Altered metal distribution in the *sr45-1* Arabidopsis mutant causes developmental defects

Short title

Altered Fe homeostasis in *sr45-1*

One sentence summary

The *sr45-1* mutation affects Fe homeostasis, which results in reproductive defects

Authors

Steven Fanara, Marie Schloesser, Marc Hanikenne* and Patrick Motte*

InBioS-PhytoSystems, Functional Genomics and Plant Molecular Imaging and Centre for Assistance in Technology of Microscopy (CAREm), University of Liège, 4000 Liège, Belgium

* Equal contribution

Corresponding authors

Steven Fanara, (steven.fanara@uliege.be; Tel: +32-4-366-38-11)

Correspondence may also be addressed to Marc Hanikenne (marc.hanikenne@uliege.be; Tel: +32-4-366-38-44)

University of Liège

InBioS-PhytoSystems

Quartier de la Vallée, 1

Chemin de la Vallée, 4 - Bât B22

B4000 Liège

Belgium

Author contributions

P.M. and M.H. conceived and directed the research. S.F. conducted most experiments, with contributions of M.S. P.M. and S.F. analyzed most of the data. M.H. and S.F. analyzed the RNA-
Seq data. P.M. and M.H. supervised experiments. S.F. made the figures. S.F., P.M. and M.H. wrote the manuscript, with comments of M.S.
Abstract

The plant SR (serine/arginine-rich) splicing factor SR45 plays important roles in several biological processes, such as splicing, DNA methylation, innate immunity, glucose regulation and ABA signaling. A homozygous Arabidopsis sr45-1 null mutant is viable, but exhibits diverse phenotypic alterations, including delayed root development, late flowering, shorter siliques with fewer seeds, narrower leaves and petals, and unusual numbers of floral organs. Here, we report that the sr45-1 mutant presents an unexpected constitutive iron deficiency phenotype characterized by altered metal distribution in the plant. RNA- Sequencing highlighted severe perturbations in metal homeostasis, phenylpropanoid pathway, oxidative stress responses, and reproductive development. Ionomic quantification and histochemical staining revealed strong iron accumulation in the sr45-1 root tissues accompanied by an iron starvation in aerial parts. We showed that some sr45-1 developmental abnormalities can be complemented by exogenous iron supply. Our findings provide new insight into the molecular mechanisms governing the phenotypes of the sr45-1 mutant.
Introduction

The precursor messenger RNA (pre-mRNA) splicing process is a crucial step in the regulation of gene expression. Splice-site selection is carried out by the binding of many trans-acting factors including the serine/arginine-rich (SR) splicing factors (Meyer et al., 2015; Jeong, 2017). Alternative splicing (AS) of a specific pre-mRNA can lead to the synthesis of multiple mRNAs and affects up to 70% of multi-exon genes in Arabidopsis (Arabidopsis thaliana) (Chamala et al., 2015). AS allows plants to cope with environmental challenges by modulating gene expression and biological processes (Palusa et al., 2007; Palusa and Reddy, 2010; Shang et al., 2017). SR proteins belong to a highly conserved family in multicellular eukaryotes and are characterized by a modular structure consisting of one or two N-terminal RNA-binding domains, called RNA recognition motifs (RRMs), and a C-terminal domain rich in arginine-serine dipeptide repeats (RS) mainly involved in protein-protein interactions (Barta et al., 2010; Manley and Krainer, 2010; Califice et al., 2012). In Arabidopsis, nineteen SR proteins constitute seven subfamilies according to their specific modular organization (Califice et al., 2012). Among these, the atypical SR45 protein contains two RS domains flanking a central and unique RRM (Golovkin and Reddy, 1999). The sr45-1 null mutant exhibits some developmental defects, including delayed root development, late flowering, shorter siliques with fewer seeds, narrower leaves and petals, and unusual numbers of floral organs (Ali et al., 2007; Zhang et al., 2017).

SR45 undergoes alternative splicing producing two isoforms: the isoform SR45.1 diverges from SR45.2 by eight amino acids due to the presence of an additional 21-nucleotides sequence (Zhang and Mount, 2009). These isoforms fulfill distinct roles during Arabidopsis development, since SR45.1 restores exclusively flowers defect and SR45.2 only complements the delayed root development when expressed in the sr45-1 background (Zhang and Mount, 2009).

SR45 was demonstrated to regulate the splicing of functionally diverse targets, thereby acting in ABA signaling and plant defense (Carvalho et al., 2010; Xing et al., 2015; Zhang et al., 2017). SR45 can act as a negative regulator of glucose and ABA signaling during early seedling development by modulation of SnRK1.1 stability through regulation of 5PTase13 splicing under glucose treatment (Carvalho et al., 2010; Carvalho et al., 2016). Knockout mutants for RS40, RS41 and SR45 all displayed ABA hypersensitivity (Carvalho et al., 2010; Chen et al., 2013). The sr34b mutation leads to the stabilization of an alternatively spliced IRON-REGULATED TRANSPORTER 1 (IRT1) mRNA. Accumulation of the IRT1 divalent cation transporter in turn...
induces an increased uptake of cadmium (Cd) ions into the root, hence a hypersensitivity to the toxic metal Cd (Zhang et al., 2014). Mutations of several SR factors in rice (Oryza sativa) also depict the critical role played by alternative splicing in plant response to mineral nutrient status (Dong et al., 2018). Plant SR proteins actively participate to the regulation of alternative splicing under abiotic stress (Laloum et al., 2018; Albaqami et al., 2019).

Iron (Fe) is an essential micronutrient and cofactor responsible for redox state modulation (Nouet et al., 2011; Thomine and Vert, 2013). Since an overproduction of reactive oxygen species (ROS) may result from Fe overaccumulation (Ravet et al., 2009), a physiological balance of Fe uptake and accumulation has to be controlled and maintained in plant. Dicots such as Arabidopsis rely on a three-step reduction-based strategy to acquire Fe (Römheld and Marschner, 1986). The H^+-ATPase AHA2 mediates proton extrusion, which leads to local soil acidification and Fe solubilization (Santi and Schmidt, 2009). With the help of chelators, such as coumarin phenolic compounds (scopoletin, fraxetin and sideretin) that greatly facilitate the acquisition of solubilized Fe from soils (especially at high pH $\geq$ 7) (Mladenka et al., 2010; Fourcroy et al., 2014; Schmidt et al., 2014; Rajniak et al., 2018; Siwinska et al., 2018; Tsai et al., 2018), the enzyme FERRIC REDUCTION OXYDASE 2 (FRO2) catalyzes the reduction of ferric [Fe(III)] to ferrous [Fe(II)] Fe (Robinson et al., 1999). The import of the ferrous Fe ions into root cells is finally performed by IRT1 (Vert et al., 2002; Castaings et al., 2016). Those three major components, as well as transcription factors from the myeloblastosis (MYB) family (MYB10 and MYB72) and several Fe homeostasis genes, are under the positive regulation of the basic helix-loop-helix (bHLH) transcription factor FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Ivanov et al., 2012; Sivitz et al., 2012). While ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE1 (EIL1) promote its stability upon Fe deficiency (Lingam et al., 2011), ZAT12, BTSL1, BTSL2 and MYC2 target FIT for proteasomal degradation to avoid intake of potentially toxic metals by IRT1 upon prolonged Fe deficiency (Le et al., 2016; Cui et al., 2018; Rodríguez-Celma et al., 2019). Together with its paralog MYB10, MYB72 regulates the expression of two NICOTIANAMINE SYNTHASE, NAS2 and NAS4, in order to facilitate the Fe redistribution in the plant by controlling the biosynthesis of the Fe chelator NA (Palmer et al., 2013). Another bHLH (POPEYE, PYE) is also transcriptionally involved in Fe redistribution, especially through the expression regulation of NAS4 and ZIF1, a vacuolar nicotianamine.
transporter (Long et al., 2010; Haydon et al., 2012). A RING E3 ubiquitin ligase, BRUTUS (BTS), compromises the Fe root-to-shoot translocation network controlled by PYE by targeting PYE-interacting transcriptional co-regulator for 26S proteosomal degradation (Kobayashi et al., 2013; Selote et al., 2015; Hindt et al., 2017). The root-to-shoot translocation of Fe involves the loading of citrate and Fe in the xylem respectively by the citrate efflux transporter FERRIC REDUCTASE DEFECTIVE 3 (FRD3) (Durrett et al., 2007) and possibly by Ferroportin 1 (FPN1) (Morrissey et al., 2009). Since endodermis suberization alters the uptake of nutrient, suberization is particularly delayed in plants growing under Fe deficiency (Baxter et al., 2009; Geldner, 2013; Kamiya et al., 2015; Barberon et al., 2016). This response is tightly regulated by the ethylene and ABA stress hormones which are, respectively, suppressor and activator of suberization and thus are positive and negative regulators of the root Fe-uptake, respectively (Barberon et al., 2016).

Alternative splicing is essential to ensure metal tolerance in plants as seen in sr rice mutants (Zhang et al., 2014; Dong et al., 2018). A case example is the ZINC-INDUCED FACILITATOR 2 (ZIF2) gene that undergoes intron retention in its 5'UTR to promote zinc tolerance through enhancement of its translation (Remy et al., 2014). Developmental alterations in the sr45-1 mutant are strikingly similar to phenotypes associated to altered metal homeostasis, such as shorter roots and shorter siliques with fewer seeds as observed in frd3-7 or opt3-2 mutants (Stacey et al., 2008; Roschztardtz et al., 2011). Although many genes involved in root responses to Fe deficiency have been identified, much less is known about the importance of their post-transcriptional processing. In this study, we explored the contribution of SR45 to Fe homeostasis in Arabidopsis roots. We show that the vegetative development of sr45-1 plants is greatly impacted under Fe deficiency. Upon Fe supply, roots are slightly shorter because of Fe accumulation in the vascular system and the production of an oxidative burst. Fe is less concentrated in the aerial part of the mutant compared to wild-type because of impaired root-to-shoot translocation. We performed RNA-Sequencing (RNA-Seq) on wild-type and sr45-1 roots upon Fe deficiency and control condition, which demonstrated dramatic transcriptional changes of genes involved in Fe homeostasis, ROS responses and reproductive development. As a consequence, a local metal imbalance appears in reproductive tissues of sr45-1, leading to shorter siliques, reduced seed number per silique, and smaller and narrower seeds. These phenotypes are fully restored in mutant plants upon exogenous Fe supply, revealing a connection between Fe
uptake and developmental defects of sr45-1.

Results

The “Metal ion transport” GO category is enriched among SR45-associated RNAs

RNA-precipitation experiments recently conducted on seedlings and inflorescences revealed that SR45 binds to and regulates functionally diverse RNAs referred to as SR45-Associated RNAs (SAR) (Xing et al., 2015; Zhang et al., 2017). Here, we submitted a merged set of SAR (7799 genes) to a functional enrichment meta-analysis to identify the putative processes and pathways controlled by SR45 (Supplemental Table S1): 404 Gene Ontology (GO) categories were significantly (p-value < 0.05) enriched among the SR45 targets and consistently illustrated the previously described phenotypes of the sr45-1 mutant. For instance, the involvement of SR45 in salt tolerance (Albaqami et al., 2019) was reflected in numerous biological processes related to ion transport. Surprisingly, a biological process called “metal ion transport” (116 genes) was significantly enriched, within which several sub-GO enrichments related to divalent cation and iron transport were further observed (Supplemental Table S1).

Moreover, 38.5% of SAR corresponded to genes identified in transcriptomic studies as differentially expressed upon varying Fe supply (hereinafter referred as “iron-responsive genes”) (Supplemental Figure S1).

Metal levels are increased in the roots of sr45-1

These observations led us to examine whether the sr45-1 mutation results in any Fe homeostasis perturbation and therefore the ionome was profiled in tissues of both sr45-1 and WT (Col-0) plants grown in Fe-deficient (0 µM) and Fe-sufficient (10 µM) conditions. In Fe-sufficient condition, the roots of sr45-1 accumulated more Fe (136%), Mn (218%) and Zn (150%) compared to the WT. In contrast, Cu levels were similar in the two genotypes (Figure 1A-D). Upon Fe deficiency, Fe and Zn accumulated similarly in sr45-1 and WT roots, but sr45-1 roots accumulated more Cu (125%) and Mn (146%). Conversely, sr45-1 accumulated less Fe (52%), Mn (38%), Zn (37%) and Cu (33%) than the WT in shoots under Fe deficiency (Supplemental Figure S2A-D). Accumulation reduction of Fe (17%), Mn (18%) and Zn (32%) was also significant in sr45-1 shoots under Fe-sufficient condition (Supplemental Figure S2A and C-D). While root macronutrients (Ca, K and Mg) levels were similar between genotypes.
under Fe deficiency and sufficiency, Ca levels were reduced by 26% in mutant shoots at 0 µM Fe but K levels were increased by 36% at 10 µM Fe, and no significant changes were observed in mutant shoots regarding Mg levels (Supplemental Figure S3A-C).

In Fe sufficiency condition, Perls’ staining revealed strong Fe accumulation in the vascular cylinder of sr45-1 roots, (Figure 1E), a phenotype strikingly similar to a ferric reductase defective3 (frd3) loss-of-function mutant (Green and Rogers, 2004; Roschztardtz et al., 2009; Scheepers et al., 2020). Because of this phenotypic similarity, we decided to concomitantly analyse frd3-7 as a control for defective Fe homeostasis (Figure 1E). No Fe accumulation in root vascular tissues occurred upon Fe deficiency in the three genotypes. It is well described that overaccumulation of Fe can trigger ROS overproduction, ultimately leading to oxidative stress (Reyt et al., 2015). Fe and ROS accumulation were shown to correlate in root vascular tissues of frd3-7 (Scheepers et al., 2020). DAB staining, revealing H$_2$O$_2$ accumulation, was accordingly detected in the central vasculature of the root in both sr45-1 and frd3-7 mutants but not in the WT (Figure 1F). Altogether, the Fe accumulation pattern in roots and shoots of sr45-1 plants are indicative of a defective metal root-to-shoot translocation of Fe in sr45-1.

The sr45-1 mutant is sensitive to the Fe status

Based on these observations, we investigated the effect of Fe supply on the sr45-1 mutant development by growing either seedlings for 14 days on Fe-depleted (0 µM Fe), control (10 µM Fe) or supplemented with Fe excess (50 µM Fe) agar solid Hoagland or 6-week-old plants in hydroponic solution in Fe-deficient and Fe-sufficient conditions (Figure 2, Supplemental Figure S4A). In seedlings, the root growth of the sr45-1 mutant was reduced by 47% under Fe deficiency and 68% under Fe excess compared to WT (Supplemental Figure S4A). The reduction of root biomass correlated with a reduction of shoot biomass with a loss of 46%, 38.5% and 21% at Fe deficiency, sufficiency and excess, respectively, compared to WT at Fe sufficiency (Supplemental Figure S4B).

In adult plants, sr45-1 root growth was significantly decreased in both deficiency and excess conditions (Supplemental Figure S4C). In response to Fe deficiency, roots of the WT and sr45-1 were 27.5% and 43% shorter, respectively, compared to Fe sufficiency. Similarly, the shoot fresh weight of both wild-type and sr45-1 mutant plants was affected by Fe deficiency with a significant biomass loss of 61.5% and 71.3%, respectively, compared to the Fe-sufficient
Although *sr45-1* development was already delayed in control condition, the *sr45-1*/WT ratio for both root length (Figure 2C) and shoot fresh weight (Figure 2D) indicated that adult *sr45-1* plants were more sensitive to Fe deficiency than the WT. Fe deficiency resulted in chlorosis in shoots of both WT and mutant. However, the aerial parts of the *sr45-1* mutant presented a more severe chlorosis (Figure 2A-B) at 0 µM Fe than the WT, confirming higher sensitivity of the mutant to Fe deficiency. Exposure to higher Fe supply (50 µM) additionally suggested that the mutant had a perturbed root-to-shoot Fe translocation: whereas the WT suffered of Fe toxicity with less biomass production in aerial parts in these conditions (significant loss of 34%), higher Fe supply did not cause further delay nor improved shoot growth of adult *sr45-1* plants (Supplemental Figure S4D). This confirmed the impaired Fe root-to-shoot distribution and inferred that increasing Fe supply would trigger toxicity in *sr45-1* roots.

**Zinc accumulation in roots of *sr45-1* is toxic**

The 2-fold and 1.5-fold increases in accumulation of respectively Mn and Zn in *sr45-1* roots upon Fe sufficiency (Figure 1C-D) may cause growth reduction as previously observed (Dučić and Polle, 2007; Lei et al., 2007; Kawachi et al., 2009; Fukao et al., 2011; Shanmugam et al., 2011; Marschner and Marschner, 2012; Millaleo et al., 2013; Scheepers et al., 2020). Thus, we investigated the influence of those metals on the growth of *sr45-1*. Contrary to Mn depletion, Zn depletion did not significantly impact the root growth of the WT in our growth condition on agar plates. In contrast, root length of *sr45-1* was significantly longer by 12% upon Zn deficiency, but roots remained 50% smaller compared to WT in control condition (Figure 3), suggesting that Zn accumulation in roots was toxic and indeed partially contributed to the growth defect of *sr45-1*. Mn and Zn deficiency did not influence Fe or H$_2$O$_2$ accumulation in the *sr45-1* root vasculature (Supplemental Figure S5A-B).

The shoot fresh weight of *sr45-1*, which was lower by 28% in control condition compared to the WT, was increasingly affected by Mn and Zn deficiencies, leading to a biomass loss of 70% and 73.5%, respectively, compared to the WT in Fe sufficiency (Supplemental Figure S5C). In Zn-deficient condition, Col-0 shoot biomass was decreased by 68.5%, resulting in the absence of significant changes between both genotypes. Altogether, these results pointed to a Zn
toxicity in roots and to a defective metal homeostasis, possibly in acquisition and mobilization of metals.

Transcriptome profiling of sr45-1 mutant roots

To uncover the molecular mechanisms underlying metal distribution in sr45-1, a transcriptomic profiling of WT and sr45-1 root tissues of adult plants grown hydroponically in Fe-deficient (0 µM) and Fe-sufficient (10 µM) conditions was conducted using RNA-Seq. A limited number of differentially expressed genes (DEGs) [fold change ≥ 2 or ≤ -2 and false discovery rate (FDR) < 0.05] were identified between WT and mutant roots in Fe-sufficient (29 DEGs) and Fe-deficient conditions (49 DEGs) (Figure 4 and Supplemental Table S2) and no enriched GO functional category was identified among either of those sets of DEGs (Supplemental Table S3). sr45-1 mutant plants presented less transcriptomic changes in response to Fe deficiency than Col-0 (1350 vs 2442 DEGs) and accordingly, the number of enriched GO biological processes was considerably reduced in the mutant (33 vs 50 and 20 vs 75 for the up- and down-regulated genes, respectively) (Supplemental Table S3). In fact, in response to Fe deficiency, Col-0 displayed extensive changes in the expression of genes contributing to diverse biological processes that were not over-represented in the mutant roots. For instance, the response of Col-0 roots to Fe deficiency involved the induction of metal ion homeostasis and transport, as well as the repression of several functions including phenylpropanoid biosynthetic process, cell wall modifications, lignin biosynthesis, biotic and hormonal responses (Supplemental Table S3). The molecular alteration in the sr45-1 mutant roots under Fe deficiency included perturbation in cellular iron ion homeostasis (FER and VTL genes) and oxidative stress response (through down-regulation of, respectively, 7 and 35 genes), as well as in anion transport and sulfate transport (through up-regulation of, respectively, 34 and 6 genes). Surprisingly, while response to ethylene was a significant over-represented function in both Col-0 and sr45-1 roots upon Fe deficiency, divergence appeared: 42 genes were under-expressed in Col-0 (against 9 of them in sr45-1) and 22 genes were over-expressed in the mutant (against 18 of them in Col-0) (Supplemental Table S3).

To further characterize sr45-1 root transcriptome and fine-tune our understanding of the metal homeostasis regulated by SR45, additional comparisons were performed to discover SR45 differentially regulated genes (SDR; DEGs significantly and specifically deregulated in sr45-1
roots in respective comparison) (Figure 4): SDR included 304 and 387 in the comparison of the Fe deficiency DEGs in the WT (2442 genes) with either (i) the Fe deficiency DEGs in the mutant (1350 genes) or (ii) the DEGs between mutant at Fe deficiency and the WT in control condition (2000 genes), respectively. The reciprocal of the latest comparison yielded 259 additional SDR. Finally, 827 SDR were obtained when intersecting the DEGs obtained when comparing a genotype (WT or sr45-1) in deficiency with the other genotype in sufficiency. Together with the DEGs identified between genotypes in Fe-sufficient (29) and Fe-deficient (49) conditions, it represented 1071 unique SDR compared to 1844 CDR (Col-0 differentially regulated genes) (Supplemental Table S4). The analysis of GO enrichment highlighted molecular contribution of both CDRs and SDRs to many similar biological pathways. However, CDRs included genes involved in biological functions that were not over-represented in the mutant, such as response to jasmonic acid and ethylene, cell wall organization, phenylpropanoid biosynthetic and metabolic processes or lignin metabolic process (Figure 4B, Supplemental Table S5). SDRs contribute additionally to two biological functions unrepresented in CDRs, namely chemical homeostasis and response to organonitrogen compound, which include genes involved in Fe, Mn, Zn, Cu and Cd homeostasis (Figure 4C, Supplemental Table S5).

These observations gave evidence that the mutant is pervasively affected in many pathways upon Fe deficiency. It raised the question whether sr45-1 mutant plants were able to accurately regulate the response of key genes or whether adult mutant plants were coping with residual induction of the metal homeostasis due to defective seedlings (as shown by the severe toxic symptoms displayed in response to Fe supply (Supplemental Figure S4).

The Fe acquisition machinery is constitutively induced in sr45-1 seedlings

As the ionome and the growth of the mutant were affected by the Fe status (Figure 1 and Supplemental Figure S4), gene expression (using qRT-PCR) and protein activity of key components of the Fe uptake machinery were examined in roots of 14-day-old seedlings, as well as 9-week-old roots grown in Fe deficiency and sufficiency conditions. The expression of the bHLH transcription factor FIT was up-regulated by Fe deficiency in both WT and sr45-1 seedling roots compared to the 10 µM Fe condition. However, FIT expression was ~47-51% higher in sr45-1 seedlings compared to Col-0 in both Fe-sufficient and Fe-deficient conditions (Figure 5A). Similarly, the FIT targets AHA2, FRO2 and IRT1 were also more highly expressed.
(124-208%) in sr45-1 roots in both conditions compared to Col-0 (Figure 5B-D), consistent with increased acidification of the medium (mostly driven by AHA2) (Supplemental Figure S6A) and ferric chelate reductase activity (mostly driven by FRO2) in sr45-1 roots (Supplemental Figure S6B). Altogether, these observations were indicative of a constitutive Fe deficiency response in sr45-1 roots upon control Fe supply (10 µM). This response was further aggravated when no Fe (0 µM) was supplied.

In adult roots, all key components of the Fe uptake machinery were induced in both Col-0 and sr45-1 upon Fe deficiency, but no significant deviation was observed between genotypes for FIT, FRO2 or IRT1 expression. However, AHA2 expression was ~36.8% higher in sr45-1 roots compared to Col-0. While FIT expression was not induced in adult mutant roots at Fe deficiency or sufficiency (Figure 5A), the expression of genes encoding the MYB72, bHLH38, bHLH39, bHLH100 and bHLH101 transcription factors, which are known to act in conjunction with FIT to induce the Fe acquisition machinery (AHA2, FRO2 and IRT1) (Yuan et al., 2008; Palmer et al., 2013; Wang et al., 2013), was significantly up-regulated in the sr45-1 mutant under Fe deficiency compared to Col-0 (Supplemental Figure S7).

Since Fe accumulation in the root vasculature was observed in both sr45-1 and frd3-7 mutants, the relative expression level of FRD3 in sr45-1 roots was also analyzed. The level of FRD3 was reduced by 29% and 25% in the mutant upon Fe-deficient and Fe-sufficient conditions, respectively (Figure 5E). In agreement with this observation, both sr45-1 and frd3-7 accumulated a higher level of citrate, the substrate of FRD3, in roots, but it was significantly lower in sr45-1 (Supplemental Figure S6C). In contrast, adult mutant roots did not show any deviation in FRD3 expression compared to the WT (Figure 5E).

All results suggested that, contrary to adult plants, sr45-1 seedlings display a constitutive iron deficiency response independently of the iron concentration used (0 µM or 10 µM Fe) and that the root-to-shoot Fe translocation may be impaired through down-regulation of FRD3 in young roots.

Influence of coumarins on the developmental defects of sr45-1 mutant seedlings
The phenylpropanoid pathway, which participates in the synthesis of coumarins involved in Fe solubilization from the soil, including through a transcriptional control by BEE1 and MYB15 (Petridis et al., 2016; Chezem et al., 2017), both identified as SDR in this study, is improperly
regulated in sr45-1 adult roots (Supplemental Table S3 and Supplemental Table S5). In fact, many genes were down-regulated in Col-0 in response to Fe deficiency, but only few of them were affected in this fashion in the mutant (Supplemental Figure S8). This suggested that Fe mobilization from the soil may be affected in the mutant. To investigate this hypothesis, Col-0 and mutant seedlings were grown on solid Hoagland media containing or not Fe-mobilizing compounds and supplemented with 10 µM Fe. A Hoagland control medium, containing Fe-HBED at pH 5.7, was used as the maximum of Fe bioavailability, and a Hoagland unavailable Fe medium, containing FeCl₃ at neutral pH, was used as the minimum of Fe bioavailability (Tsai et al., 2018). Whereas the root growth of the WT was significantly reduced (∼10%) on FeCl₃ medium, it was increased by ∼29% in the mutant (Figure 6A), which confirms that Fe in a soluble form causes toxicity in sr45-1 roots. No signal of iron accumulation nor oxidative stress was visualized in seedlings grown on the unavailable Fe medium (Figure 6B-C). This suggested that sr45-1 is less capable of mobilizing Fe from the Fe unavailable medium. We then tested whether supplementation of coumarins could help the mutant in this regard. Exogenous application of fraxetin did not affect the root growth of Col-0 nor sr45-1 (Figure 6A) but led to an increase in the Fe and H₂O₂ accumulation in the mutant roots compared to the FeCl₃ only condition (Figure 6B-C). In the absence of Fe (0 µM FeCl₃), no significant change in the length of WT roots was observed, but the mutant roots were significantly longer by 8.5% compared to the Fe unavailable condition, further indicating the Fe toxicity in the mutant. The further addition of fraxetin led to a significant decrease of both Col-0 (∼13.5%) and sr45-1 (∼12.5%) root length (Figure 6A), whereas no Fe nor H₂O₂ staining was observed in seedlings (Figure 6B-C). This suggests that coumarins tend to mobilize Fe (and possibly other metals) from the medium, which restores metal toxicity in the mutant.

In presence of unavailable Fe, the shoot fresh weight of Col-0 or sr45-1 were significantly reduced by ∼33% and ∼5.5% compared to themselves in control condition (Supplemental Figure S9A-B), confirming that the mutant shoots initially suffered of an iron deficiency that was not significantly aggravated in presence of unavailable Fe. Supplementation of coumarins affected the shoot fresh weight of the seedlings in the same fashion than the unavailability of Fe (Supplemental Figure S9A-B), which suggests that despite the improved solubilization and uptake of Fe, the mutant did not efficiently translocate Fe from roots to shoots to ensure proper
development. The total chlorophyll content of the WT or the mutant was not significantly affected in any of the tested conditions (Supplemental Figure S9C).

Although the phenylpropanoid pathway is deregulated, the function of fraxetin is not defective in the mutant. In fact, the mutant is capable of remobilizing iron from the insoluble FeCl$_3$ salt in presence of this coumarin, leading to root iron accumulation but not toxicity.

Iron supplementation fully restored all the reproductive phenotypes of sr45-1 mutant plants

We last questioned if the sr45-1 phenotypes of shorter siliques and reduction in seed yield (Zhang et al., 2017) could be rescued by Fe supplementation, as shown for the frd3-7 mutant (Roschztardtz et al., 2011). Col-0 and sr45-1 plants were therefore grown on soil until seed setting. They were daily irrigated with water without (–Fe) or with an additional treatment consisting of a weekly irrigation with sequestrene (+Fe). The sequestrene irrigation of the soil fully restored silique length (Figure 7A-B) and seed number per silique in the sr45-1 mutant (Figure 7D). The “shrunken” phenotype of the sr45-1 seeds compared to Col-0, consisting of a decreased seed length, width and mass, was also fully restored upon Fe irrigation (Figure 7C and 7E-F). However, Fe-irrigated mutants still displayed impaired growth of stem (Supplemental Figure S10A) and late flowering upon Fe treatment (Supplemental Figure S10B-C) (Ali et al., 2007). This suggested that the complementation by Fe was specific to seed development.

To examine whether defects in seed morphology were linked to perturbed metal concentrations, an ionomic analysis was conducted on –Fe and +Fe seeds. Compared to WT seeds, sr45-1 seeds displayed significantly increased in Zn, K and Mg levels but contained reduced amount of Mn. Upon Fe irrigation, these defects were completely restored for all metals but Mn (Figure 7G-J). In the case of Zn, the Fe supplementation did not modulate the Zn concentration in mutant seeds but increased the Zn concentration in the one of Col-0 (Figure 7G). Finally, no defect was detected in mutant seeds for micronutrients Fe and Cu, nor macronutrients Ca and P (Supplemental Figure S10D-G), but Fe irrigation increased the Cu content in both WT and sr45-1 seeds (Supplemental Figure S10E). Altogether, these results suggested that the mutant seeds contained abnormal concentrations of metals and that Fe supplementation was able to restore all the ion defects (but Mn) in the mutant seeds.
SR45, an atypical SR protein containing two RS domains, is an auxiliary component of the spliceosome whose biological functions remained elusive (Califice et al., 2012). It has been recently shown that this splicing factor regulates diverse biological processes including abscisic acid signaling and innate immunity, thanks to the identification of its direct targets (SARs) in two complementary studies (Xing et al., 2015; Zhang et al., 2017). Here, combining these two datasets, identifying SARs (7799) (Xing et al., 2015; Zhang et al., 2017) and SDR genes (358) in reproductive tissues (Zhang et al., 2017), with an RNA-Seq analysis of the roots of the sr45-1 mutant upon changes in Fe supply, identifying 1071 SDR genes, allowed to better describe major defects linked to the sr45-1 mutation and hence to better depict a global function of SR45. Eighteen genes emerged as shared among SARs and SDR genes, representing probable SR45 RNA targets that are constantly deregulated regardless of the tissue and stage of development of the sr45-1 mutant (e.g. MYB15, PWD/GWD2, OCT3 or AtHMP37) (Supplemental Table S6).

Loss-of-function mutants of several SDR genes identified in this study display abnormal silique length and seed set, as well as seed abortion (Supplemental Table S5 and Supplemental Figure S11), which are also strong phenotypes of the sr45-1 mutant (Zhang et al., 2017). Among these knock-out lines, the mutation of PWD/GWD2 (AT4G24450) leads to a reduced number of siliques and a decreased seed production, and pwd/gwd2 seeds are shrunken with an irregularly shaped coat (Pirone et al., 2017). The PWD/GWD2 gene is not only a putative target of SR45 splicing activity (SAR) but was also identified in a set of 918 differentially expressed genes in sr45-1 seedlings (Xing et al., 2015) and as down-regulated in roots of adult sr45-1 plants here. Even though several phenotypes, including reduced growth rate of the primary root (Pirone et al., 2017), of the pwd/gwd2 mutant are a surprising reminiscence of the root and reproductive defects we observed in sr45-1 (Figure 2 and Figure 7), it was recently shown that the down-regulation of PWD/GWD2 expression does not correlate with a protein decrease in the sr45-1 mutant inflorescences (Chen et al., 2019). However, because the PWD/GWD2 function is restricted to the companion cells (Glaring et al., 2007) and consists in sustaining the phloem loading of polysaccharides in source organs (Pirone et al., 2017), the absence of protein accumulation changes in inflorescences is not relevant in regards to the primary root cellular function played by this protein (Pirone et al., 2017). Therefore, the reproductive defects of sr45-1 might not be solely associated to the down-regulation of PWD/GWD2, which is confirmed by the fact that Fe
supplementation is able to rescue the reproductive defects of the mutant (Figure 8) in the absence of regulation of PWD/GWD2 (Supplemental Figure S11). These results strongly suggest that part of the silique and seed developmental defects is related to altered metal homeostasis in reproductive organs, which is known to tremendously affect anther dehiscence, pollen development, fruit development, seed quality and seed yield (Verbruggen and Hermans, 2013; Guo et al., 2016; Singh and Reddy, 2017).

Supporting this hypothesis, sr45-1 mutant seeds contain decreased amounts of Mn as well as increased levels of macronutrients K, Mg and Zn, possibly accounting for the reproductive defects upon water irrigation, i.e. in the absence of Fe supplementation (Figure 7). Previously, the expression of several ion transporter-encoding genes was shown to be deregulated in sr45-1 inflorescences (Zhang et al., 2017). Indeed, AtHMP37 (AT4G27590) and AtHMP27 (AT3G24450), PCR2 and HIPP22, respectively encoding two heavy metal transport/detoxification superfamily proteins (Li et al., 2020), a zinc transporter (Song et al., 2010) and a cadmium-detoxifying protein (Tehseen et al., 2010), are all up-regulated in sr45-1 inflorescences (Zhang et al., 2017). The member of the heavy metal transport/detoxification superfamily, AtHMP37, is a SDR gene (in both roots and inflorescences) identified as a putative RNA target of SR45 (SAR) (Supplemental Table S6) (Xing et al., 2015; Zhang et al., 2017) that is up-regulated in sr45-1 roots (Supplemental Figure S13). On the contrary, the NFP7.1 gene, which encodes a nitrate transporter (Babst et al., 2019), and the NRT1.7 nitrate transporter (Fan et al., 2009; Liu et al., 2017b), were shown to be down-regulated in sr45-1 inflorescences (Chen et al., 2019). It is well known that the N status affects Mg and Fe homeostasis (e.g. FIT regulation) (García et al., 2010; Curie and Mari, 2017; Liu et al., 2017a; Kailasam et al., 2018; Liu et al., 2018) and that a crosstalk exists between Zn and Fe homeostasis through at least IRT1, ZIF1 and FRD3 (Haydon et al., 2012; Charlier et al., 2015; Scheepers et al., 2020; Hanikenne et al., 2021). It is therefore possible that changes in the levels of micro- and macronutrients in seeds are the result of deregulation in the expression of nutrient transporters or vice versa, the causality between these observations being difficult to determine from our data. In fact, the mutant root growth is altered by Fe supply from the first week of development (see Supplemental Figure S12) and continues to be drastically affected upon extensive exposure (Figure 2).

Surprisingly, while Fe concentration was diminished in the sr45-1 mutant shoots upon both Fe deficiency and sufficiency (Supplemental Figure S2), it is very similar in seeds of Col-0
and sr45-1 (Supplemental Figure S10D). This suggests that the cause of the sr45-1 developmental defects in reproductive organs is not Fe starvation of the embryo but rather a consequence of intricate ionome perturbations. In fact, Zn concentration in seeds obtained from plants watered with solely tap water was higher (84.5 %) in the mutant compared to the WT, suggesting that the radicle local environment contained a higher Zn pool, which may result in a local Fe deficiency as previously observed (Dučić and Polle, 2007; Lei et al., 2007; Kawachi et al., 2009; Fukao et al., 2011; Shanmugam et al., 2011; Marschner and Marschner, 2012; Millaleo et al., 2013; Scheepers et al., 2020). The constitutive induction of the Fe uptake machinery (Figure 5) in the sr45-1 seedlings would reflect this ionome and possibly Fe defect in the seeds. However, the primary root growth of seeds collected from sr45-1 plants supplemented with Fe was not significantly improved compared to seeds collected from plants watered with tap water (Supplemental Figure S12). It is therefore still unclear how altered partitioning of metals in the mutant seeds affects the primary growth of the seedling.

It is however evident that sr45-1 seedlings and plants exhibit several alterations of metal homeostasis: (i) Fe and H$_2$O$_2$ accumulation within the root vasculature; (ii) Mn and Zn accumulation in roots and (iii) reduced shoot metal (Fe, Zn, Mn) concentrations, resulting in reduced biomass and chlorosis upon changes in Fe supply. RNA-Seq and qRT-PCR analyses suggest that mutant roots displayed a basal induction of Fe-starvation-responsive genes in control conditions with less drastic expression changes compared to Col-0 upon Fe deficiency (1350 vs 2442 DEGs) in adult plants (Figure 4, Supplemental Figures S7 and S13) and a stronger response in seedlings (Figure 5, Supplemental Figure S6).

Our working hypothesis therefore stands in favor of a scenario where the defective seed ionome affects the development of the embryo, which consequently results in an induction of the Fe uptake machinery in seedlings upon germination (Figure 5A-D) in an attempt to compensate for the metal starvation (Figure 7J). The down-regulation of FRD3 expression in seedlings (Figure 5E) would reduce root-to-shoot translocation of citrate-complexed metals that may reveal toxic when in excess in aerial parts (i.e. Fe, Zn). This ultimately results in lower shoot biomass and metal concentrations (Figure 1 and Supplemental Figure S2), together with Fe and Zn toxicity in roots (Figure 3), including the accumulation of H$_2$O$_2$ (Figure 1F and Supplemental Figure S5B). If ionome and growth perturbations persist in mutant adult plants...
(Figure 7G-J), their effect on Fe and metal homeostasis gene expression appear mitigated (Figure 4 and Figure 5).

Conclusion

We reported here that some of the sr45-1 mutant phenotype result from a severe alteration in metal mobilization, localization and transport. We also showed that exogenous application of Fe can rescue the reproductive defects of the mutant. Our data fine-tune our understanding of the physiological responses impaired in the mutant, such as the oxidative response, metal homeostasis and the phenylpropanoid pathway.

Methods

Plant material and growth conditions

All experiments were conducted under a 8-h-light (100 µmol m$^{-2}$ s$^{-1}$)/16-h-dark regime in a climate-controlled growth chamber (21°C). Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as wild-type, and seeds from sr45-1 mutant (SALK_004132, Col-0 background) and frd3-7 mutant (SALK_122235, Col-0 background) were obtained from the SALK collection. Seeds were surface-sterilized and germinated on 1/2 Murashige and Skoog (MS) medium (Duchefa Biochimie) supplemented with sucrose (1% w/v, Duchefa Biochimie) and Select Agar (0.8% w/v, Sigma-Aldrich) and stratified in the dark at 4°C for 48h. For hydroponics experiments, 3-week-old seedlings were transferred in hydroponic trays (Araponics) and cultivated for 3 weeks in control Hoagland medium, followed by 3 weeks of experimental conditions. The nutrient medium was renewed with fresh solution once a week and 3 days prior to the harvesting. The control Hoagland medium included 10 µM FeIII-HBED [N,N’-di(2-hydroxybenzyl) ethylenediamine N,N’-diacetic acid monohydrochloride], 1 µM Zn (ZnSO$_4$.H$_2$O) and 5 µM Mn (MnSO$_4$.H$_2$O) as reported in (Talke et al., 2006; Hanikenne et al., 2008; Scheepers et al., 2020). Fe, Zn and Mn were respectively added or omitted from medium as described.

Unless stated otherwise, for root length measurement (including treatments with 120 µM of DMF-solubilized fraxetin), Perls and hydrogen peroxide staining, acidification capacity assay, FeIII chelate reductase activity assay and citrate content measurement, surface-sterilized seeds were directly sown on square plastic Petri plates (Greiner Bio-One) containing modified
Hoagland supplemented with sucrose (1% w/v, Duchefa Biochimie), FeIII-HBED (0 µM to 50 µM) and agar (0.8% w/v, Agar Type M, Sigma-Aldrich), and grown vertically after stratification.

Seed number per silique, seed morphology, silique size and roots length

3-weeks-old seedlings were transferred in soil and daily watered with water (referred to as -Fe). In addition of this treatment, half of the seedlings from each genotype were weekly supplied with a sequestrene solution (referred to as +Fe, 0.1 g/L, Liro N.V.) until the completion of silique development and seed maturation. Siliques were then harvested before dehiscence, and their size was determined using the segmented line tool on ImageJ. After an incubation of two weeks in 95% ethanol at room temperature, the seed number per silique was determined under a Nikon SMZ1500 stereomicroscope. Seeds were finally photographed using a Nikon SMZ1500 stereomicroscope equipped with a Nikon Digital Sight DS-5M camera in order to determine seed length and width.

Perls and hydrogen peroxide staining

For Perls staining, roots of Arabidopsis thaliana Col-0 and sr45-1 mutant seedlings (or 9-week-old plants) were vacuum infiltrated with HCl (4%, v/v) and K-Ferrocyanide (II) (4%, w/v, Sigma-Aldrich) (1/1) for 15 minutes. The reaction was continued for an additional 30 minutes at room temperature and was then stopped by substituting the solution by distilled water (Roschzttardtz et al., 2009). Observation was realized using a Nikon SMZ1500 stereomicroscope equipped with a Nikon Digital Sight DS-5M camera.

Hydrogen peroxide (H$_2$O$_2$) was detected according to (Baliardini et al., 2015). Roots samples were vacuum infiltrated with 3-3’-diaminobenzidine tetrahydrochloride (1.25 mg/mL, DAB, Sigma-Aldrich), Tween-20 (0.05% v/v) and 200 mM Na$_2$HPO$_4$, then incubated in the same solution for a total of one hour. They were subsequently bleached in acetic-acid/glycerol/ethanol (1/1/3) during 5 minutes at 100°C, and stored in glycerol/ethanol (1/4) before further analysis. Samples were observed under a stereomicroscope as above.

Acidification capacity and Ferric (FeIII) chelate reductase activity assays

For the measure of acidification capacity, roots from a pool of 5 seedlings were incubated in 0.005% bromocresol purple (Roth) during 24 hours in the dark. A$_{433}$ of the protonated form of
the dye was then measured and expressed relative to the root weight of sample (Santi and Schmidt, 2009; El-Ashgar et al., 2012).

The ferric chelate reductase (FCR) activity was measured on roots from a pool of 5 seedlings. Samples were immerged in a reductase solution containing FeIII-EDTA (0.1 mM, Roth) and FerroZine (0.3 mM, Acros Organics) for 30 minutes in the dark. $A_{562}$ of the FeII-FerroZine complex was then determined. The final calculation included the root weight of sample and the molar extinction coefficient of the complex (28.6 mM$^{-1}$cm$^{-1}$) (Yi and Guerinot, 1996).

Citrate content

Citrate determination was performed as previously described (Schvartzman et al., 2018). Briefly, 100 mg of roots were frozen and grinded in liquid nitrogen with a mortar and pestle. Samples were resuspended in 1 mL of distilled water, and the pH was adjusted to 7 – 8 with 1 M KOH. Samples were deproteinized using 100 µL of 1 M perchloric acid, and citrate content was measured using a citric acid assay kit according to the manufacturer’s protocol (BioSentec, France).

Total chlorophyll and carotenoid content

Total chlorophyll content was determined from three to six young seedlings or the rosette of one 9-week-old plant. Plants were weighed, then incubated in the dark during 72h or seven days in 95% ethanol. Discolored plants were removed and the solution was submitted to spectroscopic analysis. The total amount of chlorophyll was calculated using the equation:

$$\text{Chl } a + b = \frac{6.1 A_{665} + 20.04 A_{649}}{\text{fresh weight}}$$

(Wintemans and de Mots, 1965). Carotenoid content (xanthophylls and carotenes) was determined for the same samples using the equation:

$$C(x + c) = \frac{1000 A_{470} - 2.13 \text{ Chl } a - 97.64 \text{ Chl } b}{209 \text{ fresh weight}}$$

(Lichtenthaler and Buschmann, 2001).

Gene expression analysis

Total RNAs were extracted from 100 mg of plant tissues (entire plants or roots) using NucleoSpin RNA Plant kit (Macherey Nagel) as per manufacturer’s instruction. cDNAs were synthesized from 1 µg of total RNAs using oligo(dT) and the RevertAid H Minus First Strand cDNA Synthesis Kit (Fisher Scientific). Quantitative PCR reactions were performed in a
QuantStudio5 (Applied Biosystems) using 384-well plates and Takyon Low Rox SYBR MasterMix dTTP Blue (Eurogentec) on material from three independent biological experiments, and a total of three technical repeats were run for each combination of cDNA and primer pair. Gene expression was normalized relative to At1g58050 as described (Pfaffl, 2001). At1g58050 expression was the more stable among all tested references (EF1α and UBQ10) (Czechowski et al., 2005; Spielmann et al., 2020). **Supplemental Table S7** shows the primers used for these experiments.

Upon harvesting, plant root tissues were blotted dry, immediately frozen in liquid nitrogen and stored at -80°C. Total RNAs were prepared using 100 mg of homogenized tissues and RNeasy Plant Mini kit with on-column DNase treatment (Qiagen). Libraries for RNA-Seq were prepared from 1 µg of total RNAs with the TruSeq Stranded mRNA Library Prep Kit (Illumina, CA, USA), multiplexed and sequenced in two runs with an Illumina NextSeq500 device (high throughput mode, 75 base single-end reads) at the GIGA-R Sequencing platform (University of Liège), yielding on average ~22 million reads per sample. Read quality was assessed using FastQC (v0.10.1, [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). Quality trimming and removal of adapters were conducted using Trimmomatic (v0.32, Bolger et al., 2014), with the following parameters: trim bases with quality score lower than Q26 in 5’ and 3’ of reads; remove any reads with Q<26 in any sliding window of 10 bases; crop 1 base in 3’ of all reads, and discard reads shorter than 70 bases. Overall quality filtering discarded between 7 and 9% of the raw reads. The Arabidopsis reference genome sequence (TAIR10) and annotation (201606 version) files were downloaded from Araport on Sept 16, 2016 ([www.araport.org](http://www.araport.org)). Read mapping on the genome was achieved using TopHat (v2.1.1), with the following parameters: --read-mismatches 2; --min-intron-length 40; --max-intron-length 2000; 2 --report-secondary-alignments; --no-novel-juncs and providing an indexed genome annotation file. Raw read counts were obtained using htseq-count (v0.6.1p1) and differentially expressed genes were identified by pairwise comparisons with the DESeq2 package (v1.12.3, Love et al., 2014). Genes were retained as differentially expressed when the log2 fold-change (FC) was > 1 or < -1, with a false discovery rate (FDR, Benjamini-Hochberg) adjusted *p*-value of < 0.05. Principal Component Analysis plots (PCA) were created with the *PlotPCA* function from R using rlog transformed data (Beginner's guide, DESeq2 package, May 13, 2014,
http://www.bioconductor.org/packages/2.14/bioc/vignettes/DESeq2/inst/doc/beginner.pdf). GO enrichment analyses were conducted using the Thalemine tool on Araport (http://www.araport.org). The heatmaps were constructed using the heatmap.2 function of the gplots R package.

### Analysis of metal content

Seed (−Fe and +Fe), root and shoot tissues of wild-type plants (Col-0) and sr45-1 mutant plants were harvested separately. Root tissues were desorbed and washed as previously described (Talke et al., 2006), and seed and shoot tissues were rinsed in distilled water. 1 to 30 mg of dried tissue were digested and prepared as described (Nouet et al., 2015). Metal content was determined by ICP-AES (inductively coupled plasma-atomic emission spectroscopy) (Vista AX, Varian).

### Statistical analysis

All data evaluation and statistics were done using GraphPad Prism 7 (GraphPad Software v7.00).

### Accession numbers and information

All gene sequences are available through The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/), with this accession number: Arabidopsis SR45 (AT1G16610), AHA2 (AT4G30190), bHLH100 (AT2G41240), bHLH101 (AT5G04150), bHLH11 (AT4G36060), bHLH18 (AT2G22750), bHLH19 (AT2G22760), bHLH20 (AT2G22770), bHLH25 (AT4G37850), bHLH38 (AT3G56970), bHLH39 (AT3G56980), FIT (AT2G28160), FRD3 (AT3G08040), FRO2 (AT1G01580), IRT1 (AT4G19690), MYB15 (AT3G23250), MYB72 (AT1G56160), NAS1 (AT5G04950), NAS2 (AT5G56080), NAS3 (AT1G09240), NAS4 (AT1G56430), and S8H (AT3G12900). The Arabidopsis thaliana sr45-1 T-DNA insertion (SALK_004132) and frd3-7 T-DNA insertion (SALK_122235) lines were available at the SALK collection. The RNA-Seq reads have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive Database with BioProject identification number [XXX].
Supplemental data

**Supplemental Figure S1.** Number of potential SR45 targets showing iron deficiency responsiveness.

**Supplemental Figure S2.** Micronutrient concentrations in sr45-1 shoots.

**Supplemental Figure S3.** Macronutrient concentrations in sr45-1 tissues.

**Supplemental Figure S4.** Phenotype characterization of wild-type and sr45-1 seedlings and plants upon iron deficiency and iron excess.

**Supplemental Figure S5.** Toxicity of manganese and zinc in roots and shoots.

**Supplemental Figure S6.** Fe uptake machinery activity in sr45-1 seedlings.

**Supplemental Figure S7.** Regulation of FIT-interacting coactivators in sr45-1 adult plants.

**Supplemental Figure S8.** Transcriptomic analysis in roots upon iron deficiency and sufficiency.

**Supplemental Figure S9.** Effect of exogenous application of coumarins on sr45-1 shoots.

**Supplemental Figure S10.** Effect of irrigation on stem height and flowering.

**Supplemental Figure S11.** SR45 differentially regulated genes (SDRs) involved in reproductive development.

**Supplemental Figure S12.** Root length of sr45-1 seedlings upon iron deficiency and iron excess.
Supplemental Figure S13. Transcriptomic analysis in roots upon iron deficiency and sufficiency.

Supplemental Table S1. Significantly enriched categories among SR45-associated RNAs.

Supplemental Table S2. Lists of differentially expressed genes from all pairwise comparisons described in Figure 4.

Supplemental Table S3. Significantly enriched categories among DEGs identified in roots of WT and sr45-1 upon iron deficiency and sufficiency.

Supplemental Table S4. Col-0 differentially regulated genes (CDR) and SR45 differentially regulated genes (SDR) from all pairwise comparisons described in Figure 4.

Supplemental Table S5. Significantly enriched categories among CDR genes and SDR genes.

Supplemental Table S6. Comparison of SDR genes identified in roots with SR45-associated RNAs and SDR genes identified in inflorescences.

Supplemental Table S7. List of primers used in this study.

Acknowledgments

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**Figure 1.** Metal concentration, iron staining and H$_2$O$_2$ staining in roots upon iron deficiency and iron excess. (A) Iron (Fe), (B) copper (Cu), (C) manganese (Mn) and (D) zinc (Zn) concentrations in roots of wild-type (Col-0) and mutant (sr45-1) plants grown hydroponically in Hoagland medium supplemented with 0 (deficiency) or 10 (control) µM Fe. Values represent means ± SEM (from one experiment representative of two independent experiments, each including 2 or 3 series of 2 plants per genotype and condition). Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means between genotypes are indicated by stars (* P<0.05, ** P<0.01, *** P<0.001) or between treatments within genotypes by different letters (P<0.05). n.s.: not significant. (E) Iron accumulation visualized using Perls staining in roots of Col-0 and sr45-1. (F) H$_2$O$_2$ accumulation stained using DAB in roots of Col-0 and sr45-1. The pictures are representative of 2 independent experiments. Arrowheads show Fe or H$_2$O$_2$ accumulation in roots. Scale bar: 100 µm.

**Figure 2.** Phenotypes of wild-type and sr45-1 plants upon iron deficiency. (A) Representative pictures and (B) quantification of total chlorophyll content of wild-type (Col-0) and mutant (sr45-1) plants grown hydroponically in Hoagland medium supplemented with 0 (deficiency) or 10 (control) µM Fe. Scale bar: 1 cm. (B) Ratios of root length and (C) shoot fresh weight in sr45-1 versus Col-0 plants. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test (B) or by a t-test (C-D). Statistically significant differences between means between genotypes are indicated by stars (* P<0.05, ** P<0.01, *** P<0.001) or between treatments within genotypes by different letters (P<0.05). n.s.: not significant. Scale bar: 1 cm.

**Figure 3.** Toxicity of manganese and zinc in sr45-1 roots. Relative root length of wild-type (Col-0) and mutant (sr45-1) plants grown vertically in petri dishes containing Hoagland medium supplemented with iron (10 µM Fe-HBED) but not manganese (0 µM MnSO$_4$) or zinc (0 µM ZnSO$_4$). Root growth is relative to Col-0 at 10 µM Fe-HBED, 5 µM MnSO$_4$ and 1 µM ZnSO$_4$. Values represent means ± SEM (from four independent experiments, each including 2 series of 6 seedlings per genotype and condition). Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means...
between genotypes are indicated by stars (**P<0.001) or between treatments within genotypes
by different letters (P<0.05).

**Figure 4.** Transcriptomic analysis in roots upon iron deficiency and sufficiency. (A) Summary
statistics of up- (↑) or down-regulated (↓) genes in different pair-wise comparisons. The direction
of comparisons is indicated by arrows. Overlaps in Venn diagrams represent deregulated genes in
both Col-0 and sr45-1 roots in respective comparisons (indicated by colors). Right circle:
Number of differentially regulated genes solely associated to sr45-1 roots (SDRs). Left circle:
Number of differentially regulated genes solely associated to Col-0 (CDRs). Bar graphs of Gene
Ontology (GO) enriched categories in differentially expressed CDR genes (B) and SDR genes
(C). The enrichment score represents –log_{10}(p-value). Dissimilar GO terms between genotypes
are colored in red (see also Supplemental Table S5).

**Figure 5.** Fe uptake machinery in sr45-1 seedlings and adult plants. Quantitative RT-PCR
analysis of expression of (A) FIT, (B) AHA2, (C) FRO2, (D) IRT1 and (E) FRD3 genes wild-type
(Col-0) and mutant (sr45-1) seedlings grown vertically in petri dishes containing Hoagland
medium supplemented with 0 or 10 μM iron (Fe) (left panel) and in roots of wild-type (Col-0)
and mutant (sr45-1) plants grown hydroponically in Hoagland medium supplemented with 0 or
10 μM Fe (RNA-seq samples) (right panel). Values represent means ± SEM (from four
biological replicates, each consisting of 2-6 plants per genotype and condition) and are relative to
At1g58050. Data were analyzed by two-way ANOVA followed by Bonferroni multiple
comparison post-test. Statistically significant differences between means between genotypes are
indicated by stars (*P<0.05, **P<0.01, ***P<0.001) or between treatments within genotypes
by different letters (P<0.05). n.s.: not significant.

**Figure 6.** Effect of exogenous application of coumarins on sr45-1 roots. (A) Relative root growth
of wild-type (Col-0) and mutant (sr45-1) plants grown vertically in petri dishes containing
Hoagland medium supplemented with 10 μM iron (Fe) at various pH (5.7 in presence of Fe-
HBED or 7 in presence of FeCl₃). Root growth is relative to Col-0 at 10 μM Fe-HBED + DMF
(pH 5.7). Values represent means ± SEM (from two independent experiments, each including 3
or 4 series of 6 plants per genotype and condition). Data were analyzed by two-way ANOVA
followed by Bonferroni multiple comparison post-test. Statistically significant differences between means between genotypes are indicated by stars (*** P<0.001) or between treatments within genotypes by different letters (P<0.05). (B) Iron accumulation and (C) H$_2$O$_2$ accumulation in roots of wild-type (Col-0) and mutant (sr45-I) plants grown vertically in petri dishes containing Hoagland medium supplemented with 0 (deficiency) or 10 (control) µM iron (Fe) at various pH (5.7 in presence of Fe-HBED or 7 in presence of FeCl$_3$). The pictures are representative of two independent experiments. Arrowheads show Fe or H$_2$O$_2$ accumulation in roots. Scale bar: 100 µm.

**Figure 7.** Iron irrigation fully rescues the development of the reproductive tissues. (A) Representative pictures of wild-type (Col-0) and mutant (sr45-I) siliques, (B) silique length, (C) representative pictures of wild-type (Col-0) and mutant (sr45-I) seeds, (D) number of seeds per silique, (E) seed mass (for pools of 100 seeds), and (F) seed length and width of plants grown in soil and daily irrigated with water without (–Fe) or with an additional treatment consisting of a weekly irrigation with sequestrene (+Fe). Values represent means ± SEM (from three independent experiments with 10-18 siliques pertaining to the main stem [4 plants per genotype and condition] (B) or with seeds obtained from 4 plants per genotype and condition (D-F)). Scale bars: 1 mm for siliques; 500 µm for seeds. (G) Zinc (Zn), (H) potassium (K), (I) magnesium (Mg) and (HJ) manganese (Mn) concentration in seeds (–Fe or +Fe) of wild-type (Col-0) and mutant (sr45-I) plants. Values represent means ± SEM (from three independent experiments with seeds obtained from 4 plants per genotype and condition). For each experiment, data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means are indicated by stars (* P<0.05, ** P<0.01, *** P<0.001) or different letters (P<0.05). n.s.: not significant. (F) was obtained by performing statistical analysis for both seed length and seed width independently and then by displaying the results in a single graph.

**Literature cited**


Figure 1

(A) Root Fe (µg g⁻¹ DW) for 0 µM Fe and 10 µM Fe.

(B) Root Cu (µg g⁻¹ DW) for Col-0 and sr45-1.

(C) Root Mn (µg g⁻¹ DW) for Col-0 and sr45-1.

(D) Root Zn (µg g⁻¹ DW) for 0 µM Fe and 10 µM Fe.

(E) Microscopic images of root systems for 0 µM Fe and 10 µM Fe.

(F) Microscopic images of root systems for 0 µM Fe and 10 µM Fe.
**Figure 1.** Metal concentration, iron staining and H$_2$O$_2$ staining in roots upon iron deficiency and iron excess. (A) Iron (Fe), (B) copper (Cu), (C) manganese (Mn) and (D) zinc (Zn) concentration in roots of wild-type (Col-0) and mutant (sr45-1) plants grown hydroponically in Hoagland medium supplemented with 0 (deficiency) or 10 (control) µM Fe. Values represent means ± SEM (from one experiment representative of two independent experiments, each including 2 or 3 series of 2 plants per genotype and condition). Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means between genotypes are indicated by stars (* P<0.05, ** P<0.01, *** P<0.001) or between treatments within genotypes by different letters (P<0.05). (E) Iron accumulation visualized using Perls staining in roots of Col-0 and sr45-1. (F) H$_2$O$_2$ accumulation stained using DAB in roots of Col-0 and sr45-1. The pictures are representative of 2 independent experiments. Arrowheads show Fe or H$_2$O$_2$ accumulation in roots. Scale bar: 100 µm.
Figure 2

A

Col-0

sr45-1

B

Total chlorophyll content (µg g⁻¹ FW)

0 µM Fe

10 µM Fe

C

Root length (ratio sr45-1/Col-0)

0 µM Fe

10 µM Fe

D

Shoot fresh weight (ratio sr45-1/Col-0)

0 µM Fe

10 µM Fe

n.s. ** ***
**Figure 2.** Phenotypes of aerial parts of wild-type and *sr45-1* plants upon iron deficiency. (A) Representative pictures and (B) quantification of total chlorophyll content of wild-type (Col-0) and mutant (*sr45-1*) plants grown hydroponically in Hoagland medium supplemented with 0 (deficiency) or 10 (control) µM Fe. Scale bar: 1 cm. (B) Ratios of root length and (C) shoot fresh weight in *sr45-1* versus Col-0 plants. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test (B) or by a t-test (C-D). Statistically significant differences between means between genotypes are indicated by stars (* P<0.05, ** P<0.01, *** P<0.001) or between treatments within genotypes by different letters (P<0.05). Scale bar: 1 cm.
Figure 3

The bar chart shows the relative root growth for different iron concentrations and treatments. The treatments include:

- 10 μM Fe
- 10 μM Fe + 0 μM Mn
- 10 μM Fe + 0 μM Zn

The bars are color-coded:
- Gray: Col-0
- Dark gray: sr45-1

Statistical significance is indicated by letters:
- A
- B
- AB
- a
- ab
- b

Significance levels are marked with asterisks:
- ***

The chart illustrates a comparison of root growth under these conditions, with significant differences highlighted by the letters and asterisks.
Figure 3. Toxicity of iron, manganese and zinc in roots. Relative root length of wild-type (Col-0) and mutant (sr45-1) plants grown vertically in petri dishes containing Hoagland medium supplemented with iron (10 µM Fe-HBED) but not manganese (0 µM MnSO₄) or zinc (0 µM ZnSO₄). Root growth is relative to Col-0 at 10 µM Fe-HBED, 5 µM MnSO₄ and 1 µM ZnSO₄. Values represent means ± SEM (from four independent experiments, each including 2 series of 6 seedlings per genotype and condition). Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means between genotypes are indicated by stars (*** P<0.001) or between treatments within genotypes by different letters (P<0.05).
Figure 4
Figure 4. Transcriptomic analysis in roots upon iron deficiency and sufficiency. (A) Summary statistics of up- (↑) or down-regulated (↓) genes in different pair-wise comparisons. The direction of comparisons is indicated by arrows. Overlaps in Venn diagrams represent deregulated genes in both Col-0 and sr45-1 roots in respective comparison (indicated by colors). Right circle: Number of differentially regulated genes solely associated to sr45-1 roots (SDRs). Left circle: Number of differentially regulated genes solely associated to Col-0. Bar graphs of Gene Ontology (GO) enriched categories in differentially expressed CDR genes (B) and SDR genes (C). The enrichment score represents $-\log_{10}(p\text{-value})$. Dissimilar GO terms are colored in red (see also Supplemental Table 5).
Figure 5. Fe uptake machinery in sr45-1 seedlings and adult plants. Quantitative RT-PCR analysis of expression of (A) FIT, (B) AHA2, (C) FRO2, (D) IRT1 and (E) FRD3 genes in wild-type (Col-0) and mutant (sr45-1) plants grown vertically in petri dishes containing Hoagland medium supplemented with 0 or 10 μM iron (Fe) (left panel) and in roots of wild-type (Col-0) and mutant (sr45-1) plants grown hydroponically in Hoagland medium supplemented with 0 or 10 μM Fe (RNA-seq samples) (right panel). Values represent means ± SEM (from four biological replicates, each consisting of 2-6 plants per genotype and condition) and are relative to At1g58050. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means between genotypes are indicated by stars (* P<0.05, ** P<0.01, *** P<0.001) or between treatments within genotypes by different letters (P<0.05).
Figure 6

A

Relative root growth

10 µM Fe-HBED + DMF
10 µM FeCl3 + DMF
10 µM FeCl3 + 120 µM Fraxetin
0 µM FeCl3 + 120 µM Fraxetin

Col-0
sr45-1

B

C

Figure 6
Figure 6. Effect of exogenous application of coumarins on sr45-1 roots. 
(A) Relative root growth of wild-type (Col-0) and mutant (sr45-1) plants grown vertically in petri dishes containing Hoagland medium supplemented with 10 µM iron (Fe) at various pH (5.7 in presence of Fe-HBED or 7 in presence of FeCl₃). Root growth is relative to Col-0 at 10 µM Fe-HBED + DMF (pH 5.7). Values represent means ± SEM (from seven semi-independent experiments, each including 6 plants per genotype and condition). Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means between genotypes are indicated by stars (*** P<0.001) or between treatments within genotypes by different letters (P<0.05). (B) Iron accumulation and (C) H₂O₂ accumulation in roots of wild-type (Col-0) and mutant (sr45-1) plants grown vertically in petri dishes containing Hoagland medium supplemented with 0 (deficiency) or 10 (control) µM iron (Fe) at various pH (5.7 in presence of Fe-HBED or 7 in presence of FeCl₃). The pictures are representative of two semi-independent experiments. Arrowheads show Fe or H₂O₂ accumulation in roots. Scale bar: 100 µm.
Figure 7
Figure 7. Iron irrigation fully rescues the development of the reproductive tissue. (A) Representative pictures of wild-type (Col-0) and mutant (sr45-1) siliques, (B) sique length, (C) representative pictures of wild-type (Col-0) and mutant (sr45-1) seeds, (D) number of seeds per sique, (E) seed mass (for pools of 100 seeds), and (F) seed length and width of plants grown in soil and daily irrigated with water without (−Fe) or with (+Fe) an additional treatment consisting of a weekly irrigation with sequestrene. Values represent means ± SEM (from three independent experiments with 10-18 siliques pertaining to the main stem [4 plants per genotype and condition] (B) or with seeds obtained from 4 plants per genotype and condition (D-F)). Scale bars: 1 mm for siliques; 500 µm for seeds. (G) Zinc (Zn), (H) potassium (K), (I) magnesium (Mg) and (HJ) manganese (Mn) concentration in seeds (−Fe or +Fe) of wild-type (Col-0) and mutant (sr45-1) plants. Values represent means ± SEM (from three independent experiments with seeds obtained from 4 plants per genotype and condition). For each experiment, data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means are indicated by stars (* P<0.05, ** P<0.01, *** P<0.001) or different letters (P<0.05). Figure 7F was obtained by performing statistical analysis for both seed length and seed width independently and then by displaying the results in a single graph.


