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# Functional characterization of *MdIMA1* from apple (*Malus domestica*) in modulating iron homeostasis

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## Abstract

Iron (Fe), an essential micronutrient for plants, critically influences crop yield and quality. The *IRON MAN* (*IMA*) gene family regulates Fe uptake, but its functional role in apple (*Malus domestica*) remains unclear. This study performed phylogenetic and tissue-specific expression analyses of the *MdIMAs* gene family in apple under Fe deficiency. Among the *MdIMAs* genes, *MdIMA1* was revealed to act as the key regulatory gene involved in Fe deficiency response. Heterologous expression of *MdIMA1* in *Arabidopsis thaliana* significantly enhanced Fe deficiency tolerance, as manifested by increased fresh weight, chlorophyll content, ferric chelate reductase (FCR) activity, plasma membrane H<sup>+</sup>-ATPase activity, and Fe concentration. Moreover, genes associated with rhizosphere acidification and Fe transport and absorption were upregulated upon *MdIMA1* overexpression, leading to promoted Fe uptake and utilization. Similarly, heterologous expression in tomato improved fresh weight and chlorophyll content, and enhanced both fruit yield and Fe concentration under Fe-deficient conditions. Meanwhile, *MdIMA1* overexpression in apple calli resulted in elevated Fe deficiency tolerance through increasing H<sup>+</sup>-ATPase activity, while silencing *MdIMA1* reduced this activity. Collectively, these results indicate that *MdIMA1* positively regulates Fe uptake and utilization, providing a valuable target gene of significance for improving Fe nutrition in apples.

**Keywords** Apple, Fe deficiency, Fe uptake, *MdIMA1*, Plasma membrane H<sup>+</sup>-ATPase

## Introduction

Iron (Fe), an essential trace element for plant metabolic activities, is critical for growth and development. It is a key component of numerous proteins and enzymes, including ferritin, ferrous oxidases, and Fe-sulfur proteins (Connorton et al. 2017). Fe also plays an indispensable role in photosynthesis, mitochondrial energy metabolism, and the formation of the core components of the electron transport chain (Connorton et al. 2017). As one of the most abundant elements in Earth's crust, Fe's bioavailability in soil is often limited, particularly in oxic and alkaline conditions, due to the low solubility of its free form. This limitation results in widespread Fe deficiencies in plants (Narender et al. 2018). Fe deficiency impairs photosynthetic efficiency and disrupts chlorophyll biosynthesis (Kroh And Pilon 2020). Early signs of

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Fe deficiency include interveinal chlorosis, where veins remain green while adjacent mesophyll tissue becomes lighter, creating a stark contrast between veins and interveinal areas (Senoura et al. 2017; Therby-Vale et al. 2022). Prolonged Fe deficiency exacerbates chlorosis, with severe cases leading to white leaves, halted chlorophyll biosynthesis, inhibited biomass accumulation, and ultimately reduced crop yield and quality (Briat et al. 2015). Moreover, Fe deficiency in crops poses a global health risk, as it is the leading cause of Fe-deficiency anemia, affecting nearly one billion people, particularly in regions where diets are dominated by Fe-poor crops (Stevens et al. 2013). To enhance plant tolerance to Fe scarcity and improve the nutritional value of edible plants, breeding or genetically engineering Fe-enriched crop varieties is crucial.

Plants employ two primary strategies to cope with Fe deficiency (Kobayashi And Nishizawa 2012). Strategy I, found mainly in dicotyledons and non-Graminaceous monocots like *Arabidopsis thaliana*, involves *ferric reductase oxidase 2* (*FRO2*), which reduces ferric Fe ( $\text{Fe}^{3+}$ ) to ferrous Fe ( $\text{Fe}^{2+}$ ), followed by iron-regulated transporter 1 (*IRT1*) that transports  $\text{Fe}^{2+}$  across the plasma membrane (Hindt And Guerinot 2012; Dubeaux et al. 2018; Gao et al. 2019). This strategy is supported by rhizosphere acidification, driven by plasma membrane  $\text{H}^+$ -ATPases, and Fe-chelating agents like coumarins or Fe-reducing substances (Sisó-Terraza et al. 2016). In *Arabidopsis*, coumarins such as sideretin and fraxetin exhibit Fe-chelating activity and reduce  $\text{Fe}^{3+}$  to bioavailable  $\text{Fe}^{2+}$ , thereby facilitating Fe uptake under deficiency conditions (Paffrath et al. 2024). Strategy II, employed primarily by Graminaceous plants such as rice (*Oryza sativa* L.), involves the secretion of uncomplexed phytosiderophores (PSs) like 2'-deoxymugineic acid (DMA), which chelate  $\text{Fe}^{3+}$  in the soil to form  $\text{Fe}^{3+}$ -PS complexes that are then taken up by membrane transporters of the yellow stripe-like (YSL) oligopeptide family (DiDonato et al. 2004; Senoura et al. 2017). Although these strategies are traditionally viewed as mutually exclusive, recent studies indicate that  $\text{Fe}^{3+}$ -activating coumarins can also be secreted by non-Graminaceous species like *Arabidopsis*, suggesting functional overlap between the two strategies (Rodríguez-Celma et al. 2013; Fourcroy et al. 2014; Schmid et al. 2014).

To adapt to fluctuating Fe availability, plants regulate the expression of genes involved in Fe absorption, transport, and homeostasis. These genes are particularly active in leaves, though the Fe deficiency response is primarily initiated in roots (Schwarz And Bauer 2020). The bHLH transcription factor POPEYE (PYE) and the FER-mediated iron deficiency-responsive transcription factor (FIT) represent distinct gene subsets that contribute

to Fe acquisition and cellular homeostasis in *Arabidopsis* (Lan et al. 2012). FIT modulates its transcriptional activity through heterodimerization with clade Ib bHLH proteins (bHLH38, bHLH39, bHLH100, and bHLH101), thereby regulating Fe uptake responses in *Arabidopsis* (Wang et al. 2013; Lei et al. 2020). Upstream of these clade Ib bHLH proteins, some members of the bHLH IVc subgroup (including bHLH34, bHLH104, bHLH105, and bHLH115) act as positive regulators by binding to the promoters of *bHLH38/39/100/101*, directly controlling their expression (Zhang et al. 2015; Li et al. 2016). In contrast to *Arabidopsis*, that utilizes Strategy I for Fe acquisition, rice adopts Strategy II. The rice iron-related bHLH transcription factor 2 (*OsIRO2*), an ortholog of *Arabidopsis* clade Ib bHLH proteins (*AtbHLH38/39*), acts as a central regulator of Fe uptake, specifically controlling the expression of the  $\text{Fe}^{3+}$ -PS transporter *OsYSL15*, but does not regulate  $\text{Fe}^{2+}$  absorption *via* *OsIRT1* (Kobayashi And Nishizawa 2012). Additionally, rice proteins *OsIRO3* (an *Arabidopsis* homolog of PYE) and *OsPRI1* (an *Arabidopsis* homolog of bHLH105) function within the same regulatory network. Iron-binding haemerythrin RING ubiquitin ligases 1 (*OsHRZ1*) and *OsHRZ2* negatively regulate Fe absorption by targeting this cascade involving *OsIRO2*, *OsIRO3*, and *OsPRI1* (Kobayashi et al. 2013, 2016). Furthermore, *iron deficiency-responsive element-binding factor 1* (*IDEF1*) and *IDEF2* genes in rice coordinately regulate overlapping sets of Fe deficiency-responsive genes (Kobayashi et al. 2009; Wang et al. 2020).

Small peptides, consisting of fewer than 100 amino acids, are key regulatory agents that mediate key physiological processes such as plant morphogenesis, organogenesis, and adaptation to abiotic stresses (Czyzewicz et al. 2013). IRON MAN (IMA) peptides represent an emerging class of bioactive polypeptides that coordinate rhizospheric Fe acquisition and cellular homeostasis in higher plants (Rout And Sahoo 2015). The IMA polypeptide family, defined by a conserved 17-amino acid C-terminal motif, operates independently of the full-length protein in many angiosperms (Grillet et al. 2018; Li et al. 2021). The C-terminus of IMA contains the BRUTUS (BTS) interaction domain (BID), which interacts with the BTS protein (Li et al. 2021). BTS, an Fe transporter protein, regulates the plant Fe deficiency response by modifying bHLH IVc through ubiquitination (Selote et al. 2015). Under Fe-sufficient conditions, BTS reduces Fe absorption and suppresses the expression of Fe uptake-related genes by degrading bHLH105/bHLH115, preventing Fe toxicity in plants (Hindt And Guerinot 2012). Under Fe-deficient conditions, AtIMAs interact with BTS to stabilize bHLH105/bHLH115, modulating the expression of Fe deficiency-responsive genes

through FIT, bHLHb, and PYE in *Arabidopsis* (Li et al. 2021). Furthermore, IMA peptides regulate Fe absorption, transport, and homeostasis by mediating long-distance Fe signal transfer from shoots to roots (Grillet et al. 2018; Li et al. 2021). In *Arabidopsis*, overexpressing *IMA* enhances Fe deficiency tolerance by inducing the expression of Fe uptake genes in the roots. Silencing all eight members of the *IMA* gene family impairs Fe uptake and causes severe chlorosis, but this wild-type phenotype can be restored by either increasing Fe availability or expressing *IMA1* (Grillet et al. 2018). In legumes (*Lotus japonicus*), *LjIMA1* and *LjIMA2* are expressed in shoots and function both systemically and locally in nodules (Ito et al. 2024). These evolutionarily conserved IMA peptides coordinate nitrogen homeostasis via nitrogen-Fe balance regulation in legumes and *Arabidopsis thaliana* (Ito et al. 2024). Additionally, IMA peptides inhibit *Cu deficiency-induced transcription factor 1* (*CITF1*) expression, leading to reduced Cu uptake gene expression and maintaining Cu homeostasis through CITF1-dependent pathways (Cai et al. 2024). IMAs also increase the tolerance of *Arabidopsis thaliana* and wheat (*Triticum aestivum*) to the heavy metal Cd (Meng et al. 2022; Wang et al. 2023a, b). However, the role of IMA peptides in regulating Fe homeostasis in apple remains unexplored.

This study successfully cloned the *MdIMA1* gene and conducted a bioinformatics analysis of the apple *MdIMAs* gene family. Functional characterization of *MdIMA1* through heterologous expression in *Arabidopsis* and tomato revealed a significant enhancement in Fe deficiency resistance. Beyond its role in Fe homeostasis, overexpression of *MdIMA1* in apple calli improved Fe deficiency adaptation. These findings demonstrate that *MdIMA1* is critical for enhancing plant resistance to Fe deficiency. This study not only highlights the key role of *MdIMA1* in regulating Fe homeostasis but also provides valuable genetic resources for improving Fe utilization in crops, including apples. The results offer a theoretical foundation for future genetic engineering and breeding strategies aimed at developing Fe deficiency-resistant cultivars.

## Materials and methods

### Plant materials and growing conditions

Roots, stems, leaves, and flowers were collected from 7-year-old 'Gala' plants (*Malus domestica*) grafted onto GL-3 rootstock. The fruits were harvested 80 days after anthesis. All tissues were immediately flash-frozen in liquid nitrogen upon collection and stored at  $-80^{\circ}\text{C}$  for subsequent gene expression analysis. To investigate the response of *MdIMA1* to Fe deficiency, 30-day-old 'tea crabapple' (*Malus hupehensis* Rehd.) seedlings were placed in liquid media with either normal Fe supply or

Fe-deficient conditions. Samples were collected at 0, 3, 6, 12, 24, and 48 hours.

'Colombian' (Col, *Arabidopsis thaliana*) seeds underwent sterilization and a 3-day vernalization at  $4^{\circ}\text{C}$  before planting on 1/2 Murashige and Skoog (MS) medium, with a photoperiod of 16-h light/8-h dark. The temperature and relative humidity were maintained at  $22^{\circ}\text{C}$  and 65%, respectively. After 3 days of germination, the seedlings were transferred to 1/2 MS media with  $50\text{ }\mu\text{M}$  Fe-ethylenediaminetetraacetic acid (Fe-EDTA; +Fe) or  $100\text{ }\mu\text{M}$  FerroZine (-Fe + FRZ) for 10 days.

Following 7 days of germination and growth on 1/2 MS medium, the *Arabidopsis* seedlings were transferred to nutrient-treated vermiculite, supplemented with  $50\text{ }\mu\text{M}$  Fe-EDTA (+Fe) or  $200\text{ }\mu\text{M}$  FerroZine without Fe-EDTA (-Fe + FRZ), and cultured for 30 days. FerroZine, in the presence of  $\text{Fe}^{2+}$ , formed a red complex, which chelated  $\text{Fe}^{2+}$ , creating a low-iron environment.

Seeds of wild-type and *MdIMA1*-overexpressing transgenic 'Micro-Tom' tomato (*Solanum lycopersicum*) were sterilized and germinated on 1/2 MS media for one week. The seedlings were then transplanted to vermiculite treated with either  $50\text{ }\mu\text{M}$  Fe-EDTA (+Fe) or  $200\text{ }\mu\text{M}$  FerroZine without Fe-EDTA (-Fe + FRZ) and grown under a 16-h light/8-h dark cycle at  $25^{\circ}\text{C}/18^{\circ}\text{C}$  (day/night) with white light at  $200\text{ mmol photons/m}^2/\text{s}$  for 15 days.

'Orin' apple calli were cultured on MS media supplemented with  $1.5\text{ mg/L}$  2,4-dichlorophenoxyacetic acid (2,4-D),  $0.5\text{ mg/L}$  6-benzylaminopurine (6-BA), and  $8\text{ g/L}$  agar at  $25^{\circ}\text{C}$  in the dark, with subculturing every 15 days. For Fe treatments, wild-type and *MdIMA1*-OE calli were transferred to MS media with or without Fe-EDTA, representing the +Fe and -Fe conditions, respectively.

### Rhizosphere acidification experiments

For rhizosphere acidification testing, apple calli or *Arabidopsis* were subjected to normal Fe supply or Fe deficiency treatment. The calli or roots were transferred to agar plates containing 0.01% bromocresol violet and  $0.2\text{ mM}$   $\text{CaSO}_4$  ( $\text{pH}=6.5$ ) and incubated for one day. The extent of acidity was assessed by observing color changes around the calli or roots.

### Phylogenetic tree, structure analysis, conserved motif, and sequence alignment

The amino acid sequences of IMA proteins from 21 species were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). A phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replications in MEGA (V.11.06) software. iTOL (v6.7.6; <https://itol.embl.de/>) was used for tree visualization. The apple GFF3 file was retrieved from the GDR database (<https://www.rosaceae.org/>). Gene structure

(intron–exon) of the four *MdIMAs* was visualized using GSDS 2.0 (<http://gsds.cbi.pku.edu.cn>). Highly conserved regions of *MdIMAs* were analyzed with WebLogo (<http://weblogo.berkeley.edu/logo.cgi>). Multiple sequence alignment of *MdIMA1*, *MdIMA2*, *MdIMA3*, and *MdIMA4* was performed with the Omega cluster (<https://www.ebi.ac.uk/Tools/>) and visualized in Jalview (<https://www.jalview.org/>) (Yue et al. 2025).

#### Plasmid construction and genetic transformation

The full coding sequence of *MdIMA1* was molecularly cloned and inserted into the plant expression vector pRI101, which carries the CaMV 35S promoter (Li et al. 2020). Using *Agrobacterium*-mediated floral dip transformation, the *MdIMA1*-overexpressing (*MdIMA1*-OE) vector was successfully integrated into *Arabidopsis* (Yang et al. 2021). Wild-type *Arabidopsis* plants at the prolific bolting stage, with numerous unopened floral buds, were selected for transformation. The *Agrobacterium* strain GV3101, containing the *MdIMA1*-pRI101 construct, was cultured in LB medium for 12 h. The bacterial cells were harvested by centrifugation, resuspended in an infiltration medium containing 5% sucrose and 0.02% Silwet L-77, and adjusted to an optical density (OD<sub>600</sub>) of 0.8. The aerial parts of the plants were immersed in the bacterial suspension for 30 s. After infiltration, the plants were placed horizontally and maintained under high humidity in the dark for 24 h, after which they were returned to standard growth conditions until seed maturation. The T1 seeds were harvested, surface sterilized, and sown on kanamycin-containing (50 mg/L) 1/2 MS selection plates. Transgenic lines were selected based on kanamycin resistance, and three independent homozygous lines exhibiting *MdIMA1* overexpression were established for phenotypic characterization (Liu et al. 2024a, b).

To genetically modify tomatoes, the *MdIMA1*-pRI101 construct was introduced into the *Agrobacterium tumefaciens* strain EHA105. This strain was used to infect the Micro-Tom tomato cultivar, generating *MdIMA1*-OE tomatoes. True leaves from sterile seedlings were excised, precultivated for 2 days, and immersed in an *Agrobacterium* suspension (OD<sub>600</sub>=0.8), followed by shaking for 15 min. The leaves were placed on MS solid media for 2 days of co-cultivation, then transferred to MS media supplemented with kanamycin, resulting in regenerated resistant plants (Deng et al. 2023).

For *MdIMA1* transgenic apple calli, the *MdIMA1*-OE vectors were introduced into 'Orin' calli via *Agrobacterium tumefaciens* strain EHA105. Fifteen-day-old apple calli were immersed in the *Agrobacterium* suspension (OD<sub>600</sub>=0.6), shaken for 15 min, and incubated on antibiotic-free MS solid media for 2 days. The calli were then transferred to kanamycin-containing media to screen

for *MdIMA1*-OE calli. For *MdIMA1*-RNAi calli, the *MdIMA1*-RNAi vector was constructed by inserting an antisense fragment of the *MdIMA1* gene into the pRI101 vector. The calli were added to the activated *Agrobacterium* suspension (OD<sub>600</sub>=0.6), vacuum infiltrated for 5 min, shaken for 20 min, and incubated on antibiotic-free MS solid media for 2 days. *MdIMA1* gene expression levels were subsequently measured (Li et al. 2020).

#### Quantitative real-time PCR (qRT–PCR) analysis

For qRT-PCR analysis, plant tissue was collected and ground in liquid nitrogen. Total RNA was extracted using the RNA Plant Plus Reagent Kit (Vazyme, Nanjing, China), and cDNA synthesis was performed with a reverse transcription kit (SparkJade, Shandong, China). Following the manufacturer's instructions, qRT-PCR was conducted using the UltraSYBR Green Master Mix. Relative transcript levels were quantified using the 2<sup>−ΔΔCT</sup> method (Yang et al. 2021). *Md18S* and *AtACTIN* served as endogenous controls for *Arabidopsis* and apple, respectively, as described by An et al. (2018). Each experiment included three biological replicates. Primer sequences are listed in Table S1.

#### Chlorophyll content determination

Chlorophyll content in tomato and *Arabidopsis* leaves was determined via spectrophotometry. Leaves were immersed in 95% ethanol overnight for decolorization, followed by complete extraction of chlorophyll. Absorbance was recorded at 470 nm, 665 nm, and 647 nm, and chlorophyll concentrations were calculated (Sun et al. 2023).

#### H<sup>+</sup>-ATPase activity assay

*Arabidopsis* and apple calli were subjected to +Fe or -Fe treatments to isolate the plasma membrane. Plasma membrane H<sup>+</sup>-ATPase activity was quantified using a previously reported method (Liu et al. 2024a).

#### Fe concentration determination

Fe concentration was determined following the protocol described by Sun et al. (2021). Tomato and *Arabidopsis* samples (0.3 g) were dried at 80°C for 48 h and ground into powder. Samples were digested overnight with 5 mL of concentrated sulfuric acid, then heated to 330°C until white smoke appeared. Afterward, 30% hydrogen peroxide was added to clarify the solution. Fe concentration was measured by inductively coupled plasma–optical emission spectrometry (ICP-OES).

#### Ferric chelate reductase (FCR) activity assay

FCR enzymatic activity was quantified using ferrous chelation spectrophotometry, as previously described

(Schikora And Schmidt 2001). *Arabidopsis* samples were desorbed for 5 min with 0.5 mM  $\text{CaSO}_4$ , then equilibrated in acidic reaction buffer (pH=5.8) containing 0.5 mM ferroquinoline, 0.5 mM  $\text{CaSO}_4$ , and EDTA. A Shimadzu UV1800 UV-Vis spectrophotometer (Kyoto) was used for spectrophotometric analysis at 562 nm. Each treatment was performed with at least three biological replicates.

### Statistical analysis

All experimental procedures were carried out with three independent biological replicates, and the data are presented as means  $\pm$  standard deviations (SDs). Statistical analysis was performed using DPS software (one-way ANOVA with the Tukey–Kramer test,  $P < 0.05$ ). Significant differences are indicated by lowercase letters. Error bars in figures represent SDs.

## Results

### Phylogenetic analysis and amino acid sequence alignment of *MdIMAs*

In *Arabidopsis*, *AtIMA* family genes play a pivotal role in regulating Fe homeostasis (Grillet et al. 2018). To investigate the function of the *MdIMA* gene family in apple, a bioinformatics analysis was performed, including phylogenetic tree construction, gene structure analysis, conserved motif identification, and amino acid sequence alignment. IMA protein sequences from 21 species were collected to explore the evolutionary relationships of the *IMA* genes, resulting in the construction of a phylogenetic tree divided into three distinct subgroups (Group 1, Group 2, and Group 3). *MdIMA1*, *MdIMA2*, and *MdIMA3* clustered within Group 3, with *MdIMA1* showing the closest evolutionary relationship to KAH0970404.1 (*Prunus armeniaca*). Conversely, *MdIMA4* and *Arabidopsis AtIMA* were grouped in Group 2, indicating a closer evolutionary relationship with *AtIMA* (Fig. S1a). Gene structure analysis revealed that *MdIMA1*, *MdIMA3*, and *MdIMA4* each contain a single exon without introns, whereas *MdIMA2* comprises three exons and two introns (Fig. S1b). Conserved motif and sequence alignment analyses demonstrated that the *MdIMAs* exhibit high sequence conservation, particularly in key functional domains (Fig. S1c, d). These results suggest that *MdIMA1*, *MdIMA2*, and *MdIMA3* are highly conserved within the apple genome.

### Tissue-specific expression and Fe response analysis of *MdIMAs*

To examine the tissue-specific expression patterns of the *MdIMAs*, qRT-PCR analysis was conducted across various apple tissues. The results showed that all four

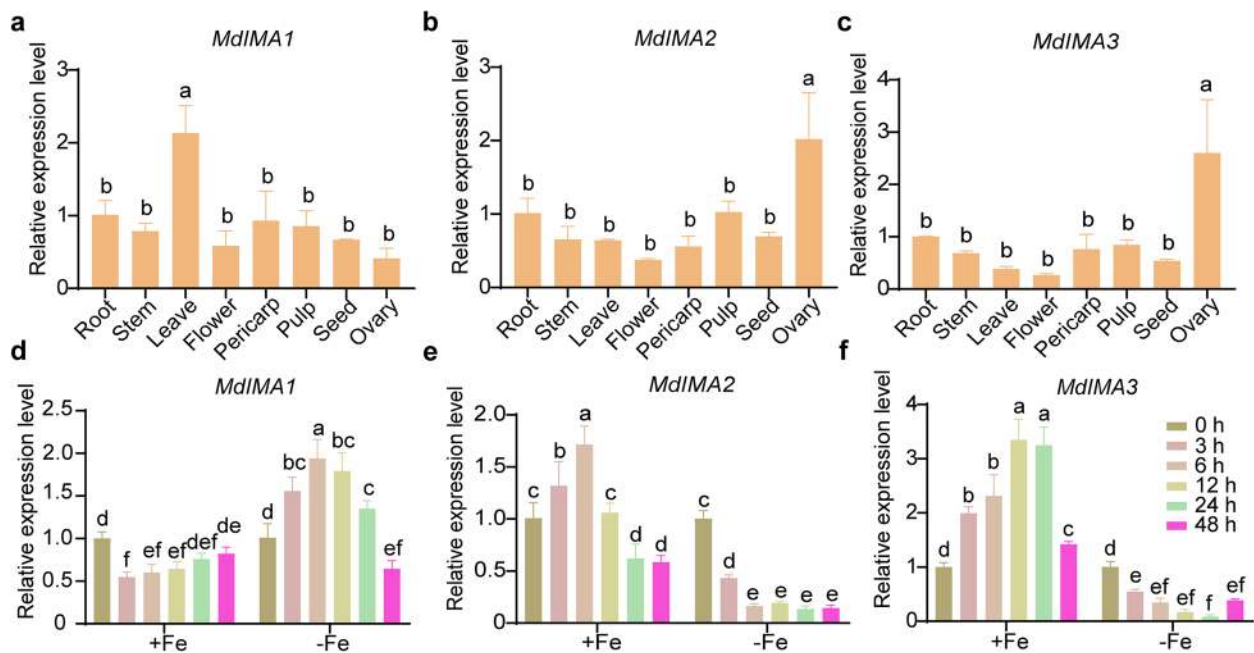
*MdIMAs* were expressed in roots, stems, leaves, flowers, pericarps, pulps, seeds, and ovaries. Notably, *MdIMA1* was most highly expressed in leaves, while *MdIMA2* and *MdIMA3* exhibited the highest expression in ovaries, with minimal expression in flowers and other tissues (Fig. 1a–c). These results suggest that *MdIMAs* are involved in multiple stages of apple development and growth.

Additionally, following Fe deficiency treatment, the expression levels of *MdIMAs* were monitored *via* qRT-PCR to assess their response to Fe. The transcript levels of *MdIMA1* in apple seedlings initially increased and then decreased after transfer from a Fe-sufficient medium to a Fe-deficient one (Fig. 1d), while the expression of *MdIMA2* and *MdIMA3* decreased (Fig. 1e, f). These results indicate that *MdIMAs* are responsive to Fe deficiency, with *MdIMA1* showing increased transcript levels in response to Fe scarcity.

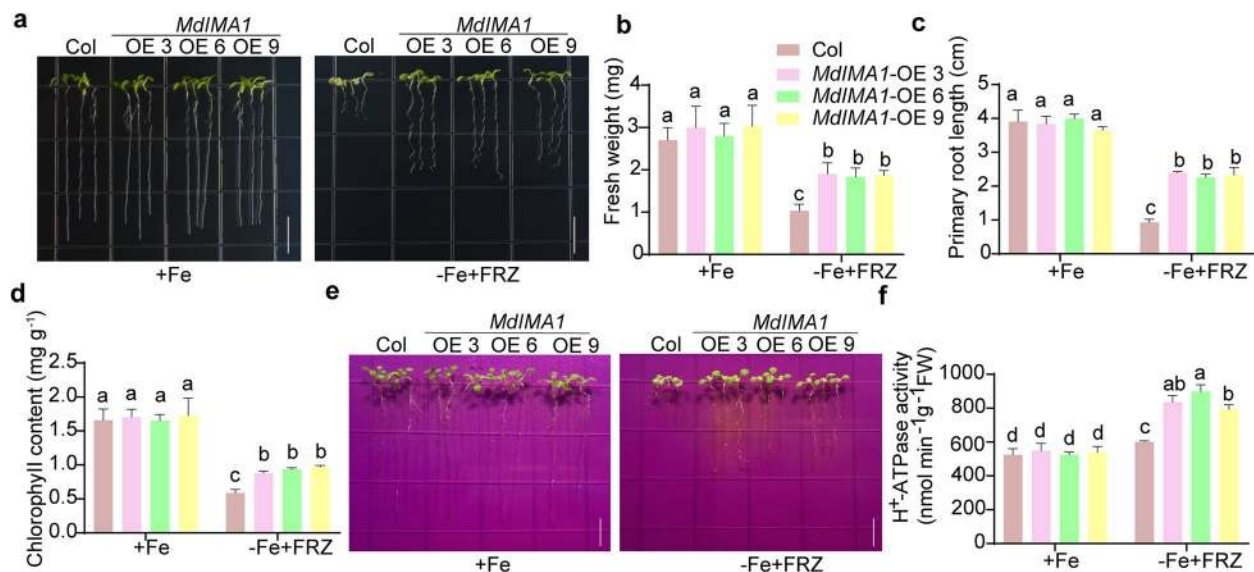
### Heterologous expression of *MdIMA1* in *Arabidopsis* enhances Fe deficiency tolerance

Plant roots enhance Fe utilization efficiency through proton transport and rhizosphere acidification (Ning et al. 2023). To evaluate the role of *MdIMA1* in these processes, *MdIMA1*-overexpressing (*MdIMA1*-OE) transgenic *Arabidopsis* plants driven by the CaMV 35S promoter (35S::GFP-*MdIMA1*) were generated, and three independent lines were selected for further analysis (Fig. S2). To investigate the impact of heterologous expression of the apple Fe regulator, the transcript levels of the endogenous *Arabidopsis IMA* homologs were analyzed under both Fe-sufficient and Fe-deficient conditions. The results showed no significant differences in the expression of *Arabidopsis IMA* homologs (*AtIMA1*, *AtIMA2*, and *AtIMA3*) between the *MdIMA1*-OE and wild-type (Col) plants under Fe-sufficient conditions. Under Fe-deficient conditions, expression of the homologous genes in both *MdIMA1*-OE and Col plants was significantly upregulated compared to Fe-sufficient conditions, but the degree of upregulation was not significantly different between the two groups (Fig. S3). Under normal Fe conditions, no significant differences were observed in the fresh weight, primary root length, or chlorophyll content between Col and *MdIMA1*-OE transgenic lines. However, under Fe-deficient conditions, Col seedlings exhibited a pronounced chlorotic phenotype, characterized by reduced chlorophyll content, as well as significant decreases in fresh weight and primary root length compared to the *MdIMA1*-OE lines (Fig. 2a–d).

In dicotyledonous plants, Fe deficiency triggers rhizosphere acidification by activating membrane proton pumps (e.g.  $\text{H}^+$ -ATPases) in root epidermal cells, which actively secrete  $\text{H}^+$  into the surrounding soil. This acidic



**Fig. 1** Expression patterns of the *Malus domestica* *IRON MAN* (*MdIMAs*). **a–c** Relative expression of *MdIMA1* (**a**), *MdIMA2* (**b**), and *MdIMA3* (**c**) in different tissues or organs via qRT-PCR. **d–f** Relative expression of *MdIMA1* (**d**), *MdIMA2* (**e**), and *MdIMA3* (**f**) under normal Fe supply (+Fe) and Fe-deficient (-Fe) conditions. The data are shown as means  $\pm$  SDs ( $n=3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey-Kramer test,  $P < 0.05$ )



**Fig. 2** Heterologous expression of *Malus domestica* *IRON MAN 1* (*MdIMA1*) in *Arabidopsis* regulates Fe deficiency tolerance. **a** Phenotypes of wild-type (Col, *Arabidopsis thaliana* 'Colombia') and *MdIMA1*-overexpressing (*MdIMA1*-OE) *Arabidopsis* seedlings (13-d-old) under +Fe and -Fe+FRZ conditions. **b–d** Fresh weight (**b**), primary root length (**c**), and chlorophyll content (**d**) of Col and *MdIMA1*-OE *Arabidopsis* seedlings. **e–f** Rhizosphere acidification (**e**) and  $H^+$ -ATPase activity (**f**) of the Col and *MdIMA1*-OE lines. The data are shown as means  $\pm$  SDs ( $n=3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey-Kramer test,  $P < 0.05$ ). FW, fresh weight; +Fe, 50  $\mu$ M Fe-EDTA; -Fe+FRZ, 100  $\mu$ M FerroZine without Fe-EDTA

microenvironment enhances the activity of enzymes such as FCR, promoting the reduction of  $\text{Fe}^{3+}$  to the bioavailable  $\text{Fe}^{2+}$  (Sun et al. 2023). *Arabidopsis* seedlings grown under +Fe and -Fe+FRZ conditions were transferred to agar plates containing bromocresol violet to assess changes in Fe uptake of roots. The results showed that, under Fe-deficient conditions, *MdIMA1*-OE plants exhibited more pronounced rhizosphere acidification than Col, as indicated by the bright yellow coloration surrounding the roots (Fig. 2e). Moreover,  $\text{H}^+$ -ATPase activity measurements under Fe-deficient conditions revealed significantly higher enzyme activity in all three *MdIMA1*-OE lines compared to Col, whereas no changes were observed under Fe-sufficient conditions (Fig. 2f).

To further investigate the developmental effects of Fe deficiency, 37-d-old *MdIMA1*-OE seedlings were subjected to both Fe-sufficient and Fe-deficient conditions, and various physiological parameters were measured. Under Fe-deficient conditions, *MdIMA1*-OE plants showed a significant increase in fresh weight and chlorophyll content compared to Col (Fig. 3a–c). The activity of FCR, a marker of Fe reduction ability, also significantly increased in *MdIMA1*-OE plants under Fe deficiency (Fig. 3d). Additionally, Fe concentration measurements indicated that *MdIMA1*-OE *Arabidopsis* exhibited a marked increase in  $\text{Fe}^{3+}$  uptake capacity under Fe-deficient conditions (Fig. 3e).

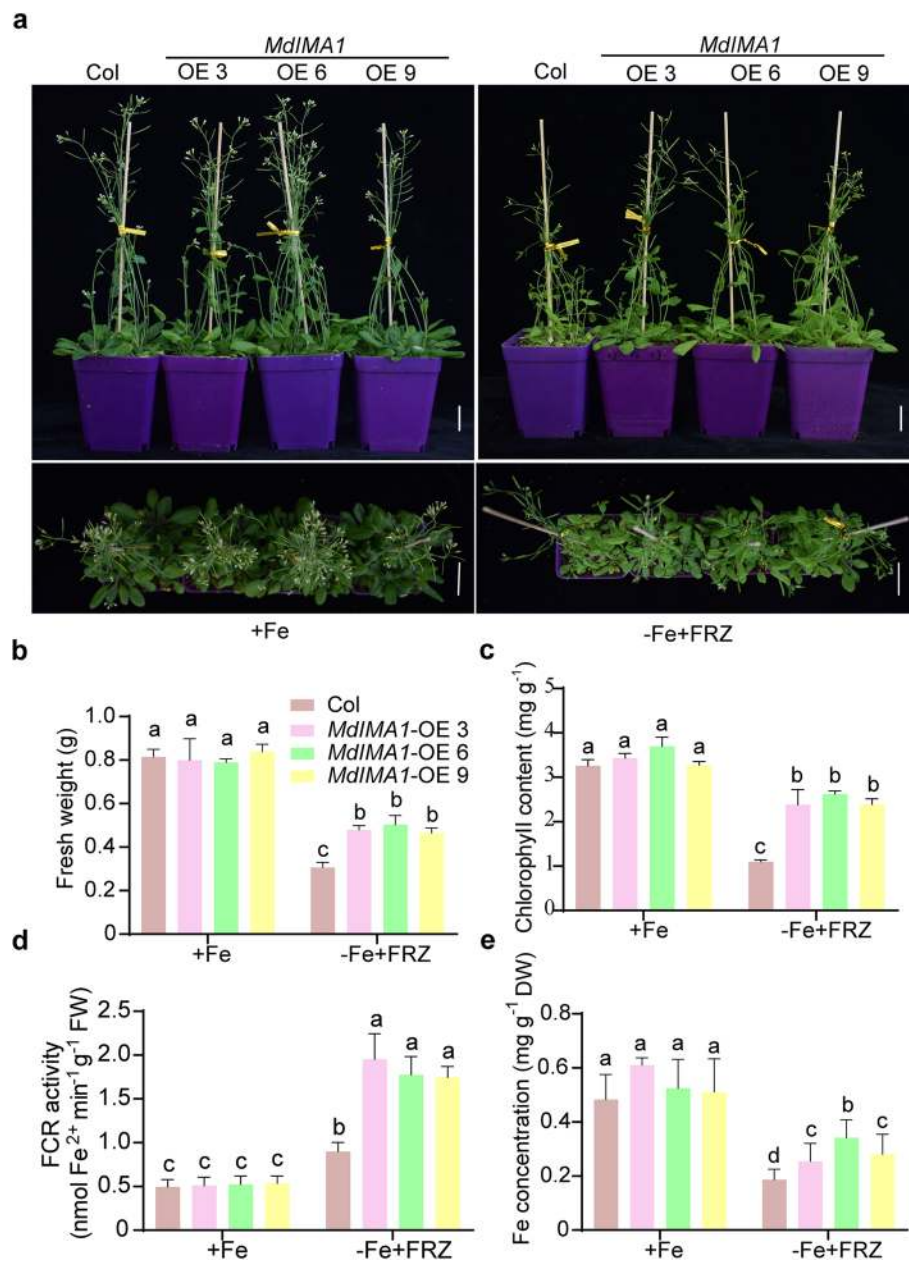
To further understand the processes influencing Fe deficiency responses, a qRT-PCR study was conducted using both wild-type (Col) and *MdIMA1*-OE *Arabidopsis* plants. The results revealed that Fe deficiency strongly stimulated the expression of the  $\text{H}^+$ -ATPase gene *AtHA7* (Fig. 4a). Additionally, the expression levels of *AtFRO2*, *AtIRT1*, *AtYSL3*, and *AtFRO3* were significantly upregulated in *MdIMA1*-OE *Arabidopsis* under Fe deficiency (Fig. 4b–e). These results suggest that *MdIMA1*-OE *Arabidopsis* promotes the expression of key genes involved in rhizosphere acidification and Fe uptake. Specifically, *MdIMA1*-OE enhances the expression of *AtHA7* gene, involved in proton secretion for acidification, as well as *AtFRO2* and *AtFRO3*, which encode ferric reductase enzymes, and the Fe transporter genes *AtYSL3* and *AtIRT1*, thus facilitating Fe absorption. However, no significant effect was observed on the expression of genes encoding *natural resistance-associated macrophage protein 1* (*AtNRAMP1*) and *nicotianamine synthase 1* (*AtNAS1*), which are involved in other Fe transport pathways (Fig. 4f–g). These results suggest that heterologous expression of *MdIMA1* enhances Fe deficiency tolerance by modulating the transcription of essential Fe homeostasis genes in *Arabidopsis*.

### Heterologous expression of *MdIMA1* in tomato enhances tolerance to Fe deficiency

The heterologous expression of *MdIMA1* was shown to enhance Fe deficiency tolerance in *Arabidopsis* (Figs. 2, 3, and 4). To further validate its function, three independent *MdIMA1*-overexpressing transgenic tomato lines were generated (Fig. S4). To assess the conserved function of *MdIMA1* in a heterologous system, the transcription of its tomato orthologs was analyzed. Under Fe-sufficient conditions, the transcript levels of two tomato IMA homologs (Solyc12g006770 and Solyc12g006760) in the *MdIMA1*-OE tomato lines were comparable to those in the wild-type plants. Upon Fe deficiency, the expression of these two *SIIMAs* homologs increased significantly in both *MdIMA1*-OE and wild-type plants, but no significant difference was observed between the two groups (Fig. S5). The *MdIMA1*-OE transgenic tomatoes were then subjected to normal Fe or Fe deficiency treatments. Under normal Fe conditions, no significant differences were observed between the *MdIMA1*-OE and wild-type tomatoes (Fig. 5a). However, under Fe-deficient conditions, *MdIMA1*-OE lines exhibited significantly greater shoot fresh weight, root fresh weight, and plant height compared to wild-type plants (Fig. 5b–d), though stem diameter remained unchanged (Fig. 5e). The chlorophyll and Fe concentrations were notably higher in the *MdIMA1*-OE lines than in wild-type plants under Fe deficiency (Fig. 5f, g). Furthermore, the yield and total Fe content of tomato fruit from *MdIMA1*-OE plants were significantly greater than those from wild-type plants under Fe deficiency (Fig. 5h, i). Overall, all measured physiological parameters of *MdIMA1*-OE transgenic tomato plants were significantly superior to those of wild-type plants under Fe-deficient conditions, suggesting that *MdIMA1* functions as a positive regulator of Fe homeostasis and enhances tolerance to Fe deprivation.

### *MdIMA1* modulates tolerance to Fe deficiency in apple calli

To verify the endogenous function of *MdIMA1* in apple, *MdIMA1*-OE transgenic apple calli were generated, and three independent lines were selected for phenotypic analysis (Fig. S6). Wild-type and *MdIMA1*-OE calli were cultured in Fe-EDTA (+Fe) or Fe-free (-Fe) media for 7 days, followed by transfer to media containing bromocresol violet for acidification analysis. The results showed that proton outflow from *MdIMA1*-OE apple calli significantly increased, causing the culture medium surrounding the calli to change from purple to yellow (Fig. 6a). Additionally,  $\text{H}^+$ -ATPase activity in *MdIMA1*-OE calli was significantly higher than in wild-type calli (Fig. 6b). To further investigate, Fe concentration was measured in both *MdIMA1*-OE and wild-type apple calli. Under

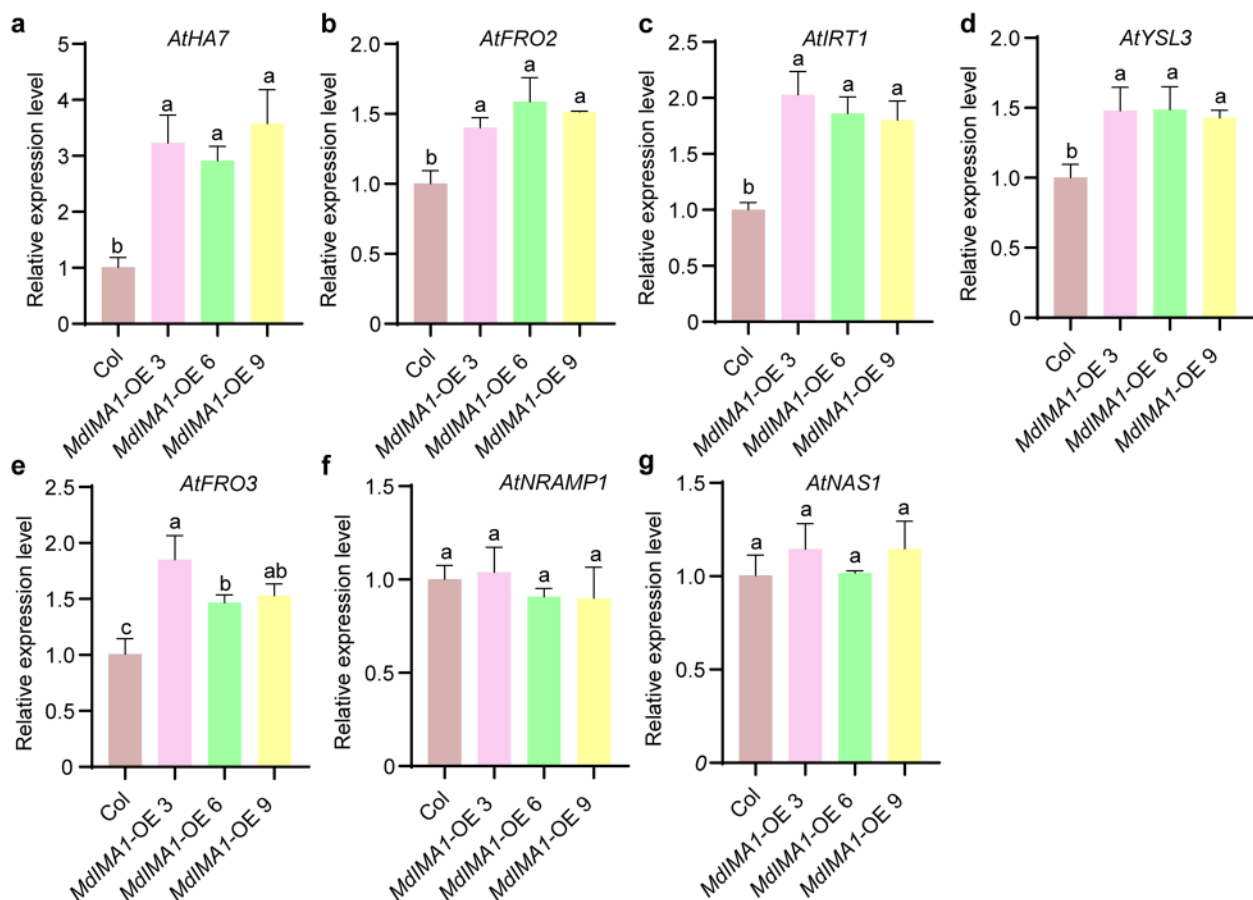


**Fig. 3** Heterologous expression of *Malus domestica* IRON MAN 1 (*MdIMA1*) enhances the tolerance of *Arabidopsis thaliana* to Fe deficiency. **a** Morphology of wild-type (Col, *Arabidopsis* 'Colombia') and *MdIMA1*-overexpressing (*MdIMA1*-OE) *Arabidopsis* plants (37-d-old) under normal Fe supply and Fe-deficient + FRZ conditions. **b–e** Determination of fresh weight (**b**), chlorophyll content (**c**), ferric chelate reductase (FCR) activity (**d**), and Fe concentration (**e**) of Col and *MdIMA1*-OE seedlings under + Fe and -Fe conditions. The data are shown as means  $\pm$  SDs ( $n = 3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). FW, fresh weight; DW, dry weight; + Fe, 50  $\mu$ M Fe-EDTA; -Fe + FRZ, 100  $\mu$ M FerroZine without Fe-EDTA

Fe-deficient conditions, *MdIMA1*-OE calli accumulated considerably more Fe than wild-type calli (Fig. 6c).

To further explore the function of *MdIMA1* in native systems, *MdIMA1*-RNAi apple calli were generated, and *MdIMA1* expression was significantly reduced in these lines (Fig. 6e). Under Fe-sufficient conditions,

H<sup>+</sup>-ATPase activity did not significantly differ between *MdIMA1*-RNAi and wild-type calli. However, under Fe-deficient conditions, H<sup>+</sup>-ATPase activity in *MdIMA1*-RNAi calli was markedly lower than that in wild-type calli (Fig. 6f). These results indicate that *MdIMA1* plays



**Fig. 4** Expression profiles of Fe-related genes in *MdlMA1*-OE *Arabidopsis thaliana* under Fe-deficient conditions. **a–g** qRT–PCR analysis of genes involved in Fe uptake, including *AtHA7* (**a**), *AtFRO2* (**b**), *AtIRT1* (**c**), and Fe transport, such as *AtYSL3* (**d**), *AtFRO3* (**e**), *AtNRAMP1* (**f**), and *AtNAS1* (**g**), in wild-type (Col, *Arabidopsis* 'Colombia') and *MdlMA1*-OE *Arabidopsis* (37-d-old). The data are shown as means  $\pm$  SDs ( $n=3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). *MdlMA1*-OE, *Malus domestica* IRON MAN-overexpressing; *AtHA7*, *Arabidopsis thaliana*  $H^+$ -ATPase 7; *AtFRO2*, FERRIC REDUCTASE OXIDASE 2; *AtIRT1*, IRON-REGULATED TRANSPORTER 1; *AtYSL3*, YELLOW STRIPE-LIKE 3; *AtNRAMP1*, NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 1; *AtNAS1*, NICOTIANAMINE SYNTHASE 1

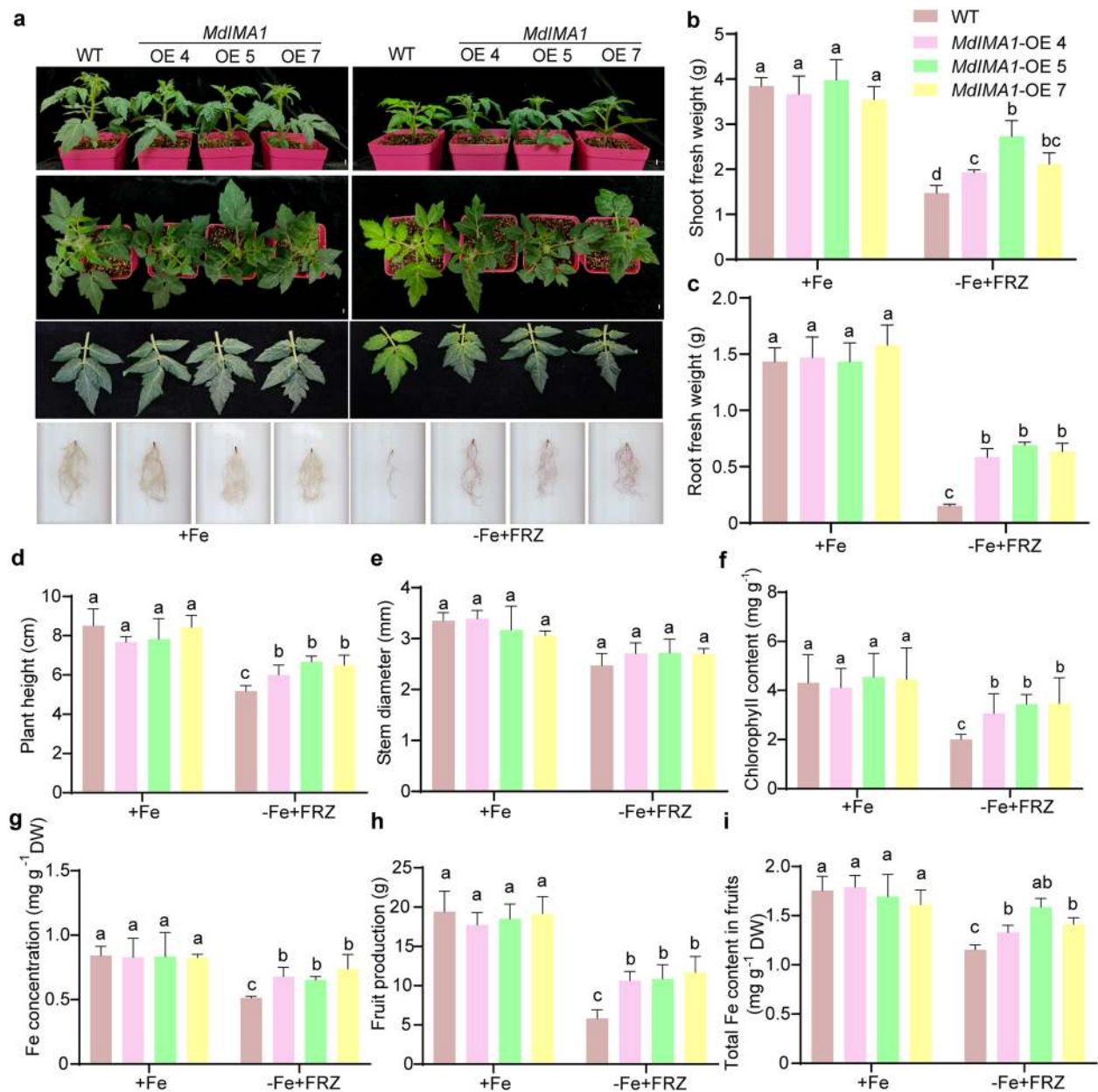
a critical role in enhancing the ability of apple calli to cope with Fe deficiency.

## Discussion

Fe, an essential micronutrient, is critical for the regular growth and development of both unicellular and multicellular organisms. Despite Fe's abundance in soil, its bioavailability is strongly influenced by environmental factors. In an oxidized soil environment, Fe primarily exists as insoluble  $Fe^{3+}$  oxide or hydroxide, forming stable crystalline minerals under alkaline conditions ( $pH > 7.5$ ). As Fe undergoes hydrolysis, its solubility decreases significantly, leading to precipitation. These processes reduce the concentration of soluble  $Fe^{2+}$  in the soil, making it difficult for plant roots to access sufficient available Fe, which ultimately causes Fe deficiency chlorosis (Hsieh And Waters 2016; Wade et al. 2021; Wang et al. 2023a, b). IMAs are highly conserved polypeptides

in angiosperms that are induced under Fe stress conditions. These proteins typically feature conserved C-terminal sequences and around 50 amino acids (Grillet et al. 2018; Wang et al. 2023a, b). IMAs play a key upstream role in regulating cellular Fe uptake under Fe deficiency conditions (Grillet et al. 2018; Wang et al. 2023a, b). Overexpression of IMAs has been shown to enhance Fe deficiency tolerance in *Arabidopsis* and is critical for Fe uptake (Grillet et al. 2018). In leguminous crops, *LjIMA1* and *LjIMA2* are expressed in the stem and root, respectively, and together regulate Fe accumulation in the rhizomes (Ito et al. 2024).

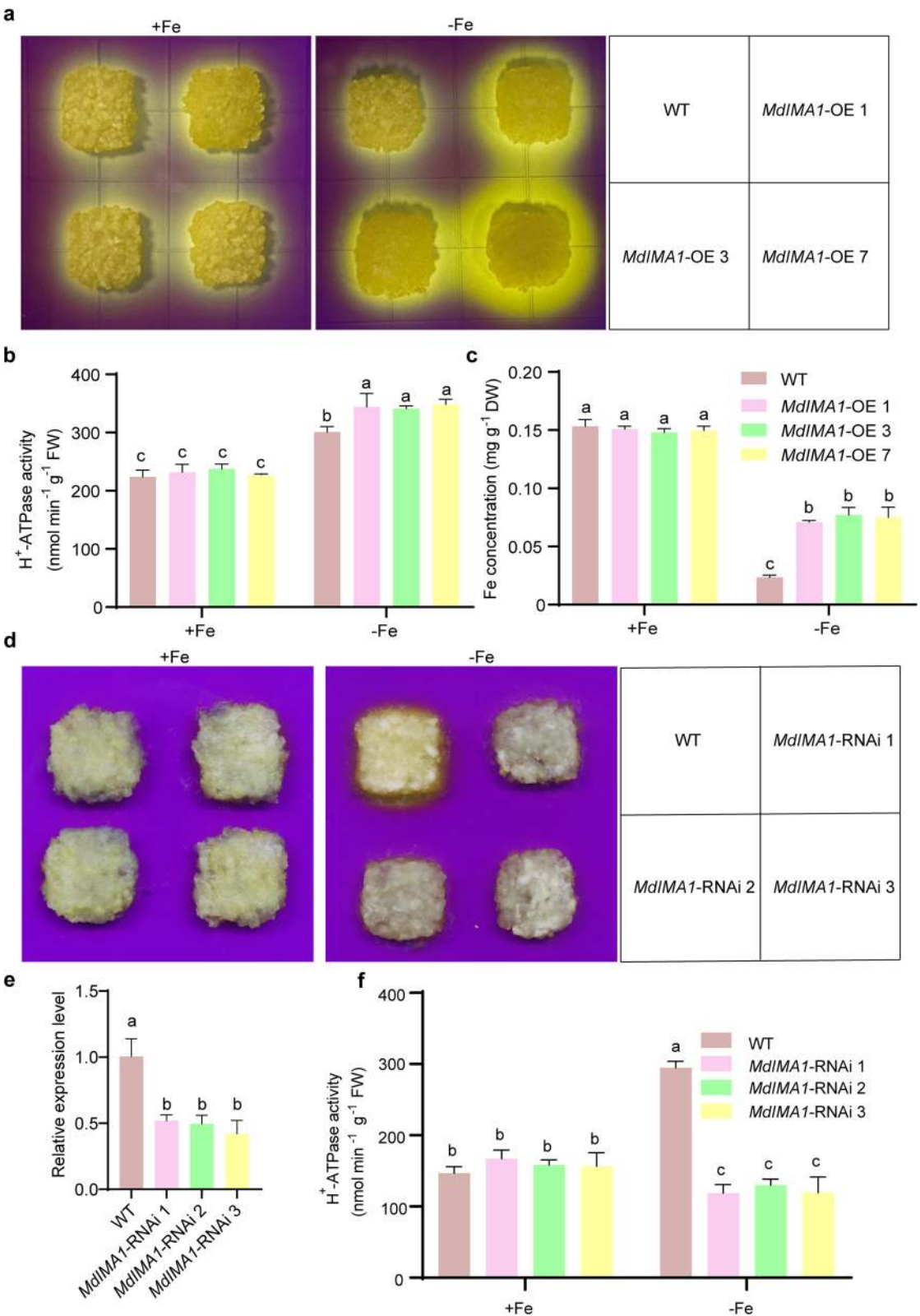
Pathogen-induced plant immune responses have been found to alleviate Fe deficiency, with *IMA1* serving as a key regulator in both Fe uptake and plant immune signaling (Cao et al. 2024). IMA polypeptides are highly conserved across angiosperms and play central roles in Fe homeostasis. *Arabidopsis* contains 8 IMA members,



**Fig. 5** Heterologous expression of *Malus domestica* IRON MAN 1 (*MdIMA1*) in tomato results in tolerance to Fe deficiency. **a** Phenotypes of wild type (WT) and *MdIMA1*-overexpressing (*MdIMA1*-OE) tomato plants (22-d-old) under normal +Fe and -Fe +FRZ conditions. **b–i** Physiological measurements of WT and *MdIMA1*-OE plants under Fe deficiency conditions, including shoot fresh weight (**b**), root fresh weight (**c**), plant height (**d**), stem diameter (**e**), chlorophyll content (**f**), Fe concentration (**g**), fruit production (**h**), and total Fe content in fruits (**i**). The tomato fruits of the WT and *MdIMA1*-OE lines were harvested after 3 weeks of growth on +Fe and -Fe +FRZ vermiculite. The data are shown as means  $\pm$  SDs ( $n = 3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). DW, dry weight; +Fe, 50  $\mu$ M Fe-EDTA; -Fe +FRZ, 200  $\mu$ M FerroZine without Fe-EDTA

(See figure on next page.)

**Fig. 6** Overexpressing and silencing of *Malus domestica* IMA IRON MAN 1 (*MdIMA1*) tolerance to Fe deficiency in apple calli. **a** Phenotype analysis of proton exocytosis in wild type (WT) and *MdIMA1*-overexpressing (*MdIMA1*-OE) apple calli. Three *MdIMA1*-OE lines were used for acidification experiments. **b, c**  $H^+$ -ATPase activity (**b**) and Fe concentrations (**c**) of WT and altered *MdIMA1*-OE calli under normal Fe supply (+Fe) and Fe-deficient (-Fe) conditions for 7 days. **d** Phenotypes of proton exocytosis in WT and *MdIMA1*-RNAi apple calli. **e** Relative expression levels of *MdIMA1* in WT and *MdIMA1*-RNAi transgenic apple calli. **f**  $H^+$ -ATPase activity of WT and *MdIMA1*-RNAi under +Fe or -Fe conditions. The data are shown as means  $\pm$  SDs ( $n = 3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). FW, fresh weight; DW, dry weight



**Fig. 6** (See legend on previous page.)

while apple has 4. Phylogenetic analysis shows that *MdIMAs* are highly conserved across angiosperms. Gene structure analysis revealed that *MdIMA1*, *MdIMA3*, and *MdIMA4* each contain a single exon with no introns, while *MdIMA2* consists of three exons and two introns. Conserved motif analysis confirmed the high sequence conservation of *MdIMAs*. Under Fe-deficient conditions, *MdIMA1/2/3* were responsive to Fe deficiency, with *MdIMA1* showing the most prominent induction. Similar to *AtIMA1*, the *MdIMA1* gene may enhance apple's ability to absorb Fe and improve its tolerance to Fe deficiency.

The heterologous expression of apple *MdIMA1* did not significantly affect the expression of IMA homologous genes in *Arabidopsis* or tomato under either Fe-sufficient or Fe-deficient conditions. This outcome is consistent with the conserved regulatory mechanisms of plant Fe metabolism (Kobayashi & Nishizawa, 2012). Under Fe-sufficient conditions, the plant Fe homeostasis network remains stable, and neither endogenous IMA genes nor heterologously expressed *MdIMA1* are significantly activated. This aligns with the functional role of IMA peptides as Fe deficiency-responsive signals rather than regulators of basal Fe balance (Grillet et al. 2018). Under Fe-deficient conditions, stress signals strongly induce the upregulation of homologous genes in both wild-type and transgenic plants. The conserved C-terminal motif of *MdIMA1* functionally overlaps with that of endogenous IMA family members, contributing to the activation of Fe deficiency response pathways (Grillet et al. 2018). However, the robust regulatory network, functional redundancy among endogenous IMA genes, and post-translational regulation (e.g. ubiquitination-mediated degradation) obscure the subtle regulatory contributions of heterologous *MdIMA1* (Kobayashi et al. 2020), resulting in no significant differences between genotypes. Functional redundancy is a common feature in other Fe metabolism-related gene families as well (Chen et al. 2021). This result confirms the cross-species functional conservation of *MdIMA1* while highlighting the robustness of plant Fe metabolism networks (Kobayashi & Nishizawa, 2012). It also suggests that optimizing *MdIMA1* expression patterns or combining it with other regulatory genes may be required to enhance plant Fe use efficiency.

Chlorophyll, a critical pigment for photosynthesis, directly influences photosynthetic efficiency. Higher chlorophyll levels are associated with increased photosynthetic rates and improved plant adaptation to abiotic stress (Mandal And Dutta 2020). Our findings show that overexpression of *MdIMA1* under Fe-deficient conditions significantly enhanced chlorophyll retention in both *Arabidopsis* and tomato, leading to increased plant

fresh weight. Furthermore, *MdIMA1* overexpression promoted primary root development and expanded the root surface area, thereby enhancing Fe reduction and translocation under Fe-deficient conditions. In contrast, wild-type *Arabidopsis* and tomato plants exhibited typical Fe deficiency symptoms, such as leaf chlorosis and growth retardation, which were alleviated in the *MdIMA1*-overexpressing plants. Additionally, the root length of transgenic *Arabidopsis* and tomato plants increased under Fe-deficient conditions, resulting in improved fruit yield and increased Fe concentration in the fruit.

In dicotyledonous and non-Graminaceous monocotyledonous plants, Fe deficiency is regulated through Strategy I. This strategy relies on three complementary processes to manage Fe homeostasis under conditions of Fe insufficiency: increased root  $H^+$ -ATPase activity, increased proton release, and subsequent soil acidification, which collectively improve Fe solubility in the soil (Kim et al. 2019). Secondary metabolites, such as coumarins and riboflavin, also contribute to this strategy by increasing root  $H^+$ -ATPase activity, thus facilitating Fe transport (Tsai et al. 2018; Robe et al. 2021a, b). Our study supports these findings, demonstrating elevated  $H^+$ -ATPase activity and root acidification under Fe-deficient conditions. Moreover, proton secretion was observed in apple calli, further supporting the role of *MdIMA1* in enhancing plant tolerance to Fe deficiency.

Dicotyledonous and non-Graminaceous monocotyledonous plants employ an efficient mechanism for Fe uptake, which involves the activation and reduction of  $Fe^{3+}$ -chelate complexes. Specifically,  $Fe^{3+}$  is reduced to  $Fe^{2+}$ , a form that is readily absorbed and utilized by plants (Wang et al. 2023a, b). Under Fe-sufficient conditions, FCR in plant roots reduces Fe from  $Fe^{3+}$  chelates, and the resulting  $Fe^{2+}$  is transported across the cytoplasmic membrane into root cells (Mai et al. 2016). The high-affinity  $Fe^{2+}$  transporter, IRT1, subsequently mediates the uptake of ferrous ions into the cell (Kobayashi And Nishizawa 2012; Dubeaux et al. 2018). In *Arabidopsis*, heterologous expression of *MdIMA1* significantly enhanced FCR activity. This improvement contributed to an increased environmental reducing capacity, activating insoluble Fe in the rhizosphere and enhancing the plant's ability to acquire Fe. These findings highlight the critical role of FCR activity and Fe transporter proteins in the efficient uptake and utilization of Fe by plants.

The  $H^+$ -ATPase enzyme indirectly supports plant Fe absorption in two stages. First, when activated by Fe deficiency signals, it pumps  $H^+$  from root cells into the rhizosphere soil, lowering the soil pH and converting insoluble  $Fe^{3+}$  into soluble, readily absorbable  $Fe^{2+}$ . Second, the outward  $H^+$  movement creates a proton gradient

across the cell membrane, which drives Fe transporters (such as IRT1) to actively transport  $\text{Fe}^{2+}$  into the cell against the concentration gradient, completing Fe uptake (Li et al. 2022). In comparison to wild-type plants, the overexpression of *MdIMA1* in apple calli and *Arabidopsis* significantly increased proton exocytosis, thereby improving Fe acquisition and elevating the Fe concentration. qRT-PCR analysis revealed that Fe deficiency (-Fe+FRZ) significantly upregulated the expression of several genes, including *AtHA7*, *AtYFRO2*, *AtIRT1*, *AtYSL3*, and *AtFRO3*, in *MdIMA1*-OE *Arabidopsis*. Among these, *AtHA7* is involved in rhizosphere acidification, while *AtYFRO2*, *AtIRT1*, *AtYSL3*, and *AtFRO3* are related to Fe transport and absorption, with *AtYSL3* playing a pivotal role in Fe transport (Li et al. 2022). Notably, *MdIMA1*-overexpressing *Arabidopsis* exhibited significantly higher FCR activity than wild-type plants, further improving Fe absorption. In conclusion, the overexpression of *MdIMA1* enhances Fe acquisition by promoting proton exocytosis, upregulating the expression of Fe-related genes, and improving root system architecture. This collectively alleviates Fe deficiency and boosts plant productivity.

Fe deficiency is a significant limitation to global crop productivity, particularly in calcareous soils with low Fe availability (Ramzani et al. 2016). The use of iron fertilizers is often inefficient and unsustainable. Functional validation of *MdIMA1*, along with its heterologous expression in tomato and *Arabidopsis*, has demonstrated that *MdIMA1* enhances Fe tolerance in apple, tomato, and *Arabidopsis*, highlighting its potential for agricultural applications. To fully harness the potential of *MdIMA1*, further research is needed to explore the molecular mechanisms underlying its regulation of Fe homeostasis, particularly its interactions with other Fe-related genes and signaling pathways. Additionally, extending this research to other crops such as rice, wheat, and maize will help assess the effectiveness of *MdIMA1* across species and under different environmental conditions (Pereira et al. 2021). Integrating *MdIMA1* into marker-assisted breeding programs could accelerate the development of crop varieties resistant to Fe deficiency. A key area of future investigation is whether *MdIMA1* also participates in the synergistic absorption of Fe alongside other essential minerals like zinc (Zn) and manganese (Mn), to prevent nutrient imbalances. Understanding these interactions can provide valuable insights into optimizing nutrient uptake and minimizing antagonisms between micronutrients (Rietra et al. 2017). Addressing these research directions could position *MdIMA1* as a critical genetic tool for enhancing Fe tolerance in crops.

## Conclusion

In conclusion, this study revealed that *MdIMA1* plays a central role in regulating Fe homeostasis in plants. The overexpression of *MdIMA1* increased fresh weight, root length, and chlorophyll content under Fe-deficient conditions. Furthermore, *MdIMA1* enhanced Fe absorption by boosting the activity of FCR and  $\text{H}^+$ -ATPase. qRT-PCR analysis demonstrated that heterologous expression of *MdIMA1* in *Arabidopsis* upregulated the expression of several Fe absorption- and transport-related genes under Fe deficiency conditions, providing further evidence that *MdIMA1* functions to enhance plant tolerance to Fe deficiency.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-025-00095-2>.

Additional file 1: Figure S1. Bioinformatics analysis of *IRON MAN* (IMAs). a Phylogenetic tree of IMA proteins from different plant species. b Gene structure analysis of the *Malus domestica* IMAs (*MdIMAs*). c Conserved motif analysis of *MdIMAs*. d Protein sequence alignment of *MdIMAs*.

Additional file 2: Figure S2. Identification of *MdIMA1*-OE transgenic *Arabidopsis thaliana*. a Identification of transgenic *Arabidopsis* via DNA electrophoresis. Wild type (Col, *Arabidopsis* 'Colombia') was used as a negative control. b Relative expression levels of *MdIMA1* in Col and *MdIMA1*-OE transgenic *Arabidopsis*. The data are shown as means  $\pm$  SDs ( $n = 3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). *MdIMA1*, *Malus domestica* *IRON MAN 1*; *MdIMA1*-OE, *MdIMA1*-overexpressing.

Additional file 3: Figure S3. Effects of heterologous *MdIMA1* expression on endogenous IMA gene expression in *Arabidopsis*. a–c qRT–PCR analysis of the endogenous genes *AtIMA1* (a), *AtIMA2* (b), and *AtIMA3* (c) in wild type (Col) and *MdIMA1*-OE *Arabidopsis* under +Fe or -Fe+FRZ conditions. The data are shown as means  $\pm$  SDs ( $n = 3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). *MdIMA1*, *Malus domestica* *IRON MAN 1*; *AtIMAs*, *Arabidopsis thaliana* IMAs; +Fe, normal Fe supply; -Fe+FRZ, Fe-deficient+FerroZine condition.

Additional file 4: Figure S4. Identification of the *MdIMA1*-OE transgenic tomato. a Identification of the transgenic tomato via DNA electrophoresis. The wild type (WT) was used as a negative control. b Relative expression levels of *MdIMA1* in WT and *MdIMA1*-OE transgenic tomato plants. The data are shown as means  $\pm$  SDs ( $n = 3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). *MdIMA1*, *Malus domestica* *IRON MAN 1*; *MdIMA1*-OE, *MdIMA1*-overexpressing.

Additional file 5: Figure S5. Effects of *MdIMA1* heterologous expression on tomato endogenous gene expression. a, b qRT–PCR analysis of the endogenous genes *Solyc12g006770* (a) and *Solyc12g006760* (b) in wild type (WT) and *MdIMA1*-OE tomato under +Fe or -Fe+FRZ conditions. The data are shown as means  $\pm$  SDs ( $n = 3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). *MdIMA1*, *Malus domestica* *IRON MAN 1*; +Fe, normal Fe supply; -Fe+FRZ, Fe-deficient+FerroZine condition.

Additional file 6: Figure S6. Identification of *MdIMA1*-OE apple calli. a Identification of transgenic apple calli via DNA electrophoresis. The wild type (WT) was used as a negative control. b Relative expression level of *MdIMA1* in WT and *MdIMA1*-OE transgenic apple calli. The data are shown as means  $\pm$  SDs ( $n = 3$ ). Different letters indicate statistically significant differences (one-way ANOVA, Tukey–Kramer test,  $P < 0.05$ ). *MdIMA1*, *Malus domestica* *IRON MAN 1*; *MdIMA1*-OE, *MdIMA1*-overexpressing.

Additional file 7: Table S1. Primers used for the semi RT–PCR and quantitative RT–PCR analyses.

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## Authors' contributions

XFW, QYY, SSL and RXL conceived and performed the experiments. YXL, JXL, HLL and XHQ analyzed the data. MKW, YNW, JL and CC provided technical assistance. QYY and SSL wrote and revised the manuscript. XFW provided financial support.

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## Data availability

The article and supplemental materials contain data that support the findings.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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