Reactive Oxygen Species coordinate the transcriptional responses to iron availability in Arabidopsis

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Highlight

We demonstrate that CAT2 enzyme modulates root H₂O₂ levels and forms a regulatory loop with the central Fe acquisition transcription factor FIT to influence Arabidopsis response to prolonged Fe deficiency.
Abstract

Reactive oxygen species play a central role in the regulation of plant responses to environmental stress. Under prolonged iron (Fe) deficiency, increased levels of hydrogen peroxide (H$_2$O$_2$) initiate signaling events, resulting in the attenuation of Fe acquisition through the inhibition of FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT). As this H$_2$O$_2$ increase occurs in a FIT-dependent manner, our aim was to understand the processes involved in maintaining H$_2$O$_2$ levels under prolonged Fe deficiency and the role of FIT in this process. We identified CAT2 gene, encoding one of the three Arabidopsis catalase isoforms, as regulated by FIT. CAT2 loss-of-function plants displayed severe susceptibility to Fe deficiency and greatly increased H$_2$O$_2$ levels in roots. Analysis of the Fe homeostasis transcriptional cascade revealed that H$_2$O$_2$ influences the gene expression of downstream regulators FIT, BHLHs of group Ib and POPEYE (PYE), however H$_2$O$_2$ did not affect their upstream regulators, such as BHLH104 and ILR3. Our data shows that FIT and CAT2 participate in a regulatory loop between H$_2$O$_2$ and prolonged Fe deficiency.

Keywords

FIT; H$_2$O$_2$; ROS; bHLH; catalase; iron uptake
Introduction

Plants are constantly confronted with a varying array of stress conditions to which they need to respond and adapt in order to survive. Aerobic metabolic processes, such as photosynthesis and respiration, as well as chemical reactions in the cell, lead to the production of reactive oxygen species (ROS), the most prominent of them being superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH·) and singlet oxygen ($^1$O$_2$). ROS can be produced in different cellular compartments and have different reactivity and stability. They can increase in amount under stress conditions and damage proteins, nucleic acids and lipids, eventually triggering cell death. On the other hand, controlled and spatially defined ROS production can trigger signaling events and initiate or contribute to specific plant stress responses (Czarnocka and Karpinski, 2018; Mittler, 2017; Noctor and Foyer, 2016; Smirnoff and Arnaud, 2019). To maintain ROS homeostasis, plants employ ROS-scavenging enzymes, such as catalases (CATs), superoxide dismutases, ascorbate peroxidases, glutathione peroxidases, and peroxiredoxins (Inupakutika et al., 2016; Khedia et al., 2019; Mittler et al., 2004). Arabidopsis thaliana (Arabidopsis) has three catalase genes, CAT1, CAT2 and CAT3, whose products dismutate H$_2$O$_2$ to H$_2$O and O$_2$, with varying contribution of the three proteins in the different plant organs (Mhamdi et al., 2010). CAT1 is mainly expressed in pollen and seeds, CAT2 in photosynthetic tissues, roots and seeds, and CAT3 in leaves and vascular tissues (Frugoli et al., 1996; McClung, 1997; Mhamdi et al., 2010). The differential contribution and partial functional redundancy of the three catalases in different organs has been recently studied (Su et al., 2018; Yang et al., 2019). CAT loss-of-function mutants have been successfully employed as a model system to study the role of ROS in abiotic and biotic stress responses (Mhamdi et al., 2010; Schmidt et al., 2020; Sewelam et al., 2014).

An abiotic stress with a profound impact on plant health and survival is the lack of sufficient iron (Fe). Fe deficiency-induced anemia affects billions of people worldwide, necessitating the study and deeper understanding of the mechanisms that plants utilize to acquire Fe from the soil, in order to combat Fe malnutrition through the development of Fe-fortified crops (Naranjo-Arcos and Bauer, 2016). Most flowering plants, except members of the Poaceae family, acquire Fe by employing a reduction-based mechanism, involving the function of a root surface ferric reductase and a ZIP-family bivalent metal transporter. In Arabidopsis, the Fe reductase activity is carried by the FERRIC REDUCTASE-OXIDASE2 (FRO2) protein and transport is achieved through the action of the IRON-REGULATED TRANSPORTER1 (IRT1), which are encoded by genes upregulated under Fe deficiency (Brumbarova et al., 2015; Ivanov et al., 2012; Kobayashi and Nishizawa, 2012). Transcriptionally, Fe acquisition is controlled by a series of regulatory events representing a hierarchical cascade of
mainly bHLH-family transcription factors (Gao et al., 2019). Gene coexpression analysis defines different Fe-related regulatory modules (Brumbarova and Ivanov, 2019; Ivanov et al., 2012; Schwarz and Bauer, 2020). One module consists of genes involved in the acquisition of Fe, such as FRO2 and IRT1, regulated by FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT). Another module comprises genes with functions in Fe homeostasis, regulated by and coexpressed with POPEYE (PYE) (Long et al., 2010). A third module contains mainly Fe storage-related genes, such as FERRITINs, also controlled by IAA-LEUCINE RESISTANT3 (ILR3) and bHLH121/UPSTREAM REGULATOR OF IRT1 (URI) (Brumbarova and Ivanov, 2019; Ivanov et al., 2012; Schwarz and Bauer, 2020). At the same time ILR3, together with URI, is involved in the regulation of PYE and the BHLH subgroup Ib genes BHLH038, BHLH039, BHLH100 and BHLH101 (Gao et al., 2020a; Kim et al., 2019; Lei et al., 2020). The subgroup Ib bHLHs interact with FIT to regulate its expression, as well as the expression of FRO2 and IRT1 (Trofimov et al., 2019; Wang et al., 2007; Wang et al., 2013; Yuan et al., 2008). Among Ib bHLHs, BHLH039 is a robust Fe deficiency marker gene upregulated by Fe deficiency (Ivanov et al., 2012; Wang et al., 2007). Its expression is not regulated by FIT, however when FIT is lacking, BHLH039 undergoes additional upregulation, following a strong Fe deficiency signal in the plant upregulating the bHLH cascade (Schwarz and Bauer, 2020; Wang et al., 2007). The result of this complex interplay of regulators is the dynamic transcriptional response ensuring the balanced acquisition, redistribution and safe storage of Fe.

With the help of hormonal signaling, the Fe acquisition domain is dynamically regulated not only in time but also in space. Thus, the expression domain of IRT1 is adjusted around the early differentiation zone of the root according to the Fe requirements of the plant through crosstalk between the phytohormones ethylene and auxin (Blum et al., 2014). ROS, and specifically H2O2, were previously shown to influence the activity of FIT through an interaction with the oxidative stress-responsive transcription factor ZINC FINGER OF ARABIDOPSIS THALIANA12 (ZAT12) (Le et al., 2016). The interaction between the two occurs under prolonged Fe deficiency, leading to FIT inactivation and attenuation of the Fe deficiency response. The specific increase in H2O2 observed under these conditions was FIT-dependent and a central prerequisite for the observed major switch in the response strategy of the plant (Brumbarova and Ivanov, 2018; Brumbarova et al., 2016b; Le et al., 2019; Le et al., 2016).

A question that remains open is which processes are involved in the maintenance of the increased H2O2 levels under prolonged Fe deficiency and in which way FIT participates in this regulation. Here, we show through histochemical co-staining of H2O2 and O2•− in the root that lack of functional FIT leads to spatially deregulated ROS distribution under prolonged Fe deficiency. FIT is involved in the
transcriptional regulation of CAT genes, specifically CAT2. CAT2 loss-of-function mutants display severely hampered Fe deficiency responses. CAT2 is a major contributor to the H$_2$O$_2$ scavenging capacity of roots under prolonged Fe deficiency. Lack of CAT2 leads to a marked increase of H$_2$O$_2$ in root hair cells of the early differentiation zone. Gene expression analysis showed that CAT2 activity affects the regulation of PYE and FIT, together with genes from their target modules. At the same time, their upstream regulators ILR3 and bHLH104 are not affected transcriptionally by the lack of CAT2, suggesting posttranslational effects of CAT2 and H$_2$O$_2$ on ILR3 and bHLH104 activity. Our data suggest the existence of a FIT- and CAT2-mediated feedback loop controlling the upstream regulators of Fe uptake and homeostasis under prolonged Fe deficiency.

Materials and methods

Plant material and growth conditions
Arabidopsis (Arabidopsis thaliana) wild type Col-0, FIT loss-of-function fit-3 mutant (GABI_108C10, Jakoby et al., 2004) and CAT2 loss-of-function cat2-1 mutant SALK_076998, N576998, named cat2-2 in Queval et al. (2007) and later renamed to cat2-1 as in Bueso et al. (2007), were used in this study. T-DNA insertion lines were in the Col-0 genetic background. Plants were grown upright for 10 days on half-strength Hoagland medium agar plates (Jakoby et al., 2004) either supplemented with 50 µM FeNaEDTA (Fe sufficient condition) or without Fe (Fe deficient condition).

Root length measurement
Images of plants grown upright on half-strength Hoagland medium agar plates were acquired and root lengths were measured using the JMicroVision software, version 1.2.7 (http://www.jmicrovision.com). Following spatial calibration, length measurements were made of freehand drawn lines covering the root on the image. The obtained lengths were averaged, and SD was calculated.

Fe reductase activity measurement
The activity of the root surface ferric reductase was measured as described in (Le et al., 2016). Briefly, roots were rinsed in 100 mM Ca(NO$_3$)$_2$ solution and incubated in a solution containing 100 µM FeNaEDTA and 300 µM ferrozine for one hour at room temperature in the dark. Enzymatic activity was calculated using the ferrozine molar extinction coefficient of 28.6 mM$^{-1}$cm$^{-1}$. The experiment was performed in six replicates. Each replicate comprised of a pool of five seedlings. The
obtained data were normalized to the sum of the root length for each replicate. Ferrredoxin activity of the six replicates per condition was averaged and SD was calculated.

**Photosynthetic pigment measurement**

For plant pigment extraction, pre-weighed rosettes of 10 days-old seedlings where shock-frozen in liquid N\(_2\) prior to tissue rupture using 1.4 mm ceramic beads and a Precellys 24 tissue homogenizer (Bertin Instruments) for 3 times 20 seconds, at 6500 rpm. Samples were frozen in between homogenization steps in liquid N\(_2\). Pigment was extracted adding 500 µl pure acetone immediately after grinding, followed by a centrifugation step (3 min, 13000 rpm). The extraction was repeated once. The supernatant was collected and absorption was measured at 646, 663, and 750 nm in a 96-well plate reader (Infinite 200 PRO, Tecan). Concentrations (c) of chlorophyll (Chl) a, Chl b and carotenoids were calculated as described in Lichtenthaler and Wellburn (1983):

\[
\begin{align*}
    c(\text{Chl }a) &= 11.75 \times E_{662} - 2.35 \times E_{645} \\
    c(\text{Chl }b) &= 18.61 \times E_{645} - 3.96 \times E_{662} \\
    c(\text{carotenoids}) &= (1000 \times E_{470} - 2.27 \times c(\text{Chl }a) - 81.4 \times c(\text{Chl }b)) / 227, \text{ in } \mu g*ml^{-1}.
\end{align*}
\]

Data was normalized to the sample fresh weight to obtain pigment content of the rosette. Six independent replicates per condition were measured, averaged and SD was calculated.

**Catalase activity measurement**

Catalase activity was measured following the protocol described in Noctor *et al.* (2016) with some modifications, as follows. Total root and shoot extracts were prepared by grinding the frozen tissue using 1.4 mm ceramic beads and a Precellys 24 tissue homogenizer (Bertin Instruments) for 3 times 20 seconds, at 6500 rpm. The powder was homogenized in 0.1 M phosphate buffer, 1 mM EDTA (pH 7.5). The buffer volume was adjusted according to the fresh weight of the samples, so that 10 µl of buffer were added to each 1 mg of sample. The suspension was centrifuged for 10 min at 4 °C, at 13000 rpm. 40 mM H\(_2\)O\(_2\) was added to 180 µl of each extract. The samples were immediately placed in a 96-well plate reader (Infinite 200 PRO, Tecan) and the disappearance of H\(_2\)O\(_2\) absorption at 240 nm was recorded over time. The data from the first one minute of the reaction was used to calculate catalase activity, applying the H\(_2\)O\(_2\) molar extinction coefficient of 40 mM\(^{-1}\)*cm\(^{-1}\). The catalase activity for three independent replicates per condition were measured, averaged and SD was calculated.
H$_2$O$_2$ content measurement

H$_2$O$_2$ in root samples was measured using the Amplex Red H$_2$O$_2$-peroxidase assay kit (Molecular Probes) as described in detail in Brumbarova et al. (2016a). The H$_2$O$_2$ content values for three independent replicates per condition were measured, averaged and SD was calculated.

Seed Fe content measurement

The Fe content of seeds was measured using a colorimetric assay, adapted for plant seeds from the protocol of Tamarit et al. (2006). Seeds were dried overnight at 100 °C, grinded in an Agate mortar (Merck) and weighed. 10-15 mg of material were resuspended in 500 µl of 3 % HNO$_3$ and incubated for 16 h at 100 °C, followed by centrifugation for 5 min at 12000 rpm. 400 µl of 3 % HNO$_3$ containing either FeNaEDTA standard or seed material were mixed with 160 µl sodium ascorbate (38 mg/ml), 320 µl Fe chelator BPDS (1.7 mg/ml) and 126 µl ammonium acetate solution (diluted 1:3 from saturated solution). The specific absorbance of the Fe-chelator complex was recorded at 535 nm after 5 min, together with a reference measurement at 680 nm for nonspecific absorbance, using a 96-well plate reader (Infinite 200 PRO, Tecan). The obtained Fe concentration was normalized to the dry weight of the sample. The Fe content values for three independent replicates per genotype were obtained, averaged and SD was calculated.

Quantitative analysis of gene expression by RT-qPCR

Gene expression analysis was performed as described previously (Gratz et al., 2019). In short, total RNA was prepared from roots of Arabidopsis plants using the Spectrum Plant Total RNA kit (Sigma-Aldrich). cDNA was prepared using Oligo dT primer and RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific). For quantitative PCR, reactions were prepared with DyNAmo Color-Flash SYBR Green qPCR Kit (Thermo Fisher Scientific) and recorded in a C100 Touch PCR Cycler containing the CFX96 Real-Time System (Bio-Rad). The CFX Manager software (Bio-Rad) was used for data analysis. Primer pairs used in this study are listed in Table S1. Mass standards for each gene were used to calculate absolute gene expression, which was normalized to the expression of the reference EF1Ba gene. The gene expression was assayed on three independent replicates, averaged and SD was calculated.
**Histochemical staining of ROS**

To compare H$_2$O$_2$ accumulation in seedlings grown under Fe sufficient or deficient conditions, 3,3'-Diaminobenzidine (DAB, Sigma-Aldrich) staining was performed. After short vacuum infiltration (5 min, -400 mbar), seedlings were incubated for 1 h in the dark under gentle agitation in DAB solution (1 mg/ml DAB in 50 mM MES buffer, pH 6.5). Samples were rinsed once with ddH$_2$O. 80 % EtOH was added and seedlings were heated to 80 °C for 20 min. Staining was documented on an Axio Imager.M2 microscope (Zeiss).

The distribution of H$_2$O$_2$ and O$_2^-$ in roots was visualized by co-staining using the fluorescent dyes 3'-O-acetyl-6'-O-pentafluorobenzenesulfonyl-2',7'-difluorofluorescein (BES-H2O2-Ac, Wako Pure Chemical Corporation) and Dihydroethidium (DHE, Sigma-Aldrich). Seedlings grown under Fe sufficient (50 mM FeNaEDTA, + Fe) or deficient (0 mM FeNaEDTA, - Fe) conditions for ten days, were incubated for 30 min in the dark at RT in liquid half-strength Hoagland medium containing 50 µM BES-H$_2$O$_2$-Ac and 10 µM DHE.

**Fluorescence microscopy and image analysis**

Images of BES-H$_2$O$_2$-Ac and DHE-stained roots were taken immediately after the 30 min incubation. For imaging, Axio Imager.M2 microscope (Zeiss) was used. BES-H$_2$O$_2$-Ac dye was visualized using Filter set 38 (HE eGFP shift free (E) [EX BP 470/40, BS FT 495, EM BP 525/50]) and DHE using Filter set 43 (HE Cy 3 shift free (E) [EX BP 550/25, BS FT 570, EM BP 605/70]). All roots were pictured at 20x magnification in tiles mode and single images were stitched to produce a composite image of the root. Same imaging settings were employed for all genotypes and replicates.

Images obtained from fluorescent ROS staining were imported into ImageJ (http://rsbweb.nih.gov/ij/) for further analysis. To mark the region of interest (ROI) for signal intensity measurement, a segmented line, synchronized for DHE- and BES-H$_2$O$_2$-Ac, channels was superimposed onto the root. Absolute signal intensity was measured and values were imported into Microsoft Excel. Signal intensity of both channels was normalized to the maximum signal intensity measured for BES-H$_2$O$_2$-Ac to ensure correct representation of the DHE-to-BES-H$_2$O$_2$-Ac signal ratio. This maximum value was set to 100 and all other values are plotted relative to it. Relative signal intensity plots represent either the signal along the root or in virtual cross sections across three zones. Each plot is an average of three biological replicates with respective SD.
Statistical analysis

Data were analyzed using one-way ANOVA followed by Fisher’s Least Statistical Difference post hoc test in the SPSS Statistics software (IBM). Statistically significant differences were estimated based on P-values (P < 0.05).

Accession numbers

Sequence data from this article can be found in the TAIR and GenBank data libraries under accession numbers: BHLH038 (AT3G56970), BHLH039 (AT3G56980), BHLH047 (PYE, AT3G47640) BHLH100 (AT2G41240), BHLH101 (AT5G04150), BHLH104 (AT4G14410), BHLH105 (ILR3, AT5G54680), CAT1 (AT1G20630), CAT2 (AT4G35090), CAT3 (AT1G20620), FIT (AT2G28160), FRO2 (AT1G01580), IRT1 (AT4G19690), ZAT12 (AT5G59820).

Results

Root H$_2$O$_2$ content increases under prolonged Fe limitation

Increased H$_2$O$_2$ content in the root was previously shown to influence responses to prolonged Fe deficiency (Le et al., 2016). We aimed to understand whether this increase affected the whole root equally or whether the observation could be linked to certain root zones, such as those responsible for the acquisition of Fe. First, we performed a quantitative measurement of endogenous H$_2$O$_2$ in plants grown for ten days under either Fe sufficient or Fe deficient conditions. As quantified in Fig. 1A, the overall H$_2$O$_2$ content of Fe deficient plants was significantly increased compared to the control Fe condition. We next performed DAB staining for visualizing H$_2$O$_2$ in the root (Fig. 1B). A general increase in staining could be observed in Fe deficient roots, consistent with the quantitative data. To confirm the specificity of the staining, cotyledons of a control plant were manually wounded with forceps. The elicited stress response caused an increase in H$_2$O$_2$ levels in the whole seedling, resulting in strong DAB staining (Fig. 1B). The data suggested a general increase in H$_2$O$_2$ content throughout the root under prolonged Fe deficiency. However, due to the low dynamic range of the staining method, it did not provide sufficient detail on potential fine differences between different root zones.

ROS distribution depends on Fe availability and plant capacity to take up Fe

Previously, it was shown that the ratio between H$_2$O$_2$ and O$_2^-$ along the root is important for defining the differentiation state of the root cells (Reyt et al., 2015; Tsukagoshi et al., 2010). Therefore, in order to obtain a detailed map of ROS distribution within the root under prolonged Fe deficiency, we performed a dual histochemical staining for H$_2$O$_2$ and superoxide (O$_2^-$, Fig. 2), using
BES-H$_2$O$_2$-Ac and DHE, respectively. This allows to distinguish the tissue-level localization of the two ROS species simultaneously and in vivo. In wild-type Fe-sufficient roots, we observed strong H$_2$O$_2$ staining in the root tip and a gradual decrease of signal towards the root base. In the differentiation zone, the signal was observed in both the central cylinder and the epidermis (Fig. 2A-C, green channel). Signal quantification confirmed this, showing that the fluorescent intensity peaked in the transition zone between the meristem and the elongation zone (Fig. 2A, B, green channel). This observation is consistent with previous reports (Reyt et al., 2015; Tsukagoshi et al., 2010). O$_2^-$ pattern, on the other hand, differed greatly along the root. We observed a small maximum at the tip of the meristem above the stem cell niche. However, the majority of the signal was present in the differentiation zone, especially in the central cylinder (Fig. 2A-C, red channel). Prolonged Fe deficiency resulted in the expansion of the H$_2$O$_2$ domain within the meristem together with a marked increase in signal intensity throughout the root (Fig. 2D-F, green channel), consistent with our DAB staining and the quantitative H$_2$O$_2$ measurement.

The FIT loss-of-function mutant fit-3 (Jakoby et al., 2004) was previously reported to lack prolonged Fe deficiency-induced H$_2$O$_2$ accumulation, suggesting that ROS homeostasis might be under the control of FIT (Le et al., 2016). Roots of fit-3 grown under control Fe condition showed distribution patterns for both H$_2$O$_2$ and O$_2^-$ comparable to the wild type (Fig. 3A-C). As expected, under Fe deficiency the H$_2$O$_2$ pattern remained unchanged compared to the sufficient Fe condition (Fig. 3D-F). A prominent effect occurred under Fe deficiency, where the lateral distribution of the O$_2^-$ signal shifted from the central cylinder and was instead very prominent in the fit-3 root periphery (Fig. 3D and F). Our findings suggest, that in absence of FIT the H$_2$O$_2$-to-O$_2^-$ ratio cannot adjust to the prolonged Fe deficiency conditions, implying a major role of the Fe deficiency regulator FIT in ROS signaling as part of the Fe deficiency response cascade.

**FIT is required for the transcriptional regulation of H$_2$O$_2$ dismutases in roots**

One possible explanation for the observed H$_2$O$_2$ localization in the root under Fe deficiency is differential activity of H$_2$O$_2$ metabolizing enzymes. FIT may affect the formation of ROS at the transcriptional level, e.g. through controlling gene expression of catalases, enzymes that eliminate H$_2$O$_2$.

We analyzed the gene expression of three catalase isoforms, CAT1, CAT2, and CAT3, due to their very prominent activity and the dependence of their products on the Fe state of the plant, being Fe cofactor-dependent enzymes (Nicholls et al., 2000). For identifying potential Fe and FIT transcriptional targets among the three catalase genes, we reasoned that the candidate regulatory
gene should be upregulated under Fe deficiency and behave oppositely in the absence of functional FIT.

Gene expression profiling revealed that CAT1 underwent downregulation under prolonged Fe deficiency in wild type, whereas, in the fit-3 mutant a reduced and non-Fe-responsive expression was observed (Fig. 4A). CAT2 showed a significant upregulation under prolonged Fe deficiency in wild type. Interestingly, in a FIT loss-of-function situation its regulation was exactly opposite to that observed in wild type (Fig. 4B), suggesting a strong dependence of CAT2 expression on the function of FIT. Furthermore, CAT2 abundance under low Fe supply is most probably a compensatory mechanism to counteract excess H$_2$O$_2$ levels, hence preventing toxic H$_2$O$_2$ effects. CAT3, similarly to CAT1, was downregulated by prolonged low Fe availability in wild type. (Fig. 4C) This tendency remained in the fit-3 mutant, however, in the absence of FIT, the overall expression of the gene was strongly reduced (Fig. 4C).

The expression analysis showed that all three catalase-encoding genes were dynamically regulated in response to prolonged Fe deprivation and their expression depended to a different extent on the presence of the central Fe acquisition transcriptional regulator FIT. The above described CAT2 expression changes under prolonged Fe deficiency suggested that the CAT2 protein might play a role in modulating H$_2$O$_2$ levels in these growth conditions.

**Perturbation of intracellular H$_2$O$_2$ homeostasis compromises root Fe acquisition**

Our gene expression analysis revealed CAT2 as a potential FIT-dependent regulator of H$_2$O$_2$ levels under prolonged Fe deficiency. To understand how CAT2 participates in the regulation of Fe deficiency responses, we investigated the Fe response in the previously characterized CAT2 loss-of-function mutant cat2-1 (Bueso et al., 2007; Queval et al., 2007). In wild-type plants, Fe deficiency resulted in the well-documented elongation of the primary root (Gratz et al., 2019; Gruber et al., 2013; Ivanov et al., 2014), which was also the case for the cat2-1 mutant. However, the length of the primary cat2-1 root was reduced by 13 % compared to the wild type. Under sufficient Fe supply, cat2-1 root length was reduced even more, by 35 % compared to the wild type (Fig. 5A, B). This could be due to the combined deleterious effects of reduced H$_2$O$_2$ scavenging capacity of the mutant and the available Fe.

Although cat2-1 roots were able to elongate under prolonged Fe deficiency, the penalty on the shoot weight of the mutant was strong under both Fe supply conditions (Fig. 5C), probably due to the photorespiratory phenotype of cat2-1, and consistent with the documented major role of CAT2 in controlling ROS homeostasis in leaves (Mhamdi et al., 2010).
The degradation of photosynthetic pigments is a well-documented effect under Fe deficiency (Ivanov et al., 2012) and we could observe it in our test system (Fig. 5D). In cat2-1 plants, the overall levels of chlorophyll and carotenoids were significantly lower than in wild type and were further reduced under prolonged Fe limitation (Fig. 5D). Root surface ferric reductase activity was tested as an indicator for the plant capacity to take up Fe. Activity levels were reduced in cat2-1 even though the ferric reductase responded to Fe limitation (Fig. 5E). We then grew plants on soil and measured the Fe content in their seeds, as a direct consequence of the plant Fe status (Fig. 5F). In confirmation to our observations so far, cat2-1 seeds contained low levels of Fe, comparable to the strongly Fe-deficient fit-3 mutant (Fig. 5F). Overall, we characterized cat2-1 loss-of-function plants as strongly Fe-deficient and unable to induce Fe uptake to counteract Fe deficiency.

**CAT2 participates in modulating root ROS accumulation under prolonged Fe deficiency**

The impaired Fe mobilization under prolonged Fe deficiency in the cat2-1 mutant suggests that enhanced H$_2$O$_2$ was responsible for this effect. In order to analyze the significance of CAT2 for ROS accumulation under prolonged Fe deficiency, we first evaluated the expression of all CAT genes in cat2-1 plants (Fig. 6A-C). As expected, CAT1 expression was reduced under Fe deficiency in the wild type, while the levels in cat2-1 remained comparable to the wild-type sufficient Fe condition. As expected, CAT2 transcript was not detected in the cat2-1 mutant, and CAT3 showed similar expression tendency as CAT1 (Fig. 6A-C). This indicated that the expression of CAT1 and CAT3 was higher than usual in the absence of CAT2 under prolonged Fe deficiency and pointed towards potential compensatory regulation of CAT1 and CAT3 for the missing CAT2, most likely in response to enhanced ROS levels. To test the effect of this potential compensation, we performed a total catalase activity measurement in shoots and roots of wild-type and cat2-1 plants. In shoots, lack of CAT2 led to a dramatic reduction in overall shoot catalase activity under both sufficient Fe and prolonged Fe deficiency (Fig. 6D), consistent with available data (Queval et al., 2007). In roots, the effect was also significant but less prominent (Fig. 6E). Fe deficient wild-type root extracts showed enhanced catalase activity compared to those from sufficient Fe-grown roots. Compared to wild type, in cat2-1, the catalase activity under sufficient Fe was similar, however, it was significantly reduced under prolonged Fe deficiency (Fig. 6E). At the same time, the fit-3 mutant showed strongly reduced catalase activity (Supplemental Figure 1), suggesting that the presence of a functional FIT positively influences the catalase function. We then tested the net outcome of these effects by measuring the total H$_2$O$_2$ content of the root. As already shown, prolonged Fe deficiency led to a marked increase in H$_2$O$_2$ levels in wild type (Fig. 6F). In cat2-1, the overall H$_2$O$_2$ accumulation was increased compared to wild type under both conditions, indicating a prominent role of CAT2 for
maintaining the ROS balance in roots (Fig. 6F). The data suggests that despite potential compensation from CAT1 and CAT3, CAT2 is the isoform utilized by roots to control the levels of intracellular \( \text{H}_2\text{O}_2 \) under prolonged Fe limitation. One must note that additional ROS scavenging enzymes might play a role in this process, explaining for example the pronounced \( \text{H}_2\text{O}_2 \) increase in cat2-1 under sufficient Fe supply.

To estimate the effect of CAT2 at the organ level, we stained cat2-1 roots for \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) (Fig. 7). Compared to wild type (Fig. 2), cat2-1 showed similar staining pattern with a \( \text{H}_2\text{O}_2 \) peak in the transition zone and a small \( \text{O}_2^- \) peak in the meristem, however, we observed major changes in the differentiation zone (Fig. 7A-C, green channel). Here, strong \( \text{H}_2\text{O}_2 \) signal was observed in the epidermis, particularly prominent in some but not all root hair cells. \( \text{O}_2^- \) signal was confined mainly in the central cylinder, however, along the length of the root we observed zones without staining (Fig. 7A, B, red channel). These zones lacking \( \text{O}_2^- \) were more prominent and more frequent under prolonged Fe deficiency, where also \( \text{H}_2\text{O}_2 \) staining was strong, especially in root-hair cells close to the root tip (Fig. 7D-F). The overall picture in the absence of CAT2 showed a disturbed ROS homeostasis, with over-pronounced \( \text{H}_2\text{O}_2 \) accumulation in the differentiation zone across the root, masking \( \text{O}_2^- \) levels, hence resulting in a disturbed balance between the ROS species under prolonged Fe deficiency. These enhanced \( \text{H}_2\text{O}_2 \) levels could provoke stress-response signals due to ROS toxicity, overpowering the signaling role of \( \text{H}_2\text{O}_2 \) in activating the Fe deficiency response network.

Coordination of transcriptional Fe homeostasis response is compromised in the absence of a functional CAT2 gene

Based on the observed phenotypes of deregulated Fe mobilization in cat2-1 loss-of-function plants, we next aimed to uncover the transcriptional regulation that underlies this susceptibility to prolonged Fe deficiency. We first investigated the expression of the Fe uptake-related genes that are under the control of FIT, namely \( \text{FIT} \) itself, \( \text{FRO2} \) and \( \text{IRT1} \) (Fig. 8A-C). In wild type, all three genes performed as previously reported, with expression upregulated under Fe deficiency. However, in the cat2-1 mutant, the expression was markedly decreased in both Fe conditions relative to wild type (Fig. 8A-C). This suggests that together with the increased \( \text{H}_2\text{O}_2 \) levels in the absence of CAT2, Fe uptake genes undergo repression or are hardly expressed, consistent with the reported effects of external \( \text{H}_2\text{O}_2 \) application and the action of ZAT12 as FIT inhibitor (Le et al., 2016). The ROS-responsive \( \text{ZAT12} \) gene, showed strong upregulation in cat2-1, as expected because of enhanced \( \text{H}_2\text{O}_2 \) levels in this mutant (Supplemental Fig. 2). We then tested the expression of the \( \text{BHLH039} \) gene encoding a FIT partner and positive Fe deficiency response regulator (Wang et al., 2007; Wang et al., 2013; Yuan et al., 2008). \( \text{BHLH039} \) transcriptional upregulation is not require FIT protein but,
instead, it is directly coupled to an Fe deficiency signaling cascade upstream of FIT, mediated by bHLH transcription factors of the subgroups IVb and IVc, such as URI and ILR3 (Gao et al., 2020a; Kim et al., 2019; Lei et al., 2020; Samira et al., 2018; Tissot et al., 2019). In absence of FIT, the BHLH039 gene is strongly upregulated, however, in the absence of ZAT12 BHLH039 expression is reduced. These expression patterns can be explained by the different Fe levels, caused by different FIT activity, in wild-type and zat12 mutant plants (Le et al., 2016). Interestingly, BHLH039 expression in cat2-1 was strongly reduced (Fig. 8D), similarly to FIT. This suggested that the link coordinating the expression of the two genes is dependent on the function of CAT2. The genes encoding the closest bHLH039 homologs, BHLH038, BHLH100 and BHLH101 were similarly downregulated in cat2-1 in comparison to the wild type (Supplemental Fig. 3A-C). Thus, the upstream bHLH signaling cascade involving the bHLH subgroup IVb and IVc transcription factors is also dependent on ROS regulation. This was confirmed when we tested additional regulatory genes, encoding the Fe homeostasis regulator PYE (Long et al., 2010), from subgroup IVb, and the upstream regulators ILR3 and BHLH104 (Zhang et al., 2015), from subgroup IVc. PYE gene expression is coregulated with that of BHLH039, and similarly to BHLH039, the expression of PYE was reduced in the cat2-1 mutant (Fig. 8E), confirming that the upstream regulation is affected by cellular H$_2$O$_2$ levels. In contrast, ILR3 (Fig. 8F) and BHLH104 (Supplemental Fig. 3D) expression remained unaffected in cat2-1 compared to wild type, suggesting that a H$_2$O$_2$-dependent posttranslational event inhibits the activity of ILR3 and its homologs in response to prolonged Fe deficiency.

Discussion

ROS signaling is one of the key determinants underlying plant environmental responses (He et al., 2018). Among ROS, H$_2$O$_2$ is directly involved in the regulation of responses to prolonged Fe deficiency through promoting the expression of the transcription factor ZAT12 and regulating its stability (Brumbarova et al., 2016b; Le et al., 2016). In this study, we demonstrate that the accumulation of H$_2$O$_2$ in Arabidopsis roots under prolonged Fe deficiency is dependent on the activity of CAT2. With the help of a CAT2 loss-of-function mutant, we were able to demonstrate that H$_2$O$_2$-dependent regulation affects not only the Fe uptake-related FIT target genes but also the genes encoding the transcription factors from subgroup Ib (bHLH038, bHLH039, bHLH100, bHLH101) and PYE. The genes encoding the upstream regulators ILR3 and bHLH104 are themselves not affected by the increased H$_2$O$_2$ levels in the cat2-1 mutant. However, ILR3 and bHLH104 protein activity is affected, a fact that points in the direction of a H$_2$O$_2$-dependent posttranslational regulation of these proteins (Fig. 9).
Our data suggests that the role of CAT2 in this complex regulatory interplay most probably is to control the upper limit of available \( \text{H}_2\text{O}_2 \) under prolonged Fe deficiency. Under this condition, an as yet unidentified \( \text{H}_2\text{O}_2 \) source is activated and CAT2 activity is enhanced to maintain a balance of higher than usual cellular \( \text{H}_2\text{O}_2 \) levels. The data suggests that even though in roots CAT2 shares activity with the two other isoforms, CAT1 and CAT3, it is still the predominant catalase isoform determining \( \text{H}_2\text{O}_2 \) degradation under prolonged Fe deficiency. This is supported by the fact that cat2-1 plants exhibit decreased catalase activity and increased \( \text{H}_2\text{O}_2 \) cellular levels compared to wild type under these conditions. In terms of gene expression, previous studies reported no compensatory effect of CAT1 and CAT3 expression in the absence of CAT2 in leaves (Queval et al., 2007). Under Fe deficiency however, the expression of CAT1 and CAT3 was increased in roots of cat2-1 compared to wild type, probably as a compensation attempt for the missing CAT2.

The obtained data supports a model in which CAT2 is deeply integrated in the cellular regulatory events under prolonged Fe limitation (Fig. 9). The CAT2 gene closely responds to the Fe status of the root and the presence of FIT, suggesting that it is either a direct FIT target or a strongly FIT-dependent gene. Indirectly, through \( \text{H}_2\text{O}_2 \) availability and potentially additional players, including FIT, the activity of CAT2 modulates the functionality of the Fe response transcriptional cascade. As CAT2 gene is dependent on the function of this cascade, this also functions as a feedback loop for the regulation of CAT2. An additional level of interplay between CAT2 and Fe response comes from the end result of the transcriptional cascade, namely the production of the IRT1 transporter, that provides Fe necessary for the enzymatic activity of CAT2.

An interesting question concerns the localization of the CAT2-derived effects, both on subcellular and whole organ level. CAT2 was known as an exclusively peroxisomal protein, however recent studies clearly demonstrate the presence, function and significance of catalases outside the peroxisome. Examples are available for additional CAT roles in the cytoplasm and the nucleus (He et al., 2018). It has also been proposed that CATs can relocate in the cell in response to redox signals (Foyer et al., 2020). At this point, it is not possible to speculate if a specific compartment might harbor catalase activity orchestrating the responses to prolonged Fe limitation. At the same time however, our data clearly suggests the domains within the root where the described regulatory events might take place. In wild-type plants under prolonged Fe deficiency, the \( \text{H}_2\text{O}_2 \) levels in the differentiation zone of the root was clearly increased, especially in regions where \( \text{O}_2^- \), visible in the central cylinder, reached a plateau. \( \text{H}_2\text{O}_2 \) presence in this region, which we refer to as the early differentiation zone, is very pronounced in root hair cells, coinciding very well with the reported expression of the principal Fe transporter IRT1 (Barberon et al., 2011; Blum et al., 2014). These observations suggest that the root hair cells of the early differentiation zone are an important
location where the described regulatory crosstalk occurs. This is supported by the fact that under prolonged Fe deficiency, *cat2-1* plants show strong \( \text{H}_2\text{O}_2 \) signal in root hair cells of this region, suggesting that in root hair cells the role of CAT2 is to metabolize excess \( \text{H}_2\text{O}_2 \) to maintain ROS balance and coordinate Fe responses.

Excess Fe supply is another condition that requires adjusting the Fe uptake capacity of the plant to avoid Fe toxicity. It was previously demonstrated that the balance between \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) in the root meristematic and differentiation zones partially mediated the response of the root system architecture to Fe excess (Reyt *et al.*, 2015).

At present, the details of the mechanistic role of \( \text{H}_2\text{O}_2 \) in the process remain unclear. FIT and its target genes are dependent on the presence of bHLH039. BHLH39 and PYE are co-expressed, and their promoters are upregulated in response to Fe deficiency by the bHLH subgroup IVc transcription factors (Gao *et al.*, 2019). Subgroup IVc bHLHs ILR3 and BHLH104 are transcriptionally upregulated by Fe deficiency (Samira *et al.*, 2018; Wang *et al.*, 2017), similarly to FIT and subgroup Ib bHLHs. Very interestingly, however, their gene expression was not affected by ROS in the *cat2-1* mutant, while their protein activity, judged by the expression of their direct target genes *BHLH039* and *PYE*, was inhibited by ROS in *cat2-1*. Low subgroup Ib transcription factor activity leads to lower FIT activity and Fe acquisition downstream of FIT in *cat2-1*. Our data conform with previous observations from *BHLH039* overexpression in plants (Naranjo-Arcos *et al.*, 2017). It was shown that increased Fe levels due to *BHLH039* overexpression resulted in a downregulation of all BHLH subgroup Ib genes and *PYE*, indicating that the Fe deficiency response cascade acting upstream of these genes was not active. At the same time, *BHLH039* overexpressing plants had high levels of ROS and accordingly elevated ZAT12 expression along with symptoms of oxidative stress. Here, we show that the presence of ROS, even in the absence of Fe, is responsible for switching off the Fe deficiency response. ZAT12 may be one of the regulators mediating the inhibition through FIT interaction.

Therefore, in our model, we propose that \( \text{H}_2\text{O}_2 \) posttranslationally inhibits transcriptional regulators, such as ILR3 and bHLH104, and therefore causes the observed downstream transcriptional changes. Redox-dependent conformational changes have been shown to affect protein activity and nuclear localization, e.g., of NONEXPRESSOR OF PATHOGENESIS-RELATED GENE 1 (NPR1), involved in pathogen response (Mou *et al.*, 2003; Tada *et al.*, 2008). Alternatively, or additionally, redox-sensing mechanisms may involve differential binding to a partner protein, altered protein stability and DNA binding activity (He *et al.*, 2018). Further studies are needed to determine whether the transcription factor proteins ILR3, bHLH104 or other subgroup IVc proteins or URI are directly controlled by ROS. Their activity is regulated by Fe-dependent posttranslational control mechanisms. These include for
example E3 ligases, determining bHLH transcription factor protein abundance, and their activity might be as well ROS-controlled (Hindt et al., 2017).

Previous studies have proposed that ROS regulation displays considerable interplay with other compounds, such as reactive nitrogen species (Wang and Chu, 2020), among which nitric oxide has been shown to affect plant responses to Fe limitation (Graziano and Lamattina, 2007; Meiser et al., 2011). Another interesting aspect of ROS is their interplay with calcium (Ca$^{2+}$) signaling (Kollist et al., 2019). The signal relay between ROS and Ca$^{2+}$ in the central cylinder may result in the generation of O$_2^•$ through the action of RESPIRATORY BURST OXIDASE HOMOLOGUES (RBOHs). As this radical is short-lived and quickly converts to H$_2$O$_2$ (Jimenez-Quesada et al., 2016), it is of great interest as a potential H$_2$O$_2$ donor for the above-described signaling events. The subcellular origin of H$_2$O$_2$ production is important, as it may influence the transcriptional outcome (Sewelam et al., 2014). Excess of H$_2$O$_2$ might inhibit the Ca$^{2+}$-ROS cascade and such an inhibition might be the reason for the “gaps” we observed in the O$_2^•$ staining in the central cylinder of cat2-1 plants. Due to this, we cannot exclude that disturbed Ca$^{2+}$ signaling might contribute to the Fe response phenotypes of CAT2 loss-of-function plants, as Ca$^{2+}$ is documented to play a role in both transcriptional (Gratz et al., 2019) and posttranslational regulation of Fe import (Dubeaux et al., 2018; Khan et al., 2019), targeting FIT and IRT1 activity, respectively.

In summary, here we described a role for CAT2 in modulating the root responses to prolonged Fe deficiency. This is achieved through the regulation of H$_2$O$_2$ levels in root epidermis cells, where the Fe deficiency-induced increase of generated H$_2$O$_2$ is counteracted by CAT2 to a level that prevents toxic H$_2$O$_2$ effects and yet allows maintaining sufficient H$_2$O$_2$ amounts to coordinate the transcriptional Fe response. Through mutual regulation, CAT2 and FIT represent components of a regulatory loop for regulation of plant responses to Fe deficiency, where H$_2$O$_2$ functions as an adjustable signaling intermediate.
Author contributions
RI, TB, PB conceived the project; CS, RI, ME, TB performed experiments; CS, RI, TB analyzed data; all authors discussed results; CS, RI, TB wrote the original draft and prepared the figures; all authors contributed to finalizing the manuscript; PB acquired funding.

Data Availability
The data supporting the findings of this study are available from the corresponding author, Tzvetina Brumbarova, upon request.

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Figure legends

Fig. 1. Root H$_2$O$_2$ content is increased under prolonged Fe deficiency. (A) Quantitative H$_2$O$_2$ measurement in wild-type roots grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply (n = 3). Bars represent mean values ± SD. Different letters indicate statistically significant differences (P < 0.05). (B) Histochemical H$_2$O$_2$ staining of wild-type roots grown for 10 days under +Fe or -Fe. Positive control: seedling hurt by wounding to induce H$_2$O$_2$ accumulation. Size bars: 0.5 cm.

Fig. 2. Histochemical H$_2$O$_2$ and O$_2^•$− staining in wild-type roots grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply. (A) Staining example of a root grown under +Fe. (B) Longitudinal signal intensity scan of roots grown under +Fe, ± SD. (C) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (A) of roots grown under +Fe. (D) Staining example of a root grown under -Fe. (E) Longitudinal signal intensity scan of roots grown under -Fe. (F) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (D) of roots grown under -Fe. All graphs represent averaged data from five roots, ± SD. Size bars in (A) and (D): 0.5 cm.

Fig. 3. Histochemical H$_2$O$_2$ and O$_2^•$− staining in fit-3 roots grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply. (A) Staining example of a root grown under +Fe. (B) Longitudinal signal intensity scan of roots grown under +Fe supply, ± SD. (C) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (A) of roots grown under +Fe. (D) Staining example of a root grown under -Fe. White arrowheads highlight the predominant O$_2^•$− signal in the peripheral root zones. (E) Longitudinal signal intensity scan of roots grown under -Fe. (F) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (D) of roots grown under -Fe. White arrowheads in the top graph highlight the predominant O$_2^•$− signal in the peripheral root zones and correspond to the arrowheads in (D). All graphs represent averaged data from five roots, ± SD. Size bars in (A) and (D): 0.5 cm.

Fig. 4. Expression of the genes encoding three Arabidopsis catalase isoforms, (A) CAT1, (B) CAT2 and (C) CAT3, in roots of 10 days-old wild-type and fit-3 mutant plants grown under sufficient (+Fe) or deficient (-Fe) Fe supply (n = 3). Bars represent mean values ± SD. Different letters indicate statistically significant differences (P < 0.05).
Fig. 5. Fe-related phenotypes of cat2-1 mutant plants. (A) Representative images of wild-type and cat2-1 plants grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply. Size bars: 1 cm. (B) Root length quantification (n = 27 to 32). (C) Shoot weight quantification (n = 6). (D) Photosynthetic pigment quantification (n = 6). (E) Ferric reductase activity (n = 6). (F) Seed Fe content of soil-grown wild-type, cat2-1 and fit-3 plants (n = 3). (B-E) Bars represent mean values, ± SD. Different letters indicate statistically significant differences (P < 0.05).

Fig. 6. Role of CAT2 in root catalase activity and H$_2$O$_2$ accumulation under prolonged Fe deficiency. (A-C) Gene expression of (A) CAT1, (B) CAT2 and (C) CAT3 in wild-type and cat2-1 plants grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply (n = 3). (D) Shoot catalase activity in wild type and cat2-1 (n = 3). (E) Root catalase activity in wild type and cat2-1 (n = 3). Bars represent mean values, ± SD. Different letters indicate statistically significant differences (P < 0.05).

Fig. 7. Histochemical H$_2$O$_2$ and O$_2^-$ staining in cat2-1 roots grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply. (A) Staining example of a root grown under +Fe. (B) Longitudinal signal intensity scan of roots grown under +Fe, ± SD. (C) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (A) of roots grown under +Fe. (D) Staining example of a root grown under -Fe. (E) Longitudinal signal intensity scan of roots grown under -Fe. (F) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (D) of roots grown under -Fe. All graphs represent averaged data from five roots ± SD. Yellow arrowheads in (A) and (D) indicate regions along the central cylinder lacking O$_2^-$ signal. Size bars in (A) and (D): 0.5 cm.

Fig. 8. Regulation of Fe acquisition and homeostasis genes in response to increased H$_2$O$_2$ caused by the absence of CAT2. (A-F) Gene expression of (A) FIT, (B) FRO2, (C) IRT1, (D) BHLH039, (E) PYE and (F) BHLH105 (ILR3) in wild-type and cat2-1 plants grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply (n = 3). Bars represent mean values, ± SD. Different letters indicate statistically significant differences (P < 0.05).

Fig. 9. Proposed model for the role of FIT, CAT2 and H$_2$O$_2$ in the regulation of Fe acquisition and homeostasis under prolonged Fe deficiency. Subgroup IVC proteins ILR3 and bHLH104, together with other transcription factors (not depicted), form a complex with PYE and regulate the expression of downstream Fe homeostasis genes (dark punctated arrows), including PYE itself and subgroup Ib BHLHs (BHLH038, BHLH039, BHLH100, BHLH101). Subgroup Ib bHLHs interact with FIT, a complex
which positively regulates FIT, Fe acquisition genes, such as FRO2 and IRT1, and CAT2. CAT2 protein is a heme-containing H$_2$O$_2$ dismutating enzyme, which requires Fe, imported with the help of FRO2 ferric reductase and the transporter IRT1. Under prolonged Fe deficiency, H$_2$O$_2$ production is increased and acts in two directions (red punctated lines). First, high H$_2$O$_2$ levels negatively influence the positive regulation by PYE-ILR3/bHLH104. Thus, transcription of PYE and subgroup lb BHLH genes is downregulated. This depletion of subgroup lb bHLH proteins is the first factor that negatively influences the formation of the positive Fe acquisition transcriptional complex FIT-bHLH lb and results in attenuation of FRO2 and IRT1 expression, and therefore Fe import. The second effect of H$_2$O$_2$ accumulation is the activation of the ROS-responsive transcription factor ZAT12. ZAT12 can interact with FIT, forming an inactive FIT complex. This is the second factor that prevents the formation of the FIT-bHLH lb positive regulatory complex. The role of CAT2 would be to limit the H$_2$O$_2$ levels by dismutating the excess H$_2$O$_2$ to balance the efficiency at which the transcriptional regulatory cascade functions.
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Fig. 1. Root H$_2$O$_2$ content is increased under prolonged Fe deficiency. (A) Quantitative H$_2$O$_2$ measurement in wild-type roots grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply (n = 3). Bars represent mean values ± SD. Different letters indicate statistically significant differences (P < 0.05). (B) Histochemical H$_2$O$_2$ staining of wild-type roots grown for 10 days under +Fe or -Fe. Positive control: root hurt by wounding to induce H$_2$O$_2$ accumulation. Size bars: 0.5 cm.
Fig. 2. Histochemical H$_2$O$_2$ and O$_2^-$ staining in wild-type roots grown for 10 days under sufficient (+Fe) or deficient Fe supply (-Fe). (A) Staining example of a root grown under +Fe. (B) Longitudinal signal intensity scan of roots grown under +Fe, ± SD. (C) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (A) of roots grown under +Fe. (D) Staining example of a root grown under -Fe. (E) Longitudinal signal intensity scan of roots grown under -Fe. (F) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (D) of roots grown under -Fe. All graphs represent averaged data from five roots, ± SD. Size bars in (A) and (D): 0.5 cm.
Fig. 3. Histochemical $H_2O_2$ and $O_2^{−}$ staining in fit-3 roots grown for 10 days under sufficient (+Fe) or deficient Fe supply (-Fe). (A) Staining example of a root grown under sufficient Fe supply. (B) Longitudinal signal intensity scan of roots grown under sufficient Fe supply ± SD. (C) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (A) of roots grown under sufficient Fe supply. (D) Staining example of a root grown under deficient Fe supply. White arrowheads highlight the predominant $O_2^{−}$ signal in the peripheral root zones. (E) Longitudinal signal intensity scan of roots grown under deficient Fe supply. (F) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (D) of roots grown under deficient Fe supply. The white arrowheads in the top graph highlight the predominant $O_2^{−}$ signal in the peripheral root zones and correspond to the arrowheads in (D). All graphs represent averaged data from five roots ± SD. Size bars in (A) and (D) represent 0.5 cm.
Fig. 4. Expression of the genes encoding three Arabidopsis catalase isoforms, (A) CAT1, (B) CAT2 and (C) CAT3, in roots of 10 days-old wild-type and ft-3 mutant plants grown under sufficient (+Fe) or deficient (-Fe) Fe supply (n = 3). Bars represent mean values ± SD. Different letters indicate statistically significant differences (P < 0.05).
Fig. 5. Fe-related phenotypes of cat2-1 mutant plants. (A) Representative images of wild type and cat2-1 plants grown for 10 days under sufficient (+Fe) or deficient Fe supply (-Fe). (B) Root length quantification of at least 150 plants of each genotype and condition. (C) Shoot weight quantification of at least 150 plants of each genotype and condition. (D) Photosynthetic pigment quantification of wild type and cat2-1 shoots. (E) Ferric reductase activity of of wild type and cat2-1 shoots. (F) Seed Fe content of soil-grown wild type, cat2-1 and fit-3. Size bars in (A) represent 1 cm. In (B-E), bars in (D-F) represent mean values of three to five independent experiments ± SD. Different letters indicate statistically significant differences (P < 0.05).
Fig. 6. Role of CAT2 in root catalase activity and H$_2$O$_2$ accumulation under prolonged Fe deficiency. (A-C) Gene expression of (A) CAT1, (B) CAT2 and (C) CAT3 in wild-type and cat2-1 plants grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply (n = 3). (D) Shoot catalase activity in wild type and cat2-1 (n = 3). (E) Root catalase activity in wild type and cat2-1 (n = 3). (F) Root H$_2$O$_2$ content in wild type and cat2-1 (n = 3). Bars represent mean values, ± SD. Different letters indicate statistically significant differences (P < 0.05).
Fig. 7. Histochemical \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) staining in cat2-1 roots grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply. (A) Staining example of a root grown under +Fe. (B) Longitudinal signal intensity scan of roots grown under +Fe, ± SD. (C) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (A) of roots grown under +Fe. (D) Staining example of a root grown under -Fe. (E) Longitudinal signal intensity scan of roots grown under -Fe. (F) Lateral signal intensity scans at three different positions, indicated as “a”, “b” and “c” in (D), roots grown under -Fe. All graphs represent averaged data from five roots ± SD. Yellow arrowheads in (A) and (D) indicate regions along the central cylinder lacking \( \text{O}_2^- \) signal. Size bars in (A) and (D): 0.5 cm.
Fig. 8. Regulation of Fe acquisition and homeostasis genes in response to increased H$_2$O$_2$ caused by the absence of CAT2. (A-F) Gene expression of (A) FIT, (B) FRO2, (C) IRT1, (D) BHLH039, (E) PYE and (F) BHLH105 (ILR3) in wild-type and cat2-1 plants grown for 10 days under sufficient (+Fe) or deficient (-Fe) Fe supply (n = 3). Bars represent mean values, ± SD. Different letters indicate statistically significant differences (P < 0.05).
Fig. 9. Proposed model for the role of FIT, CAT2 and H$_2$O$_2$ in the regulation of Fe acquisition and homeostasis under prolonged Fe deficiency. Subgroup IVc proteins ILR3 and bHLH104, together with other transcription factors (not depicted), form a complex with PYE and regulate the expression of downstream Fe homeostasis genes (dark punctated arrows), including PYE itself and subgroup Ib BHLHs (BHLH038, BHLH039, BHLH100, BHLH101). Subgroup Ib BHLHs interact with FIT, a complex which positively regulates FIT, Fe acquisition genes, such as FRO2 and IRT1, and CAT2. CAT2 protein is a heme-containing H$_2$O$_2$ dismutating enzyme, which requires Fe$_3^+$ imported with the help of FRO2 ferric reductase and the transporter IRT1. Under prolonged Fe deficiency, H$_2$O$_2$ production is increased and acts in two directions (red punctated lines). First, high H$_2$O$_2$ levels negatively influence the positive regulation by PYE-ILR3/bHLH104. Thus, transcription of PYE and subgroup Ib BHLH genes is downregulated. This depletion of subgroup Ib bHLH proteins is the first factor that negatively influences the formation of the positive Fe acquisition transcriptional complex FIT-bHLH Ib and results in attenuation of FRO2 and IRT1 expression, and therefore Fe import. The second effect of H$_2$O$_2$ accumulation is the activation of the ROS-responsive transcription factor ZAT12. ZAT12 can interact with FIT, forming an inactive FIT complex. This is the second factor that prevents the formation of the FIT-bHLH Ib positive regulatory complex. The role of CAT2 would be to limit the H$_2$O$_2$ levels by dismutating the excess H$_2$O$_2$ to balance the efficiency at which the transcriptional regulatory cascade functions.