Altered levels of AtHSCB disrupts iron translocation from roots to shoots

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Abstract

Key message Plants overexpressing AtHSCB and *hscb* knockdown mutants showed altered iron homeostasis. The overexpression of AtHSCB led to activation of the iron uptake system and iron accumulation in roots without concomitant transport to shoots, resulting in reduced iron content in the aerial parts of plants. By contrast, *hscb* knockdown mutants presented the opposite phenotype, with iron accumulation in shoots despite the reduced levels of iron uptake in roots. AtHSCB play a key role in iron metabolism, probably taking part in the control of iron translocation from roots to shoots.

Abstract Many aspects of plant iron metabolism remain obscure. The most known and studied homeostatic mechanism is the control of iron uptake in the roots by shoots. Nevertheless, this mechanism likely involves various unknown sensors and unidentified signals sent from one tissue to

another which need to be identified. Here, we characterized *Arabidopsis thaliana* plants overexpressing AtHSCB, encoding a mitochondrial cochaperone involved in [Fe–S] cluster biosynthesis, and *hscb* knockdown mutants, which exhibit altered shoot/root Fe partitioning. Overexpression of AtHSCB induced an increase in root iron uptake and content along with iron deficiency in shoots. Conversely, *hscb* knockdown mutants exhibited increased iron accumulation in shoots and reduced iron uptake in roots. Different experiments, including foliar iron application, citrate supplementation and iron deficiency treatment, indicate that the shoot-directed control of iron uptake in roots functions properly in these lines, implying that [Fe–S] clusters are not involved in this regulatory mechanism. The most likely explanation is that both lines have altered Fe transport from roots to shoots. This could be consistent with a defect in a homeostatic mechanism operating at the root-to-shoot translocation level, which would be independent of the shoot control over root iron deficiency responses. In summary, the phenotypes of these plants indicate that AtHSCB plays a role in iron metabolism.

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Introduction

Iron-sulphur clusters are amongst the simplest cofactors used by (almost) all living organisms. These clusters are implicated in a myriad of vital processes such as respiration, photosynthesis, sulphur and nitrogen assimilation, amino acid and purine metabolism, plant hormone and coenzyme biosynthesis and DNA repair and translation, among others (Balk and Pilon 2011; Lill 2009). Although clusters can

be synthesized non-enzymatically in vitro (Beinert 2000), their biosynthesis is catalyzed by specific enzymes. In bacteria, three complete [Fe-S] biogenesis systems have been discovered, i.e., NIF (nitrogen fixation), SUF (mobilization of sulfur) and ISC (iron-sulphur cluster); several of the proteins in these systems are encoded by operons. The genes comprising the *isc* operon are conserved and widely distributed, and homologues of the *isc* components are found in the mitochondria of yeast and higher eukaryotes (Balk and Lobreau 2005; Muhlenhoff and Lill 2000).

In yeast mitochondria, the release of sulphur from cysteine is catalyzed by the cysteine desulphurase Nfs1, and sulphur is transferred to the scaffold protein Isu1, where the iron-sulphur clusters are assembled (Balk and Lobreau 2005; Balk and Pilon 2011; Johnson et al. 2005). The nature of the iron source is still under debate, although in several organisms, such as *E. coli*, *S. cerevisiae* and *H. sapiens*, there is substantial evidence pointing towards frataxin (Yfh1) (Stemmler et al. 2010), a protein that also appears to have a regulatory effect on the ISC pathway (Pastore and Puccio 2013). In the last stage of this pathway, in a process specifically assisted by a dedicated chaperone system comprising the Hsp70 ATPase Ssq1 and the specialized DnaJ-like cochaperone Jac1 (Cupp-Vickery et al. 2004; Dutkiewicz et al. 2004) among other additional factors, the mature [Fe-S] cluster is transferred and inserted into apo-proteins. This complex formation is thought to induce a structural change in Isu1, thereby weakening Fe-S-cluster binding, thus facilitating cluster dissociation and transfer to apo-proteins (Bonomi et al. 2008; Lill 2009; Markley et al. 2013).

In *Arabidopsis thaliana* plants, frataxin (AtFH) and cysteine desulphurase (AtNFS1) homologues are thought to be involved in early steps of Fe-S cluster formation in mitochondria, specifically iron loading and sulphur transfer to the scaffold proteins (Gerber et al. 2003). We previously characterized the *Arabidopsis* frataxin homologue and demonstrated that it is essential for full activity of mitochondrial [Fe-S] proteins and hemeproteins (Busi and Gomez-Casati 2012; Busi et al. 2004, 2006; Maliandi et al. 2011) and that it participates in the Fe-mediated oxidative stress response (Maliandi et al. 2007; Martin et al. 2009). In addition, we recently reported that AtNFS1 interacts with AtFH and that this interaction modulates the kinetic properties of the former protein (Turowski et al. 2012). These findings indicate that AtFH plays a direct role in regulating mitochondrial cysteine desulphurase activity and that both proteins function in early steps of [Fe-S] cluster biogenesis (Busi and Gomez-Casati 2012; Busi et al. 2006; Turowski et al. 2012). The second stage of the [Fe-S] cluster assembly pathway in plant mitochondria likely resembles that of yeast mitochondria, involving the transfer of the nascent cluster from AtISU1 to the target proteins, probably mediated by the

chaperone/cochaperone system AtHSCA1-2/AtHSCB. In addition, the cochaperone AtHSCB from *Arabidopsis* can functionally complement the yeast *jac1* knockout mutant, suggesting that AtHSCB plays a role in [Fe-S] cluster biogenesis in plants. Furthermore, an AtHSCB T-DNA insertion mutant shows reduced seed set and abnormal trichome development, as well as dramatically reduced activities of the [Fe-S] enzymes aconitase (ACO) and succinate dehydrogenase (SDH) (Xu et al. 2009). Moreover, we previously demonstrated that AtHSCB can physically interact with AtISU1 and AtHSCA2, providing more evidence for its involvement in the biosynthesis of [Fe-S] clusters in plants (Leaden et al. 2014). However, to date, little is known about the in vivo function of the cochaperone AtHSCB in plants. Nevertheless, HSC20, the human AtHSCB homologue, has been shown to physically interact with a specific subset of recipient [Fe-S] proteins, such as SDHB (the complex II [Fe-S] subunit) and other accessory factors that assist cluster delivery through their tripeptide LYR motif, whose presence appears to be the basis for substrate discrimination (Maio et al. 2014).

Iron-sulphur biogenesis represents an important sink for iron in living organisms. Plants have to acquire this metal from soils where, although abundant, it usually has very low bioavailability. To cope with this problem, dicots and non-graminaceous monocots utilize strategy I for iron uptake, which involves proton extrusion to solubilize Fe^{3+} in the soil, reduction of Fe^{3+} to Fe^{2+} and transport of Fe^{2+} across the root epidermal cell membrane. In *Arabidopsis*, a member of H^+ -ATPase family (AHA2) likely mediates proton efflux (Santi and Schmidt 2009), the ferric-chelate reductase oxidase 2 (FRO2) is responsible for the reduction of Fe^{3+} (Robinson et al. 1999) and there is ample evidence that the iron regulated transporter 1 (IRT1) transports Fe^{2+} into root cells (Barberon et al. 2011; Connolly et al. 2003; Eide et al. 1996). To acquire enough Fe while avoiding toxicity, Fe uptake in non-graminaceous plants is tightly controlled (Vert et al. 2002; Yi and Guerinot 1996). The key regulator of root iron acquisition in strategy I plants, FIT, plays crucial roles in positively regulating FRO2 and IRT1 under Fe deficiency (Colangelo and Guerinot 2004; Jakoby et al. 2004), in conjunction with two other basic helix-loop-helix transcription factors (bHLH38 and bHLH39) (Yuan et al. 2008). Recently, in *Arabidopsis* it has been described an ATP-binding cassette transporter ABCG37 (alias PDR9) that is involved in secretion of phenolic compounds from roots into the medium in response to Fe deficiency, indicating the presence of a secondary strategy to Fe uptake in non-graminaceous plants (Fourcroy et al. 2014; Rodriguez-Celma and Schmidt 2013). In contrast to the abundant data on root iron uptake and its regulation, little is known about Fe transport to the shoot, remobilization and distribution in different tissues and subcellular translocation and storage.

Three proteins that are clearly involved in iron transport are the nicotianamine synthase NAS4 (Koen et al. 2013), the citrate transporter FRD3 (Rogers and Gueriot 2002) and the xylem iron transporter FPN1 (Morrissey et al. 2009). While considerable progress has been made over the past few years in elucidating the mechanism underlying iron transport, some of the key components of iron metabolism that are yet to be deciphered include (i) the full set of transcription factors that regulate and fine-tune iron homeostasis, (ii) the precise mechanisms and transporters involved in transport into and out of xylem and phloem vessels and (iii) signals and receptors (sensors) that regulate Fe nutritional status (Darbani et al. 2013; Hindt and Gueriot 1823; Ivanov et al. 2012; Vigani et al. 2013).

Evidence suggests that there is an intimate connection between Fe homeostasis and [Fe-S] cluster biosynthesis. Fungi, fruit flies, mice and animal cells that fail to biosynthesize iron-sulphur clusters exhibit abnormalities in intracellular iron distribution and misregulated activation of high-affinity cellular iron uptake (Anderson et al. 2005; Kaplan and Kaplan 2009; Kim et al. 2001; Kisfalvi et al. 1999; Lill and Muhlenhoff 2008). This connection has been unravelled in mammals, where the bifunctional protein ACO/IRP1 plays a central regulatory role in iron homeostasis by controlling RNA stability or mRNA translation of genes involved in iron metabolism. Formation and loss of the IRP1 [4Fe-4S] cluster regulates RNA binding and provides unique links between iron metabolism and cellular [Fe-S] cluster biogenesis (Anderson et al. 1823). In yeast, the association between Fe and [Fe-S] metabolism has also been confirmed. Mitochondrial [Fe-S] cluster biogenesis is required for both Aft1/Aft2-dependent activation of the iron uptake response (among other actions against iron deficiency) and Yap5-dependent activation of high iron response genes (Li et al. 2012; Poor et al. 2014), providing a direct link between high and low iron sensing in *S. cerevisiae* via [Fe-S] clusters. Neither of these Fe regulatory mechanisms is conserved in plants (Arnaud et al. 2007). Nonetheless, as the Fe sensor in plants remains to be uncovered, and in light of the available evidence, the possibility of an [Fe-S]-dependent Fe-sensing mechanism in plants cannot be ruled out.

In this study, we characterized the phenotypes of *A. thaliana* plants overexpressing AtHSCB and *hscb* knockdown mutants showing altered iron homeostasis. The overexpression of AtHSCB led to activation of the iron uptake system and iron accumulation in roots without concomitant transport to shoots, resulting in reduced iron content in the aerial parts of plants. By contrast, *hscb* knockdown mutants presented the opposite phenotype, with iron accumulation in shoots despite the reduced levels of iron uptake in roots.

Taken together our data suggest that AtHSCB play a key role in iron metabolism, probably taking part in the control of iron translocation from roots to shoots.

Materials and methods

Plant materials, molecular cloning and culture conditions

Arabidopsis thaliana ecotype Col-0 was used in this study. *Arabidopsis* mutant lines SALK 099684 (*hscb1*) and SALK 020271 (*hscb2*) were obtained from the Arabidopsis Biological Resource Center. T-DNA insertion lines were confirmed by PCR with T-DNA left border primer (LBa1) and gene-specific primers (Up-*hscb*-prom and Rv-*hscb*-prom, see Table S1).

Arabidopsis plants overexpressing AtHSCB were obtained by cloning AtHSCB cDNA by PCR using Pfu polymerase (Promega, <https://www.promega.com>) and the primer pair AtHSCB-myc-fw/AtHSCB-myc-rv. The resulting PCR product was cloned into the pDonr221 vector by recombination (Gateway Technology, Invitrogen, <http://www.invitrogen.com.ar>). The AtHSCB insert was transferred into the pC-TAPa plasmid by recombination (Rubio et al. 2005) using the LR Clonase reaction to give rise to p-AtHSCB-myc. *Arabidopsis* was transformed with p-AtHSCB-myc by the floral dip method (Clough 1998). Gentamicin selected plants were confirmed by PCR with the primer pair HSCB-mycfw/RtpCtaparv, and after three generations, homozygous lines were obtained.

For general plant growth, surface-sterilized seeds were plated on half-strength MS medium in Petri dishes. After stratification, the plants were grown under a 16 h light/8 h dark cycle at 22 °C in a growth chamber for 15 days. Three iron treatments were imposed: (a) Iron deficiency: half-strength MS medium was prepared without iron, 20 µM FeNaEDTA was added, and the plants were grown for 15 days; (b) Foliar iron: plants grown for 14 days on half-strength MS medium were sprayed with 50 µM Fe-EDTA as described previously (Garcia et al. 2013), and samples were taken after 24 h; (c) 48 h iron depletion: plants grown for 13 days under standard conditions were transferred to iron depleted medium (half-strength MS medium prepared without iron plus BPS) for 48 h and then harvested. In the citrate treatment, plants grown for 13 days under standard conditions were transferred to either citrate supplemented medium (half-strength MS medium with the addition of 3 mM sodium citrate) or fresh standard medium, and harvested after 2 days.

RNA extraction and quantitative real time PCR (qPCR)

Three biological replicates, consisting each sample of leaves or roots pooled from 10 to 15 plants, were analyzed. Total RNA was extracted from rosette leaves and roots using the SV Total RNA Isolation System (Promega). Complementary DNA was synthesized using M-MLV reverse transcriptase (Promega) and random hexamers. The qPCR reactions were performed in a MiniOPTICON2 system (BioRad, <http://www.bio-rad.com>) using SYBR Green I (Invitrogen) as the detection reagent as previously described (Busi et al. 2011). Gene-specific primers for amplification of 150–250 bp products were designed using primer3 software. Melt curve analysis were conducted to confirm the specificity of each assay. Gene expression levels were calculated using the comparative Ct method ($2^{-\Delta\Delta C_t}$) (Pfaffl 2001).

Protein quantification and western blot

Total protein concentrations were determined as described (Bradford 1976). SDS–PAGE was performed using 10% P/V gels (Laemmli 1970). Protein bands were electro-blotted onto a nitrocellulose membrane (Bio-Rad), which was incubated with a-myc antibody (Santa Cruz, <http://www.scbt.com>). The antigen–antibody complex was visualized with alkaline phosphatase-linked a-mouse IgG, and BCIP and NBT staining (Bollag et al. 1996).

Rosette area and lateral root length measurements

Rosette area and lateral root (LR) length/number were assessed in 15-day-old seedlings by image analysis with Image J (<http://imagej.nih.gov/ij/>).

Chlorophyll Assay

Plants grown on half-strength MS medium for 15 days were harvested, and total chlorophyll was extracted from the shoots and assayed spectrophotometrically as previously described (Lichtenthaler 1987).

Enzymatic activities

Aconitase and succinate dehydrogenase activities were determined as previously reported (Busi et al. 2006) adding a desalting step of the protein extracts previous to each activity determination, measuring the formation of *cis*-aconitate at 240 nm, and the oxidation of 2,6-dichlorophenolindophenol at 609 nm, respectively. Malate dehydrogenase (MDH) activity was assayed spectrophotometrically in the oxaloacetate-reducing direction as described (Tripodi and Podestà 2003). All the activity determinations were performed in

samples of pooled roots and shoots, at least in triplicate, and the mean values $\pm$ standard deviation (SD) were reported.

Ferric-chelate reductase activity was monitored in whole intact root systems as described (Yuan et al. 2008). Five roots of 15-day-old seedlings, were rinsed with distilled pure water and submerged in assay solution (200 μ M CaSO_4 , 100 μ M Fe(III)NaEDTA , 200 μ M phenantroline, 5 mM MES, pH 5.5). After 1 h, an aliquot of the assay solution was removed and its absorbance was determined spectrophotometrically at 510 nm. The Fe(II) -phenantroline concentration was calculated against a calibration curve of $\text{Fe(NH}_4\text{)(SO}_4\text{)}_2$.

Perls staining, in situ Perls/DAB intensification and iron quantification

Iron stainings and quantification were performed on 15-day-old plants grown under iron sufficient conditions. Perls staining was performed as described in (Roschztardt et al. 2009), with minor modifications. Seedlings were washed three times with distilled water (5 min each wash), vacuum infiltrated with Perls stain solution (4% HCl, 4% potassium-ferrocyanide 1:1) for 5 min. and left in the staining solution for 30 more min. at room temperature and in the dark. The stained samples were washed three times with distilled water and subjected to microscopic analysis [SMZ-2T microscopy connected to a TV Lens C-0.45 $\times$ (Nikon, <http://www.nikon.com>)].

For histochemical iron localization, seedlings were fixed with ethanol/glacial acetic acid/formaldehyde (50/5/3.7%) for 15 h., washed with 0.01 M phosphate buffer (pH 7.4) three times, and dehydrated in successive baths of 50, 70, 90, 96, and 100% ethanol, 100% isopropanol, and 100% xylene. Then, the roots were embedded in paraffin, and thin Sections (3 μ m) were made. Finally, the sections were deposited on glass slides and stained with the Perls/DAB iron intensification protocol described in (Roschztardt et al. 2009).

For iron quantification, roots were separated from shoots and completely acid digested with HNO_3 . Total iron contents were measured spectrophotometrically with batho-phenanthroline as indicated in Tarantino et al. (2010).

Citrate quantification

Citrate quantification was performed by gas chromatography mass spectrometry–based metabolite profiling. Extraction, liquid partition, and derivation prior to GC–MS analysis were performed as described by Lisec et al. (2006). For analysis, three biological replicates with a second group of technical replicates were utilized (six total data points). Citrate was unambiguously assigned using retention index and mass spectrum as the two most important identification

criteria, and by running a standard of sodium citrate processed in the same way as the samples. Peak heights were normalized using the amount of the sample fresh weight and ribitol for internal standardization. Relative metabolite contents were informed.

Results

Phenotypic characterization of *A. thaliana* plants with modified levels of AtHSCB

To analyze the physiological role of AtHSCB, we first identified and characterized two homozygous *hscb* knock-down mutant plants from the Salk Institute Genomic Analysis Laboratory database of sequenced T-DNA (*hscb1*: SALK_099684 and *hscb2*: SALK_020271), which carry single T-DNA insertions (Fig. S1a). Secondly, to strengthen our data, we generated transgenic plants overexpressing AtHSCB under the control of the cauliflower mosaic virus 35S promoter (Fig. S1b). Two independent homozygous overexpressing lines, designated *HSCB1* and *HSCB2*, were

selected in the T3 generation. We analyzed AtHSCB transcript levels in roots and leaves of 15-day-old seedlings grown on half-strength MS agar plates by quantitative real-time PCR (Fig. 1b). Compared with wild-type plants, AtHSCB transcript levels in the *hscb* mutant lines were reduced by 40–50 % in roots and 65–85 % in shoots, whereas AtHSCB transcript levels increased more than 10- to 20-fold in roots and one- to four-fold in leaves in *HSCB*-overexpressing lines. Western blot analysis using antibodies against the *myc* peptide confirmed the presence of recombinant AtHSCB-myc protein in *HSCB1*- and *HSCB2*-overexpressing seedlings (Fig. 1c).

After 15 days of growth on half-strength MS agar plates, both overexpressing and knockdown mutant lines developed smaller seedlings than the wild-type line (Fig. 1a). Rosette area measurements confirmed the reduced seedling size (Fig. 1d), with an approximately 20 % decrease in whole leaf area for all lines. In addition, the overexpressing and knockdown lines were chlorotic under this growing condition (Fig. 1a). Total chlorophyll quantification validated this observation: *hscb2* had a 22 % reduction in chlorophyll content, and the chlorophyll contents

Fig. 1 Phenotypic characterization of *HSCB1*, *HSCB2*-overexpressing, *hscb1* and *hscb2* knockdown seedlings. **a** Phenotypic comparison of plants vertically grown on agar plates. **b** Quantitative real-time PCR analysis of AtHSCB transcript levels in seedlings of wild-type, *hscb1*, *hscb2*, *HSCB1* and *HSCB2* lines (n=3). *Actin2* was used as an internal control. **c** Immunodetection of recombinant AtHSCB-myc protein expression in seedlings of wild-type, *HSCB1* and *HSCB2* lines

using a-myc peptide antibody. **d** Rosette area measurements, **e** total chlorophyll quantification, **f** LRT number, **g** LR length. Each analysis was performed on 15-day-old plants grown on half strength MS agar plates. 10–15 biological replicates were analyzed for each line. Bars with the same letter are not significantly different ($P < 0.05$) by One Way Anova test

in the other lines were less than half of wild-type levels (Fig. 1e). One of the most striking traits in the overexpressing plants was their root system architecture. Primary root growth in the mutant and overexpressing lines was not significantly different from that in wild-type seedlings. However, whereas the LR morphology of *hscb* seedlings was similar to that of wild type, the LR number (Fig. 1f) and LR length (Fig. 1g) of overexpressing lines were strongly reduced.

Arabidopsis *AtHSCB* T-DNA insertion mutants have reduced activities of the iron–sulphur enzymes ACO and SDH, suggesting that *AtHSCB* plays a role in iron sulphur biogenesis in plants (Xu et al. 2009). To reassess the knockdown mutant plants and to survey the [Fe–S] cluster assembly pathway in the overexpressing lines, we measured ACO and SDH activities in the leaves and roots of these lines. As a control, we measured the activity of MDH, a tricarboxylic acid (TCA) cycle enzyme without [Fe–S] cofactors, to confirm that any difference in activity observed would be due to an alteration in [Fe–S] assembly and/or [Fe–S] protein functions. Our results validate the previous findings, as in both roots and leaves of *hscb* lines, the activities of [Fe–S] enzymes were reduced by more than 50% compared to wild type (Fig. 2a, b), while MDH activity remained unchanged (Fig. 2c). In the roots of *HSCB* (*AtHSCB*—overexpressing) lines, both ACO and SDH activities were higher than those of wild type (Fig. 2a, b). These results were not surprising, as these lines strongly overexpress a cochaperone of the [Fe–S] biosynthetic pathway, but unexpectedly, ACO and SDH activities in *HSCB* leaves were highly reduced relative to those of wild type. Since MDH activity was the same in each tissue and line, all differences observed in [Fe–S] enzymes activities appear to be due to a reduction in cofactor biosynthesis.

AtHSCB affects total iron content, distribution and uptake

Several lines of evidence point towards an alteration in iron metabolism as a possible cause for the phenotypes observed. First, since Fe is a substrate of [Fe–S] cluster biogenesis, it is feasible that low iron content or availability may lead to low cofactor assembly in the *HSCB* lines. In other taxa such as fungi and metazoans, an intimate connection exists between Fe homeostasis and [Fe–S] cluster biogenesis (Anderson et al. 1823; Lill et al. 2012). Furthermore, root system architecture is the combined result of genetically determined patterns and the environmental challenges to which plants are exposed, including nutrient accessibility, and iron availability can affect LR development (Giehl et al. 2012). Consequently, to establish the cause of the reduced ACO and SDH activity in *HSCB* leaves, we analyzed the iron contents in all lines under investigation using various techniques.

First, we harvested 15-day-old seedlings grown under Fe sufficient condition and performed qualitative histochemical detection by Perls staining, revealing that *HSCB* transgenic lines contained a significantly greater amount of ferric iron in their roots than wild-type and knockdown seedlings (Fig. 3a). Conversely, ferric iron levels in *hscb* roots were not significantly different from those of wild-type roots. However, upon closer inspection, iron tended to be concentrated outside the vascular bundle in the *HSCB* lines, whereas in *hscb* lines, the metal appeared to be concentrated in vessels.

Second, to quantify total Fe content, we harvested and acid digested the plants and measured iron levels using a spectrophotometric technique. As shown in Fig. 3b, under Fe sufficient conditions, *HSCB* seedlings accumulated approximately fourfold more Fe in their roots than did the wild type. We did not detect any difference in Fe content

Fig. 2 The effect of *AtHSCB* on [Fe–S] enzymatic activity in roots and shoots. Plants were grown on half-strength MS agar medium for 15 days and harvested, and **a** ACO, **b** SDH, and **c** MDH activities were measured in pooled root and shoot tissues of wild-type, *HSCB*

and *hscb* lines. MDH, an enzyme lacking [Fe–S], was used as a control. Data are representative of means $\pm$ SD from three independent experiments. Bars with the same letter are not significantly different ($P < 0.05$) by One Way Anova test

Fig. 3 Altered levels of *AtHSCB* affect iron partitioning between roots and shoots. Wild-type, *HSCB* and *hscb* plants were grown on half-strength MS agar medium (iron sufficient conditions) for 15 days and harvested. **a** Histochemical Fe detection in roots by direct Perls staining. Bar represents 0.1 mm. **b** Total Fe content in roots and shoots.

Three biological replicate sets of pooled roots and shoots were subjected to iron analysis by a spectrophotometric technique. **c** Quantitative real time PCR analysis of *AtFER1* transcript levels in roots and leaves ($n=3$). *Actin2* was used as a control gene. Bars with the same letter are not significantly different ($P < 0.05$) by One Way Anova test

between wild-type and *hscb* plant roots. Conversely, Fe levels in knockdown line shoots were approximately twofold higher than in wild type, while the iron content in *HSCB* shoots was nearly half that of wild-type shoots. These results clearly indicate that Fe distribution between (and probably within) roots and shoots in both the overexpressing and knockdown mutant lines was highly altered.

Finally, we analysed ferritin expression by qPCR to address the question of whether this anomalous iron content is localized inside or outside the cells in roots and shoots. *A. thaliana* has four ferritin genes, *AtFER1–4* (Petit et al. 2001); *AtFER1* is the most highly expressed ferritin gene in vegetative tissues (Reyt et al. 2015). Since *AtFER1* is usually used as a marker of excess iron or free iron-induced cell oxidative stress (Ravet et al. 2009), measuring its transcript levels by qPCR provides an indirect way to measure symplastic iron levels. As shown in Fig. 3c, *AtFER1* was highly expressed in *HSCB* roots, indicating that the high levels of iron in these roots were primarily located inside the cells. However, although *hscb* leaves exhibited elevated iron levels, they also exhibited slightly reduced *AtFER1* expression.

This result might indicate that either (i) iron accumulated in the apoplastic space in leaves or (ii) that iron was present inside the cells but did not reach the chloroplasts, the organelle to which ferritins are mainly targeted [reviewed in Briat et al. (2010)]. Perls/DAB staining performed on 15-day-old plants grown on iron sufficient media seems to confirm both hypothesis (Fig. S2). *hscb* leaves displayed increased iron deposits around vascular tissues, pointing towards accumulation in the apoplastic space. Nevertheless, part of the leaf iron must be incorporated in mesophyll cells because some structures, probably the nucleus or nucleolus (Roschztardt et al. 2011), appeared deeply labeled. Wild-type and *HSCB* leaves did not show these last iron dyed structures. The reduced *FRO2* activity exhibited by *hscb* plants (see Fig. 4b) also suggests high availability of symplastic iron in leaves.

In an attempt to determine the mechanism by which overexpression and knockdown of *AtHSCB* lead to abnormal iron content and distribution, we investigated the expression of *FIT*, *FRO2*, and *IRT1*; these genes are involved in root iron uptake and are commonly used as markers of

Fig. 4 Iron uptake is elevated in *HSCB* lines and reduced in *hscb* lines. Wild-type, *HSCB* and *hscb* lines were grown on half-strength MS agar medium for 15 days. **a** Expression levels of *FIT*, *IRT1* and *FRO2*. **b** Ferrochelatase reductase activity. At least 20 roots were pooled and subjected to enzyme activity analysis (n=5). Bars with the same letter are not significantly different ($P < 0.05$) by One Way Anova test

iron deficiency in *Arabidopsis* due to their rapid induction under this condition (Eide et al. 1996). When the seedlings were grown under iron sufficient conditions for 15 days, *FIT* transcript levels were approximately twofold higher in *HSCB* lines than in wild-type plants. Conversely, *hscb* lines exhibited similar or lower levels of *FIT* transcripts than the wild type (Fig. 4a). Also, *IRT1* and *FRO2* transcript levels were high in *HSCB*-overexpressing lines, whereas in the knockdown lines, the transcript levels of these genes were not statistically different from those of wild-type plants. The only exception was *hscb1*, which exhibited reduced *FRO2* transcript levels. To establish whether the *FRO2* transcript levels in *HSCB1/HSCB2* and *hscb1/hscb2* were correlated with protein abundance, we analyzed *FRO2* activity. Consistent with its transcript accumulation, *FRO2* activity was more than two- and three-fold higher in *HSCB1* and *HSCB2* roots, respectively, compared with wild-type roots. On the other hand, in the roots of *hscb* seedlings, *FRO2* activity was one-half that of wild-type plants (Fig. 4b). These findings indicate that the iron uptake machinery of *HSCB*-overexpressing plants is constitutively activated when grown under iron sufficient conditions and that these plants behave like iron deficient plants. Conversely, knockdown mutant plants probably incorporate iron at a lower rate than the wild type because Fe(III) reduction is assumed to be the rate-limiting step in iron uptake (Connolly et al. 2003); these plants function as though they were growing under iron excess conditions.

Correct functioning of the shoot-directed system controlling the iron deficiency response in roots

Many studies suggest that the aerial parts of plants play an important role in regulating Fe deficiency responses (Enomoto et al. 2007; Lucena et al. 2006; Maas et al. 1988; Vert et al. 2003), particularly in the induction of key proteins in Strategy I iron uptake, such as *FRO2* and *IRT1*. Nevertheless, it is not clear which pool of iron is sensed (apoplastic, cytoplasmic, vacuolar, mitochondrial, chloroplastic and so on), which molecular components are involved in the

sensing mechanism and how the response is transduced to the root. The proper functioning of the shoot-directed system controlling root Fe deficiency responses is usually verified by foliar application of iron to Fe-deficient plants; when this system functions correctly, a strong decrease in *FRO2* activity is observed (Garcia et al. 2013).

The altered gene transcript levels of the major elements in the root iron uptake system and *FRO2* enzymatic activity are in accordance with the abnormal leaf iron contents detected in the lines under investigation. We next examined whether AtHSCB interferes with signal transduction when leaf iron contents are altered. We sprayed the leaves of *HSCB*-overexpressing and mutant lines with Fe-EDTA and measured *FRO2*, ACO, SDH and MDH activity 24 h later. After the Fe-EDTA treatment, *FRO2* activity did not change significantly in *hscb* and wild-type roots, whereas it decreased approximately 50% in *HSCB* roots compared to the respective controls (Fig. 5a). Leaf SDH and MDH activity did not change in either line in response to Fe-EDTA treatment, nor did ACO activity in *hscb* and wild-type leaves. However, ACO activity increased more than 50% in *HSCB1*-overexpressing leaves, compared to their control conditions (Fig. 5b). These and the above results indicate that the aerial part of the plants control iron uptake in roots. *FRO2* activity was high in *HSCB*-overexpressing plants due to the low iron content in leaves, and it was low in knockdown mutant roots due to the high levels of iron in leaves (Fig. 3b). Moreover, when the leaf iron content increased in *HSCB*-overexpressing seedlings due to spray application of Fe-EDTA, *FRO2* activity decreased, demonstrating that the foliar iron sensing mechanism functions correctly, as does the response system controlled by the aerial part of the plant. In addition, ACO activity increased when Fe was sprayed onto *HSCB* leaves, indicating that the low ACO activity in the shoots of these lines is due to the low leaf iron content, which probably limits [Fe-S] cluster assembly.

The mechanism that controls iron uptake in plants has been well studied. However, little is known about how iron is transported from roots to shoots and how this process is regulated. To shed some light on this issue, we measured

Fig. 5 The shoot-directed control of iron deficiency responses in roots functions properly. Leaves of plants grown for 14 days under iron-sufficient conditions were sprayed with 50 μ M Fe-EDTA or water (control). **a** Ferrochelatase reductase activity in pooled roots ($n=5$) and

b ACO, SDH and MDH activities in pooled shoots ($n=3$) at 24 h after treatment. Bars with the same letter are not significantly different ($P<0.05$) by Two Way Anova test

the gene expression levels of the best-characterized players in iron transport and remobilization metabolism. As shown in Fig. S3, *FPN1*, *FRD3* and *NAS4* were induced in *HSCB* plants, while their expression was downregulated (*FPN1*) or unaltered in *hscb* seedlings. These results were expected, as at least *FRD3* and *NAS4* respond to iron deficiency (Klatte et al. 2009; Roschztardt et al. 2011).

The root iron accumulation phenotype in *frd3* mutant plants is rescued by citrate supplementation in the medium; and due to the iron transport and leaf iron assimilation, the constitutive high FRO2 activity is decreased (Durrett et al. 2007). Considering the *HSCB* resembling phenotype, we performed a 48 h citrate supplementation treatment on 13-day-old plants. Results showed that *HSCB* seedlings responded like *frd3* mutants reducing root iron content (Fig. 6a), increasing their leaf iron levels, which approach those of wild type plants grown under iron sufficient conditions (Fig. 6b) and, consequently, lowering their FRO2 activity to Col 0 levels (Fig. 6c). Wild type plants presented results comparable to those of *HSCB* on citrate-containing media, while in *hscb* knockdown seedlings iron was redistributed from shoots to roots (Fig. 6a, b). Although this was a striking finding there are many reports, in different species and with different degrees of intensity, of iron transport from leaves to roots (Carrasco-Gil et al. 2016; Nikolic and

Romheld 2003; Rodriguez-Lucena et al. 2009; Roosta and ohsenian 2012). Moreover, *hscb* FRO2 activity increased after citrate treatment in agreement with the reduced leaf iron content (Fig. 6c). These results confirm once again that the leaf homeostatic mechanism controlling iron uptake is working properly.

Which other iron homeostatic mechanism is affected in plants with altered *AtHSCB* levels?

A clue about a possible way in which anomalous iron distribution is alleviated came from observation of the lines under iron-deficient conditions. The foliar areas of overexpressing and knockdown mutant lines grown under Fe-deficient conditions (20 μ M Fe) for 15 days were 50 and 30% larger, respectively, than those of wt lines, indicating that *HSCB* and *hscb* lines grew better under these conditions than wild type (Fig. 6). We therefore reasoned that the absence of iron might unlock (*HSCB* lines) or normalize (*hscb* lines) iron transport.

To investigate this notion, we studied Fe contents in roots and leaves of *HSCB* and *hscb* plants that were grown for 13 days under iron sufficiency and transferred to medium devoid of iron. After 48 h of iron absence, all lines exhibited a general decrease in root iron levels, but consistent

Fig. 6 Citrate supplementation modifies iron distribution in roots and shoots. Plants grown for 13 days under iron-sufficient conditions were transferred to half-strength MS medium supplemented with 3 mM sodium citrate or fresh half-strength MS agar medium for 48 h. **a** Histological Fe detection in roots by direct Perls staining. Bar represents 0.1 mm. **b** Total Fe content in shoots. Three biological replicate

sets of pooled shoots were subjected to iron analysis by a spectrophotometric technique. **c** Ferrochelatase reductase activity in pooled roots ($n=5$). **d** Relative abundance of citrate in roots of 15-day-old plants grown on iron sufficient conditions (half strength MS medium). Bars with the same letter are not significantly different ($P < 0.05$) by One Way Anova test

with our hypothesis, this decrease was more pronounced in overexpressing lines: between 30–40% in wt, *hscb1* and *hscb2* vs. 50–60% in *HSCB1* and *HSCB2* lines, respectively. Moreover, wt and *hscb* lines exhibited reductions in iron levels in leaves of approximately 50% compared to the controls, whereas these levels increased 40 and 50% in *HSCB1* and *HSCB2* leaves, respectively (Fig. 7a). The results suggest that the shoot-directed control of iron uptake by the root continues operating properly, as FRO2 activity increased 3.7-fold in wild-type roots and twofold in *hscb* roots (reduced leaf iron content), whereas in *HSCB* roots, FRO2 activity decreased 50% (increased leaf iron content; Fig. 7b). These results confirm the notion that in the absence of Fe, the knockdown lines accumulated lower levels of leaf iron (as expected) and, more importantly, the overexpressing lines were able to transport the iron trapped in their roots to the shoots. Taken together, these results indicate that the decrease in root iron levels, or some signal or metabolic

consequence derived from this decrease, unlocks iron transport from root-to-shoot in *HSCB*-overexpressing plants.

Discussion

AtHSCB is involved in [Fe-S] biosynthesis and affects iron metabolism

To date, few studies have investigated the biological function of AtHSCB and its participation in the formation of [Fe-S] clusters (Leaden et al. 2014; Xu et al. 2009). In the current study, we examined the role of this protein in vivo, making use of plants with gain- and loss-of-function of AtHSCB. The activities of the [Fe-S] enzymes ACO and SDH were markedly reduced in the knockdown lines, whereas these enzyme activities increased in the roots of the overexpressing lines. On the contrary, the activity of

Fig. 7 Iron deficiency restores Fe transport from roots to shoots in *hscb* and *HSCB* plants. Plants grown for 13 days under iron-sufficient conditions were transferred to iron-deficient (no added Fe) or fresh half-strength MS agar medium for 48 h. **a** Total Fe contents in roots

and shoots were determined in three biological replicate sets of pooled tissues. **b** Ferrochelatase reductase activity in pooled roots ($n=5$). Bars with the same letter are not significantly different ($P<0.05$) by Two Way Anova test

the Krebs cycle enzyme MDH, which is devoid of [Fe-S], was unaffected in both lines, demonstrating that only iron-sulphur enzyme activities were altered (Fig. 2). Thus, our results clearly show that modifications in the expression of the cochaperone AtHSCB alter [Fe-S] cluster biogenesis, confirming its role in this plant biosynthetic pathway.

In addition, the *hscb* lines were chlorotic and grew slowly, both of which are common features of plants with deficient [Fe-S] biosynthesis [*atm3* (Kushnir et al. 2001), *isu1*, *nfs1* (Frazzon et al. 2007), *atfh1* (Busi et al. 2006)]. While the reasons for the chlorosis are somewhat difficult to unravel, and only *atm3* lines have been investigated in some detail (Bernard et al. 2009), the slow growth phenotype might be explained by the impact of low ACO and SDH activity on TCA cycle flux (Balk and Schaedler 2014). *HSCB* plants were also chlorotic and smaller than wild-type lines. Nevertheless, the ultimate cause of both phenotypic traits is the low leaf iron content in these plants. Low iron availability affects chlorophyll biosynthesis (Msilini et al. 2013) and likely iron-sulphur biogenesis, which in turn reduces the activity of ACO and SDH, among other [Fe-S] enzymes, with similar effects on Krebs cycle flux to that of *hscb* plants. This finding was clearly confirmed in the current study: when leaf iron contents increased in *HSCB* plants due to foliar application or by reduced or no iron in the medium, [Fe-S] enzyme activities recovered (Fig. 5b) and the plants grew better (Fig. S4).

Another unusual characteristic of the *HSCB*-overexpressing plants was their root architecture system (Fig. 1a, f, g). Root system architecture is the combined result of a genetically determined pattern and the environmental challenges to which plants are exposed, including limited nutrient availability. Morphological changes in the LR system in response to varying Fe availability in the media have been reported (Giehl et al. 2012). LR length increases in a dose-response manner up to conditions of iron sufficiency (approximately 25–50 µM Fe), allowing the plant to explore different niches. At these Fe concentrations, the

dose response is reversed and LR length decreases (Giehl et al. 2012), probably to prevent excessive iron uptake. LR number and density are also altered by inner iron availability, although less drastically. These changes are triggered by locally accumulated Fe in the root tissue, which is thought to be integrated into a sensing mechanism to adjust LR system architecture (Giehl et al. 2012). These observations may explain the root architecture of *HSCB*: the high iron content in these roots may reduce LR initiation (number or density) and severely inhibit LR extension. On the contrary, *hscb* seedlings exhibit typical root system architecture because their root iron content is normal.

Regulation of iron metabolism: shoot-directed control of root iron deficiency responses

In vascular plants, communication between different plant organs is necessary to avoid imbalances in the nutrient supply and to integrate the nutritional demands of the whole plant (Schmidt 2003). Most studies suggest that the aerial part of the plant plays a key role in regulating Fe deficiency responses (Enomoto et al. 2007; Lucena et al. 2006; Maas et al. 1988; Vert et al. 2003), and these signals appear to be predominant over root iron responses (Enomoto et al. 2007; Garcia-Mina et al. 2013). A model has been proposed describing how both promotive and repressive signals (Vert et al. 2003), sent from shoots to roots in response to foliar iron status or to some related metabolite, regulate Fe deficiency responses, most likely through the phloem (Curie and Briat 2003; Lucena et al. 2006; Maas et al. 1988). In light of this model, to help elucidate the reason for the imbalanced Fe distribution in *HSCB* and *hscb* seedlings, we investigated the main iron uptake system. *HSCB* plants, containing low levels of foliar iron, exhibited high activation of the iron uptake system, with increased transcript levels of *FIT1*, *FRO2* and *IRT1* and increased *FRO2* activity, whereas *hscb* plants, with high shoot iron contents, exhibited reduced *FRO2* activity (Fig. 4). Thus, our results are consistent with

the model describing how the aerial part of the plant regulates Fe uptake in roots (Vert et al. 2003).

Changes in foliar iron availability induce the biosynthesis and/or transport of a signalling molecule that conveys this information to the root via the phloem (Schmidt 2003). Previous studies have demonstrated that foliar application of iron to Fe-deficient wild-type *Arabidopsis*, pea and tomato plants drastically reduces FRO2 activity (Enomoto et al. 2007; Garcia et al. 2013; Maas et al. 1988). To investigate whether shoot-to-root signalling takes place in the *HSCB* lines to deactivate FRO2, we performed foliar treatment of *HSCB* and *hscb* lines grown under Fe sufficient condition with Fe-EDTA. After 24 h, FRO2 activity was reduced in the *HSCB* lines (Fig. 5), indicating that in these plants, the iron sensing and signalling mechanisms function correctly, and signals coming from shoots are properly sent to roots.

The foliar regulatory system presumably involves two unknown sensors: one for sensing the nutrient status of the shoot and one in the root that perceives the long-distance signals coming from the shoot (Schmidt 2003). The results of the foliar iron treatment experiment indicate that in *HSCB* plants, these two sensors function properly. Moreover, the results of the citrate supplementation assay (Fig. 6) and the iron deficiency treatment experiment (Fig. 7) reinforce this conclusion and extend it to *hscb* seedlings. In both *HSCB* and *hscb* lines, after 48 h of citrate supplementation or iron deficiency, FRO2 activity changed in accordance with the resulting variations in foliar iron levels: *HSCB* plants (with increased foliar iron levels) exhibited reduced FRO2 activity, whereas *hscb* seedlings (with reduced shoot iron content) exhibited increased FRO2 activity. This last result also implies that [Fe-S] clusters are not involved in these two sensing mechanisms, as the *hscb* lines, which are deficient in [Fe-S] biogenesis, responded to changes in shoot iron levels as expected. Moreover, [Fe-S] clusters are neither the sensed nutrient signal in the shoot nor implicated in the signal sent to the root.

Iron redistribution: effect of citrate treatment

In general, our results are consistent with an alteration in iron transport between roots and shoots. *HSCB* plants actively incorporate iron from the medium (Fig. 4), and although genes related to Fe transport and redistribution, such as *FRD3*, *FPNI* and *NAS4*, are induced (Fig. S3), iron is trapped in roots and not delivered to leaves, which are Fe deficient (Fig. 3). By contrast, in *hscb* seedlings, iron uptake is reduced and *FPNI* is repressed, but iron accumulates in leaves. Little is known about Fe transport from roots to shoots, and many questions remain. Nonetheless, studies of the *Arabidopsis frd3* mutant have provided molecular evidence for the role of citrate in long-distance Fe transport (Durrett et al. 2007; Green and Rogers 2004; Rogers

and Guerinot 2002). Iron is thought to be transported in the xylem as different complexes with citrate and malate (Flis et al. 2016; Grillet et al. 2014), because free Fe ionic forms can be toxic and are also prone to precipitation at the neutral or slightly acidic pH values typical of xylem sap (Abadia et al. 2011). The TCA cycle provides abundant organic acids, including citrate and malate (Lopez-Millan et al. 2000). The TCA cycle possesses two Fe-containing enzymes, ACO and SDH, and under Fe deficiency, it can shift from circular to linear mode (Steuer et al. 2007) due to the reduced activity of both enzymes (Vigani 2012). In these situations, increased citrate export from the mitochondria to the cytosol has been observed in several plant species (Vigani et al. 2009), which may enhance Fe transport from roots to shoots.

ACO and SDH activity were considerably reduced in the *hscb* lines (Fig. 2), and indeed root citrate content was higher than in wild type line (Fig. 6d). Nevertheless elevated citrate levels were probably not the cause of the high iron contents in their shoots. Citrate supplementation assay, which is expected to increase citrate content even more, switched iron distribution in these plants instead of reinforcing the phenotype (Fig. 6a, b). Conversely, the increase in ACO and SDH activity observed in *HSCB* roots suggests that this alteration in the TCA cycle could lead to imbalanced organic acid content. It would be conceivably to assume a decline in citrate levels as the cause of the decreased iron transport from roots to shoots. Moreover, in light of the results from the citrate supplementation assay, this notion would be reinforced. However, altered citrate levels were presumably not responsible for the phenotype since *HSCB* and Col 0 plants showed the same root citrate content in iron sufficient conditions (Fig. 6d).

Regulation of iron metabolism: checkpoint at the root-to-shoot translocation level

It has become evident that shoot-directed control of root iron deficiency responses is not the only iron homeostatic mechanism in plants (Schmidt and Buckhout 2011). A number of transcription factors and other regulatory proteins, such as PYE, HRZs/BTS, bHLH100 and bHLH101, are controlled by iron starvation, many of which appear not to be regulated by FIT, demonstrating the existence of additional regulatory networks driving responses to low iron (Long et al. 2010; Sivitz et al. 2012). Cell-type-specific transcriptional profiling has revealed that the stele is the most responsive cell type to iron deprivation within the root and that many genes induced under these conditions encode transcription factors (Dinnen et al. 2008). Therefore, the vascular bundle may serve as a transcriptional regulatory centre for the iron deficiency response (Long et al. 2010). On the other hand, elevated iron contents can become extremely toxic, causing symptoms such as brown and necrotic degenerative leaves

(Lucena et al. 2015). It has been observed that leaf iron levels are not affected by 48 h and 1 week of excess Fe treatment in *Arabidopsis* (Reyt et al. 2015) and rice (Liang et al. 2014), respectively, indicating that Fe in these plants is not transported from root to shoot. These findings suggest that different pathways exist for inter-organ regulation of the responses to iron excess, and that a checkpoint operating at the root-to-shoot translocation level may exist (Reyt et al. 2015). However, unlike animals, fungi and bacteria, in which ionic Fe or [Fe-S] clusters are the main elements sensed in iron metabolism (Anderson et al. 1823; Lee and Helmann 2007; Poor et al. 2014), our understanding of iron sensors in plants remains limited, and it is not clear which signals regulate these homeostatic pathways.

Our results show that *HSCB* lines have reduced Fe contents in shoots, whereas *hscb* lines have increased Fe contents in shoots, suggesting that Fe transport from roots to shoots in these lines is altered. We also observed that under iron deficient conditions both *HSCB* and *hscb* plants grew better than Col 0 plants (Fig. S4) and we reasoned that iron levels might affect a checkpoint controlling iron translocation to shoots. BTS, a known iron sensor and regulatory protein highly expressed in vascular tissues, whose stability is controlled by iron binding to its hemerythrin domains (Selote et al. 2015), also possesses a rubredoxin domain. AtHSCB contains such a domain as well, which is conserved in all higher eukaryotes but not in yeast or bacteria (Bitto et al. 2008). The rubredoxin-type fold binds metal ions including Fe and Zn, as well as [Fe-S] clusters (Sieker et al. 1986) and it is probably responsible for the stability of HSC20, the AtHSCB human homolog. Mutant HSC20, in which the four conserved cysteines of the rubredoxin domain were replaced by serines showed a clearly lower abundance than the native protein upon transient expression in HeLa cells (Uhrigshardt et al. 2010). However, little is known about the role of this domain beyond its involvement in various functions distantly related to iron metabolism, such as redox processes (Hagelueken et al. 2007) and accumulation of photosystem II in oxygenic phototrophs (Calderon et al. 2013). It might be hypothesized that upon iron binding AtHSCB would be stabilized, and would control iron translocation from root to shoot. High iron levels -or high AtHSCB levels, as in the overexpressor lines- might reduce iron transport to the shoot. We observed that after 48 h of iron deficiency, the shoot Fe content decreased in wild-type and *hscb* plants and increased in *HSCB* seedlings; thus, the iron trapped in *HSCB* roots could be transported to the shoots (Fig. 7). This finding supports the idea that a checkpoint of iron transport might operate in *Arabidopsis* roots. Under iron deficiency, AtHSCB would be destabilized, and transport from roots to shoots would increase to satisfy metabolic needs in the shoot. Conversely, under Fe excess, transport from root to shoot would be inhibited,

in an AtHSCB dependent mechanism, to avoid shoot toxicity. Indeed, it has been observed that after iron excess treatment *HSCB* transcripts are induced in rice roots (Liang et al. 2014). This AtHSCB regulatory mechanism might be performed in the cytosol, where small amounts of AtHSCB and HSC20 have been detected (Uhrigshardt et al. 2010; Xu et al. 2009).

In summary, our results provide new evidence supporting the involvement of AtHSCB, a protein from the [Fe-S] cluster mitochondrial biosynthetic pathway, in iron translocation from roots to shoots.

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Author contributions LL, MAP, MB performed the required experiments and analyzed the data. MAP, MVB and DFGC designed the conception and delineation of the study, wrote the manuscript and reviewed it before submission. All authors read and approved the final manuscript.

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