

The overexpression of ATPS1 gene, a homodimeric enzyme involved in sulfur assimilation, confers Fe-deficient tolerance in *Malus halliana*

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Abstract

Iron (Fe) deficiency is one of the most common micronutrient deficiencies limiting crop production globally, especially in arid regions because of decreased availability of iron in alkaline soils. S supply increases Fe availability to plants, ATP sulphatase plays an important role in the synthesis of organic sulfur in plants, which is considered to be an important rate-limiting enzyme in sulphur uptake. However, the mechanism of whether it responds to Fe deficiency in plants remains unclear. Here, we identified a ATP sulphatase gene *MhATPS1* from *Malus hallinan* based on qRT-PCR and then genetically transformed it into tobacco and apple calli, which showed that transgenic tobacco and overexpressed apple calli secreted more H^+ content compared to the wild type (WT), resulting in increased inter-root acidification. In addition, the transgenic tobacco and apple calli showed better growth under Fe deficiency conditions. Furthermore, increased Fe^{2+} content and ferric chelate reductase (FCR) activity in the transgenic tobacco and overexpressed calli indicated improved Fe uptake, which is also confirmed by up-regulation of Fe uptake genes, e.g. FRO2, IRT1, FER and FIT. Under Fe deficiency stress, having more strengthened antioxidant capacity in transgenic tobacco, and the same results were shown in overexpressed apple calli. At last, the NBT and DAB staining results also verified the greater scavenging capacity of reactive oxygen species in transgenic tobacco. In summary, the *MhATPS1* gene may play a positive role in Fe deficiency stress both tobacco and apple calli.

Introduction

Iron (Fe), as an essential trace element for plant growth and development, is involved in a variety of biological processes, including photosynthesis, respiration and chlorophyll biosynthesis [1]. Although the total amount of Fe on earth meets the needs of plants, but most of it exists in the form of insoluble chelates, especially in saline soils, and cannot be effectively absorbed and utilized by plants [2–3]. Among them, fruit trees are susceptible to suffer from Fe deficiency and trigger chlorosis, resulting in yield reduction, quality decline, and nutritional imbalance [3].

In response to the low availability of Fe in the soil, plants have evolved two different strategies for obtaining Fe from the soil, including ‘Strategy I’ and ‘Strategy II’ [4]. ‘Strategy I’ plants including dicotyledons and non-gramineous plants, which through increasing the secretion of inter-root H^+ ions and increasing ferric chelate reductase (FCR) activity, Fe^{3+} is reduced to Fe^{2+} , and then IRT1 (Fe-regulated transporter) transports Fe^{2+} into the root cells for plant uptake and use [5–6]. In contrast, gramineous plants use ‘Strategy II’ to access iron via a chelation mechanism [7].

The supply of S in a Fe-deficient environment improves the utilisation of Fe by the plants, studies in oilseed rape [8], maize [9], and tomatoes [10] also argue for this view. ATP sulphatase (ATP sulfurylase, ATPS) plays an important role in the synthesis of organic sulfur in the plant. It is the first enzyme to play a role in sulphate assimilation in plants. Sulphate ion and ATP are catalyzed by the enzyme to produce adenosine-5-phosphate sulfate (APS) and pyrophosphate (PPi), which are then reduced to cysteine and other sulfur-containing amino acids [11–12]. Several lines of evidence have found that ATP sulphatase participate in the stress tolerance response of plants. For example, the overexpressed ATPase genes can increase the GSH content in plants to enhance the resistance of soybean [13], maize [14] and mustard [15] under stress by maintaining the integrity of plant cell membrane structure and defending membrane lipid peroxidation caused by free radical accumulation. Chan et al. (2013) have found that overexpressed ATP sulfate can improve the drought resistance of plants by regulating the synthesis of osmoregulatory substances, resistance signaling substances, and antioxidants in plants [16]. In addition, overexpression of the ATPS gene had a positive effect on plant resistance to heavy metal stress, whose high expression is conducive to the production of more GSH quench free radicals, thus enhancing the tolerance to chromium stress [17], and this finding was used to solve soil heavy metal pollution.

ATP sulfurylase have been shown to play an important role in abiotic stresses in plants [13,17]. However, the mechanism of whether it responds to Fe deficiency in plants remains elusive. Therefore, we reveal and study its function in Fe-deficient environments. First, based on qRT-PCR, ATPS1 was found to be strongly induced by Fe deficiency, and dramatically up-regulated. Subsequently, the ATPS gene was cloned from *Malus halliana*, and overexpressed apple calli and heterologously expressed transgenic tobacco were obtained by genetic transformation to determine the function of the gene in Fe-deficient surroundings, which will lay the foundation for further studies on the function of ATP sulfatase in woody plant stress and provide a reference for research on stress tolerance mechanisms in perennial fruit trees.

Material And Methods

Plant Materials And Treatments

Malus halliana seedlings were selected with cultivating for one month on Murashige and Skoog (MS) medium containing 0.5 mg/L 6-benzyl amino purine (6-BA) and 0.2 mg/L naphthalene acetic acid (NAA) to root culture with 1/2 MS medium including 0.5 mg/L IBA. Rooted plants were cultured as previously described by Sun et al. (2016) and then treated with different Hoagland nutrient solutions as follows. Fe sufficient (40 μ M Fe, Na-EDTA), Fe deficiency (0 μ M Fe, Na-EDTA).

Tobacco (*N. tabacum* L.) was subcultured at a 30-day interval on Murashige and Skoog (MS) medium at 25°C with a 16-h/8-h light/dark period. Whereas ‘Wanglin’ (*Malus Domestica* Borkh.) apple calli were subcultured at a 20-day interval on MS medium that contained 1.0 mg/L 2,4-D and 0.5 mg/L 6-BA at 26°C in dark [19]. They refreshed every 20 days before the apple calli and tobacco leaves were used for genetic transformation.

Gene Sequence Analysis

ATPS gene sequences from multiple plant species were downloaded from the NCBI database ([HTTPS://www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/)). The physical and chemical properties and hydrophobicity predicted proteins were analyzed using ExPASy online tools ([HTTPS://web.expasy.org/protparam/](https://web.expasy.org/protparam/) and [HTTPS://web.expasy.org/protscale/](https://web.expasy.org/protscale/)). Their subcellular localization was predicted and analyzed with the WOLF PSORT online tools ([HTTPS://wolfpsort.hgc.jp/](https://wolfpsort.hgc.jp/)), and conserved protein motifs were analyzed using the MEME suite online tool ([HTTPS://meme-suite.org/tools/meme](https://meme-suite.org/tools/meme)). *Cis*-acting promoter elements were predicted and analyzed using the PLACE database ([HTTPS://www.dna.afrc.go.jp/PLACE](https://www.dna.afrc.go.jp/PLACE)). DAMAN and MEGAX software were used for multiple sequence alignment and phylogenetic tree construction.

Quantitative Real-time Pcr

Primers were designed using DNAMAN based on sequences retrieved from the NCBI database. Real-time PCR primer pairs are listed in Table S1. *GAPDH* was used as a reference. Quantitative data analysis was performed using the $2^{-\Delta\Delta Ct}$ method [20]. The reaction system is referenced in Li's [20] paper. Three replicates were performed for each sample.

Gene Cloning And Vector Construction

ATPS genes induced by Fe deficiency were identified in the transcriptome database and compared to sequences at NCBI (<https://www.ncbi.nlm.nih.gov/>) to obtain the apple *MhATPS1* gene. Therefore, the coding region of *MhATPS1* was cloned using the primers shown in Table S1. The PCR product was obtained by electrophoresis on a 1.5% agarose gel. The target gene fragment was cut out for gel recovery, and the cloned vector pMD19-T was ligated for sequencing. The correctly sequenced *MhATPS1* plasmid was extracted, and *NdeI* and *KpnI* were used for the extraction. We performed double-digestion and simultaneous digestion of the overexpression vector pRI101. T4 ligase was used to ligate the two fragments overnight at 16°C to transform Trans 5a competent cells, which were screened on 100 mg L⁻¹ kanamycin. Resistance-positive clones were obtained as plasmids for overexpression vectors.

Agrobacterium-mediated transformation of tobacco and apple calli

A bacterial solution was used to infect tobacco leaves from which the main veins and petioles had been removed. After dark culture, bud induction, and rooting stage culture, kanamycin-resistant regenerated seedlings were obtained. The regenerated shoot DNA was extracted and identified by PCR. Infection of apple calli was based on the previously described method by Xie [21]. After 15 days, calli of the same culture state were immersed in infection solution with an OD value between 0.6 and 0.8 and cultured in the dark (120 r min⁻¹) for 15–20 min, then filtered. The calli were placed on a solid MS medium for 2 days and spread evenly on 250 mg L⁻¹ cephalosporin and 100 mg L⁻¹ kanamycin resistance medium. They were screened for about 30 days on the plate until a transgenic callus was obtained.

Determination of Fe and chlorophyll content

The Fe content was determined as described by Gong et al. (2013). Specifically, the test material was washed and dried, and the material was dried in an oven at 65°C. After grinding, 0.1 g of the sample was weighed and added to 5 mL HNO₃ and left to stand for 30 minutes. The material was digested in a microwave digester at 180°C for 25 minutes. The Fe content was determined using a plasma spectrometer ICP-mass spectrometer (ELAN DRC-e, Perkin Elmer, Toronto, ON, Canada). Each set of tests was repeated three times.

The chlorophyll concentration of fresh leaves was measured according to the method of Yi et al. (1994). After treatment, 0.1g of the treated transgenic or WT tobacco leaves were washed with water and dried, the veins were removed and the tissue was cut, 0.1g of the fresh leaf was cut into small pieces and homogenized with 15 mL alcohol: acetone (1:1; V/V) for 20 h. The mixture was centrifuged at 5000 rpm for 10 min, and then the absorbance of the supernatant was recorded at 645nm, 663 nm, and 470nm respectively. Finally, the total chlorophyll concentration was calculated. Each group of tests was repeated three times.

Ferric chelate reductase (FCR) activity determination

According to the method of Schikora and Schmidt (2001) to test the FCR activity in apple calli and tobacco. The main steps were as follows: Plant tissue was washed in 0.5 mM CaSO₄ for 5 min, which was washed off against deionized water, and then transferred to the chromogenic solution (0.5 mM ferrozine, 0.5 mM Fe, Na-EDTA, and 0.5 mM CaSO₄ PH 5.8) for 120 min in the dark, with manual rotation every 10 min. The activity of reduction was measured at 562 nm by a spectrophotometer (UV 1800, Shimadzu, Japan); and the assay solution without the experimental material was used as a blank control, data represent the means of three biological replicates.

Quantitative measurement of H₂O₂ content and 3,3-diaminobenzidine (DAB) staining

The content of H₂O₂ was determined as described by Sairam and Srivastava (2002). Briefly, fresh tissue was ground in precooled acetone on ice and centrifuged, and the supernatant was transferred into new centrifuge tubes. Next, 5% titanium sulfate and concentrated ammonia water were added to the precipitate in sequence. After three rinses with acetone, the precipitate was redissolved in 2 M H₂SO₄. The absorbance of the supernatant was measured at 415 nm. Measurements were performed for three replicates per treatment. The amount of H₂O₂ was located visually by incubating fresh tissues in 1 mg/mL DAB (pH 3.8) at room temperature overnight in the dark. Leaf pigments that would interfere with the determination were removed with absolute ethanol. The samples were photographed as described above.

Quantitative measurement of O²⁻ content, and nitrotetrazolium blue chloride (NBT) staining

The O²⁻ productivity rate was quantified according to the method of Huang et al. [26]. In situ O²⁻ accumulation in leaves was examined via histochemical staining with NBT [27]. Fresh leaves of wild and transgenic tobacco were placed in a final concentration of 1 mg/mL NBT (pH = 7.5) staining solution and infested for 4 hours in the dark at room temperature, decolorized by boiling with anhydrous ethanol, and then photographed for observation.

ROS-scavenging enzyme activity assays

Superoxide dismutase activity was assessed using the method of Beauchamp and Fridovich (1971), by measuring the photochemical inhibition of SOD at 560 nm [28]. CAT activity was measured according to Xu et al. (2013) by monitoring the consumption of H₂O₂ at 240 nm for 4 min [29]. The APX activity was determined according to Xu et al. (2014). Ascorbate oxidation was measured spectrophotometrically based on the decrease in absorption at 290 nm, using the absorption coefficient of 2.8 mM cm [30]. All measurements were conducted in triplicate.

Acidification capacity determination

Acidification assays were performed as described by Yi et al. (1994). WT and transgenic apple calli or tobacco lines were grown on Fe-sufficient media for 10 days and then transferred to Fe-deficient medium for 5 days. They were finally transferred to a 1% agar plate containing 0.006% bromocresol purple and 0.2 mM CaSO₄ (pH adjusted to 6.5 with NaOH) for 24-48h [23]. Acidification is indicated by the yellow color around the tobacco roots or calli.

Statistical Analyses

Treatment effects were assessed by analysis of variance, and means were compared using Duncan's test ($P < 0.05$). Statistical analyses were performed in SPSS version 22.0 (IBM, Armonk, NY, USA), and figures were prepared using Origin 8.0 software (OriginLab, Hampton, MA, USA). Significant differences were established by posthoc comparisons (HSD test of Tukey) at $P < 0.01$ or < 0.05 .

Result

Analysis Of Apple Atps Protein Physical And Chemical Properties

Firstly, we used the ATPS protein sequences in *Arabidopsis thaliana* for BLAST in the apple genome database (<http://genomics.research.iasma.it/>), and the obtained sequences were analyzed by SMART (<https://smart.embl.de/>) and Pfam (<http://pfam.xfam.org/null>) tools, and finally, four ATPS genes were identified in the whole apple genome.

The physical and chemical properties of four ATPS family members were analyzed (Table 1). The numbers of encoded amino acids were relatively equal, with an average of 475.5. Isoelectric points (pIs) of the proteins ranged from 7.01 to 8.82, positive residues ranged from 57 to 61, and aliphatic indices ranged from 83.91 to 89.47. Molecular masses ranged from 52,211.86 to 54,394.26 kDa, and negative residues ranged from 57 to 59. The hydrophobicity of the four proteins was negative, indicating that they were hydrophilic, although they varied in the degree of hydrophilicity. Secondary structure analysis of the ATPS proteins by the SOPMA program showed that they are mainly comprised of alpha helices (30.54–32.90%) and random coils (47.94–51.61%).

Table 1
List of ATPS family genes and information on their encoded proteins

Accession No	Gene name	Amino acid	Molecular mass (kDa)	P I	Positive residues	Negative residues	Aliphatic index	Protein hydrophobicity	Alpha Helix (%)	Random Coil (%)
MD13G1201500	LOC103410737	465	52,284.97	8.82	61	57	84.75	-0.397	32.90%	49.89%
MD15G1014100	LOC103450392	486	54,195.03	7.01	57	58	89.47	-0.340	30.86%	47.94%
MD16G1201400	LOC103403953	465	52,211.86	7.81	59	58	83.91	-0.375	30.54%	51.61%
MD08G1014800	LOC103440334	486	54,394.26	7.34	59	59	88.87	-0.370	30.86%	48.56%

The numbers in the table indicate the amount of expression in different cellular compartments

Subcellular Location Predictions For Apple Atps Proteins

Table 2
Subcellular location predictions of ATPS family proteins

Gene	Cytoplasmic	Chloroplast	Nuclear	Mitochondrial	Periplast	Nuclear and cytoplasmic	Golgi apparatus	Nuclear and plasma membrane
MD13G1201500		11						
MD15G1014100	1	11						
MD16G1201400		10						
MD08G1014800		5			8			

Cloning Of Atps1 Gene

In combination with qRT-PCR analysis of the above four genes in *Malus halliana* under an Fe-deficient environment, ATPS1, also known as MD13G1201500, was expressed at the highest level in the roots of *Malus halliana* compared to the other three genes (Fig. 1). As a result, ATPS1 was selected for follow-up studies.

The deduced amino acid sequence of the *MhATPS1* gene was compared with the amino acid sequences of ATP sulphatases of other species registered in the GenBank database using MEGAX software and was found to have the highest homology of 99.57% with *Malus sylvestris*, Homology with *Prunu mumes* lowest (Fig. S2).

The amino acid sequences of ATPS1 from different plant species were compared with each other using the MEGE X software and found to be very conserved (Fig. 4). The amino acid sequences of ATP sulfatases from different plant species were found to have a highly conserved ATPS domain, which contained three sub-structural domains, denoted as Block I, Block II and Block III, respectively (Fig. S3).

Analysis of cis-acting elements of promoters

Plant CARE analysis revealed that the *MhATPS1* promoter sequence contains several regulatory elements related to stress resistance, including the low-temperature element LTR; the drought stress-related element MBS, and multiple hormone-responsive elements such as the part of an auxin-responsive element TGA-box and the MeJA-responsiveness cis-acting element TGACG-motif.

Table 3
cis-elements in the genomic sequence 2000 bp upstream of the *MhATPS1*

Regulator sequence	Sequence	Function of site
SP1	GGGCGG	Light responsive element
TGA-box	TGACGTAA	Part of an auxin-responsive element
LTR	CCGAAA	Cis-acting element involved in low-temperature
ARE	AAACCA	Cis-acting regulatory element essential for the anaerobic induction
MBS	CAACTG	MYB binding site involved in drought-inducibility
TGACG-motif	TGACG	Cis-acting regulatory element involved in the MeJA-responsiveness
CAT-box	GCCACT	Cis-acting regulation element regulation to meristem expression

Identification Of Overexpressed Atps1 Gene In Tobacco And Apple Calli

As shown in Fig. 2, the expression levels of *ATPS1* in transgenic tobacco and overexpressed calli were determined by qRT-PCR and transgenic strains contained higher expression levels compared with the WT strains.

Overexpression of MhATPS1 gene in tobacco improves Fe deficiency tolerance

To investigate whether the *MhATPS1* gene plays a role in coping with Fe deficiency, transgenic tobacco and the WT tobacco were allowed to grow for 30 days under Fe-sufficient conditions and then shifted to Fe-deficient conditions for a further 20 days. The results showed that both transgenic tobacco and WT tobacco grew normally under Fe-sufficient conditions. Under Fe deficiency treatment, Fe deficiency limits the growth of tobacco plants, starting with the newborn leaves showing chlorosis, which was manifested by the loss of green between the veins of the upper leaves, while the lower leaves remain green, however, the WT tobacco showed more severe chlorosis in appearance (Fig. 3a). Measurement of chlorophyll content in transgenic and WT tobacco revealed lower chlorophyll contents in the unfolding young leaves in the WT tobacco than in transgenic tobacco (Fig. 3b).

Fe is an essential component or cofactor of various antioxidant enzymes and Fe deficiency can lead to oxidative stress [31]. O_2^- and H_2O_2 are the two prominent ROS molecules that usually accumulate under abiotic stresses [32], thus, Histochemical staining with 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) were performed used to check if ROS were production in WT tobacco or transgenic tobacco. Under the Fe Fe sufficient conditions, there were no detectable differences in the accumulations of O_2^- and H_2O_2 between the transgenic tobacco and the WT tobacco. In contrast, under Fe-deficient surroundings, the results showed that the leaves of WT tobacco appear to be darker in color than those of the transgenic tobacco, suggesting that less ROS accumulation in transgenic tobacco resulted in fewer polymerization oxidation products (Fig. 4a, Fig. 4b). To further verify the production of ROS, the levels of O_2^- and H_2O_2 were measured, and showed that the transgenic tobacco produced less O_2^- and H_2O_2 in leaves than WT tobacco (Fig. 4c). As the Fig. 4(d) shown that tobacco showed increased SOD and APX activity and reduced POD and CAT activity, but the antioxidant activities of POD, SOD, CAT, and APX were higher in transgenic tobacco than those of WT, indicating that overexpression of the *MhATPS1* gene can enhance ROS scavenging ability, maintain redox homeostasis under Fe deficiency.

Previous studies have shown that plants in a Fe-deficient environment will lower soil pH through plasma membrane H^+ -ATPase-mediated proton exocytosis, converting insoluble Fe ions to soluble Fe and thereby increasing Fe availability[23]. To test whether *MhATPS1* influences the rhizosphere PH in response to Fe deprivation, transgenic tobacco and WT tobacco were incubated in a Fe-sufficient environment for 10 days and then transferred to a Fe-deficient environment for 5 days, after which they were transferred to a medium containing the PH indicator bromocresol purple for staining. The results showed that under Fe-deficient conditions, the transgenic tobacco had a degree of inter-root acidification in the WT tobacco, with a yellowish colour around the root system.(Fig. 5a), indicating that overexpression of *MhATPS1* could promote the accumulation of Fe in tobacco.

To determine how the response of the *MhATPS1* gene to Fe deficiency was mediated, the activity of the FCR activity in plants was measured. No visible differences were observed in the FCR activity between the WT and the transgenic tobacco under Fe-sufficient conditions, while FCR activity was significantly increased in the transgenic tobacco under Fe-deficient conditions (Fig. 5b). In addition, the Fe^{2+} content was measured in leaves, which displayed that transgenic tobacco accumulated much higher Fe content than WT tobacco under Fe-deficient conditions (Fig. 5b). These results suggest that the overexpression of *MhATPS1* resulted in significantly strengthening tolerance to Fe deficiency in transgenic tobacco.

At last, we measured the expression levels of genes related to Fe uptake and transport. The results showed that the relative expression of *NtIRT1*, *NtFRO2*, and *NtFER1* in the transgenic tobacco was significantly higher than that of WT under Fe deficiency (Fig. 5c), indicating that the transgenic tobacco may promote Fe uptake and utilization to alleviate the stress.

Tolerance of *MhATPS1* transgenic apple calli under Fe deficiency

As shown in Fig. 6(a), WT and overexpressed calli grew uniformly on Fe-sufficient media, while the WT calli grew slowly, compared with the overexpressed apple calli on Fe-deficient media. Next, the activities of antioxidant enzymes, including SOD, POD, CAT, and APX were measured, and these activities of overexpressed calli were dramatically enhanced in apple calli compared to the WT calli Fe deficiency stress (Fig. 7a).

Another important indicator of Fe uptake is the activity of inter-root trivalent Fe reductase (FCR) activity [33]. Therefore, we first measured the activity of FCR, which is responsible for Fe reduction during Fe deficiency treatment, and the results showed that the FCR activity of the WT calli was significantly lower than that of the overexpressed calli in both Fe-sufficient and Fe-deficient media (Fig. 7b). Subsequently, we then investigated the rhizobial acidification capacity of the WT calli and the overexpressed calli. We grew them on a Fe-sufficient medium for 10 days, transferred them to a Fe-deficient medium for 5 days, and then placed them on agar plates containing the PH indicator bromocresol violet for 1 day, showing that the cultures exhibited a significant increase in rhizobial H⁺, which caused the medium to turn yellow around the cultures, regardless of the Fe treatment; however, under Fe-deficient conditions, the yellow color around the overexpressed apple calli was more pronounced than the WT calli, indicating that the overexpressed calli pump out more H⁺ content, compared to WT calli (Fig. 6b).

The key gene for Fe absorption, Fe transporter, and hydrogen secretion have been reported extensively [47]. We therefore further analyzed the changes in the expression of these genes. Next, we examined the expression of genes induced by Fe deficiency, respectively, which were AHA8, FRO2, FIT, and IRT1. As Fig. 7(c) showed that the transcription expression of FRO2 was higher in WT calli than in overexpressed calli under Fe-deficient media. Similarly, the expression of IRT1, a key transporter delivering rhizosphere Fe²⁺ into cells, is also lower in the WT calli than in the overexpressed calli. The above results could explain that overexpressed calli confer a much more powerful Fe uptake than WT calli. Furthermore, quantification of Fe²⁺ content showed that Fe²⁺ content was significantly higher in overexpressed calli than the WT calli under both normal and Fe-deficient conditions (Fig. 7b).

Discussion

Fe is an essential micronutrient for plant growth, development, and reproduction. However, approximately one-third of the world's cultivated land is calcareous, where Fe levels are very low.

To survive in Fe-deficient conditions, plants have evolved strict network systems to control iron uptake and transport, in which genes and transcription factors were involved in iron homeostasis are gradually identified and characterized, they have been used to enhance Fe uptake and storage in plants. For example, overexpression of genes such as bHLH104 [3], FRO1 [5] and IRT1[6] promotes iron uptake by plants in iron-deficient environments.

ATP sulphatase genes play an important role in plant stress resistance. Many ATP sulphatase genes have been identified in plants. For example, ATP sulphatase genes from maize [16], *Arabidopsis thaliana* [40], and soybean [39]. Studies in mustard have shown that overexpression of ATP sulphatase can increase plant resistance to salt stress [8]. Chan et al. (2013) confirmed that overexpressed ATP sulphatase enhanced tolerance to drought resistance [13]. Here we reported that the *MhATPS1* gene, and found that the relative expression of *MhATPS1* was increased in transgenic tobacco and overexpressed apple calli following treatment with Fe deficiency stress (Fig. 2), suggesting that *MhATPS1* may have a regulatory role during Fe deficient stress.

Although Fe is not a component of chlorophyll, it is indispensable in the synthesis of chlorophyll precursors [41]. The degree of chlorosis caused by Fe deficiency in crops can be expressed in terms of chlorophyll content and can therefore be used as a direct indicator of how plants are affected by Fe deficiency stress [41]. In our experiment, When transgenic and WT tobacco were grown in Fe sufficiency condition for 30 weeks and transferred to Fe-sufficient or Fe-deficient hydroponic solution for another 20 days, leaf chlorosis was observed in newly emerging tobacco leaves, but the WT tobacco showed more severe chlorosis in appearance (Fig. 3a). At the same time, Chlorophyll content also differed significantly between transgenic and WT tobacco under Fe deficient, with higher chlorophyll content in transgenic tobacco than in WT tobacco (Fig. 3b).

Plants in a Fe-deficient environment can increase effective Fe content by acidifying the soil through the plasma membrane H⁺-ATPase, thereby promoting Fe uptake by the plant. Santi et al. (2009) showed that *Arabidopsis thaliana* with strong inter-root acidification grew better [42]; Wu et al. (2012) also found that *Malus xiaojinensis* released more H⁺ from plant roots in Fe-deficient environments, promoting the uptake of Fe [43]. In the present experiment, it was also found that overexpression of the *MhATPS1* gene enhanced H⁺ release and promoted Fe uptake in transgenic tobacco and overexpressed apple calli. Therefore, *MhATPS1* may have a positive regulatory effect on PM H⁺-ATPase activity and the uptake and homeostasis of Fe. It was found that 11 PM H⁺-ATPases encoding AHA genes are present in *Arabidopsis*, some of which are involved in Fe acquisition and homeostasis, particularly AHA2, which plays an integral role in regulating plant root tumor acidification [44]. Zhao (2016) found that of the 18 PM H⁺-ATPases from apples, AHA8 was the closest homolog with *Arabidopsis* AHA2 [45]; Therefore, we measured the *MhAHA8* content of overexpressed apple calli and found that AHA8 was highly expressed after Fe deficiency stress. It is possible that high expression of AHA8 enhances H⁺-ATPase activity and large proton efflux, thereby promoting Fe uptake and utilization, which is consistent with the conclusions of

Fig. 7(c). As mentioned above, the results showed that the transgenic tobacco and overexpressed apple calli displayed a larger acidified zone compared to WT, as evidenced by a more conspicuous yellow color in agar plates containing the pH indicator bromocresol violet (Fig. 5a, Fig. 6b).

Fe deficiency can cause damage to the chloroplast and mitochondrial structures of plants, leading to blockage of the electron transport chain and possibly causing oxidative damage through the production of excess reactive oxygen species in plants [46–47]. On the other hand, Fe is a component of the antioxidant enzyme system (SOD, POD, CAT, and other enzymes) in plant cells [34], and Fe deficiency are prone to oxidative stress, which affects the plant's resistance to stress [48]. Ranieri et al. (2001) found that Fe deficiency stress increased ROS levels in plants [49], and Manchanda et al. (1999) suggested that rapidly up-regulated antioxidant enzyme (CAT or APX) activities could counteract oxidative damage to cells caused by ROS bursts [50]. SOD acts as a major enzyme to protect cell membrane lipids from oxidation and it is closely associated with plant senescence and resilience [46]; POD, which oxidises IAA and scavenges H₂O₂ formed by oxygen radicals in plants, can both be associated with Fe deficiency tolerance and growth in plants [49–50]. Ranieri et al. (2003) showed that SOD and POD activities in both roots and leaves were enhanced in *Pisum sativum* under Fe deficiency [51], which is a normal response in plant metabolism, but Fe deficiency may also lead to disturbances in normal metabolism, resulting in a disruption of the protein system, when SOD and POD activities are reduced. In summary, it is clear that the SOD and POD activities content of plants increase in response to Fe deficiency. The results of this experiment showed that the antioxidant enzyme activities of the plants were significantly higher under Fe deficiency, but the transgenic tobacco and overexpressed calli had higher antioxidant enzyme activities compared to the WT material, indicating that they had stronger ROS scavenging ability (Fig. 4c, Fig. 4a). The results of DAB and NBT staining also confirmed the conclusion that overexpression of the *MhATPS1* gene resulted in higher antioxidant enzyme activity in plants under Fe deficiency stress, thereby inhibiting the accumulation of ROS and reducing oxidative damage, thereby improving plant tolerance to Fe deficiency stress (Fig. 4a, Fig. 4b). which is consistent with the previous reports [52]. The results confirm that the increased activity of the plant antioxidant enzyme system facilitated the scavenging of excess reactive oxygen species, reduced oxidative damage, and improved tolerance to Fe deficiency.

FCR activity of transgenic tobacco and overexpressed apple calli was increased under Fe deficient conditions; and Fe contents also displayed the same trend (Fig. 5b, Fig. 7b). Among them, the average increase in Fe content of transgenic tobacco lines was 35.78% in leaves and 25.75% in apple calli compared with that of WT strains. Further, the expression of Fe acquisition genes that are involved in Fe uptake and proton release was also affected. The qRT-PCR analysis of *MhFRO2*, *MhIRT1*, and *MhFIT* revealed a dramatic increase in the expression of overexpressed calli grown on Fe deficiency (Fig. 7c). At the same time, overexpression of the above genes in transgenic tobacco promotes the uptake of Fe in tobacco, such as *NTFRO2*, *NTIRT1*, and *NTFER1* (Fig. 5c). All in all, these results indicate that *MhATPS1* promoted Fe acquisition.

Conclusion

In summary, our studies showed that *MhATPS1* functions as a positive regulator of Fe deficiency tolerance in tobacco and apple calli. It can improve the utilization of Fe in tobacco and apple calli under Fe-deficient surroundings by promoting inter-root proton secretion, enhancing FCR activity, and improving the expression of related genes such as FRO2, FIT, and IRT1 et al, resulting in increased Fe content and thus enhanced antioxidant enzyme activity to reduce the damage caused by Fe deficiency.

Declarations

Ethical Approval and Consent to participate Not applicable.

Human and Animal Ethics Not applicable.

Consent for publication All co-authors gave consent for publication of this manuscript.

Availability of supporting data All datasets analyzed in this study are presented in this article.

Conflict of interest The authors declare that have no competing interests.

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Authorship contributions JC performed the experiments and wrote the main manuscript text. ZXZ, YLG and YXW made many constructive suggestions on the experimental process. YJD, CLI, XLX, and LD carried out the experiments. All authors reviewed the manuscript.

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Figures

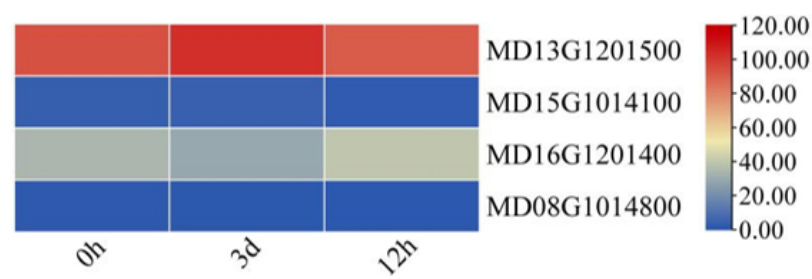


Figure 1

Expression of genes in transcriptome data

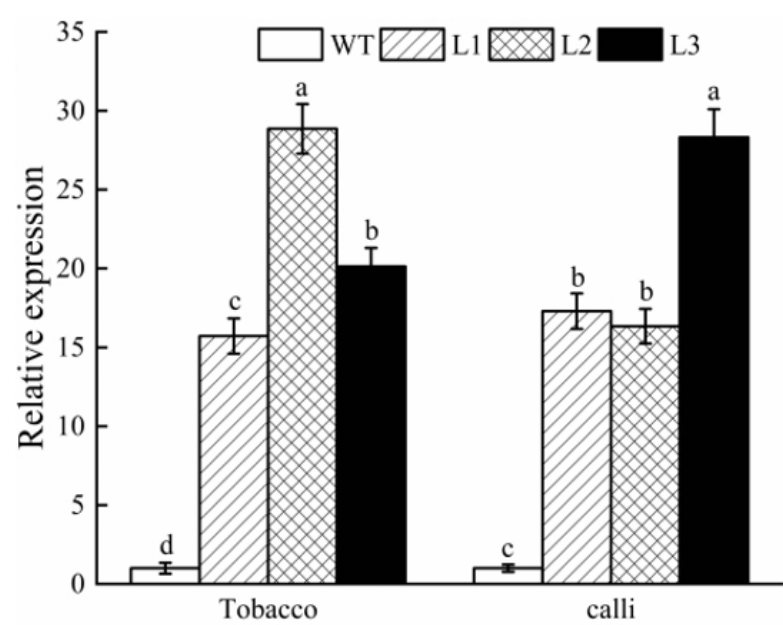


Figure 2

Expression levels of the *ATPS1* gene in different overexpressed lines. L1, L2, L3 *ATPS1* overexpression line; WT, wild-type. Different letters above the bars indicated significant differences ($p < 0.05$) as assessed by one-way ANOVA and the least significant difference (LSD) test.

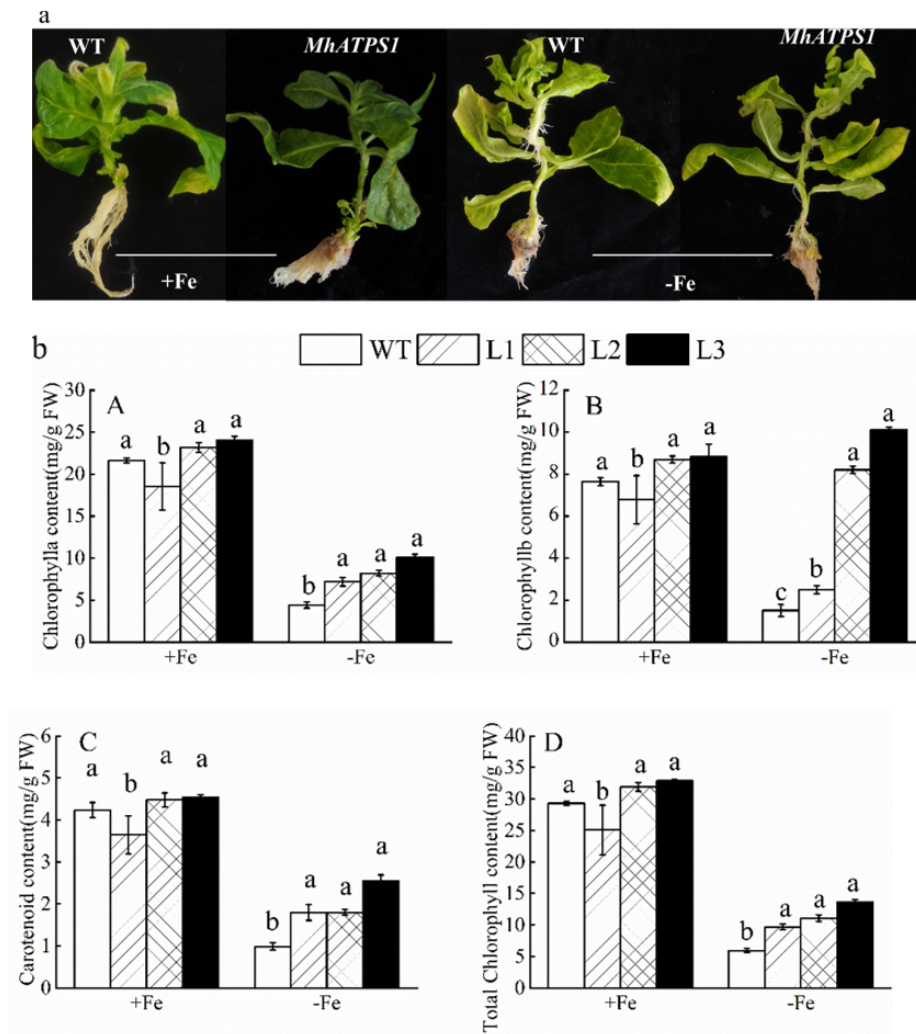


Figure 3

Growth and photosynthetic response to Fe deficiency in tobacco in hydroponic culture. a, growth of transgenic tobacco and WT tobacco under Fe sufficient (+Fe) or Fe-deficient (-Fe) conditions. b, contents of chlorophyll a, chlorophyll b, Carotenoid contents in leaves, and Total Chlorophyll content.

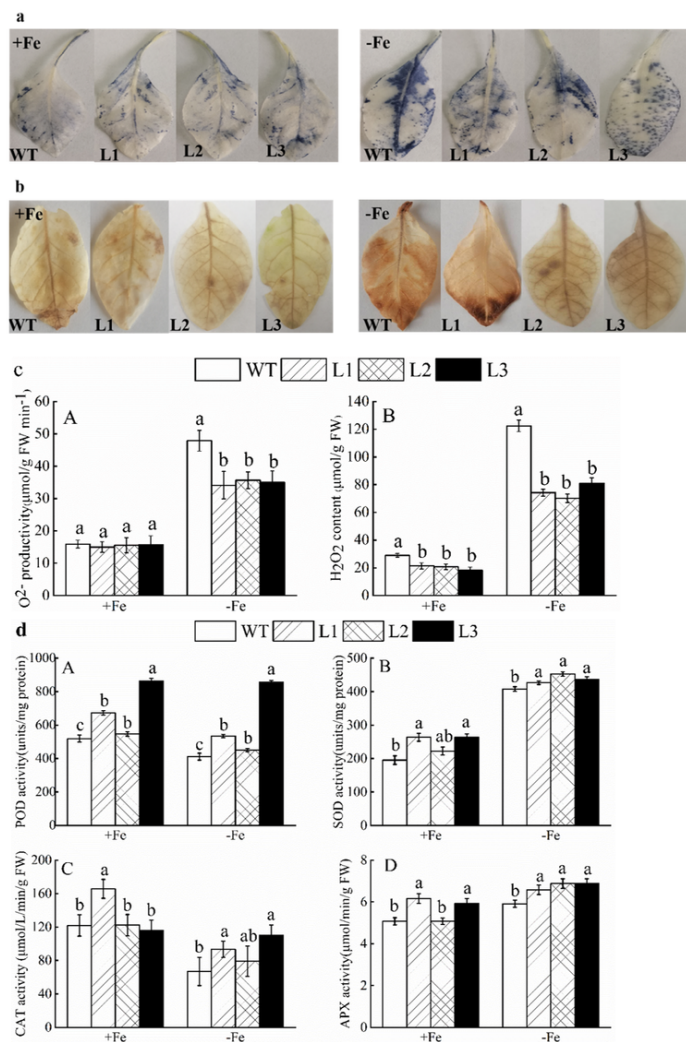


Figure 4

Antioxidant activity analysis in tobacco under Fe-sufficient (+Fe) and Fe-deficient (-Fe) conditions. a, H₂O₂ and O₂⁻ content in the leaves; b, NBT and DAB Tissue staining. c, enzymatic activity of SOD, POD, APX and CAT.

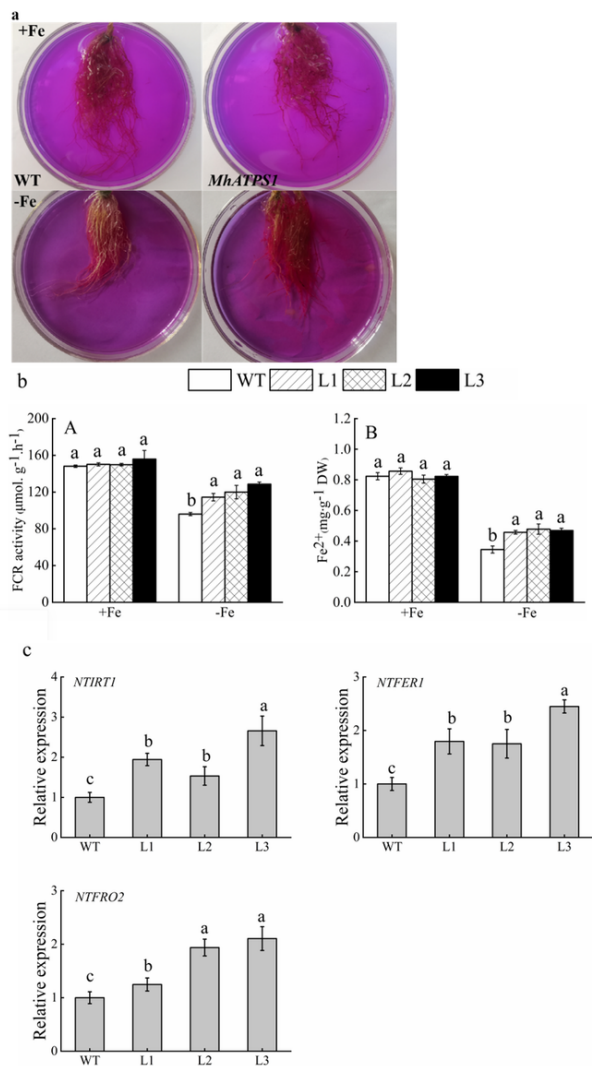


Figure 5

Fe acquisition analysis of tobacco in response to Fe deficiency. a, Rhizosphere acidification assay using agar plates containing bromocresol purple for 1 d; root H^+ content assay of tobacco grown on (+Fe) medium or (-Fe) medium. b, ferric chelate reductase (FCR) activity and Fe^{2+} content in the leaves of tobacco grown under +Fe or -Fe conditions. c, expression of genes related to Fe uptake and transport in tobacco leaves in a Fe-deficient environment.

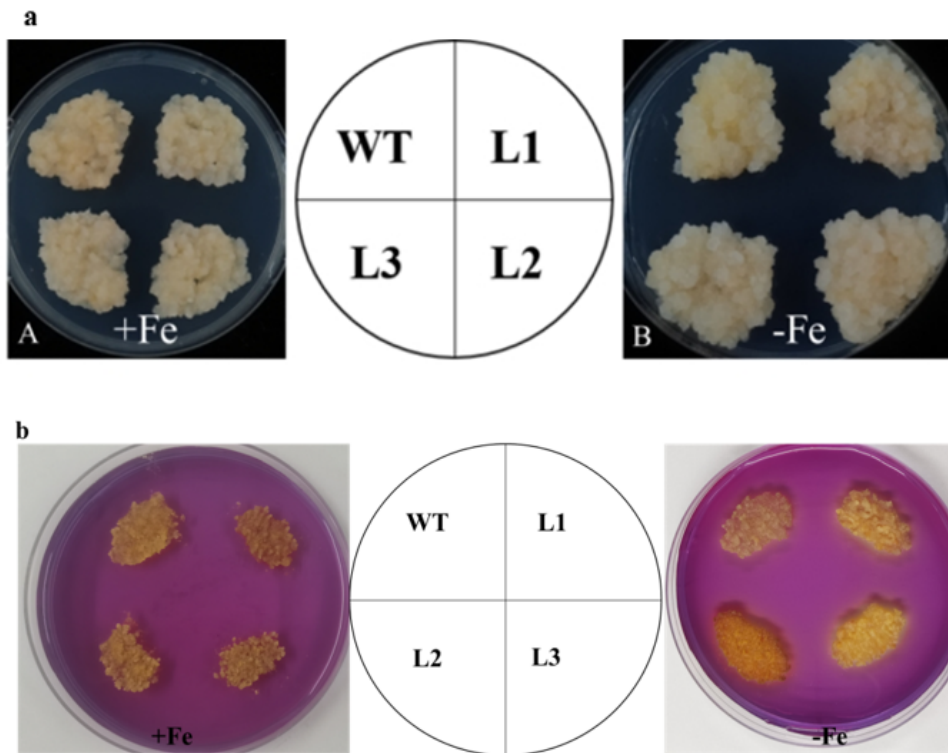


Figure 6

a, Phenotypes of *MhATPS1* transgenic and WT apple calli grown on Fe-sufficient and Fe-deficient media for 20 days. b, Rhizosphere acidification assay using agar plates containing bromocresol purple for 1d.

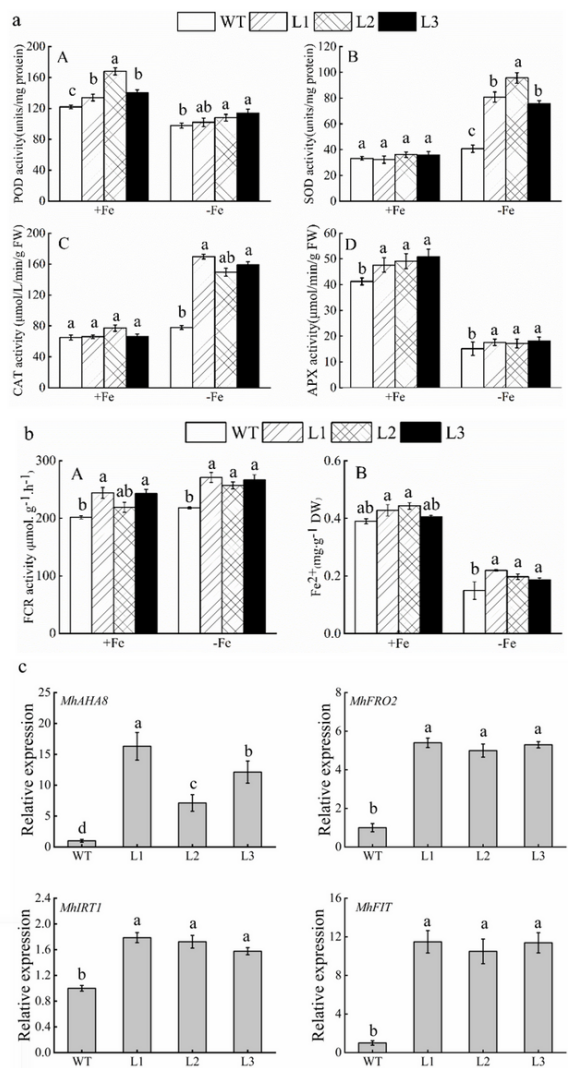


Figure 7

a, Antioxidant activity analysis in apple calli under Fe-sufficient (+Fe) and Fe-deficient (-Fe) conditions. enzymatic activity of POD SOD, APX, and CAT. b, Fe content and FCR activity in apple calli. c, Fe accumulation and expression profile of Fe uptake genes in apple calli grown under Fe-sufficient (+Fe) and Fe-deficient (-Fe) conditions.

Supplementary Files

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