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ORIGINAL ARTICLE

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Differential involvement of the IDRS *cis*-element in the developmental and environmental regulation of the *AtFer1* ferritin gene from *Arabidopsis*

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Abstract Four different ferritin genes have been identified in *Arabidopsis thaliana*, namely *AtFer1*, *2*, *3* and *4*. *AtFer1*, which strongly accumulates in leaves treated with excess iron, contains in its promoter an *I*ron-*D*ependent *R*egulatory *S*equence (IDRS). The IDRS sequence is responsible for repression of *AtFer1* transcription under conditions of low iron supply. *Arabidopsis* plants transformed with a 1,400-bp *AtFer1* promoter, with either a wild-type or a mutated IDRS fused to the β-glucuronidase (GUS) reporter gene, enabled us to analyze the activity of the *AtFer1* promoter in different tissues as well as during age-dependent or dark-induced senescence. Our results show that IDRS mediates *AtFer1* expression during dark-induced senescence while it does not affect *AtFer1* expression during age-dependent senescence or in young seedlings. Photoinhibition promoted either by high light or chilling temperature, or wounding, does not activate the *AtFer1* promoter. In contrast, *AtFer2*, *AtFer3*, *AtFer4* transcript abundances are increased in response to photoinhibition and *AtFer3* transcript abundance is increased upon wounding. Taken together, our results indicate that other *cis*-elements, different from the IDRS, regulate the territory-specific or developmental expression of *AtFer1* gene. Expression of this gene appears insensitive to some of the environmental stresses tested, which instead upregulate other members of the *Arabidopsis* ferritin gene family.

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Abbreviations At1400IDRS line: *Arabidopsis* line transformed with a construct containing the wild-type *AtFer1* 1,400-bp promoter upstream of the GUS reporter gene · At1400m*IDRS line: *Arabidopsis* line transformed with a construct containing the *AtFer1* 1,400-bp promoter with the mutated IDRS sequence · F_0: initial fluorescence of dark-adapted leaves · F_m: maximal fluorescence of dark-adapted leaves · F_v: variable fluorescence ($F_m - F_0$) of dark-adapted leaves · GUS: β-glucuronidase · JA: jasmonic acid · IDRS: iron dependent regulatory sequence · RT–PCR: reverse transcription–polymerase chain reaction · WT: wild type

Introduction

Iron is essential for all living organisms. However, in a free ionic state, it can be very noxious as a catalyst of the production of hydroxyl radicals ($OH^{\cdot}$), one of the most toxic reactive oxygen species (ROS) known (Bowler et al. 1992; Guerinot and Yi 1994).

Ferritins are ubiquitous, multimeric, iron-storage proteins formed by 24 subunits spatially organized to form a cavity able to sequester up to 4,500 iron atoms in a safe, bioavailable form; they contribute to the regulation of intracellular free-iron levels (Andrews et al. 1991; Laulhere and Briat 1993; Guerinot and Yi 1994; Harrison and Arosio 1996; Briat and Lobreaux 1997).

Four ferritin genes have been identified in *Arabidopsis*, namely *AtFer1*, *2*, *3*, *4* (Gaymard et al. 1996; Petit et al. 2001a). *AtFer1* ferritin strongly accumulates upon treatment with excess iron, through a nitric oxide-mediated pathway (Murgia et al. 2002). Functional analysis of the *AtFer1* promoter (Petit et al. 2001b) revealed a common iron-dependent regulatory mechanism in plants and animals, although the target of such regulation is mRNA in animals and DNA in plants (Cairo and

Pietrangelo 2000; Kim and Ponka 2000; Wei and Theil 2001; Petit et al. 2001b). The *AtFer1* promoter contains a 15-bp *I*ron *D*ependent *R*egulatory *S*equence (IDRS), which is responsible for gene repression under low iron supply (Petit et al. 2001b). Most likely, a proteic factor, not yet identified, inhibits *AtFer1* transcription under conditions of low iron content, by binding to the IDRS.

Sequences very similar to the *AtFer1* IDRS element have also been identified in the other three *Arabidopsis* ferritin genes *AtFer2*, *AtFer3* and *AtFer4*, although the functionality of such IDRS-like elements has not been tested yet (Petit et al. 2001b).

The aim of this work was to gain better insight in the physiological role of *AtFer1* expression, in particular in those tissues where an endogenous rise in intracellular free-iron levels is expected. We performed an in situ localization of *AtFer1* expression in young seedlings and roots of two *Arabidopsis* transgenic lines (Petit et al. 2001b). These two lines had been obtained by transformation with the 1,400-bp *AtFer1* promoter sequence, with either a wild-type (WT) or a mutated IDRS sequence, fused to the β-glucuronidase (GUS) reporter gene. By using these lines, we also quantified the activity of the *AtFer1* promoter in senescing leaves and the dependence of that activity on the IDRS element.

Finally, evaluation of the activity of the *AtFer1* promoter and of *AtFer1–4* transcripts abundance in such transformed lines upon photoinhibitory treatments or after wounding enabled us to determine the involvement of the ferritin genes in response to those stresses.

Materials and methods

Arabidopsis growth

Production of At1400IDRS and At1400m*IDRS *Arabidopsis* transgenic lines has been already described (Petit et al. 2001b). *Arabidopsis* transgenic plants, as well as WT (*Col-0*) plants, were grown at 21–25 °C, 150 μmol photons m^{-2} s^{-1} (OSRAM lamps L36 w/11-860 Lumilux plus), with a 14 h/10 h light/dark photoperiod, on sterilized Technic n.1 soil (DueEmme, Reggio Emilia, Italy) in Ara baskets (Beta Tech, Gent, Belgium). Deionized water was used for watering the plants.

In age-dependent senescence experiments, At1400IDRS and At1400m*IDRS plants were grown under standard conditions; leaves Nos.5 to 8 (No.1 being the youngest) were sampled weekly starting from day 22.

In dark-induced senescence experiments, At1400IDRS and At1400m*IDRS plants, grown under standard conditions till day 22, were transferred to a dark room and kept there till the end of the experiment; leaves Nos.5 to 8 (No.1 being the youngest) were sampled every 2 days.

Photoinhibitory treatments

Leaves from 22-day-old At1400IDRS and At1400m*IDRS plants, grown under standard conditions, were cut at the petiole level, dipped and kept in distilled water during all photoinhibitory treatments, which were performed by using a facility previously described (Tarantino et al. 1999). At the end of each treatment the photochemical efficiency of photosystem II (PSII), expressed as F_v/F_m, was calculated by evaluating the emission of chlorophyll fluorescence with a portable plant efficiency analyzer (Hansatech Instruments, Norfolk, UK).

Wounding, jasmonic acid or iron treatments

Leaves from 22-day-old At1400IDRS plants were wounded with 2- to 4-mm-long cuts, four cuts/leaf. Only two leaves of each rosette were wounded; after that plants were kept in the dark. Wounded and intact (non-wounded) leaves were sampled after 4 h, for RNA extraction and GUS activity assays.

Leaves of 22-day-old At1400IDRS plants were sprayed with 200 μM jasmonic acid (JA), kept in the dark and leaves were sampled after 6 h, for RNA extraction and GUS activity assays.

Leaves from 22-day-old *Arabidopsis* plants (*Col-0*) were infiltrated, as previously described (Murgia et al. 2002), with 500 μM Fe-citrate; 50 mM Fe-citrate stock solution was prepared fresh by mixing equal amounts of 100 mM $FeSO_4$ (in 0.06 N HCl) and 200 mM Na-citrate. Leaves were kept in the dark and sampled after 3 h for RNA extraction.

GUS histochemical staining and GUS activity assays

5-Bromo-4-chloro-3-indolyl β-D-glucuronide was used as substrate for the GUS histochemical staining, as described by Jefferson et al. (1987). Stained organs were embedded in hydroxyethylmethacrylate (Technovit 7100; Heruas-Kulzer, Wehrheim, Germany) prior to cutting 3-μm thin cross-sections using a Leica RM 2165 microtome. Cross-sections were counterstained with Schiff dye and observed with an Olympus BH2 microscope.

Leaf extracts were prepared, and GUS enzyme activity assayed and quantified as previously described (Murgia et al. 2002).

Reverse transcription–polymerase chain reaction (RT–PCR

Total RNA was extracted using Trizol reagent (Gibco) according to the manufacturer's instructions.

The primers used to amplify cDNA fragments from the *AtFer1* (Accession No. At5G01600), *AtFer2* (Accession No. At3G11050) *AtFer3* (Accession No. At3G56090) and *AtFer4* (Accession No. At2G40300) gene products were as follows:

- *AtFer1* forward, 5′-AATCCCGCTCTGTCTCC-3′
- *AtFer1* reverse, 5′-AAACTTCTCAGCATGCCC-3′
- *AtFer2* forward, 5′-ACGTCTCGTATGTCTACCATGC-3′
- *AtFer2* reverse, 5′-AAACCTCATCATTGAGAAGC-3′
- *AtFer3* forward, 5′-AAGAGTTCAACCACTACC-3′
- *AtFer3* reverse, 5′-ACTGAGGCAACACCATGGG-3′
- *AtFer4* forward, 5′-TTTCCATGGCGTGAAGAAGG-3′
- *AtFer4* rev 5′-ATGTTCAAACTCTGAAAGAGGC-3′

An equal amount of RNA was used in each sample. As a positive control, the *TUB4* gene, coding for β-tubulin4 (Accession No. At5G44340) was amplified by RT–PCR (Marks et al. 1987; Kim et al. 1998) with the primers:

- *TUB4* forward, 5′-AGAGGTTGACGAGCAGATGA-3′
- *TUB4* reverse, 5′-CCTCTTCTTCCTCCTCGTAC-3′

The accumulation of *TUB4* transcript appeared constant under all conditions tested. *Thi2.1* (Accession No. AtG72260), a thionin gene, was used as a positive control for JA treatment (Vignutelli et al. 1998) by using the primers:

- *Thi2.1* forward, 5′-TCCAACCAAGCTAGAAATGGC-3′
- *Thi2.1* reverse, 5′-CGACGCTCCATTCAGAATTTC-3′

RT–PCRs were performed using the kit "Access RT–PCR System" (Promega). Annealing reactions were performed at 52 °C

for *AtFer3*, *AtFer4* and *Thi2.1*; at 54 °C for *AtFer1*; at 55 °C for *AtFer2* and at 60 °C for *TUB4*.

Results

Localization of *AtFer1* expression in roots

GUS activity under the control of the *AtFer1* promoter sequence in the At1400IDRS line is observed in the roots with a maximum intensity just above the root tips (Fig. 1a, b, g). This root region corresponds to the most active part of the root for iron acquisition. Such an intense GUS staining is also observed at the branching points of secondary roots (not shown). After longer staining times all the secondary roots are GUS-stained, whereas the primary root is only stained in the vicinity of secondary root emergence. Cross-sections just above the root tip reveal that *AtFer1* promoter expression is restricted to the endoderm (Fig. 1c). However, in older root parts, the GUS staining is not restricted to the endoderm as it is also observed within the pericycle, the stele and root hairs (Fig. 1d). In contrast, GUS staining appears rapidly throughout the roots in the case of the At1400m*IDRS line (Fig. 1h). Intense GUS staining is also observed at the branching point of secondary roots (Fig. 1e), as in the At1400IDRS line. In addition, root cross-sections reveal that the At1400m*IDRS line expresses GUS in the cortex in addition to endoderm, pericycle and stele (Fig. 1f).

Localization of *AtFer1* expression in leaves

The *AtFer1* promoter is not expressed in the hypocotyl, except at the branching zone with cotyledons. In young At1400IDRS plants, major veins of cotyledons and leaves are GUS stained, as well as the hydathodes (Fig. 2a). GUS expression in older leaves is similar to that observed in younger leaves: veins and hydathodes specifically express the *AtFer1* promoter (Fig. 2b). Staining of At1400m*IDRS young leaves gives the same results (Fig. 2c).

Ferritin expression during age-dependent or dark-induced senescence

Expression of the *AtFer1* promoter is induced during age-dependent senescence as GUS activity increases by a factor of 10 in the At1400IDRS line (Fig. 3a). The same

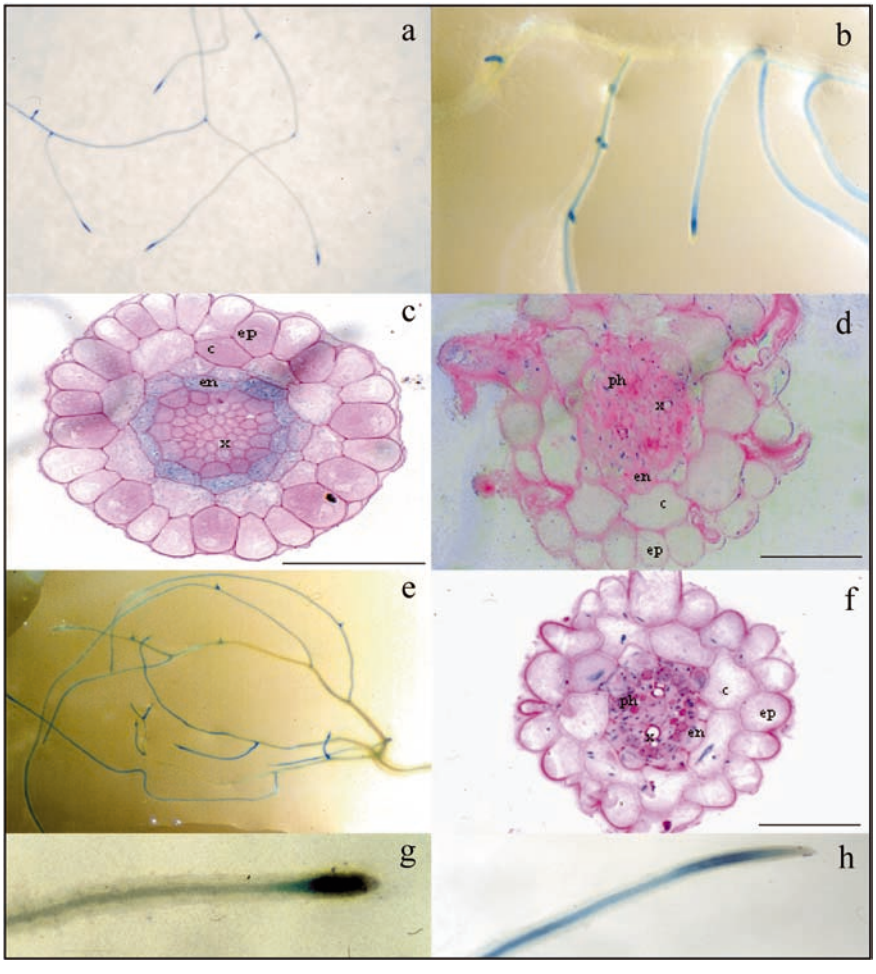

Fig. 1a–h Histochemical localization of GUS expression driven by the *AtFer1* promoter in roots of *Arabidopsis thaliana* plants. **a–d, g** At1400IDRS line; **e, f, h** At1400m*IDRS line. **a, e** Root. **b** Detail of secondary roots. **c** Cross-section a few millimeters above the root tip. **d, f** Cross-section in an older root part. **g, h** Detail of root apical part. *ep* Epiderm, *c* cortex, *en* endoderm, *x* xylem, *ph* phloem. Bars = 50 μm

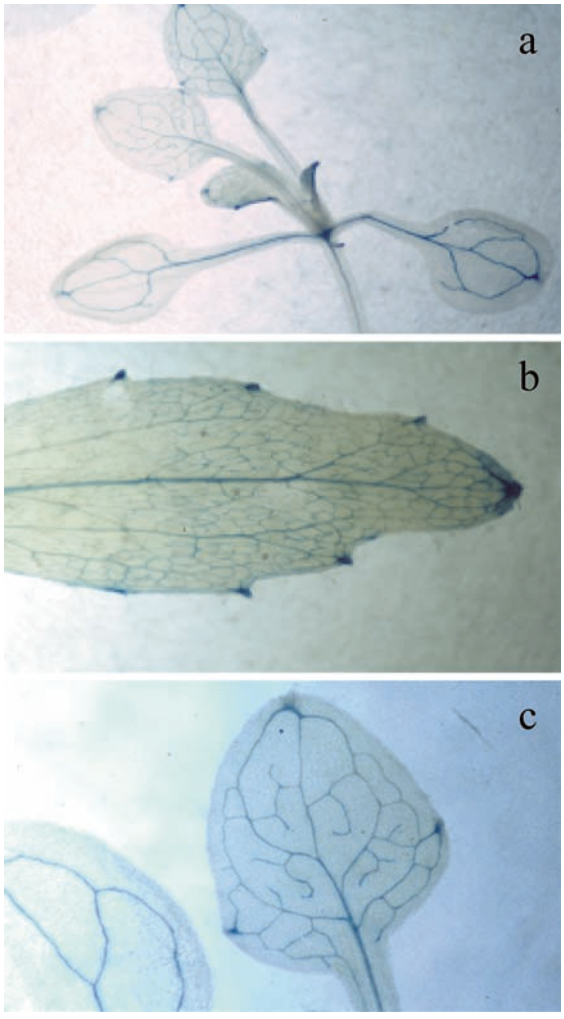

Fig. 2a–c Histochemical localization of GUS expression driven by the *AtFer1* promoter in *Arabidopsis* leaves. **a** Leaves and cotyledons of a young At1400IDRS plant. **b** Mature cauline leaf of an At1400IDRS plant. **c** Young leaf of an At1400m*IDRS plant

is true also for the At1400m*IDRS line, indicating that *AtFer1* activation during senescence is insensitive to mutagenesis of the IDRS element. Notably, such activation of *AtFer1* promoter expression precedes any visible senescence symptoms (Fig. 3b) although leaves show a decline in chlorophyll and protein contents (Fig. 3c, d), a hallmark of the onset of the senescence program (Lohman et al. 1994). Photochemical efficiency starts to decrease only at later stages of senescence, i.e. at day 57 (Fig. 3e), at which time the *AtFer1* promoter has been active for 4 weeks (Fig. 1a).

Under the same conditions, there is no accumulation of *AtFer2*, *3* or*4* transcripts when primers specific for *AtFer2*, *3* and *4* are used for RT–PCR amplifications (not shown).

In contrast, prolonged dark treatment causes a rapid yellowing of the leaves (Fig. 4b), a phenomenon called dark-induced senescence (Becker and Apel 1993; Oh et al. 1997): in such a case, chlorophyll and proteins are quickly degraded (Fig. 4c, d) and photochemical efficiency

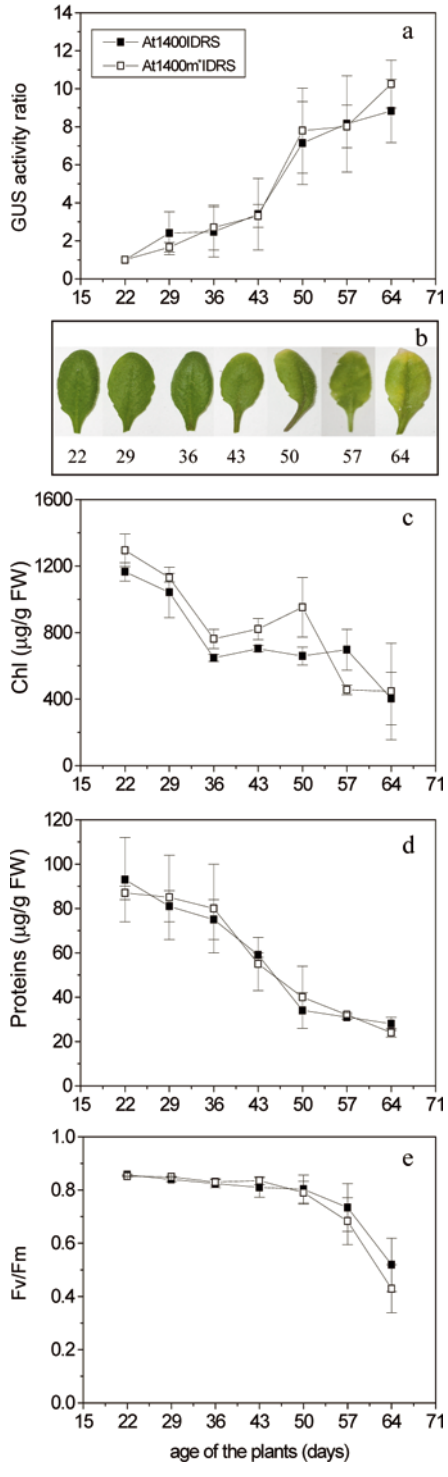

Fig. 3a–e Senescence in leaves of At1400IDRS (■) and At1400-m*IDRS (□) lines sampled at different ages (expressed in days from sowing) of the plants. **a** GUS activity ratio of senescing leaves; each sample consists of four leaves from four different plants grown under standard conditions. The GUS activity ratio is the ratio of the GUS activity of senescing leaves to that of control leaves (day 22). Data are the mean ± SD of two independent samples. **b** Phenotype of leaves at different ages. **c, d** Chlorophyll (**c**) and protein (**d**) contents of leaves at different ages. **e** Photochemical efficiency (F_v/F_m) of leaves at different ages

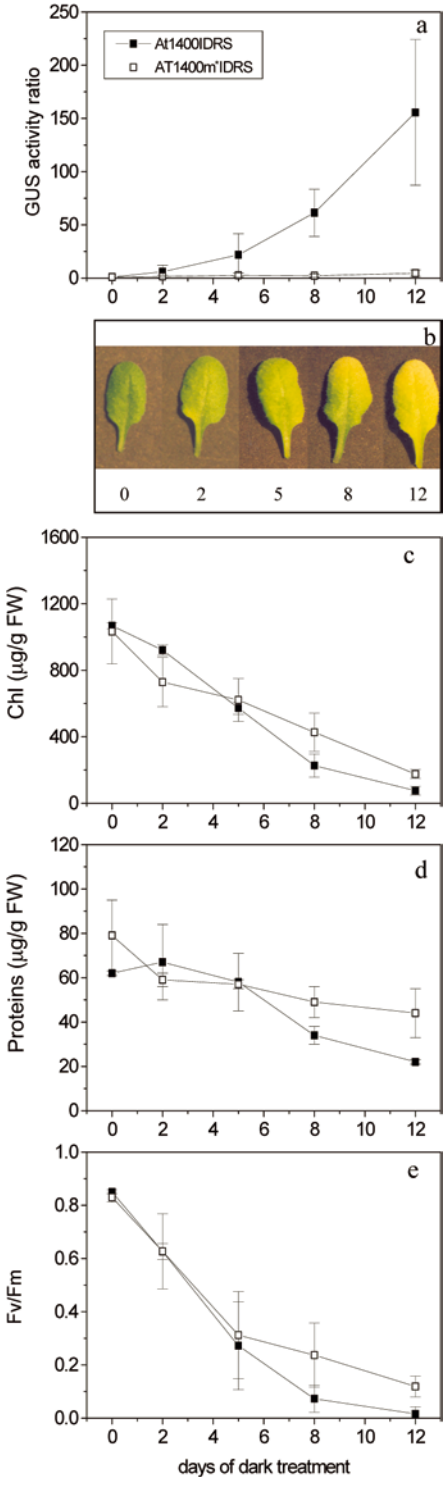

Fig. 4a–e Dark-induced senescence in leaves of At1400IDRS (■) and At1400m*IDRS (□) lines sampled at different days from beginning of dark treatment. **a** GUS activity ratio of dark-treated leaves; each sample consists of four leaves from four different plants grown for 22 days under standard conditions and then transferred to the dark. The GUS activity ratio is the ratio of the GUS activity of dark-treated leaves to that of control leaves. Data are the mean ± SD of two independent samples. **b** Phenotype of dark-treated leaves. **c, d** Chlorophyll (**c**) and protein (**d**) contents of dark-treated leaves. **e** Photochemical efficiency (F_v/F_m) of dark-treated leaves

decreases dramatically (Fig. 4e). During dark-induced senescence, the increase in *AtFer1* promoter activity is IDRS-mediated, as GUS activity dramatically increases only in the At1400IDRS leaves, but not in the At1400-m*IDRS ones (Fig. 4a).

Ferritin expression during photoinhibition

Photoinhibition caused by plant exposure to high intensity of white light, i.e. 700 µmol photons m^{-2} s^{-1}, at room temperature, reduces photochemical efficiency (Fig. 5a). This treatment does not activate the *AtFer1* promoter as GUS activity does not increase in the At1400IDRS line or in the At1400m*IDRS one (not shown). In accordance with these results, *AtFer1* transcript does not accumulate (Fig. 5b) whereas, in the same conditions, *AtFer2*, *AtFer3* and *AtFer4* transcripts accumulate (Fig. 5b). In a control RT–PCR experiment,

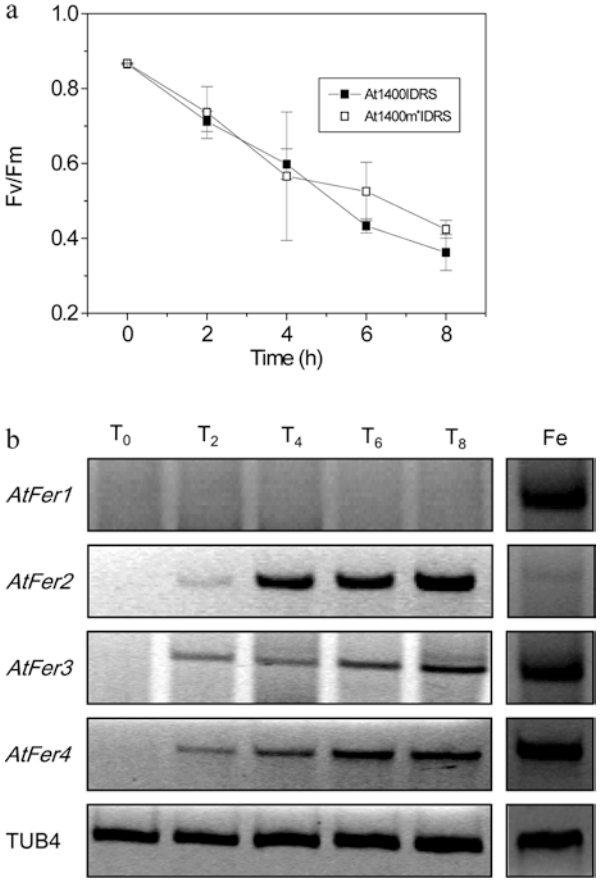

Fig. 5a, b Ferritin expression during photoinhibition at high light and room temperature. **a** Photochemical efficiency (F_v/F_m) of At1400IDRS (■) and At1400m*IDRS (□) leaves photoinhibited for 8 h at 700 µmol photons m^{-2} s^{-1} and 22 °C. Each point is the mean ± SD of five independent measurements. **b** RT–PCR analyses of *AtFer1*, *AtFer2*, *AtFer3*, *AtFer4* transcripts in photoinhibited At1400IDRS leaves, or in WT (*Col-0*) leaves infiltrated with 500 µM Fe-citrate. *Lane* T_0 Leaves prior to photoinhibition; *lanes* T_2–T_8 leaves after 2–8 h photoinhibition; *lane Fe* WT leaves after 3 h infiltration with 500 µM Fe-citrate. *TUB4* is the positive control for the equal addition of RNA in each sample

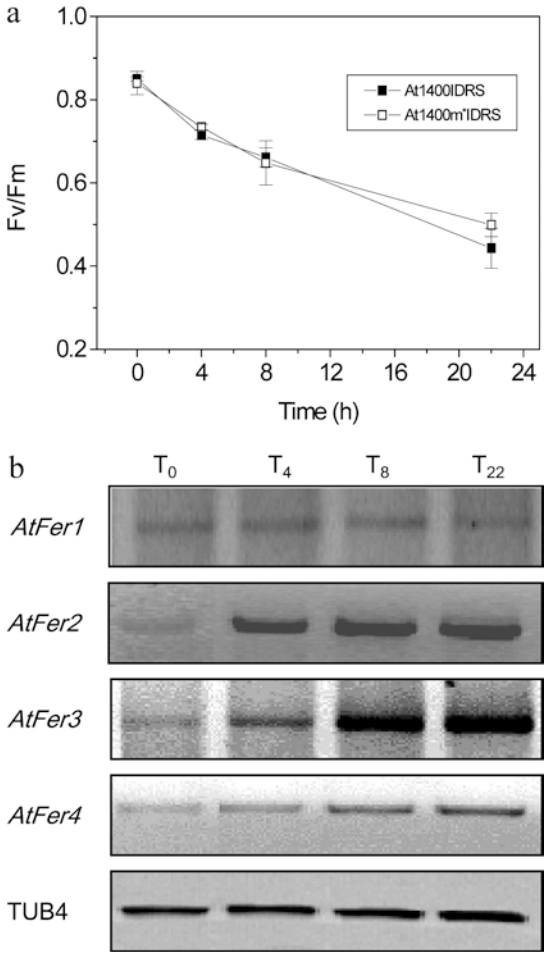

Fig. 6a, b Ferritin expression during photoinhibition at chilling temperature. **a** Photochemical efficiency (F_v/F_m) of At1400IDRS (■) and At1400m*IDRS (□) leaves photoinhibited for 22 h at 150 μmol photons m^{-2} s^{-1} and 2 °C. Each point is the mean ± SD of five independent measurements. **b** RT–PCR analysis of *AtFer1*, *AtFer2*, *AtFer3*, *AtFer4* transcripts in photoinhibited At1400IDRS leaves. *Lane* T_0 Leaves prior to photoinhibition; *lanes* T_4–T_{22} leaves after 4–22 h photoinhibition. *TUB4* is the positive control for the equal addition of RNA in each sample

AtFer1 transcripts, together with *AtFer3* and *AtFer4* transcripts, accumulate upon infiltration of WT leaves with 500 μM Fe-citrate (Fig. 5b), in full agreement with previously published results (Petit et al. 2001a).

Photoinhibition caused by plant exposure to growth light at chilling temperatures (2–4 °C) reduces photochemical efficiency (Fig. 6a) but again does not activate the *AtFer1* promoter because GUS activity does not increase in the At1400IDRS line or in the At1400m*IDRS one (not shown). In accordance with these results, the *AtFer1* transcript does not accumulate (Fig. 6b) whereas, in the same conditions, *AtFer2*, *AtFer3* and *AtFer4* transcripts accumulate (Fig. 6b).

Ferritin expression after wounding

Wounding does not activate the *AtFer1* promoter as it does not cause, in the first 4 h, any increase in GUS activity in the At1400IDRS or At1400m*IDRS lines (not shown). However, *AtFer3* transcript accumulates after wounding (Fig. 7a). Such accumulation is not systemic as it is detected only in wounded leaves and not in intact leaves of the same plant (Fig. 7a).

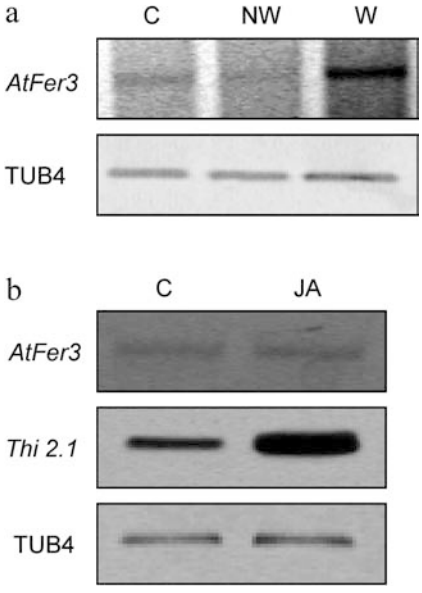

Fig. 7a, b Ferritin expression after wounding or JA treatment. **a** RT–PCR analysis of *AtFer3* transcript in 22 day-old At1400IDRS plants after wounding. *Lane C* Control leaves; *lane NW* non-wounded leaves of a wounded plant; *lane W* wounded leaves. *TUB4* is the positive control for the equal addition of RNA in each sample. **b** RT–PCR analysis of *AtFer3* transcript in At1400IDRS plants sprayed with 200 μM JA (*lane JA*). *Lane C* Control leaves. *Thi2.1* is the positive control for the efficacy of JA treatment

Jasmonic acid (JA) accumulates upon wounding through the systemic response activated by an 18-amino-acid peptide called systemin (Wasternack and Parthier 1997; Buchanan et al. 2000). Treatment with JA confirms that accumulation of *AtFer3* transcript in response to wounding is not part of a systemic response: JA is indeed unable to increase *AtFer3* transcript abundance (Fig. 7b). In contrast, accumulation of the *Thi2.1* gene transcript (Fig. 7b), known to be induced by JA (Epple et al. 1995; Vignutelli et al. 1998), confirms the efficacy of JA treatment. Taken together, these data suggest that a JA-independent *AtFer3* induction occurs upon wounding as part of a local, non-systemic response.

Discussion

Both free-iron excess or iron deficiency are noxious to plants. Regulation of cellular iron homeostasis is necessary, and ferritins contribute to such regulation by acting as iron-stores, sequestering or releasing iron atoms upon demand (Laulhere and Briat 1993). We investigated the localization of *AtFer1* expression in different plant tissues and the role of the IDRS element in the tissue and/or cell specificity of the *AtFer1* expression.

In the leaves, *AtFer1* promoter activity is mainly expressed in the vicinity of the vessels and the disruption of the IDRS element does not alter such localization. In the roots the *AtFer1* promoter activity is mainly localized in the endoderm. However, *AtFer1* promoter activity is observed also in the cortex and epiderm of older secondary roots; also, the disruption of the IDRS in the roots results in expansion of *AtFer1* promoter activity to the cortex and epiderm. Such observations indicate that under conditions of standard iron nutrition the IDRS could be involved in the repression of expression of the *AtFer1* gene in the cortex and epidermal cells of young roots. In the absence of a functional IDRS, *AtFer1* repression would not occur anymore, and this could explain the expanded expression territories observed in roots. Such a hypothesis would mean that the IDRS could control, at least in part, the territories of *AtFer1* expression in roots, in coordination with still uncharacterized tissue and/or cell specific *cis*-elements.

During senescence, cells undergo distinct metabolic and structural changes prior to cell death, which contribute to plant fitness by mobilizing nutrients towards still growing tissues (Buchanan-Wollaston 1997; Buchanan-Wollaston and Ainsworth 1997; Matile 2001). This active process is regulated by a complex net of distinct signalling pathways (Lohman et al. 1994; Oh et al. 1997; Hinderhofer and Zentgraf 2001; Woo et al. 2001). We show that *AtFer1* promoter is activated in leaves at a very early point of age-dependent leaf senescence, in an IDRS-independent manner. This evidence suggests that other signals, different from bare fluctuations in intracellular free-iron levels, regulate *AtFer1* during senescence. In particular, *cis*-elements other than the IDRS are required for the territory-specific or developmental expression of *AtFer1*. Such IDRS-independent developmental expression of *AtFer1* during the age-dependent senescence program is not observed during dark-induced senescence. The events triggered by dark treatment are in fact different from those activated during natural ageing: photochemical efficiency declines rapidly just as chlorophyll is rapidly degraded. Most probably a rise in free-iron levels deriving from disassembly of photosystems is responsible for the IDRS-dependent *AtFer1* promoter expression during dark treatment. These results indicate that it is therefore possible to uncouple the environmental response of the *AtFer1* promoter to excess iron, from its developmental regulation.

Our findings confirm that different environmental stimuli trigger leaf senescence through distinct pathways (Oh et al. 1997) and are in accordance with the differences in gene expression observed in barley leaves during age-dependent or dark-induced senescence (Becker and Apel 1993).

Two other different environmental stresses, photoinhibition and wounding, were also tested on ferritin gene expression, besides dark-induced leaf etiolation. By using a 600-bp *AtFer1* cDNA probe, we previously showed (Murgia et al. 2001) that *AtFer1* transcript accumulates upon photoinhibition. In fact, results obtained in this study show that the *AtFer1* promoter is insensitive to photoinhibition and that the *AtFer1* transcript does not accumulate, whereas *AtFer2*, *AtFer3* and *AtFer4* respond to this kind of stress. We conclude, therefore, that our previous results were probably due to the ability of the *AtFer1* probe to hybridize with some of the other ferritin gene products. This is consistent with their high sequence homologies and with the observation that *AtFer2–4* are expressed in photoinhibited leaves. In this work, the problem of probe specificity has been bypassed by designing specific primers for each gene product to be analyzed (i.e. *AtFer1*, *AtFer2*, *AtFer3* and *AtFer4*), which have been used for RT–PCR experiments.

A functional analysis of *AtFer2–4* promoters will give an integrated view of the regulated expression of the various members of the ferritin gene family from *Arabidopsis*.

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