triton-X 100.

Concentrations of ferritin greater than 1 $\mu g\,ml^{-1}$ solution could be detected in this assay. In comparison to the radial immuno-diffusion method as described by Mancini (1965), a good correlation was found. No influence was found by varying the concentrations of total protein of the samples (Scott and Burnett 1978). Ferritin iron was measured according to a method based on an immunological assay developed by Zuyderhoudt et al. (1978). Extracts were incubated with antibodies against ferritin coupled to Sepharose 4B gel material. Bound ferritin was then separated from the extract by centrifugation and the ferritin iron value was determined after reduction.

Preparation of the bean extract. Seeds were soaked for at least 2 days in water at 4° C. After removal of the seed coat the cotyledons were weighed and homogenized twice in a Waring Blendor for 2 min in 50 mM Tris-HCl, pH 7.5, buffer with 5 mM β-mercapto-ethanol. The homogenate was adjusted to 50 mM $MgCl_2$, centrifugated at 2000 *g* for 5 min, and the supernatant dialyzed against 50 mM Tris-HCl, pH 7.5.

Preparation of leaf extracts. Depending on their age, 10 to 100 pairs of leaves were ground in an IKA-WERK, type 10, homogenizer for 30 s in the presence of 20% (w/w) polyvinyl-polypyrolidone (PVPP), 50 mM Tris-HCl, pH 7.5, buffer, and 5 mM β-mercapto-ethanol. Further homogenization was obtained with a mortar and an all-glass homogenizer. The total iron content of the leaves was measured in a sample taken from this homogenate. Cell wall material and PVPP were removed by filtering over a 10-µm nylon gauze filter (MLO Nubolt) under suction. The filtrate was adjusted to 50 mM $MgCl_2$; starch and part of the membrane-bound proteins were removed by centrifugation at 2000 *g* for 5 min.

The supernatant was dialyzed against 50 mM Tris-HCl buffer, pH 7.5, extracts were stored at 4° C in the presence of 0.02% thimerosal (Sigma). The reliability of the extraction procedure was controlled by addition of a known amount of ferritin prior to homogenization. Re-extraction of the filter mass with 0.5% sodium deoxycholate did not give rise to a significant increase in ferritin yield. Non-ferritin iron was only partially extracted by this procedure. The ultimate extract of grean leaves, dark grown leaves, and bean seeds contained 55%, 70% and 80%, respectively, of the total iron content of the tissues extracted. Ferritin did not lose iron during the extraction procedure (data not shown). Total protein, total iron, ferritin protein, and ferritin-iron content of the leaf and bean extracts were determined.

Total iron determinations. Up to 1.5 ml of the homogenate, the bean or leaf extract was freeze dried and ashed at 650° C; iron was determined by the method of Scott (1944).

Chlorophyll content of the extracts. Chlorophyll was quantitatively recovered in the final extract and assayed according to Bruinsma (1963).

Protein determination. Protein concentration of the extracts was estimated by the Lowry method with bovine serum albumin as a standard.

Results

Specificity of the antiserum. The IgG fraction of the antiserum raised against bean ferritin produced one precipitation line in the double diffusion technique of Ouchterlony and the crossed immunoelectrophoresis test, with bean ferritin or primary leaf extract as antigens (Fig. 1a). Protein and iron staining (not shown) gave the same precipitation line. In tandem-crossed electrophoresis with ferritins from bean and leaf, a continuous precipitation line was formed without spur (Fig. 1b). This indicates that there is no antigenic difference between the two ferritins. We therefore used quantitative immunoelectrophoresis as a specific assay for ferritin in all extracts.

Iron and ferritin in cotyledons. The importance of ferritin in the storage of iron in the cotyledons of *Phaseolus vulgaris* was investigated. It appeared that 56% of the iron extracted was stored by ferritin. Related to the total iron content of the cotyledons this percentage is 42%. The average iron content of the ferritin in cotyledons turned out to be $1{,}880 \pm 180$ atoms iron per molecule.

The role of ferritin in the development of the primary leaf. In order to investigate the role of ferritin in the growing leaf it is important to know the changes in total iron content. There appeared to be a continuous influx of iron in the developing leaf under all light conditions (Fig. 2a). For comparison the chlorophyll content of primary leaves as a function of time is presented (Fig. 2b), which suggests that the amount of iron in a leaf is related to its chlorophyll content (see discussion). The major source of the leaf iron is most probably the cotyledons since their iron content was concomitantly reduced (data not shown, see Tiffin et al. 1973).

The results of the experiments to elucidate the role of leaf ferritin are summarized in Table 1. The ferritin content in relation to the total protein in leaves appears to be rather constant under all conditions (Columns a, b and c). In the dark, the leaves gradually accumulate ferritin iron (Column d). The

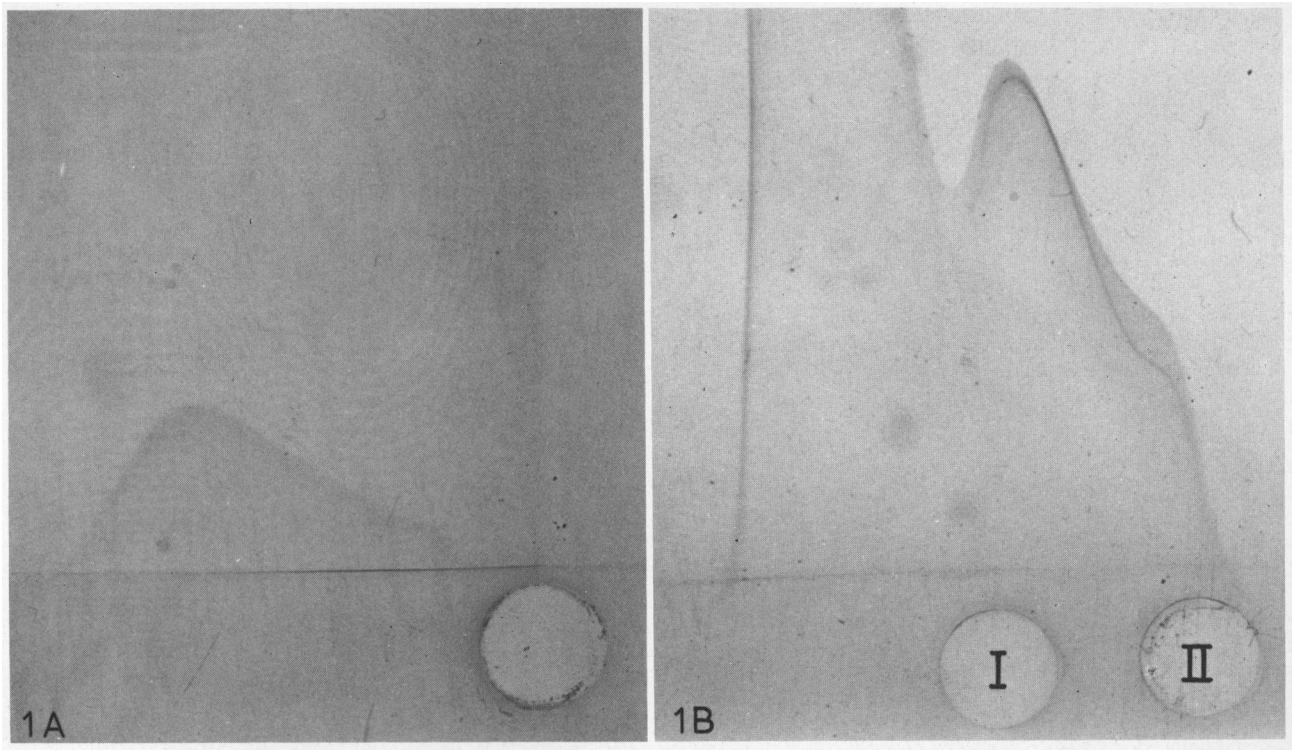

Fig. 1 A, B. Crossed electrophoresis of *Phaseolus* ferritins. After electrophoresis in the first direction the gelstrip was embedded in agarose containing antiserum raised against purified bean ferritin and electrophoresis was repeated perpendicularly to the first direction. **A**. Crossed electrophoresis of an extract of 6 d old etiolated leaves made as described in Materials and methods. **B**. Tandem crossed electrophoresis. Well *I* contained seedferritin (2.5 µg), well *II* partially purified leaf ferritin (1.5 µg)

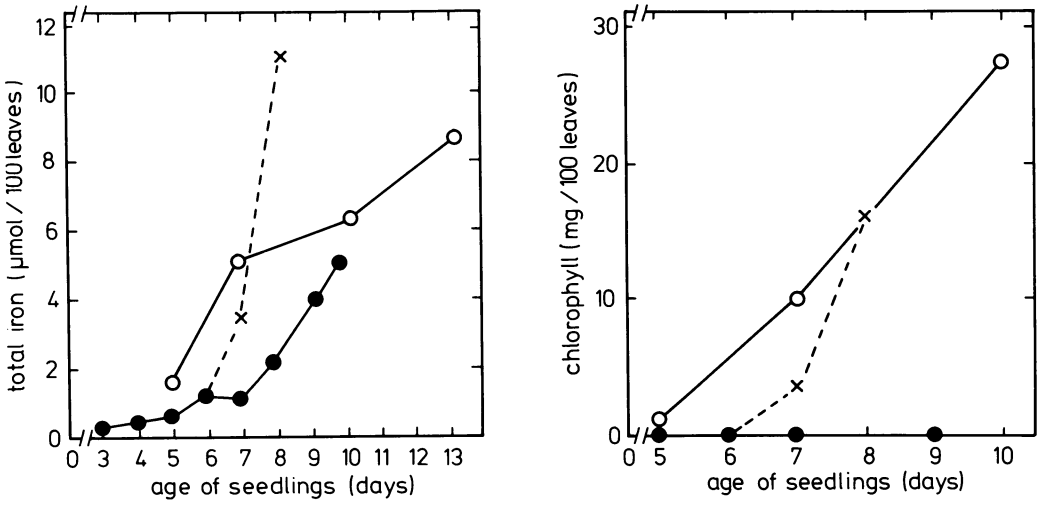

Fig. 2a, b. Total iron and chlorophyll content of primary leaves of *Phaseolus vulgaris* grown under various light conditions. ●—● grown in the dark; ○—○ grown under a light regime of 16 h light and 8 h dark; ×—× de-etiolation in continuous light after 6 d in the dark. **a** total iron content; **b** chlorophyll content

iron content of this ferritin increases steadily (Column g) but remains low, less than 600 atoms iron per molecule ferritin, compared with the mean iron content of seed ferritin (1,880 atoms iron/molecule). During the first seven days of the development in the light the leaves store iron in ferritin (Column f). After the 5th or 6th day, however, the iron content of the ferritin molecules seems to decrease (Column i), although the total amount of ferritin iron still increases until at least day 7 (Column f). Finally, on day 10, ferritin iron is reduced to a very low level.

Plants that are put in the light after five days

Table 1. Ferritin protein and ferritin iron in extracts of primary bean leaves under various light conditions

Age of seedlings in days	Ferritin µg/100 µg protein			Ferritin-Fe µmol Fe in 100 leaves			Ferritin Fe content[a] atoms Fe/molecule		
	dark grown (a)	greening (b)	light grown (c)	dark grown (d)	greening (e)	light grown (f)	dark grown (g)	greening (h)	light grown (i)
3	0.17	–	–	0.02	–	–	180	–	–
4	0.24	–	–	0.06	–	–	275	–	–
5	0.34	–	0.21	0.17	–	0.23	315	–	560
6	0.33	0.32	–	0.31	0.33	–	310	350	–
7	0.32	0.28	0.18	0.36	0.85	0.44	340	375	420
8	0.29	0.26	–	0.56	1.61	–	370	420	–
9	0.25	–	–	1.11	–	–	560	–	–
10	0.32	–	0.14	1.43	–	<0.1	540	–	< 25
13	–	–	0.22	–	–	<0.1	–	–	< 25

[a] Ferritin iron content was calculated for a protein molecular weight of 480,000

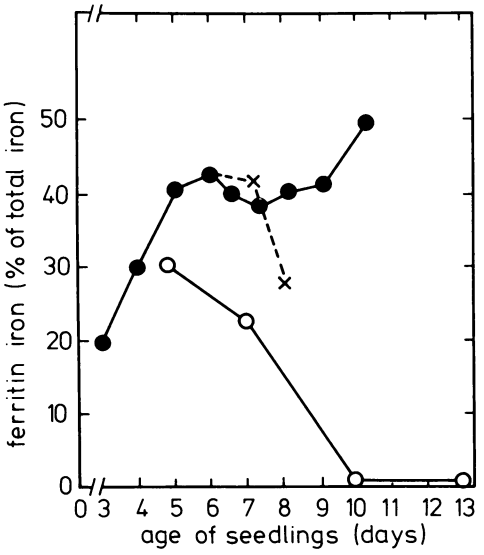

Fig. 3. Ferritin-iron as a fraction of total iron in extracts of primary bean leaves, grown under various light conditions. ●—● grown in the dark; o—o grown under a light regime of 16 h light; and 8 h dark; x—x de-etiolation in continuous light after 6 d in the dark

of growth in the dark show a rapid increase in the iron content of the leaf (Fig. 2a), with a parallel increase in the ferritin iron (Table 1, Column e). Thus, after two days of de-etiolation, ferritin iron is more than doubled with respect to the dark control (Column d). Eventually, the level of ferritin-iron will probably decrease to the level found in plants grown in the light from the movement of sowing.

Figure 3 shows ferritin iron as a fraction of the total extractable iron. In leaves grown in the dark ferritin stores about 40% to 50% of the extractable iron. In light-grown and de-etiolating leaves the ferritin-iron as part of the extractable iron is gradually reduced. This indicates that during development of functional chloroplasts a relatively decreasing quantity of iron is stored by ferritin.

Discussion

The storage function of ferritin is well demonstrated by the fact that 40% to 50% of the total extractable iron in older dark-grown leaves is ferritin iron. The question is whether in leaves with developing chloroplasts the required iron is derived from ferritin, because in such leaves there is a rapid influx of iron from the cotyledons into the primary leaves (Fig. 2a). Also, it is shown that in light-grown plants, the ferritin-iron content doubles from day 5 to day 7, while after illumination of plants grown for 6 days in the dark, ferritin-iron increases 5 fold in 2 days (Table 1, Columns f and e). So in both cases a part of the total iron supply appears to be stored in a nonfunctional state in ferritin.

The picture that emerges from these data for the role of ferritin in the development of the primary leaf can be formulated as follows: iron is transported to the developing leaves at a rate which is strongly dependent on the light regime, but in a non-straightforward manner. The development of the plastids, and thus the need for iron in the growing leaf, is not necessarily parallel to the rate of iron influx. Ferritin, being located in the cell, serves as a buffer between iron influx and utilization. In this way it spares the individual cell from an overdose of iron, e.g., at illumination after prolonged growth in the dark. In a later phase it can give up its iron: after ten days of growth in the light, almost no ferritin iron is present in the primary leaves.

Our findings do not support the model of Sprey et al. (1978). They suggested that upon illumination

iron, present in etioplasts of tobacco cotyledons as nonferritin iron-phosphate complexes, is taken up by ferritin, after which it is released again for the synthesis of iron-containing enzymes. This would imply a light-dependent increase of ferritin iron content upon illumination. Our data, however, show that in greening tissue the ferritin-iron content is only slightly (about 10%) higher than in the parallel dark control (Table 1, Columns h, g). This slight, if at all significant elevation, can be explained as a consequence of the tremendous influx of iron after illumination.

As calculated from the ferritin-protein and -iron determinations, the iron content of ferritin in leaves is rather low, never more than 600 iron atoms per molecule ferritin. Whatley (1977) observed sporadically ferritin iron cores in plastids of light- and dark-grown leaves of *P. vulgaris* only during the first two days of growth. The average low iron content of the ferritin as found in our immunological determinations can explain the fact that ferritin-iron cores visible in the electron microscope disappear.

Low ferritin iron content is not an unknown phenomenon. Huebers et al. (1976) isolated ferritin from intestinal mucosa with a mean iron content of 550 iron atoms per molecule. Also, in proliferating malignant cells and regenerating cells of the liver, a low iron content of ferritin was found (Linder et al. 1970). As stated by the authors the low iron content of ferritin in these two last cases is correlated with active cell division. According to Dale (1964), in *P. vulgaris,* the increase of cell number in the primary leaf is exponential between days 2 and 8. This also means that in the case of developing primary leaves the low iron content of ferritin coincides with active cell division.

An intriguing phenomenon is the existence of almost empty ferritin in older green leaves, especially because in this stage of growth ferritin is still synthesized. Stiefel and Watt (1979) reported that *Azotobacter* cytochrome $b_{557.5}$ is a bacterioferritin, which indicates that another role for ferritin distinct from iron storage is possible. Whether this is the case in *P. vulgaris* remains to be demonstrated.

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