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Abscisic acid is involved in the iron-induced synthesis of maize ferritin

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The ubiquitous iron storage protein ferritin has a highly conserved structure in plants and animals, but a distinct cytological location and a different level of control in response to iron excess. Plant ferritins are plastid-localized and transcriptionally regulated in response to iron, while animal ferritins are found in the cytoplasm and have their expression mainly controlled at the translational level. In order to understand the basis of these differences, we developed hydroponic cultures of maize plantlets which allowed an increase in the intracellular iron concentration, leading to a transient accumulation of ferritin mRNA and protein (Lobréaux,S., Massenet,O. and Briat,J.F., 1992, *Plant Mol. Biol.*, 19, 563–575). Here, it is shown that iron induces ferritin and RAB (Responsive to Abscisic Acid) mRNA accumulation relatively with abscisic acid (ABA) accumulation. Ferritin mRNA also accumulates in response to exogenous ABA. Synergistic experiments demonstrate that the ABA and iron responses are linked, although full expression of the ferritin genes cannot be entirely explained by an increase in ABA concentration. Inducibility of ferritin mRNA accumulation by iron is dramatically decreased in the maize ABA-deficient mutant *vp2* and can be rescued by addition of exogenous ABA, confirming the involvement of ABA in the iron response in plants. Therefore, it is concluded that a major part of the iron-induced biosynthesis of ferritin is achieved through a pathway involving an increase in the level of the plant hormone ABA. The general conclusion of this work is that the synthesis of the same protein in response to the same environmental signal can be controlled by separate and distinct mechanisms in plants and animals.

Key words: abscisic acid/ferritin/iron stress/maize

Introduction

The use of iron, an essential element for almost all living organisms, is dependent on biological molecules which prevent the problems of insolubility and toxicity of iron in the presence of oxygen. Among these molecules, ferritins, a class of ubiquitous multimeric proteins, play a key role because of their ability to sequester several thousand iron atoms in their central cavity in a soluble, non-toxic bioavailable form (Theil, 1987; Harrison *et al.*, 1989; Crichton, 1990; Andrews *et al.*, 1992).

Eukaryotic ferritins (plant and animal) arise from a common ancestral gene which is illustrated by their high sequence homology (Ragland *et al.*, 1990; Lescure *et al.*, 1991; Spence *et al.*, 1991; Andrews *et al.*, 1992; Lobréaux *et al.*, 1992). This homology implies an extraordinary conservation of their three-dimensional structure (Andrews *et al.*, 1992). Plant and animal ferritins, however, do not share the same cytological location and the control of their synthesis in response to iron overload does not take place by the same mechanism. First, plant ferritins are found within plastids (chloroplasts and non-green plastids), while animal ferritins are cytoplasmic proteins (Seckbach, 1982; Van der Mark *et al.*, 1983b; Ragland *et al.*, 1990; Lescure *et al.*, 1991). Secondly, iron induction of ferritin synthesis is mainly controlled at the translational level in animals, while transcription has been shown to be the principal target of the iron response in plants (Zahringer *et al.*, 1976; Van der Mark *et al.*, 1983a; Klausner and Harford, 1989; Proudhon *et al.*, 1989; Theil, 1990; Lescure *et al.*, 1991). These differences need to be explained.

Concerning the transcriptional control of plant ferritin synthesis in response to iron, two possibilities have to be considered. First, iron by itself could be an effector turning on and off plant ferritin gene transcription by direct interaction with some unknown *trans*-acting factors. For example, in yeast, transcription activation of the metallothionein gene CUP1 occurs only when copper binds to ACE1 (Fürst *et al.*, 1988) and, in *Escherichia coli*, the aerobactin operon is repressed by the Fur protein when it binds Fe^{2+} (Bagg and Neilands, 1987). Alternately, high environmental iron concentrations could generate a stress response inducing ferritin gene transcription, under the control of an integrated plant transduction pathway. Control of expression of genes in response to stress is often mediated by a transduction pathway which involves the plant hormone abscisic acid (ABA) (for a review see Skriver and Mundy, 1990). Under standard iron nutrition conditions, plant ferritins are not detected in vegetative organs (roots and leaves), they accumulate in seeds during their maturation and are degraded in the first days of growth following germination (Lobréaux and Briat, 1991). Furthermore, manipulating the iron concentrations of the culture medium of plant cell suspensions and of hydroponically grown plantlets results in iron loading, and in transient induction of ferritin synthesis in these cells (Lescure *et al.*, 1991), as well as in roots and leaves of plantlets (Lobréaux *et al.*, 1992). This developmental and environmental control of plant ferritin synthesis is reminiscent of the expression pattern of RAB genes (Responsive to Abscisic Acid) and led us to postulate that ABA could be involved in the control of ferritin synthesis in response to iron in plants. It should be noted that, in plants, RAB genes are specifically expressed during embryogenesis

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and can be induced in vegetative organs under various stress conditions, among which water and salt stresses are the best documented. Using an inducible system of ferritin synthesis in maize (Lobréaux *et al.*, 1992), we tested this hypothesis and demonstrated that ABA is involved in the iron-stress response leading to the accumulation of this plastid-located iron storage protein.

Results

Iron induces RAB and ferritin mRNA accumulation

RAB genes are known to be induced by ABA during embryogenesis and under various stress conditions (Skriver and Mundy, 1990). Therefore, if iron induction of ferritin synthesis is mediated, at least in part, by ABA, as we postulate, it is conceivable that RAB genes could also respond to iron stress. In order to test this hypothesis, we measured the level of ferritin and two RAB mRNAs, namely pMAH9 (Gomez *et al.*, 1988; Didierjean *et al.*, 1992) and pMA12 (Villardel *et al.*, 1990), immediately following iron treatment of hydroponically grown maize plantlets (Lobréaux *et al.*, 1992). Northern analysis of root and leaf RNA indicated that ferritin mRNA is rapidly induced after iron treatment with maximum accumulation of ferritin mRNA at 6 h (Figure 1). As an internal control, the RNAs from the same preparation were probed with a cytoplasmic ribosomal protein (CS11) riboprobe, known to be constitutively expressed at the same level in roots and leaves (Thompson *et al.*, 1992). It is clear in Figure 1 that the CS11 mRNA level is constant in roots and leaves of maize plantlets at different times after iron treatment. Kinetics of ferritin mRNA accumulation are rather different in roots than in leaves. In roots, the amount of transcript is very low at 3 h while it is already strongly induced, and close to its maximum accumulation, in leaves. It is interesting to note that at zero time, ferritin mRNA is already present in both organs and that its concentration in roots can even be higher than what is observed at 3 h after iron treatment (Figure 1). This appears to not always be the case. This apparent drop in ferritin mRNA levels occurs only when plants are harvested at the beginning of the photoperiod. Ferritin mRNA is much less abundant at zero time if samples are collected during the photoperiod (see lane 1 in Figures 3 and 5, and Figure 4 in Lobréaux *et al.*, 1992). In such a situation, the amount of ferritin mRNA detected in roots is the same at zero time and after 3 h post-iron treatment (not shown). This particular point will be developed further in the Discussion. Ferritin mRNA accumulation in response to iron is transient and has already decreased to, or below, initial levels in both leaves and roots 24 h after iron treatment (Figure 1). pMAH9 mRNA also accumulates transiently in leaves, where it reaches its maximum at 6 h, whereas its level gradually increased in roots throughout the first 24 h after treatment (Figure 1). pMA12 transcript was not induced in roots. However, in leaves, pMA12 mRNA reached a maximum 15 h after the treatment, followed by a decrease at 24 h (Figure 1). It is important to note that the same RNA preparation has been used to perform the Northern blot analysis presented in Figure 1. Thus, two different maize RAB genes, as well as ferritin, are rapidly induced by an iron stress. It is important to note that each transcript displayed a unique pattern of accumulation. Furthermore, this pattern can also vary, for the same transcript, between roots and leaves. The significance of these observations will be discussed later (see the Discussion).

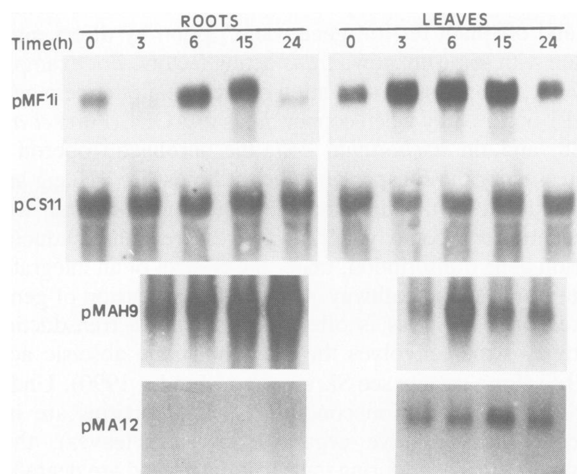

Fig. 1. Effect of an iron stress on the accumulation of ferritin and RAB mRNA in maize plantlets. 10 μg of total RNA purified from roots or leaves at 0 (plantlets were harvested during the first 30 min of the photoperiod), 3, 6, 15 and 24 h post-iron treatment were loaded in each lane (zero time was omitted in the blots probed with pMAH9 and pMA12). As an internal control, RNAs were also probed with a cytoplasmic ribosomal protein (CS11) riboprobe. RNA probes were synthesized as described in Materials and methods from pMF1i [*Sac*I–*Stu*I fragment of ferritin FM1 cDNA from Lobréaux *et al.* (1992) cloned in pKSII], pMAH9 [*Eco*RI fragment from Gomez *et al.* (1988) cloned in pKSII], pMA12 [*Pst*I fragment from Villardel *et al.* (1990) cloned in pKSII] or pCS11 [*Eco*RI–*Rsa*I fragment from Lebrun and Freyssinet (1991) cloned in pKSII] and used as indicated. RNAs from the same preparation were used in this experiment.

Iron induces ABA synthesis

The above observation has shown that the expression of known RAB genes is induced by an iron stress in maize plantlets. Such a result could imply that the iron treatment leads to ABA synthesis and accumulation, correlatively to an increase in ferritin mRNA. In order to address this point,

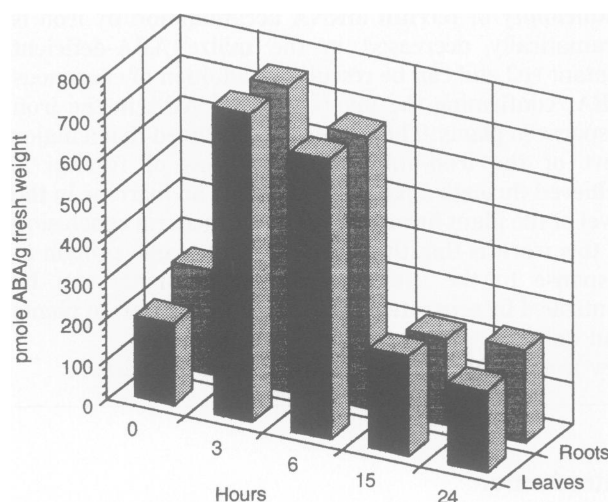

Fig. 2. Concentrations of ABA in roots and leaves of maize plantlets at different times after the addition of iron. Preparation of samples was as described in Materials and methods. ABA dosage used the Phytodetek (Sunnyvale, CA) ABA determination kit. Each value results from the mean of six measurements performed with organs harvested from two independent cultures. Standard error was <10%.

the ABA concentration was determined by an immunoassay using S-ABA monoclonal antibody (Gomez *et al.*, 1988; Yamaguchi-Shinozaki *et al.*, 1990).

First, the ABA concentration in roots and leaves was determined at different times after iron treatment (Figure 2). It is clear that iron stress can lead to the same transitory increase in ABA concentration, in both organs, with a maximum observed 3–6 h after iron addition in the culture medium. This increase has been estimated to be 4.5-fold. Secondly, the iron concentration dependence of ferritin synthesis has been assessed by Northern analysis of total leaf RNA prepared 12 h after the addition of increasing amounts of iron to the plantlet culture medium (Figure 3). It is clearly apparent that the amount of ferritin mRNA increases when iron-EDTA concentrations are increased from 0 to 250 μM. A further increase of iron-EDTA to 500 μM does not lead to another increase in ferritin mRNA level. This indicates that ≥250 μM iron is saturating this system during a treatment of 12 h. At a saturating concentration of iron (500 μM), the CS11 cytoplasmic ribosomal mRNA remains constant in roots and leaves of maize plantlets after a treatment of 3, 6, 15 and 24 h (Figure 1). Determination of the ABA concentration in leaves, 3 h after iron addition, revealed an increase of 4.7-fold over the phytohormone level of the sample for which no iron was added (Figure 3). A good correlation appears to exist between ferritin mRNA accumulation and ABA concentration increases. Therefore, it may be concluded that an iron-dependent transient accumulation of both ABA and ferritin mRNA occurs in our system.

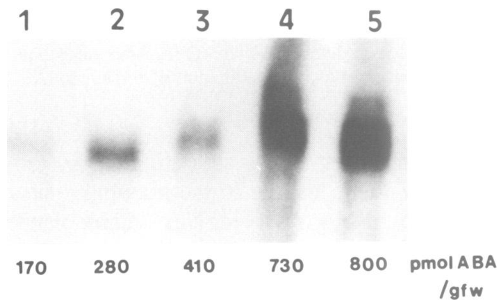

Fig. 3. Iron dependence of ferritin mRNA and ABA accumulation. Maize plantlets were induced for ferritin synthesis by using increasing amounts of iron. Leaves were then collected 3 h after iron treatment for ABA determination and 12 h after treatment for ferritin mRNA visualization by Northern blot. **Lane 1**: no iron added (plantlets were harvested >3 h after the photoperiod had begun); **lane 2**: 50 μM iron–EDTA / 7.5 μM iron-citrate; **lane 3**: 100 μM iron-EDTA / 15 μM iron-citrate; **lane 4**: 250 μM iron-EDTA / 37.5 μM iron-citrate; **lane 5**: 500 μM iron-EDTA / 75 μM iron-citrate. ABA concentration is the mean of three measurements; standard error was <10%.

Exogenous ABA induces ferritin synthesis

These results do not prove that ABA, by itself, can induce ferritin synthesis. In order to provide proof of this point, 10 μM ABA were added, after iron starvation, to the culture medium instead of the iron mixture. The level of ferritin mRNA was then monitored by Northern analysis at different times after hormone addition. The pMAH9 probe was used as a positive control for ABA treatment. Results of such an experiment are shown in Figure 4A. Ferritin mRNA was induced both in leaves and roots of ABA-treated plants. The maximum level was reached 15 h after treatment. Furthermore, it is shown in Figure 5 that the level of leaf ferritin mRNA increases in response to a 12 h treatment with increasing concentration of exogenous ABA. In such an experiment, the amount of the CS11 cytoplasmic ribosomal protein mRNA remains constant, whatever the concentration of exogenous ABA used (Figure 5). pMAH9 transcripts were also induced in roots and leaves after ABA treatment (Figure 4A). The profile of pMAH9 mRNA accumulation was identical to that observed for ferritin. A difference is observed between the kinetics of accumulation for both mRNA species between leaves and roots. In roots, the amount of ferritin and pMAH9 mRNAs decreases only slightly at 24 h, while a dramatic drop occurs in leaves at this particular time. Exogenous ABA is, therefore, able to induce ferritin mRNA accumulation in iron-starved maize plantlets.

In order to examine the fate of the ferritin protein

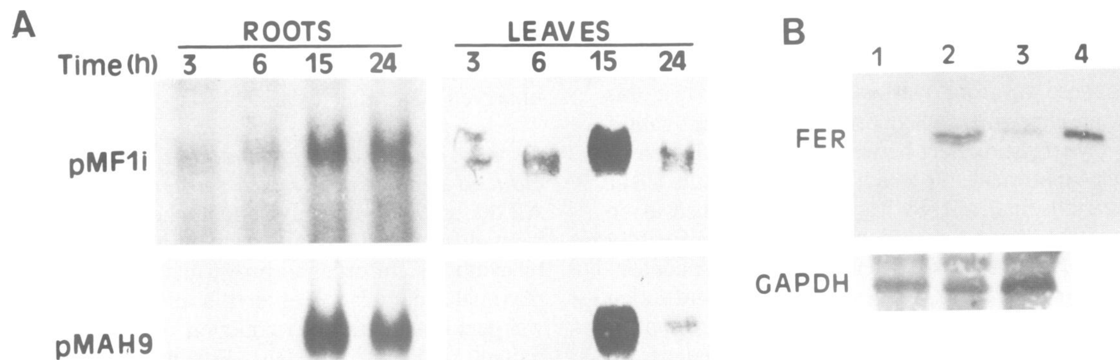

Fig. 4. Induction of ferritin synthesis by exogenous ABA. **(A)** Accumulation of ferritin and pMAH9 transcripts from maize roots and leaves in response to ABA. Culture conditions were as described in Materials and methods except that 10 μM ABA were added to the culture medium instead of iron. After 3, 6, 15 and 24 h, RNA was extracted from roots and leaves, and 10 μg RNA from each sample were analysed by Northern blot hybridization using RNA probes produced either from pMF1i (ferritin) or pMAH9 (RAB). **(B)** Ferritin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, GapC) subunit accumulation as determined by immunodetection using polyclonal antibodies raised either against maize seed ferritin or maize GAPDH, after blotting of 40 μg of total protein extracted from maize leaves. Lane 1: no iron added; lane 2: 24 h after the addition of 500 μM iron-EDTA / 75 μM iron-citrate; **lane 3**: 24 h after the addition of 200 μM ABA; **lane 4**: 20 ng pure maize seed ferritin. This experiment was repeated three times, giving the same result.

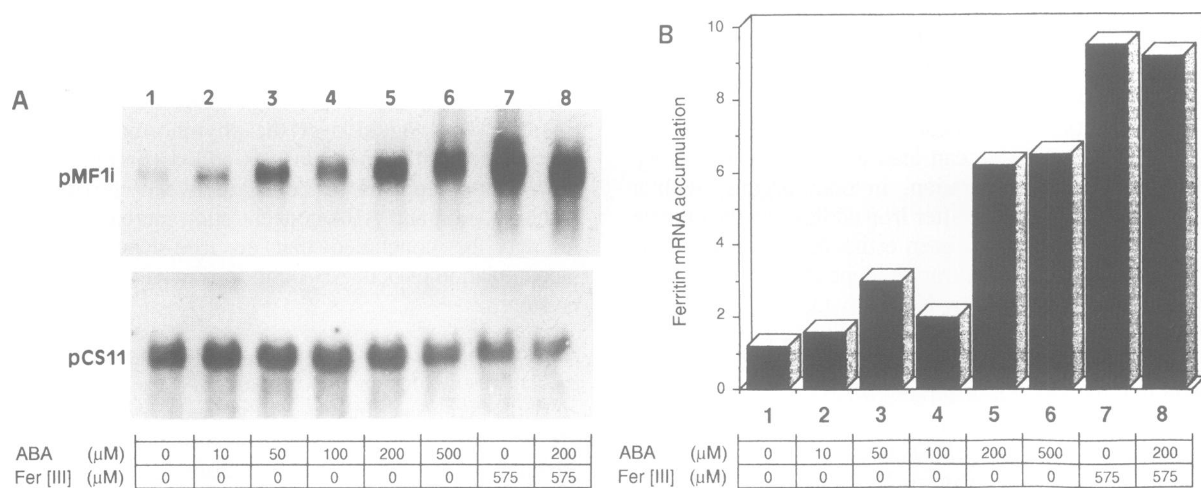

Fig. 5. Synergistic effect of iron and ABA on ferritin mRNA accumulation. **(A)** Northern blots were performed after electrophoresis of 10 μg of total RNA purified from plantlets either untreated (**lane 1**: plantlets were harvested >3 h after the photoperiod had begun) or treated for 12 h with increasing concentrations of ABA from 0 to 500 μM (**lanes 2–6**), with 500 μM iron-EDTA / 75 μM iron-citrate (**lane 7**) and with a mixture of 200 μM ABA plus 500 μM iron-EDTA / 75 μM iron-citrate (**lane 8**). RNAs from the same preparation were probed with both ferritin riboprobe (pMF1i) and CS11 cytoplasmic ribosomal riboprobe (pCS11). **(B)** Quantification of the Northern blots hybridized with the ferritin riboprobe was performed by computer image analysis after scanning the autoradiographs. Numbers indicate arbitrary units of the amount of ferritin RNA.

accumulation under such conditions, an immunodetection experiment was performed using total protein extracted from leaves 24 h after treatment with either exogenous ABA or iron. As shown in Figure 4B, lane 2, iron induces ferritin protein accumulation in leaves 24 h after its addition, as previously reported (Lobréaux *et al.*, 1992). Exogenous ABA treatment of iron-starved plantlets is also responsible for an increase in the amount of ferritin protein subunit (Figure 4B, lane 3); this relative increase is, however, much lower than that in response to iron treatment. Probing the same protein extracts by Western blot, using a polyclonal antibody raised against the maize cytoplasmic glyceraldehyde 3-phosphate dehydrogenase (GapC), has shown that the amount of this protein was not affected by iron or exogenous ABA treatment (Figure 4B).

Exogenous ABA and iron responses are not additive

All the results presented above strongly support the hypothesis that ABA is involved during ferritin gene activation in response to iron overload. To further test this hypothesis, an experiment was performed to investigate if there is any synergistic effect between iron and ABA treatment. In this experiment, the steady-state level of the CS11 cytoplasmic ribosomal mRNA has been determined as an internal control and does not change significantly (Figure 5A). In a first step, we determined the ABA concentration which is necessary to induce a maximal accumulation of ferritin mRNA in response to this hormone, when applied to maize plantlets for 12 h. The ferritin mRNA level from leaves of iron-starved maize plantlets incubated with various ABA concentrations was measured by Northern analysis and quantified by scanning of the autoradiographs (Figure 5). It is clearly apparent that the amount of ferritin mRNA increases when ABA concentrations are increased from 0 to 200 μM in the culture medium. For an unknown reason, the amount of ferritin mRNA induced by 100 μM ABA is slightly lower than that at 50 μM. When the hormone concentration is further increased to 500 μM, the level of ferritin mRNA detected does not increase any more. Thus, ≥200 μM exogenous ABA is saturating this system during a treatment of 12 h, leading to the maximum steady-state level of ferritin mRNA observed. Next, maize plantlets were exposed to either iron alone or simultaneously to iron and 200 μM ABA. The amount of ferritin transcript accumulated in response to both treatments varied only 3% (Figure 5B, lanes 7 and 8), which is not significant. This observation indicates that there is no additivity of exogenous ABA and iron responses on ferritin mRNA accumulation. We can, therefore, conclude that the iron stress performed saturates the transduction pathway, leading to ferritin mRNA accumulation in response to ABA. It strongly suggests the involvement of this hormone in the iron-induced synthesis of maize ferritin. However, when plantlets are incubated in 200 μM ABA, the accumulation of ferritin mRNA observed is only 68% of the level measured when maize is treated with iron. This result indicates that an event other than an increase in ABA level is involved during iron stress, and is responsible for 32% of the ferritin mRNA accumulation observed 12 h after treatment.

Genetic evidence of involvement of ABA in the iron-induced synthesis of maize ferritin

All these results strongly support the hypothesis that ABA is involved as an hormonal relay during iron-induced ferritin biosynthesis. In order to prove that ABA is necessary for maximal accumulation of ferritin mRNA during iron stress, we performed an iron induction experiment in a maize mutant deficient in ABA. For this purpose, we used *viviparous 2* plantlets; this mutant has a defect in the carotenoid biosynthetic pathway, which produces the precursors for ABA synthesis (Zeevaart and Creelman, 1988; Pla *et al.*, 1989). Homozygous *vp2* plantlets were obtained *in vitro* and transferred to hydroponic culture for iron starvation, followed by a 12 h iron treatment. The inducibility of ferritin transcript accumulation in leaves was quantified by Northern analysis in *vp2* and wild-type plantlets. In the ABA-deficient mutant, a 1.4-fold increase in ferritin mRNA level is observed in response to iron

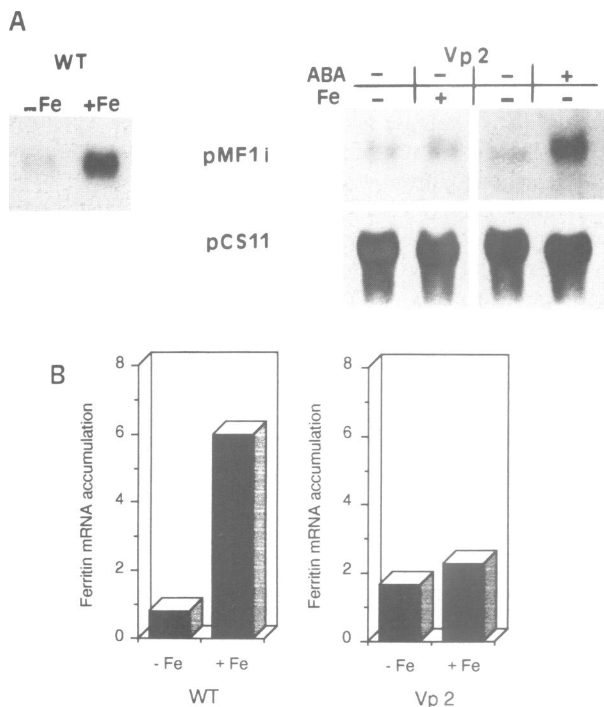

Fig. 6. Steady-state level of ferritin mRNA in response to iron in maize leaves from wild-type (WT) and *viviparous 2* (*vp2*) mutant. **(A)** 10 μg of total leaf RNA purified from wild-type and *vp2* plantlets, grown without iron or with 500 μM iron-EDTA / 75 μM iron-citrate for 12 h, were probed on the same Northern blot by using the ferritin riboprobe (pMF1i). In addition, 10 μg of total leaf RNA purified from control *vp2* plantlets (grown without iron or ABA) and *vp2* plantlets harvested 12 h after the addition of 500 μM exogenous ABA were also probed by Northern blots using the ferritin riboprobe (pMF1i). As an internal control, the various RNA samples purified from *vp2* plantlets were also probed using the CS11 cytoplasmic ribosomal riboprobe (pCS11). The RNA probes were synthesized as described in Materials and methods from pMF1i and from pCS11. Exposure was for 15 h at −70°C using a Kodak Royal X O-Mat film. **(B)** Quantification of Northern blots from wild-type and *vp2* leaf RNAs purified from plantlets grown without iron (−Fe) or after the addition of 500 μM iron-EDTA / 75 μM iron-citrate (+Fe) for 12 h, and probed with the ferritin riboprobe. This quantification was performed by computer image analysis after scanning the autoradiographs. Numbers indicate arbitrary units of the amount of ferritin RNA. The same result was obtained in two independent experiments.

(Figure 6), while in wild-type plantlets the amount of ferritin mRNA transcript is 7.5 times higher in iron-treated maize than in iron-starved plantlets. The failure of iron induction in the *vp2* mutant cannot be attributed to an independent defect additional to ABA deficiency, since addition of exogenous ABA to *vp2* plantlets induces ferritin mRNA accumulation (Figure 6A). The level of the CS11 cytoplasmic ribosomal mRNA concentration remains constant in *vp2* plantlets with or without addition of iron or ABA (Figure 6A). It is interesting to note that after iron starvation, and prior to iron induction, the amount of the transcript is 2.1-fold higher in *vp2* than in wild-type plantlets. The strong decrease observed in the inducibility of ferritin mRNA accumulation in *vp2* plants proves that ABA is essential for maximum synthesis of ferritin induced by iron.

Discussion

Based on the pattern of expression of plant ferritin in response to iron and during growth and development (Lobréaux and Briat, 1991; Lobréaux *et al.*, 1992), we postulate that ABA could be involved in the control of ferritin synthesis. Indeed, exogenous ABA is able to induce ferritin mRNA accumulation (Figures 4A and 5). Concentrations of exogenous ABA giving this response are in the range of those described for other systems (Mundy and Chua, 1988; Pla *et al.*, 1989). The decrease in this response at 24 h, mainly in leaves, could be due to ABA degradation during its transport and has already been observed (Mundy and Chua, 1988). At the protein level, exogenous ABA allows accumulation of the ferritin subunit, but to a lower extent than is observed after iron treatment (Figure 4B). It makes sense because treatment with exogenous ABA does not allow full accumulation of ferritin mRNA, as does iron treatment (Figure 5 and see below for discussion). Furthermore, it is important to be reminded that iron or ABA addition are achieved after 9 days of iron starvation, therefore the response to exogenous ABA occurs at a very low intracellular iron concentration, which has been previously reported (Lobréaux *et al.*, 1992). It has been known for a long time that, in animal systems, iron is necessary to increase the stability of ferritin molecules (Crichton, 1971; Drysdale and Shafritz, 1975). It could be the same in plants and it would explain the relatively low ferritin protein accumulation in response to exogenous ABA under conditions of iron starvation.

Because iron and ABA are both able to induce ferritin accumulation, the possibility that the iron and the ABA responses are linked was a legitimate question to answer. Iron treatment leads to an increase in ABA concentration which is dependent on the iron concentration in the culture medium (Figures 2 and 3). RAB genes are also induced by iron treatment (Figure 1). However, differences in the kinetics of ferritin and RAB mRNA accumulation in response to iron (Figure 1) indicate that multiple events are probably involved in regulating expression of these genes. This is particularly evident from the differences in mRNA accumulation between roots and leaves for the same transcript. However, it does not imply that entirely different pathways control the expression of these genes. For example, pMA12 and pMAH9, which have very different kinetics of accumulation in response to iron (Figure 1), are both regulated by ABA (Gomez *et al.*, 1988; Villardel *et al.*, 1990). More than one pathway could be involved in the control of each gene. The same common pathway could be utilized until a certain step preceding branching of secondary pathways, specific for each gene and/or for each tissue. Such a complexity is clearly demonstrated for ferritin mRNA. As a first attempt to link the iron and ABA responses, an iron treatment was performed in the presence of gibberellic acid (GA3), a known antagonist of RAB gene activation (Pena-Cortez *et al.*, 1989). In such an experiment we observed that GA3 partially suppressed the iron-induced accumulation of ferritin mRNA in leaves, 6 h after the iron treatment (not shown). However, for an unknown reason, this suppression was found to be variable from culture to culture. A synergistic ABA/iron experiment (Figure 5) reveals, indeed, that ABA and iron pathways are linked, but that the regulation of ferritin mRNA accumulation in response to iron cannot be entirely explained by the pathway involving an increase in ABA concentration, since full expression of the iron response cannot be obtained by a saturating exogenous ABA concentration. Such an observation could explain the difference in ferritin mRNA

accumulation between roots and leaves (Figure 1). The ABA concentrations are very similar in both organs at the various times analyzed after iron addition, and peak between 3 and 6 h (Figure 2). However, it is important to notice that after 3 h ferritin mRNA has not yet accumulated in roots, while it is already close to its maximum in leaves (Figure 1). This indicates that a second ABA-independent pathway could operate at 3 h in leaves. Alternately, an increase in the ABA sensitivity of leaf cells, mediated by iron, cannot be ruled out. Such an effect has recently been reported by Bostok and Quatrano (1992), showing that salt can increase the sensitivity of rice cells to ABA, affecting Em gene expression.

During the course of these kinetic experiments, we observed variations in the level of ferritin mRNA at zero time, according to the particular moment when samples were collected (beginning versus a few hours of photoperiod). This could be explained by circadian regulation of the expression of ferritin genes and/or by differences in the compartmentalization of ABA, which is known to change according to the light/dark status of the plant (Zeevaart and Creelman, 1988). Further evidence of ABA involvement in the iron-induced ferritin synthesis was gained by testing the iron response in a maize ABA-deficient mutant. Inducibility of ferritin mRNA accumulation by iron in *vp2* homozygous plantlets, which lack ABA due to blocking in the carotenoid biosynthesis pathway (Zeevaart and Creelman, 1988), is 5.4-fold less than in the wild type (Figure 6B). Interestingly, addition of exogenous ABA to *vp2* plantlets induces ferritin mRNA accumulation (Figure 6A). This result is in agreement with the fact that the failure of iron induction in the *vp2* mutant is due to ABA deficiency and not to an additional independent defect. In *vp2*, iron treatment still induces ferritin mRNA accumulation, but to a much lower extent than in wild type (Figure 6). This observation could be related to the fact that part of the iron response could be independent of the ABA response (Figure 5). Although the ferritin mRNA level, before adding iron, is always higher (~2-fold) in *vp2* than in wild-type plantlets, making a direct comparison between the two genetic backgrounds difficult.

So far, developmental and environmental regulation of RAB genes have, as a common effector, variations in osmotic pressure due to either dessication or salt and water stress (Skriver and Mundy, 1990). The observation that ferritin gene expression responds to ABA could be relevant for understanding the regulation of ferritin synthesis during development. Plant ferritins, under normal conditions of iron nutrition, are iron storage proteins which accumulate during seed formation and are degraded during seed germination (Lobréaux and Briat, 1991). This is analogous to the behaviour of known RAB protein. However, iron stress is not likely to be related to an osmotic stress (Mundy and Chua, 1988; Skriver and Mundy, 1990) because of the micromolar range of iron salt we used. It is more likely to be related to an oxidative stress because of the role metals, such as iron, can have in the conversion of reduced oxygen into hydroxyl radicals, one of the most reactive species known, through Haber–Weiss reaction (Halliwell, 1987; Imlay and Linn, 1988). Consistent with this point is the report that an oxidative stress generated by radiation raises the ABA concentration in wheat seedlings (Degani and Itai, 1978). Whether ferritin synthesis during seed development is regulated both by ABA and an additional pathway, as during iron stress, remains to be determined. It has to be remembered that environmental and developmental controls are not necessarily mediated by the same pathway. In the case of the proteinase inhibitor *pin2* gene, ABA is involved in the wounding response, but not during developmental control (Pena-Cortez *et al.*, 1991).

It has been reported that the bulk of ABA in plants is found within plastids, where it may be synthesized by the cleavage of violaxanthin to give xanthoxin as a first step (for a review see Zeevaart and Creelman, 1988). In plants, ferritins are also localized within plastids (Seckbach, 1982). In this context, it is tempting to postulate that during iron stress, which can be a natural toxicity problem in flooded acidic soils, an increase in iron uptake by plastids could occur, leading to an oxidative stress that raises the ABA concentration through photochemical and/or enzymatic cleavage of xanthophylls. As a consequence ABA, through a transduction pathway, could activate ferritin gene expression. Ferritin is then targeted to plastids where it is assembled in order to store excess iron. Experiments are in progress to test this hypothesis.

Materials and methods

Plant cultures

Maize plantlets (*Zea mays*, var. MO17) were grown under hydroponic culture in an iron-free medium as previously described (Lobréaux *et al.*, 1992). After 9 days of iron starvation, different treatments were performed. Iron induction was achieved by adding 500 μM Fe-EDTA, 150 μM Na_3-citrate and 75 μM $FeSO_4$ in the culture medium. For ABA treatment, ABA (Sigma) was added to the medium, instead of the iron mixture, by dilution of a 100 mM stock solution in ethanol. Root and leaf samples were harvested at different times after treatment, frozen in liquid nitrogen and stored at −70°C.

Viviparous 2 homozygous plantlets can only be obtained by selfing of heterozygous plants. Therefore, plants were first obtained from heterozygous seeds (kindly provided by Dr M.Pages, Barcelona) in the greenhouse. The growth conditions were 24°C during the day and 18°C at night, with natural light supplemented with artificial light to ensure a minimum of 300 $\mu E/m^2/s$ during a 16 h photoperiod. At ~30 days after pollination, ears with white kernels were harvested, surface sterilized with a 6.25% sodium hypochlorite solution containing a small amount of detergent as a surfactant, and then rinsed in three changes of sterile deionized water. White kernels were isolated and put in germination in individual plastic jars with 30 ml of N6 medium (Chu *et al.*, 1975). *In vitro* conditions were 24°C constant, a 16 h photoperiod (80 $\mu E/m^2/s$ fluorescent lighting 'Fluora', Osram) and 80% relative humidity. When *vp2* plantlets were ~10 cm high, they were transferred to hydroponic culture medium without iron for 7 days prior to iron treatment with 500 μM Fe-EDTA, 150 μM Na_3-citrate, and 75 μM $FeSO_4$.

RNA extraction and analysis

Total RNA extraction was performed as previously described (Lobréaux *et al.*, 1992). Northern blot analysis of total RNA samples was achieved using RNA probes, according to Lobréaux *et al.* (1992). For ferritin mRNA detection, the pMF1i ferritin probe was used (Lobréaux *et al.*, 1992). Clones pMAH9 (Gomez *et al.*, 1988; Didierjean *et al.*, 1992), pMA12 (Villardel *et al.*, 1990) and pCS11 (Lebrun and Freyssinet, 1991) were gifts, respectively, from Dr G.Burkard (Strasbourg), Dr M.Pages (Barcelona) and Dr M.Lebrun (Lyon). *Eco*RI insert from pMAH9, *Pst*I insert from pMA12 and the *Eco*RI–*Rsa*I fragment from pCS11 were subcloned in the appropriate restriction sites of Bluescript II KS. For quantification of mRNA levels, autoradiographs were scanned using a 256 grey level scanner (Apple) and images were analysed with the software Image 1.3.7. (NIH, Bethesda, USA).

Protein extraction and analysis

The preparation of pure maize seed ferritin and rabbit polyclonal antibodies raised against its subunit have already been reported (Lobréaux *et al.*, 1992). Polyclonal antibodies raised against maize cytoplasmic glyceraldehyde 3-phosphate dehydrogenase (GapC) were a generous gift of Pr. R.Cerff (Brunschweig University, Germany).

Total protein preparation from leaves, protein concentration measurements,

SDS–polyacrylamide gel electrophoresis and immunodetection were as already described (Lobréaux *et al.*, 1992).

ABA dosage

Plants were grown hydroponically as described above. At various times post-iron treatment, roots and leaves were ground in liquid nitrogen. Extracts were prepared according to Yamaguchi-Shinozaki *et al.* (1990) and the ABA concentration was determined using the Phytodetek-ABA kit (Idetek Inc., Sunnyvale, CA) according to the manufacturer's instructions.

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