# Resveratrol Improves the Iron Deficiency Adaptation of Malus Baccata Seedlings by Regulating Iron Absorption, Phytohormone Content, and ROS Migration

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# Abstract

Resveratrol (Res), a phytoalexin, has been widely reported to participate in plant resistance to fungal infections. However, little information is available on its role in abiotic stress, especially in iron deficiency stress. *Malus baccata* is widely used as apple rootstock in China, but it is sensitive to iron deficiency. In this study, we investigated the role of exogenous Res in *M. baccata* seedings under iron deficiency stress. Results showed that applying 100 μmol exogenous Res could alleviate iron deficiency stress. The seedlings treated with Res had a lower etiolation rate and higher chlorophyll content and photosynthetic rate compared with the apple seedlings without Res treatment. Exogenous Res increased the iron content in the roots and leaves by inducing the expression of *MbAHA* genes and improving the $H^+$-ATPase activity. As a result, the rhizosphere pH decreased, iron solubility increased, the expression of *MbFRO2* and *MbIRT1* was induced, and the ferric-chelated reductase activity was enhanced to absorb large amounts of $Fe^{2+}$ into the root cells under iron deficiency conditions. Moreover, exogenous Res application increased the contents of IAA, ABA, and GA3 and decreased the contents of DHZR and BL for responding to iron deficiency stress indirectly. In addition, Res functioned as an antioxidant that strengthened the activities of antioxidant enzymes and thus eliminated reactive oxygen species production induced by iron deficiency stress. These findings are expected to enhance the application and examination of the physiological role of Res under iron deficiency stress in apples.


# Introduction

Iron (Fe) is one of the most essential micronutrients for plant growth, and it plays a vital role in several important physiological processes, such as chlorophyll biosynthesis, photosynthesis, and antioxidative defense (Li and Lan 2017; Lima et al. 2014). Although Fe is abundant in soil, it mainly exists in the form of low-bioavailability ferric iron ($Fe^{3+}$), especially in alkaline calcareous soils where high bicarbonate contents reduce the availability of Fe severely (Lucena 2000). About 30% of the world's arable land is potentially Fe-deficient, and Fe deficiency stress is one of the widest-ranging abiotic stresses that constrain crop yield and quality (Dobbels and Lorenz 2019). Apple (*Malus domestica* Borkh.) is one of the most valuable horticultural fruit crops cultivated worldwide. However, one-third of apple orchards in China suffer from Fe deficiency stress with a significant chlorosis phenotype (Zhang et al. 2020a). Fe deficiency leads to incomplete development of the chloroplast structure, resulting in leaf yellowing and severe loss of crop yield and quality (Guo et al. 2020). Fe fertilizer is widely used in apple orchard production to supply deficient $Fe^{2+}$. However, long-term over-application of fertilizer can degrade soil biological characteristics in apple orchards and seriously affect the yield and quality of the fruit (Wang et al. 2016; Zhang et al. 2020b). Therefore, improving the utilization efficiency of Fe effectively and enhancing apple rootstock tolerance to Fe deficiency stress have become research hotspots.

Plants develop a series of mechanisms during their long-term struggle with environmental stresses. To cope with Fe deficiency stress, plants adopt two strategies for absorbing and translocating Fe (Lucena 2000; Zhou et al. 2019). Plants that adopt Mechanism I usually include dicotyledons and non-

gramineous monocots (Kobayashi and Nishizawa 2012). These plants absorb Fe mainly through three processes. First, the $H^+$-ATPase (AHA) enzyme on the root plasma membrane secretes protons to decrease the rhizosphere pH for increasing Fe solubility. Second, ferric reductase oxidase 2 (FRO2) converts invalid $Fe^{3+}$ to effective $Fe^{2+}$. Third, the high-affinity Fe transporter (IRT1) transports $Fe^{2+}$ into the root cells (Kobayashi and Nishizawa 2012; Vert et al. 2002). Plants that adopt Mechanism II, including gramineous plants, synthesize and secrete mugineic acid family phytosiderophores (MAs) to form an MA-$Fe^{3+}$ complex (Inoue et al. 2009; Lucena 2000). Apple trees are dicotyledons, and they use Mechanism I to absorb and translocate Fe. At the transcriptional regulatory level, the Fer-like Fe deficiency-induced transcription factor (FIT), the core transcription factor, directly regulates the absorption of Fe (Yuan et al. 2008). Moreover, FIT can form a heterodimer with other basic Helix-Loop-Helix transcription factor (bHLH), including bHLH38/39/100/101, to control the ferric reduction response and Fe transport into the root by directly regulating the transcription of *FRO2* and *IRT1* genes under Fe deficiency stress (Wang et al. 2013b). In apples, MdbHLH104 is activated by the SUMO E3 ligase MdSIZ1 and targets *MdAHA8* to promote plasma membrane $H^+$ exocytosis (Zhao et al. 2016; Zhou et al. 2019).

Several studies have found that phytohormones are also involved in plants' responses to Fe deficiency stress. Abscisic acid (ABA), gibberellin (GA), and salicylic acid (SA) help alleviate the damage caused by Fe deficiency stress, whereas jasmonate (JA), cytokinin (CTK), and brassinolide (BL) have the opposite effect (Brumbarova et al.2015; Wang et al. 2015; Wild et al. 2016). Exogenous ABA application can considerably improve the shoot Fe content and recover the chlorosis phenotype (Lei et al. 2014). SA can interact with NO to promote the absorption, transport, and activation of Fe in an Fe-deficient environment (Kong et al. 2014). JA suppresses the Fe absorption of plant roots by inhibiting the expression of *IRT1* and *FRO2* (Maurer et al. 2011). Recent studies have shown that application of BL to rice can aggravate the symptoms of Fe deficiency (Wang et al. 2015).

The application of plant growth regulators is an effective approach for improving the Fe deficiency tolerance of crops because of the important role of phytohormones in plants' response to Fe deficiency stress (Lei et al. 2014; Maurer et al. 2011). Resveratrol (Res), a stilbenoid compound, has been identified in more than 70 plant species, including grapes, peanuts, and knotweed (Liu et al. 2019; Yu et al. 2005). Res is classified as an antimicrobial phytoalexin that contributes to plant response to biotic and abiotic stress (Liu et al. 2019). Most of the studies on Res focused on improving its resistance to fungal infections. Romeropérez et al (Romeropérez et al. 2001). demonstrated that the Res level in grape berries is significantly induced after infection by powdery mildew. Res can also help improve the resistance of apple leaves to *Venturia inaequalis* (Schulze et al. 2005). Overexpressing exogenous stilbene synthase (STS) genes, as the key enzyme in the Res synthesis pathway, in tobacco, wheat, rice, apple, and grape can enhance resistance to fungal pathogens (Dai et al. 2015; Leckband and Lo¨rz 1998; Szankowski et al. 2003). With regard to the role of Res in plants' response to abiotic stress, previous studies have reported that Res content is induced by wounding or UV light (Cantos et al. 2003). Grimmig et al (Grimmig et al. 2002). also found that Res production is related to plants' response to ozone. In citrus seedlings, Res and its combination with α-tocopherol can mediate salt adaptation (Kostopoulou et al. 2014).

However, whether Res is involved in plants' response to Fe deficiency and the underlying molecular and physiological mechanisms remain unknown.

In this study, we investigated the effects of different concentrations of exogenous Res on *Malus baccata* seedlings under Fe deficiency stress. Then, we explored the potential physiological and molecular mechanisms through which Res influences the photosynthetic system, Fe absorption, rhizosphere pH, antioxidase activity, and hormone content. The expression of the key function genes and the transcription factors related to Fe absorption in apple were also determined under Fe deficiency stress and exogenous Res treatment. Our findings can provide a theoretical basis for analyzing the mechanism of Res in apple under Fe deficiency stress.

# Results

### Effects of exogenous Res on apple seedlings under Fe deficiency stress

Res with different concentrations was sprayed on the leaves of the apple seedlings under Fe deficiency stress to determine the effects of exogenous Res on the resistance of *M. baccata* under Fe deficiency stress. As shown in Fig. 1a, the young leaves of the apple seedlings exhibited serious leaf chlorosis under Fe deficiency stress for 24 days compared with the control. However, different concentrations of exogenous Res could alleviate the chlorosis to different degrees. When low-concentration (10 μmol) and high-concentration (200 μmol) Res were applied to the apple seedlings under Fe deficiency, the young leaves were greener than the leaves of the stressed seedlings and had a much lower etiolation rate than the control (Fig. 1b). When 100 μmol Res was applied, the young leaves maintained normal growth and had the lowest etiolation rate (24.67%) compared with the seedlings with Fe deficiency stress (85.33%) (Fig. 1b). Moreover, the fresh weight of the young leaves under Fe deficiency stress decreased considerably from 1.84 g to 1.46 g. When 100 μmol Res was applied, the fresh weight recovered to 1.78 g (Fig. 1c). Therefore, 100 μmol of Res was selected for further research.

### Effects of exogenous Res on chlorophyll content and photosynthetic rate under Fe deficiency stress

The chlorophyll content and photosynthetic rate of the apple seedlings were also determined to examine the chlorosis phenotype caused by Fe deficiency stress. The chlorophyll content decreased from 29.9 SPAD to 20.7 SPAD after 24 days of Fe deficiency stress. However, the application of exogenous Res alleviated the decline in chlorophyll content, which recovered to as high as 27.2 SPAD (Fig. 2a). Similarly, the photosynthetic rate of the apple seedlings dramatically decreased from 15.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to 8.7 $\mu mol \cdot m^{-2} \cdot s^{-1}$ after Fe deficiency stress. When exogenous Res was applied, the photosynthetic rate of the apple seedlings improved by 55.9% and reached 13.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 2b). These results show that the application of exogenous Res effectively increased the chlorophyll content and photosynthetic rate under Fe deficiency stress.

### Effects of exogenous Res on Fe content under Fe deficiency stress

Perls staining was performed to detect the Fe content in roots and explore whether exogenous Res could affect the Fe content under Fe deficiency stress. The Fe content under Fe deficiency stress was much lower than that in the control, and the staining results were considerably shallower. However, exogenous Res significantly enhanced the Fe content under Fe deficiency stress and produced deep staining results (Fig. 3a). These staining results are consistent with the results of the quantification of Fe contents in the roots. The Fe content in the roots decreased from 790.22 mg/kg DW to 491.88 mg/kg DW after Fe deficiency stress for 24 days. When exogenous Res was applied, the Fe content increased to 644.51 mg/kg DW (Fig. 3b). Aside from the Fe content in the roots, the Fe content in the leaves was also investigated. The Fe content in the leaves decreased from 586.51 mg/kg DW to 231.22 mg/kg DW under Fe deficiency stress, but exogenous Res increased this content to as high as 462.47 mg/kg DW (Fig. 3c). These results indicate that the application of exogenous Res increased the Fe content in the roots and leaves under Fe deficiency stress.

### Effects of exogenous Res on rhizosphere pH and FCR activity under Fe deficiency stress

Rhizosphere pH staining was performed to explore the mechanisms of the positive effect of exogenous Res on Fe deficiency stress. The results showed that the rhizosphere pH stain was very shallow under Fe deficiency stress. When exogenous Res was applied, the rhizosphere pH stain became even shallower than that under Fe deficiency stress (Fig. 4a). Furthermore, the activity of root $H^+$-ATPase was determined. The results showed that the $H^+$-ATPase activity increased under Fe deficiency stress from 2.84 μmol/h/g FW to 4.44 μmol/h/g FW. Exogenous Res could even enhance the $H^+$-ATPase activity to 6.46 μmol/h/g FW (Fig. 4b). In addition, FCR activity was examined. Unlike $H^+$-ATPase activity, FCR activity decreased significantly under Fe deficiency stress from 2.72 nmol/min/g FW to 0.54 nmol/min/g FW. However, when exogenous Res was applied, FCR activity was induced and reached 1.20 nmol/min/g FW (Fig. 4c).

### Effects of exogenous Res on oxidative damage and antioxidant enzyme activities under Fe deficiency stress

The oxidative damage induced by Fe deficiency stress was determined. As shown in Fig. 5a, the $O_2{\cdot}^-$ and $H_2O_2$ contents were considerably increased by Fe deficiency stress. However, exogenous Res could decrease the $O_2{\cdot}^-$ and $H_2O_2$ levels. The content of MDA, as the indicator of lipid peroxidation damage, under Fe deficiency stress was 44.17% higher than that of the control group. When exogenous Res was applied, the MDA content recovered to the normal level (Fig. 5b).

The activities of antioxidant enzymes SOD, POD, and CAT were also examined to determine the role of Res in antioxidant enzymes under Fe deficiency stress. A similar variation tendency was exhibited by the three antioxidant enzymes. The activities of SOD, POD, and CAT were significantly inhibited under Fe deficiency stress. However, exogenous Res could recover the activities of the three antioxidant enzymes, especially CAT (Fig.s 5c–5e). The CAT activity after Fe deficiency stress for 24 days was only 6.29 U/mg protein, which is much lower than that of the control group (11.46 U/mg protein). Exogenous Res application could increase such activity by 68.20% to as high as 10.58 U/mg protein (Fig. 5e). These

results demonstrate that the application of exogenous Res alleviated the oxidative damage under Fe deficiency stress.

### Effects of exogenous Res on electrolyte leakage and osmolytes under Fe deficiency stress

Electrolyte leakage was determined under Fe deficiency stress. Electrolyte leakage was significantly increased by Fe deficiency stress from 14.3% to 29.7%. When 100 μmol exogenous Res was applied, the electrolyte leakage was reduced to 24.07% (Fig. 6a).

The contents of osmolytes, including proline, soluble sugar, and soluble protein, were also measured. Fe deficiency stress significantly increased the soluble sugar and proline contents but decreased the soluble protein content. When exogenous Res was applied, the contents of soluble sugar and soluble protein increased significantly, but the content of proline exhibited almost no change (Fig.s 6b–6d).

### Effects of exogenous Res on endogenous hormone content under Fe deficiency stress

Previous studies have shown that plant hormones are involved in plants' response to Fe deficiency stress. IAA, GA, and ABA play a positive role, whereas CTK, JA, and BR play a negative role. However, the effect of exogenous Res on endogenous hormone content under Fe deficiency has never been reported (Fig. 7a). Thus, the endogenous hormone contents under Fe deficiency stress and exogenous Res treatment were examined in this study.

Under Fe deficiency stress, the contents of ABA and GA3 increased significantly, whereas the IAA content was inhibited. When exogenous Res was applied, the contents of IAA, ABA, and GA3 increased by 31.04%, 85.73%, and 18.10%, respectively (Fig. 7b). The contents of JA-Me, DHZR, and BL also increased under Fe deficiency stress. When exogenous Res was applied, the levels of DHZR and BL decreased from 4.85 ng/g to 3.32 ng/g and from 5.98 ng/g to 5.29 ng/g, respectively. The content of JA-Me exhibited no significant change (Fig. 7c).

### Effects of exogenous Res on the expression of Fe-deficiency responding genes

To elucidate the mechanism of exogenous Res involvement in the Fe deficiency response, we performed qPCR to detect the expression of Fe-related genes under Fe deficiency stress and exogenous Res treatment.

As shown in Fig. 8, exogenous Res significantly upregulated the expression of *MbAHA1*, *MbAHA3*, and *MbAHA9*, as $H^+$-ATPase (AHA) enzyme family genes, by 6.63, 2.09, and 2.19 times, respectively (Fig.s 8a–8c). The expression of *MbFRO2* and *MbIRT1* was significantly downregulated under Fe deficiency stress. However, when exogenous Res was applied, the expression of *MbFRO2* and *MbIRT1* increased by 1.24 and 0.67 times, respectively (Fig.s 8d and 8e). For the bHLH-type transcription factors, the expression of *MbPYE* and *MbBHLH104* was significantly downregulated under Fe deficiency stress. When exogenous Res was applied, the expression of *MbPYE* was continuously suppressed, whereas the expression of *MbBHLH104* was induced (Fig.s 8f and 8g). Meanwhile, the expression of *MbBHLH105* had no

significant change under Fe deficiency stress and exogenous Res treatment (Fig. 8h). For the SUMO E3 ligase protein, the expression of *MdSIZ1* was significantly upregulated by Fe deficiency stress, and exogenous Res could further increase its expression (Fig. 8i).

# Discussion

Fe deficiency is one of the most widespread micronutrient deficiency stresses faced by plants, and it severely limits crop quality and yield (Briat et al. 1995; Zhang et al. 2020a). *M. baccata*, which is native to the Greater Higgnan Mountains, is widely used as an apple rootstock in northern China (Yang et al. 2011). It has high resistance to low temperatures and can be used as a raw material for breeding cold-tolerant apple rootstock. However, *M. baccata* is sensitive to Fe deficiency stress (Wang et al. 2013a). Fe deficiency stress induces chlorosis of *M.baccata* leaves, decreases tree vigor, and results in huge economic losses (Sun et al. 2020). Therefore, the Fe deficiency tolerance of *M. baccata* seedlings must be enhanced. The application of plant growth regulators is an effective approach for improving the Fe deficiency tolerance of crops (Akhtar et al. 2018; Kong et al. 2014). Res is a phytoalexin that contributes to plant biotic responses (Liu et al. 2019; Schulze et al. 2005). Exogenous application of Res reduces postharvest decay in several fruit types, such as tomatoes, grapes, and avocado pears (Jimenez et al. 2005). However, its role in plants' response to Fe deficiency stress has never been reported. In this study, we investigated the role of different concentrations of Res in *M. baccata* seedlings under Fe deficiency stress. The effect of applying 100 μmol of Res on Fe deficiency tolerance was much better than the effect of applying 10 μmol (low concentration) and 200 μmol (high concentration) of Res; it also produced the lowest etiolation rate and highest fresh weight (Fig. 1). Plant growth regulators usually affect plant growth and development in a dose-dependent manner. Su et al (Su et al. 2020). reported that the effect of applying 0.2 mg/L of BL on improving the salt tolerance of apple seedlings is much better than the effect of applying BL concentrations of 0.05 and 1.0 mg/L. Our result indicates that 100 μmol Res is an appropriate concentration for enhancing the Fe deficiency tolerance of *M. baccata* seedlings.

The Fe contents in the roots and leaves under Fe deficiency stress and exogenous Res treatment were detected to determine how Res improves Fe deficiency tolerance. Our result indicates that 100 μmol of exogenous Res significantly increased the Fe content in the leaves and roots under Fe deficiency stress. This result is consistent with the Fe staining result (Fig. 3). A previous study also reported that exogenous ABA application can significantly improve the shoot Fe content by inducing the expression of *AtNRAMP3* in *Arabidopsis* (Lei et al. 2014). In higher plants, most of the leaf Fe is located in the chloroplasts, and abundant Fe is essential for maintaining the function of the chloroplasts and photosynthetic system (Kobayashi et al. 2012; Zhou et al.2016). Chlorophyll is the major component of chloroplasts; Fe deficiency could lead to a reduction in chlorophyll content and photosynthetic rate, which causes the yellowing leaf phenotype (Graziano and Lamattina 2007). In this study, Fe deficiency decreased the leaf chlorophyll content and photosynthetic rate of the apple seedlings. However, the application of exogenous Res increased the chlorophyll content and photosynthetic rate under Fe deficiency stress, thereby alleviating leaf chlorosis (Fig. 2). These results indicate that exogenous Res exerted a positive effect on the absorption of Fe and protected the chlorophyll and photosynthetic system from Fe

deficiency stress. A similar study also reported that exogenous melatonin can improve Fe deficiency tolerance by increasing the absorption of Fe and the chlorophyll content in *Arabidopsis* (Zhou et al. 2016). This is the novel role of Res in plants' response to Fe deficiency stress.

We analyzed how Res enhances Fe uptake in Fe-deficient apple seedlings via the mechanism of plant response to Fe deficiency stress. Apples are dicotyledons that adopt the typical Mechanism I strategy to absorb and translocate Fe (Zhang et al. 2019; Zhou et al. 2019). Under Fe deficiency stress, first, plants develop a proton pump that acidifies the rhizosphere to increase the solubility of Fe through the upregulation of plasma membrane (PM) $H^+$-ATPase (AHA) family genes, such as *CsAHA1*, *AtAHA2*, and *MdAHA8* (Sun et al. 2020; Zhao et al. 2016). In the present study, we found that rhizosphere pH was significantly reduced, whereas the activity of $H^+$-ATPases increased after exogenous Res was applied under Fe deficiency stress (Fig. 4). Moreover, exogenous Res application significantly enhanced the expression of the *MbAHA* family, including *MbAHA1*, *MbAHA3*, and *MbAHA9* (Fig. 8). These results indicate that Res increased the amount of soluble Fe by acidifying the rhizosphere and upregulating the expression of *MbAHA* family genes. Second, FRO2 and ferric-chelated reductase convert $Fe^{3+}$ to $Fe^{2+}$, which is then transported into the roots via IRT1 (Kobayashi and Nishizawa 2012; Vert et al. 2002; Zhou et al. 2019). Our results indicate that exogenous Res significantly increased the activity of FCR and induced the expression of *MbFRO2* and *MbIRT1* (Fig.s 4 and 8). Therefore, we speculate that exogenous Res can increase the expression of *MbFRO2* and *MbIRT1* and enhance the activity of FCR to utilize the soluble Fe in the rhizosphere and transport $Fe^{2+}$ to cells under Fe deficiency stress. Third, transcriptional regulation is one of the most common ways to regulate the function of genes involved in Fe uptake (Wang et al. 2013b; Yuan et al. 2008; Zhou et al. 2019). bHLH TFs have been reported to regulate the Fe deficiency response (Kobayashi and Nishizawa 2012). AtbHLH104 and AtbHLH105 positively regulate Fe absorption and rhizosphere acidification by directly activating the transcription of *bHLH38/39/100/101* (Zhang et al. 2015). Fe transport-related genes and *FRO3* are up-regulated in *pye-1* mutant under Fe-deficient conditions, suggesting that the PYE bHLH protein functions as a negative regulator under Fe deficiency stress in *Arabidopsis* (Long et al. 2010). In apple, MdbHLH104 has been identified as a positive regulator by directly binding to the promoter of *MdAHA8* to activate PM $H^+$-ATPase activity and regulate rhizosphere acidification under Fe deficiency stress.[15] In the present study, exogenous Res significantly increased the expression of *MbbHLH104* and decreased the expression of *MbPYE1* under Fe deficiency stress. Meanwhile, no significant expression change was observed for *MbbHLH105* (Fig. 8). In addition, MdSIZ1 enhances the stability of MdbHLH104 protein and promotes PM $H^+$ exocytosis and rhizosphere acidification in the Fe deficiency response (Zhou et al. 2019). Our results also showed that exogenous Res significantly increased the expression of *MbSIZ1* under Fe deficiency stress (Fig. 8). These results are consistent with the result that a high Fe content was detected in the apple seedlings with exogenous Res treatment under Fe deficiency stress (Fig. 3). Therefore, we infer that exogenous Res can enhance the Fe uptake under Fe deficiency stress mainly by regulating the signal response genes in the Mechanism I strategy.

Plant hormones are also involved in the Fe deficiency response. Exogenous IAA can regulate Fe uptake through the accumulation of NO under Fe deficiency stress (Brumbarova et al. 2015). OsARF16, a transcription factor that regulates auxin redistribution, is required for Fe deficiency response in rice (*Oryza sativa* L.) (Shen et al. 2015). GA positively responds to Fe deficiency by regulating the expression of Fe-related genes, such as *bHLH038*, *bHLH039*, *FRO2*, and *IRT1* (Matsuoka et al. 2013; Wild et al. 2016). Moreover, ABA and SA play a positive role in Fe deficiency response (Kong et al. 2014; Lei et al. 2014). In our study, the ABA and GA3 contents were significantly increased by Fe deficiency stress, whereas the IAA content was reduced. However, the IAA, ABA, and GA3 contents increased after the application of exogenous Res (Fig. 7). Meanwhile, CTK negatively regulates the root Fe uptake and inhibits the expression of *FRO2* and *IRT1* through a growth-dependent pathway in *Arabidopsis* (Séguéla et al. 2008). JA and BR were also negatively regulated Fe homeostasis (Maurer et al. 2011). Exogenous BL decreases the expression of *OsNAS1*, *OsNAS2*, and *OsYSL2* in the stem and inhibit Fe transport in the phloem (Wang et al. 2015). Our results indicate that the application of exogenous Res decreased the content of DHZR and BL but produced no significant change in JA-Me (Fig. 7). Overall, we infer that aside from the Mechanism I strategy, exogenous Res can also enhance Fe uptake under Fe deficiency stress by increasing the contents of IAA, ABA, and GA3 and decreasing the contents of DHZR and BL.

Fe is an essential component of electron transport chains in mitochondria and chloroplasts. Fe deficiency stress can induce oxidative damage (Le et al. 2016). In the present study, we investigated the influence of Fe deficiency stress on ROS level and MDA content. Our results showed that the $O_2^{\cdot-}$, $H_2O_2$, and MDA contents were sharply increased by Fe deficiency stress. This result agrees with that of Sun et al (Sun et al. 2016). who reported that Fe deficiency can trigger ROS and $H_2O_2$ production at the early Fe-deficient stage in *M. xiaojinensis*. Our study also revealed that when exogenous Res was applied, the ROS level and MDA content decreased significantly. The activities of the antioxidant enzymes, such as SOD, POD, and CAT, increased significantly under Fe deficiency stress (Fig. 5). Exogenous Res can decrease the ROS level and MDA content in citrus seedlings by enhancing the activities of SOD, POD, and APX (Kostopoulou et al. 2014). Our results revealed that Res has the same function as antioxidants under Fe deficiency stress. Electrolyte leakage is an important indicator of plant cell permeability. When oxidative damage or ion stress occurs, electrolyte leakage is affected by abiotic stresses. In the present study, electrolyte leakage was sharply induced by Fe deficiency stress, whereas exogenous Res decreased it through the accumulation of soluble sugar and soluble protein (Fig. 6). A previous study reported that exogenous Res can increase electrolyte leakage through the accumulation of proline and soluble sugar under salt stress (Kostopoulou et al. 2014). However, unlike in the presence of salt stress, the proline content in the current study showed no significant change when exogenous Res was applied under Fe deficiency stress.

In conclusion, our study found that exogenous Res significantly alleviated the Fe deficiency stress of the apple seedlings. The molecular and physiological mechanisms of such alleviation were examined. First, exogenous Res improved the absorption of Fe by promoting the Mechanism I strategy. Second, exogenous Res responded to Fe deficiency stress by regulating the contents of plant hormones. Lastly, Res functioned as an antioxidant to cope with the oxidative damage caused by Fe deficiency stress.

These findings provide a theoretical basis for analyzing how Res application improves the Fe deficiency tolerance of apples and elucidate the physiological role of Res under Fe deficiency stress.

# Materials And Methods

### Plant materials and growth conditions

Seeds of apple (*Malus baccata*) were sown in wet vermiculite after cold stratification. When the seedlings developed four leaves, they were transferred to pots with dimensions of 7 cm × 7 cm × 10 cm (length, width, and height) and irrigated with complete Hoagland's nutrient solution. The apple seedlings were grown under the condition of 23 °C ± 2 °C in a 16/8 h light/dark cycle, and the light intensity was 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After two weeks, the apple seedlings were used for Fe deficiency and exogenous Res treatment.

### Fe deficiency and exogenous Res treatment

A total of 180 apple seedlings with similar growth conditions were randomly divided into five groups. The seedlings in Group I, as the control, were watered with a complete nutrient solution (40 μmol Fe). For the Fe deficiency treatment, the seedlings in Group II were watered with Fe deficiency nutrient solution (4 μmol Fe). The foliar of seedlings in Groups III, IV, and V were sprayed with 10, 100, and 200 μmol of exogenous Res (Sangon, Shanghai, China), respectively. Res was dissolved in ethanol at a concentration of 10 mM and stored at −20 °C. Res was sprayed every two days. The seedlings from all the groups were photographed, collected after Fe deficiency/Res treatment for 24 days, immediately frozen in liquid nitrogen, and stored at −80 °C in a DW-86L388J ultra-cold storage freezer (Haier, Qingdao, China). The experiment was repeated three times.

### Measurements of chlorophyll content and photosynthetic rate

Thirty apple seedlings were randomly selected from each group to measure the chlorophyll content and photosynthetic rate after Fe deficiency and exogenous Res treatment for 24 days. A SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan) was used for the determination of chlorophyll content. The photosynthetic rate was measured with an LI-6400XT meter (LI-COR, Lincoln, USA). The light intensity was set to 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with 50% humidity. The temperature was 22 °C. Each experiment was independently repeated three times.

### Determination of Fe content

The apple seedlings from Groups I, II, and IV were collected for Fe content determination after 24 days of Fe deficiency and exogenous Res treatment. For the staining of Fe in roots, Perls staining was conducted using a Prussian blue Fe stain kit (Solarbio, Beijing, China). Samples were dehydrated at 105 °C for 30 min and baked at 80 °C for 72 h for the quantification of Fe content in roots and leaves. Then, the kiln-dried samples were digested with 12 mL of $HNO_3$ and $HClO_4$ and diluted with deionized $H_2O$ to 25 mL.

Elemental analysis of Fe was performed via inductively coupled plasma–optical emission spectrometry (PerkinElmer, Waltham Massachusetts, USA) as described by Su et al (Su et al. 2020). Each experiment was independently repeated three times.

### Rhizosphere pH staining

The roots from Groups I, II, and IV were collected for rhizosphere pH staining after 24 days of Fe deficiency and exogenous Res treatment. Rhizosphere pH staining was conducted as described by Zhao et al (Zhao et al. 2016). Each experiment was independently repeated three times.

### Determinationofroot $H^+$-ATPase and ferric-chelated reductase (FCR) activity

A total of 0.2 g of the roots of the *M. baccata* seedlings were collected after 24 days of Fe deficiency and exogenous Res treatment for the detection of root $H^+$-ATPase and FCR activity by using $H^+$-ATPase and FCR activity extraction kits, respectively (Suzhou Geruisi Biotechnology Co., Ltd., Suzhou, China). Each experiment was independently repeated three times.

### Measurements of reactive oxygen species (ROS) level and malondialdehyde (MDA) content

The leaves in Groups I, II, and IV after Fe deficiency and exogenous Res treatment for 24 days were used to determine the ROS level and MDA content. Dying of superoxide anions ($O_2^{\cdot -}$) and hydrogen peroxide ($H_2O_2$) contents was conducted by following the procedure of Zheng et al (Zheng et al. 2017). MDA content was detected using a plant MDA extraction kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Each experiment was independently repeated three times.

### Detection of antioxidant enzyme activities

A total of 0.2 g of apple leaves were ground in phosphate buffer with a concentration of 100 mM (pH 7.4), transferred into 1.5 mL tubes afterward, and centrifuged at 4,000 × *g* for 10 min at 4 °C. The supernatants were used for the detection of SOD, POD, and CAT enzyme activities at the absorbance of 560, 470, and 240 nm, respectively. Enzyme activities were detected using plant SOD, POD, and CAT extraction kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Each experiment was independently repeated three times.

### Determination of electrolyte leakage and osmolytes

A total of 0.3 g of the leaves of the *M. baccata* seedlings were collected after 24 days of Fe deficiency and exogenous Res treatment for the detection of electrolyte leakage and osmolytes, respectively. Electrolyte leakage was determined as described by Su et al.[33] Osmolytes were determined with plant proline, soluble sugar, and soluble protein extraction kits (Suzhou Geruisi Biotechnology Co., Ltd., Suzhou, China). Each experiment was independently repeated three times.

### Measurement of endogenous hormone content

A total of 0.5 g of the leaves of the *M. baccata* seedlings were collected after 24 days of Fe deficiency and exogenous Res treatment for the detection of endogenous hormones, including IAA, GA3, ABA, DHZR, BL, and JA-Me. Measurement of the endogenous hormone content was performed using electrospray ionization–high-performance liquid chromatography–tandem mass spectrometry in accordance with the method described by Zhuo et al (Zhuo et al. 2019). Each experiment was independently repeated three times.

### Quantitative RT-PCR (RT-qPCR) assay

Total RNA was extracted from the roots of the *M. baccata* seedlings by using a FastPure Plant Total RNA Isolation Kit (Vazyme, Nanjing, China), and the cDNA was synthesized from 2 μg of total RNA by using 5× All-In-One RT MasterMix (ABM, Sydney, Australia). LightCycler® 480 SYBR Green Master (Roche, Mannheim, Germany) with a LightCycler® 480 II system (Roche, Rotkreuz, Switzerland) was used for the qPCR assay, and the primers are listed in Table S1. The primer sequences for qPCR were designed in accordance with the coding sequence of genes in Primer 5 software and checked using a BLAST search in the apple genomic database. The relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method. Each experiment was independently repeated three times.

### Statistical analysis

The data were subjected to ANOVA followed by Fisher's LSD or Student's *t*-test analysis. Statistically significant differences were indicated by $P<0.05$. Statistical computations were conducted using SPSS software (IBM, Armonk, NY, USA).

# Declarations


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### Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### Author contributions

Y. T. and C. W. planned and designed the research. X. Z., H. C., Q. S., G. S., C. M., Z. S., X. Y., and X. L. performed experiments, conducted fieldwork and analyzed data etc. X. Z. and Y. T. wrote the manuscript.

# Abbreviations

Res, Resveratrol; ROS, Reactive oxygen species; MDA, Malondialdehyde; SOD, Superoxide dismutase; POD, Peroxidase; CAT, Catalase; ABA, Abscisic acid; GA, gibberellin; SA, salicylic acid; JA, jasmonate; CTK, cytokinin; BL, brassinolide.

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# Supplemental Data

Supplemental Table S1 not available with this version

# Figures

**Figure 1**

Effects of exogenous Res on apple seedlings under Fe deficiency stress. (a) The phenotype resulting from the application of different concentrations of exogenous Res (10, 100, 200 μmol) to Malus baccata seedlings under Fe deficiency stress at day 0 and day 24. The etiolation rate (b) and fresh weight (c) of the apple seedlings after Fe deficiency and exogenous Res treatment for 24 days. Data represent the means ± SD of triplicate experiments. Different lowercase letters indicate significant differences, according to Fisher's LSD ($P < 0.05$).




**Figure 2**

Effects of exogenous Res on chlorophyll content and photosynthetic rate under Fe deficiency stress. The chlorophyll content (a) and photosynthetic rate (b) of the apple seedlings after Fe deficiency and exogenous Res treatment for 24 days. Data represent the means ± SD of triplicate experiments. Different lowercase letters indicate significant differences, according to Fisher's LSD ($P < 0.05$).

**Figure 3**

Effects of exogenous Res on Fe content under Fe deficiency stress. (a) Perls staining of Fe in roots. Total Fe content of roots (b) and leaves (c) after Fe deficiency and exogenous Res treatment for 24 days. Data represent the means ± SD of triplicate experiments. Different lowercase letters indicate significant differences, according to Fisher's LSD ($P < 0.05$).




**Figure 4**

Effects of exogenous Res on rhizosphere pH and FCR activity under Fe deficiency stress. (a) The rhizosphere pH staining of the roots after Fe deficiency and exogenous Res treatment for 24 days. The arrows indicated the rhizosphere. The activities of H+-ATPase (b) and FCR (c) in roots after Fe deficiency and exogenous Res treatment for 24 days. Data represent the means ± SD of triplicate experiments. Different lowercase letters indicate significant differences, according to Fisher's LSD ($P < 0.05$).

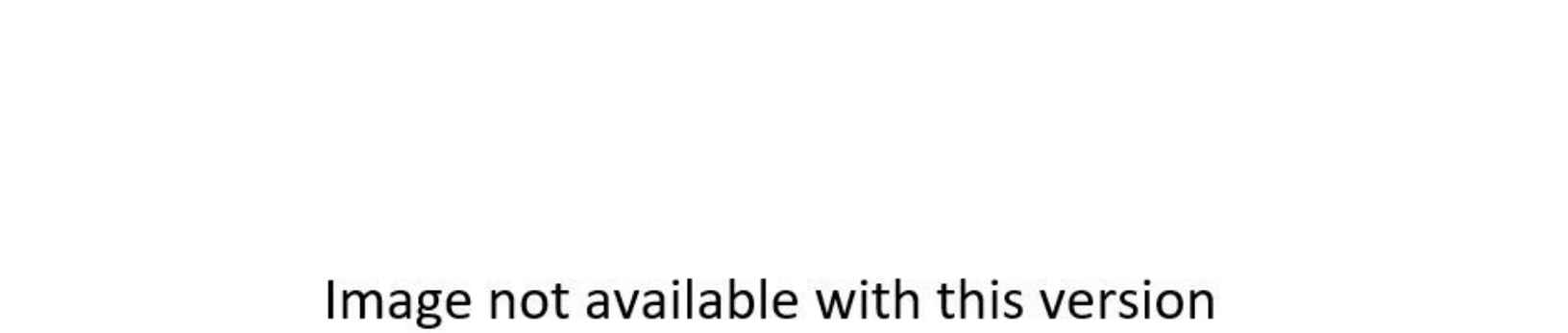


**Figure 5**

Effects of exogenous Res on oxidative damage and antioxidant enzyme activities under Fe deficiency stress. Effects of exogenous Res application on the levels of $O_2^{\cdot-}$ and $H_2O_2$ (a) and MDA content (b) under Fe deficiency stress. Effects of exogenous Res application on the activities of SOD (c), POD (d) and CAT (e) under Fe deficiency stress. Data represent the means ± SD of triplicate experiments. Different lowercase letters indicate significant differences, according to Fisher's LSD ($P < 0.05$).




**Figure 6**

Effects of exogenous Res on electrolyte leakage and osmolytes under Fe deficiency stress. Effects of exogenous Res application on electrolyte leakage (a), proline content (b), soluble sugar content (c) and soluble protein content (d) under Fe deficiency stress. Data represent the means ± SD of triplicate experiments. Different lowercase letters indicate significant differences, according to Fisher's LSD ($P < 0.05$).

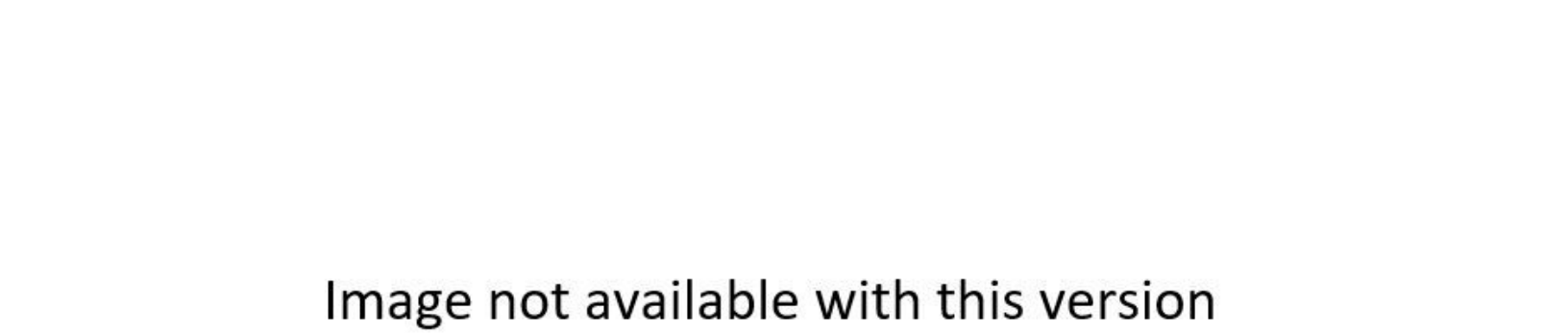


**Figure 7**

Effects of exogenous Res on endogenous hormone contents under Fe deficiency stress. (a) Mechanisms of hormones response to Fe deficiency stress. Effects of exogenous Res on IAA, GA3, ABA contents (b) and JA-Me, DHZR, BL contents (c) under Fe deficiency stress. Data are means ± SD of triplicate experiments. Asterisks (*) indicate significant differences from the control (Student's t-test, *P < 0.05, **P < 0.01).




**Figure 8**

Effects of exogenous Res on the expression of Fe deficiency responding genes in roots. The expression of MbAHA1 (a), MbAHA3 (b), MbAHA9 (c), MbFRO2 (d), MbIRT1 (e), MbPYE1 (f), MbBHLH104 (g), MbBHLH105 (h), and MbSIZ1 (i) in roots after Fe deficiency and exogenous Res treatment for 24 days. Data represent the means ± SD of triplicate experiments. Different lowercase letters indicate significant differences, according to Fisher's LSD (P < 0.05).