The long-distance *OPT3* movement is selective between herbaceous and woody plants as shown using *Malus* and *Arabidopsis thaliana* plants

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Abstract

Long-distance mobile mRNAs play key roles in gene regulatory networks controlling plant development and stress resistance. However, the mechanisms underlying species-specific delivery of mRNA still need to be elucidated. Here, the use of grafts involving highly heterozygous apple (*Malus*) genotypes allowed us to demonstrate that *MdOPT3* mRNA can be transported over a long distance, from the leaf to the root, to regulate Fe uptake; whereas, *AtOPT3*, the mRNA of the *MdOPT3* homolog from *Arabidopsis thaliana*, does not move from shoot to root. Reciprocal heterologous expression of the two types of mRNAs showed that the immobile *AtOPT3* moved from the shoot to the root in *Malus* and *Populus* plants, while the mobile *MdOPT3* became immobile in *A. thaliana* and *Solanum lycopersicum*. Furthermore, we demonstrate that the different transmissibility of *OPT3* in *A. thaliana* and *Malus* might be caused by the divergent RNA-binding *proteins* (RBPs) located in herbaceous and woody plants. This study provides insights into mechanisms underlying differences in mRNA mobility and validates the important physiological functions associated with this process.

Background

Plant grafting is a vegetative propagation technique for horticultural trait improvement, such as stress resistance, development and flowering characteristics¹⁻³. Experiments with various horticulturally important plant species have shown that abiotic stress can be alleviated by grafting scions to stress resistant rootstocks⁴⁻⁸. Such studies have also revealed that physiological and morphological features can be altered by rootstock-scion interactions⁸. Grafting-induced phenotypic changes have been proposed to be associated with the actions of mobile plant hormones, RNAs and proteins^{8,9}, but the underlying mechanisms are not well understood.

Previous studies have shown that grafts involved in two different species or a transgenic plant and its corresponding wild-type (WT) can be used to study long-distance transmission of specific signals¹⁰. Grafting experiments have confirmed that endogenous RNA molecules exist in phloem sap¹¹ and mRNAs move in both the stock-to-scion and scion-to-stock directions. The long-distance transport of RNAs is facilitated by specific proteins that prevent RNAs from being degraded during transport. For example, it is known that CmPP16 binds to its own RNA to form a ribonucleoprotein (*RNP*) complex in pumpkin (*Cucurbita maxima*). This complex interacts with the plasmodesmata protein, NCAPP1, thereby increasing the size exclusion limit of the plasmodesmata that connect companion cells and sieve tubes and facilitating entry into the phloem for long-distance transport^{12,13}. Proteome analyses have indicated that > 10% of phloem sap proteins have RNA binding properties, and it is believed that the binding of phloem-residing RNAs to RNA-binding *proteins* (RBPs) may play important roles in loading, unloading and long-distance transport of RNA in the phloem sieve system¹⁴⁻¹⁶. CmRBP50 from pumpkin has been shown to specifically bind to RNAs harboring the polymer-pyrimidine CUCU domain to form the core of the RNP complex, which then assists long-distance RNA transport in the phloem by binding to additional proteins¹⁷. StPTB1 and StPTB6, the RBP50 homologs in potato (*Solanum tuberosum*), have been shown

to be able to bind to the 3' UTR region of *StBEL5* mRNA and facilitate long-distance phloem transport of the transcripts to improve tuber yield¹⁸.

Enormous amount of mRNAs have been discovered to be mobile during nutrient stress. For example, a total of 3,546 distinct mRNAs were identified to be transported from a cucumber (*Cucumis sativus*) scion to a watermelon (*Citrullus lanatus*) stock in response to phosphorous deficiency¹⁹. In another heterografting experiment using two *Arabidopsis thaliana* ecotypes (COL and PED), although 2,006 different mRNAs were discovered to be mobile in the shoot-root system under normal conditions, only 90 and 91 transcripts were found to be mobile in response to phosphorus and nitrogen deficiency, respectively²⁰. In addition, Xia et al. (2020) established a *Nicotiana benthamiana*/tomato heterograft system to identify shoot-to-root mobile mRNAs that are produced in response to low N, P or Fe¹⁰. Although the significant amount of mobile mRNAs identified from these studies imply the potential roles of these transcripts in adapting plants to low minerals, a definite biological function of these mRNAs has not been determined.

Iron (Fe) is an essential micronutrient for plant growth²¹ and insufficient availability often limits plant development. Previous studies, including physiological²², transcriptomic²³, metabolic²⁴, and signal transmission²⁵ analyses, have improved our understanding of mechanisms involved in Fe homeostasis, and substantial evidence exists for shoot-to-root signalling in response to varying iron conditions^{23,26-28}. *AtOPT3*, one of the most studied genes related to Fe deficiency responses, is mainly expressed in vascular tissues and might regulate the long-distance transport and distribution of Fe in *A. thaliana*²⁹. In the *opt3-2* mutant, the expression of ferric chelate reductase *FRO2* and Fe/Zn/Mn uptake transporter *IRT1* in roots was up-regulated and the accumulation of Fe in the roots and leaves was increased in comparison with WT, indicating that an *OPT3* mediated long distance mobile signal produced in the aboveground parts of the plant negatively regulates the uptake of Fe in roots^{30,31}. However, the identity of the signal remains elusive and whether the long-distance movement of *AtOPT3* mRNA participates in this regulatory process is unknown.

One of the pre-requisites for the identification of mobile mRNAs using the heterografting method is to have certain degree of genetical difference between the scion and the rootstock³². Self-incompatible apple (*Malus*) species have particularly high levels of genetic variation and the use of highly heterozygous *Malus* genotypes allowed us to investigate the long-distance movement of *MdOPT3* mRNA from the leaf to the root to regulate Fe uptake, and to contrast this with the movement of *AtOPT3* mRNA in *A. thaliana*. These observations were then associated with differences in RBP profiles between herbaceous and woody plants, which suggests a mechanistic basis for the observed differential transmissibility of *OPT3* mRNAs between herbaceous and woody plants.

Results

***MdOPT3* mRNA moves from shoots to regulate Fe uptake in roots of *Malus* plants**

To gain insight into long-distance mRNA signal transduction in apple plants in response to Fe stress, we established a heterologous apple grafting system (Fig. 1a, b). Long-distance movement of RNAs in the shoot-root system was determined using a scion of 'Golden Delicious' (*Malus domestica*) and two rootstocks: *Malus xiaojinensis* (Mx), which is Fe uptake efficient, and *Malus baccata* (Mb), which has a much lower Fe uptake efficiency³³. To obtain a profile of the mobile transcripts, we conducted RNA-Seq analysis on the hetero-grafted samples (Fig. 1c, d) (Supplementary Table 1). A total of 1,421 distinct mobile mRNAs were identified, of which 14 participated in bi-directional movement. We next searched for transmissible mRNAs that were specifically associated with the Fe deficiency treatments and identified 193, comprising 150 moving from leaves to roots and 43 in the opposite direction (Supplementary Table 1).

Using the population of the specifically transmissible mRNAs, we performed a weighted gene co-expression network analysis (WGCNA) and identified 14 distinct modules (Supplementary Figure 1). One of the 14 modules is closely related to the Fe deficiency response and was of particular interest for this study. In this module, *OPT3* (MD00G1163400) was found to be the hub gene and showed high connectivity in the network with transcripts that were transmitted over long distances in the *Malus* grafts (Fig. 2a). The abundances of *MdOPT3* transcripts were found to be more significantly accumulated in roots of *Md/Mx* and *Md/Mb* grafts subjected to Fe deficiency treatment than those in control condition. Interestingly, this overaccumulation of *MdOPT3* transcripts in roots coincided with the reduced accumulation of the same transcripts in leaves (Fig. 2b). We next performed in situ hybridization and localized the *MdOPT3* mRNA in the phloem in stem cross-sections (Fig. 2c). Since *OPT3* has been linked to phloem-specific Fe transport in *A. thaliana*^{30,31,34}, we then tested whether it is involved in Fe accumulation in *Malus*. We found that *MdOPT3* suppressed *Malus* plant grown under normal Fe or Fe-deficient conditions showed weaker Fe³⁺ staining compared with the control plants (Fig. 2d). This suggested that the *MdOPT3* contributes to the coordination of Fe accumulation in *Malus* plant.

To further validate *MdOPT3* mRNA mobility in *Malus* grafts, we analyzed the *OPT3* gene sequences from cv 'Golden Delicious', *M. xiaojinensis* and *M. baccata*. A single nucleotide polymorphism (SNP) (T/C) was identified at +995 bp downstream from the ATG start site, comprising a *MdOPT3^T* allele present in 'Golden Delicious', a *MbOPT3^C* allele in *M. baccata*, and a heterozygous *MxOPT3^{T/C}* allele in *M. xiaojinensis* (Fig. 3a, Supplementary Figure 2a). A combination of *OPT3* homozygous genotypes of 'Golden delicious' (*MdOPT3^T*) and *Malus baccata* (*MbOPT3^C*) allowed us to distinguish the origin of *OPT3* in *Md/Mb* grafts accurately. In this heterograft, the heterozygous *MdOPT3^{T/C}* was identified in roots under normal growth condition (Fig. 3b). However, after Fe deficiency treatment of grafted seedlings, *MdOPT3^T* was the only form of transcript detected in the roots of *Md/Mb* grafts (Fig. 3b). This showed that, under Fe deficient growth condition, the expression of the root derived *OPT3* was prohibited and the detected *OPT3* in the root originated from the scion via the leaf-to-root long distance movement.

The movement of nutrients and signaling molecules from the leaf-to-root normally take places in phloem³⁵. To further verify whether *MdOPT3^T* is mobile from the leaf-to-root in the *Malus* grafts, phloem

girdling, a technique that disrupts the phloem transport, was employed. We found that *OPT3* mRNA was barely detected in the roots of the girdled *Md/Mb* heterografts (Fig. 3c). This result indicated that the *MdOPT3*^T mRNAs were indeed originated from the leaf and transported from the *Md* scion to the *Mb* rootstocks via the phloem.

AtOPT3* mRNA does not move from shoots to roots in *A. thaliana

AtOPT3 was shown to promote the movement of Fe from xylem to phloem in *A. thaliana* and regulate the redistribution of Fe signal and associated signaling in developing and mature tissues³⁶. The discovery of the movement of the *Malus OPT3* inspired us to investigate whether the long distance movement of the *OPT3* mRNA is a common phenomenon in plants. We reciprocally grafted shoots of the *AtOPT3* knock-down mutant (*opt3-2*) onto WT rootstocks (*opt3-2*/WT) and WT shoot scions onto *opt3-2* rootstocks (WT/*opt3-2*), and determined *AtOPT3* expression in the two graft combinations. Compared to the WT/WT control, the expression of *AtOPT3* was hardly detected in the roots of WT/*opt3-2* (Fig. 3d), indicating that the *AtOPT3* mRNAs in WT leaves were not transported to the *opt3-2* roots in *Arabidopsis*.

The mechanistic basis of the differences in *OPT3* mRNA mobility between herbaceous and woody plant species

The above results showed that *OPT3* mRNAs from *A. thaliana* and *Malus* plants differ in their mobility. The alignment analysis between *MdOPT3* and *AtOPT3* showed that the two sequences shares 73% identity at the cDNA level. To determine whether the mRNA sequence differences affect their mobility, transient expressions of *MdOPT3* in *A. thaliana* rosette leaves or *AtOPT3* in apple leaves were conducted (Fig. 4). Real time (RT)-PCR primers that can distinguish the two transcripts were used to measure the expression levels of *OPT3* in the roots of control and transgenic plants (Supplementary Table 3). We found that *AtOPT3* mRNAs could be detected in the roots of the transgenic apple seedlings but not in the control, although *AtOPT3* mRNA levels in apple roots were lower than those in the shoot (Fig. 4). In contrast to the results from the transgenic apples, the *MdOPT3* transcripts could not be detected in the *Arabidopsis* roots although the transcripts could be detected in high abundance in leaves (Fig. 4). Taken together, these results indicated that factors other than the sequence specialty of *OPT3* conferred the differential shoot-to-root mobility in the two species.

In plants, RBPs play a key role in mRNA transport³⁷, and the 3' UTR of mobile mRNAs was demonstrated to be sufficient for the long-distance movement of various mRNAs. To identify putative *MdOPT3* RBPs, we used a 3' UTR *MdOPT3* sequence as a probe (Fig. 5a), and performed RNA-pull down coupled with mass spectrometry. A total of 472 proteins from *M. domestica* were identified to be able to interact with *MdOPT3* mRNA (Supplementary Table 2). The candidate proteins were functionally annotated and a gene ontology (GO) enrichment analysis revealed that the most enriched category belongs to 'response to stimulus' (Fig. 5b). These 472 proteins included five RNA binding proteins (RBP): MD04G1208800, MD12G1223200, MD05G1230700, MD07G1160000 and MD14G1031800 (Fig. 5c, d), of which

MD04G1208800 and MD12G1223200 expression were upregulated in roots of both *Md/Mx* and *Md/Mb* grafted seedlings subjected to Fe deficiency treatment (Fig. 5c).

To quantify the binding affinities of *Malus* and *A. thaliana* RBPs with their associated mRNAs, we used surface plasmon resonance (SPR) assays with immobilized oligonucleotides. We expressed five *Malus* RBPs and the corresponding *A. thaliana* proteins in *Escherichia coli*, each as a fusion with glutathione S-transferase (GST), and purified the recombinant fusion proteins (Fig 6a). The two *Malus* RBPs (MD04G1208800 and MD12G1223200) were found to be bound to the 3' UTR of the *MdOPT3* and *AtOPT3* probes with high affinity. Whereas the corresponding *A. thaliana* RBP (AT3G26420.1) had a much lower binding value than the *Malus* RBPs, suggesting the low affinity of the *A. thaliana* RBP with the 3' UTR of the *MdOPT3* and *AtOPT3* probes. The affinity differences of RBPs between the two species may explain the observations on the differential mobility of *OPT3* mRNAs in the two species (Fig. 6b, c, d).

We further performed a sequence homology analysis of RBPs from multiple herbaceous and woody plant species and discovered that MD14G1031800 and MD12G1223200 formed separate clusters between herbaceous and woody plants (Supplementary Fig. 3), indicating that the functionality of these RBPs may differ between the two types of plants. To validate our hypothesis, we conducted transient expressions of *MdOPT3* in *Solanum lycopersicum* leaves and *AtOPT3* in *Populus tremula* leaves (Supplementary Fig. 2b). Similar to our discovery in the *Malus* transient expression study, we found that *AtOPT3* mRNAs could be detected in the roots of the transgenic *Populus tremula* (Supplementary Fig. 2c). On the contrary, the *MdOPT3* transcripts could not be detected in roots of transgenic *Solanum lycopersicum* although the transcripts were in high abundance in leaves (Supplementary Fig. 2c). These results led us to conclude that the differences between *A. thaliana* and apple in the transmissibility of *OPT3* mRNAs may be associated with the different functions of the RBP proteins present in herbaceous plants and woody plants.

Mobile *MdOPT3* mRNAs are required for the Fe deficiency response in *Malus* plants

It was previously reported that *Arabidopsis* mutant *opt3-2* exhibited a constitutive Fe deficiency response in the root³¹. Consistent with this early finding, our results showed that the expressions of the *IRT1* and *FIT* in roots of the *opt3-2* mutant were constitutively up-regulated, indicative of an iron-deficiency response. To explore whether *MdOPT3* is involved in Fe-deficiency responses in apple plants, we measured the expression levels of the two Fe-deficiency marker genes, *MdIRT1* and *MdFIT*, in *MdOPT3*-suppressed apple plants. To our surprise, both *IRT1* and *FIT* were downregulated in the suppressed plants when compared with the control plants (Fig. 7b, c). Moreover, we observed a significant decrease of Fe contents in the suppressed plants compared with the control plants grown under normal Fe or Fe-deficiency treatment (Fig. 7d). The contrast response to the suppressed expression of *OPT3* in leaf between *Malus* and *Arabidopsis* inspired us to hypothesize that *OPT3* mobility could play a role in this physiological process.

It is known that *OPT3* is located on the vascular tissues of both roots and shoots³¹. To distinguish the importance of leaf and root *AtOPT3* in response to Fe deficiency, a few graft combinations, i.e., *opt3-2/WT*, *opt3-2/opt3-2*, and *WT/opt3-2*, were created and the expressions of *IRT1*, *FIT* and *FRO2* in roots were measured. Higher expression levels of the marker genes were found in both *opt3-2/WT* and *opt3-2/opt3-2*, whereas *WT/opt3-2* grafts had lower expressions of these genes (Fig. 8e, f, g). These results indicated that the Fe-deficiency responses in *Arabidopsis* roots were stimulated by a signal from the shoot mediated by *OPT3* but *OPT3* transcript is not the mobile signal. To investigate whether the *OPT3* mRNA could be the mobile signal in apple plants under Fe-deficiency, we created another two graft systems, taking *Md* as the rootstock, and *Mx* or *Mb* as the scion (Fig. 8a, b, d, d). The lack of the *Mx* derived *OPT3^C* mRNA in the rootstock *Md* indicated the mRNA in the *Mx* leaves couldn't be transferred to the roots, whereas the detection of the *Mb* derived *OPT3^C* mRNA in the rootstock *Md* indicated that the mRNA in the *Mb* leaves could be transferred. Furthermore, we found the Fe-deficiency response related genes (*FIT*, *IRT1*, *FRO2*) were upregulated in the *Mb/Md* grafts in which *OPT3* was mobile; whereas the expression levels of these genes in the *Mx/Md*, in which the *OPT3* transcript was not mobile, were not upregulated. Taken together, our results showed that the long distance movement of *OPT3* mRNA from shoots-to-roots is essential to regulate Fe deficiency responses in roots of *Malus* plants.

Discussion

Based on the data presented in this study, we propose a model regarding the mobility of *OPT3* mRNA between herbaceous and woody plant-using *Malus* and *Arabidopsis* plant as examples. *MdOPT3* moves long distance from shoots to regulate Fe uptake in roots of *Malus* plants while this is not the case in *Arabidopsis*. We further provided evidence to demonstrate that the differential transmissibility of *OPT3* in *Arabidopsis* and *Malus* might represent a generic phenomenon in herbaceous and woody plants and this could be related to the existence of RBPs with different functions between the two types of plants (Fig.9).

At the genome level, various graft-transmissible mRNA orthologs were found to be shared among different plant species; however, each plant has a unique phloem mRNA population. For example, 2,006, 3,333 and 3,546 graft-transmissible mRNAs were identified in *A. thaliana*, grape (*Vitis vinifera*) and cucumber phloem, respectively^{20,38,39}, but only 38% and 33% of the mobile mRNA orthologs from *A. thaliana* and grape, respectively, were also detected in cucumber³⁸. The core sieve tube system (STS) mRNAs may play roles that are essential for phloem function and therefore are conserved during plant vascular tissue evolution. Conversely, the species-specific STS-mRNAs may reflect differences in growing conditions, or evolutionary adaptations, such as those exhibited by rosette plants (*A. thaliana*), annual plants and perennial vines (grape)¹¹. The difference in the transmissibility of *OPT3* between apples and *A. thaliana* may be related to differences in the development of vascular bundles, and specifically phloem, between herbaceous and woody plants. In addition to *Malus* and *Arabidopsis*, we further selected two other herbaceous and woody plants which demonstrated that the originally immobile *AtOPT3* can be transported from the shoot to the root in *Populus tremula*, while the originally mobile *MdOPT3* does not show such movement in *Solanum lycopersicum*. Multiple factors, e.g., expression site,

association with RBPs, etc., have been suggested to be associated with the mobility of mRNAs⁴⁰⁻⁴². The non-cell-autonomous phloem RBP CmPP16 has been shown to bind to mRNAs in a non-specific manner, increase the plasmodesmata size exclusion limit, and transmit as an RNP complex between cells¹². Phloem sap proteomic analysis showed that > 10% of phloem sap consist of RBP proteins, which may play an important role in the long-distance transport of RNA in the phloem^{14,16}. Thus, it is legitimate to assume that the variation in RBP orthologs between apple and *A. thaliana* may contribute to differences in phloem transcript profiles between the two species.

Potato StBEL5 mRNA was shown to move via the phloem in the veins to the tip of the subsurface stolon^{41,42}, and increasing the long-distance transmission was able to improve tuber yield⁴¹. The UTR of *StBEL5* was found to be related to its long-distance transmission and other studies also have shown the importance of the 3' UTR in binding to RBPs for long distance RNA movement³⁵. We analyzed the 3' UTR of *OPT3* from *Mx*, *Mb* and *Md*, and identified a deletion of 15 bp specific to *MxOPT3* (Supplementary Fig. 2). We observed that *OPT3* mRNAs in *Mx* leaves were mobile whereas *OPT3* mRNAs in *Mb* leaves were immobile. The variation in transmissibility of *OPT3* mRNAs between *Md*, *Mx* and *Mb* may reflect variation in the 3' UTR sequences. It was demonstrated that a potential secondary structure motif resembling tRNA in the 3' UTR is sufficient for long-distance mRNA movement⁴⁶. The deletion of 15 bp in the 3' UTR in *Mx* may affect the tRNA structure of *MxOPT3*. Moreover, our results show that variation in the 3' UTR sequences may affect the mobility of the *mRNA*, which might represent a general tool for precisely regulating communication between scion and rootstock.

The plant *OPT3* gene was first described in *A. thaliana*³⁴, and in subsequent studies of its function the focus was mainly on the tissue localization of expression changes as a consequence of Fe deficiency, as well as the identification of *opt3-2* mutants⁴⁷. It was also found that that *AtOPT3* is a key component of the iron-signaling network connecting shoots to roots³⁶. Through phloem localization and radioactive Fe tracer tests, *OPT3* were shown to mediate the transport of Fe or Fe ligand complexes from the xylem to the companion cells and then to the phloem for long-distance transport³⁶. Even though phloem RNAs are known to play important developmental roles^{15,48,49}, and *AtOPT3* is mainly transcribed and expressed in the phloem^{31,36,47}, through an interactive grafting test involving *opt3-2* and WT, we found no evidence that the *OPT3* mRNA can move from the shoot to the root in *A. thaliana* and conclude that the factor mediating the Fe signal transduction from shoot to root in *Arabidopsis* is less likely the mRNA form of *OPT3*.

Different from *Arabidopsis*, mobile *MdOPT3* mRNA is required for the Fe deficiency response in *Malus* plants. Our results showed *MdOPT3* mRNA acts as a Fe signal transduced from shoot to root. It is unknown why the shoot-to-root movement of *OPT* mRNAs is needed and how the root-arriving *OPT3* participates in the Fe-deficiency responses. Small peptides have diverse functions in cellular signaling in many organisms⁵⁰⁻⁵². In addition to Fe, OPT proteins can transport diverse substrates which are peptide-containing compounds or peptide derivatives. Future experiments are needed to verify whether the oligopeptide transport function plays a role in regulating Fe deficiency response in *Malus* plants.

Methods

Plant materials. *Malus xiaojinensis* and *Malus baccata* as rootstocks, and *Malus domestica* cv. 'Golden Delicious' as scion were propagated on Murashige & Skoog (MS) medium⁵³ containing 0.5 mg/L 6-benzylaminopurine and 0.5 mg/L indole-3-butyric acid for 1 month (growth temperature $25 \pm 2^\circ\text{C}$ day/ $21 \pm 2^\circ\text{C}$ night, $250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity, 16h/8h light/dark photoperiod). The seedlings were transferred to MS medium with 1.0 mg/L indole-3-butyric acid for rooting⁵⁴. Seedlings were grown for 3 weeks prior to experimental treatments. Iron sufficient (+Fe) or iron deficient (-Fe, 0 μM FeNaEDTA) treatments were carried out for 3 days. The cultivation methods and conditions of *Populus tremula* are the same as those of *Malus plants*.

Arabidopsis thaliana lines included WT (Col-0) and *OPT3* deletion mutants (*opt3-2*)³⁰. The seeds were surface-sterilized, stratified at 4°C for 48 h in the dark, and seeded on solid 1/2 MS medium (pH 5.8) with an aseptic pipette suction head under sterile conditions then placed at room temperature (16 h/8 h, light/dark cycle, temperature 22°C) and cultured vertically for 6 days. Fe-sufficient and Fe-deficient media were prepared as previously described⁵⁵.

Tomato (*Solanum lycopersicum*) seeds (no mechanical damage) were surface-disinfected with 2% NaClO for 4 min, rinsed with sterile water 6 times, and then sowed in a petri dish (90 mm diameter) with two layers of filter paper, after germination moved to nutrient soil for cultivation. The cultivation conditions were similar to *Malus plants*.

Grafting experiments. Before grafting, *Malus* plants were grown in MS medium for one month in a controlled environment as described above. Rootstocks and scions with similar stem diameters were selected for cleft grafting⁵⁶, and the grafted materials were transferred to rooting medium (0.5 mg/L indole butyric acid, IBA). After one and a half months, the grafted plants were used for a Fe deficiency treatment in hydroponic culture as described above. *A. thaliana* grafting was performed using a flat surface collar-free method as previously described⁵⁷. Grafting combinations were as follows: WT Col-0/WT Col-0, *opt3-2/opt3-2*, WT Col-0/*opt3-2*, *opt3-2/WT Col-0*.

RNA sequencing and mobile mRNA bioinformatic analysis. Total RNA was extracted from grafted tissues using an RNAPrep Pure Plant Plus Kit (TIANGEN, DP441). Library preparation was as described in our previous study⁵⁸.

The mobile mRNA transcripts were derived from grafted (and un-grafted) scion or rootstock samples and compared with the reference genome (ftp://ftp.bioinfo.wsu.edu/species/Malus_x_domestica/Malus_x_domestica-genome_GDDH13_v1.1/assembly) using the HISAT2 software⁵³ (<https://ccb.jhu.edu/software/hisat2/index.shtml>). Before comparing to the reference genome, we determined four groups of sequences (non-grafted, *Md/Mn* 0h, +Fe *Md/Mn* and -Fe *Md/Mn*) in the rootstocks and scion raw data, referred to as reference I, II, III and IV, respectively. These were compared to the reference genome, and unmapped reads were removed. We next compared (1) *Mn* (r) vs. *Md/Mn* (r)

(*Mn*: *Mx* or *Mb*, r: root, L: leaf), and searched for SNPs and InDels (insertion-deletions) using *Mn* (r) as a control, and compared the SNP/InDels detected in *Md*/*Mn* (r) with the control. SNP/InDels detected in *Md*/*Mn* (r) but not in *Mn* (r) were considered as candidates for transmission from the leaf to the root in *Md*/*Mn*. Similar criteria were applied to the *Md* (L) vs. *Md*/*Mn* (L) comparison. (2) We next compared the SNP/indels in sequences from *Md*/*Mn* (r) grown under normal Fe conditions with those from sequences grown under Fe deficiency conditions. SNPs and InDels were identified and mapped to the apple genome⁵⁴ using GATK (<http://www.broadinstitute.org/gatk/>). The GATK identification criteria used were: (1) No more than three single base mismatches in the range of 35 bp; (2) Compared with the reference genome, all unique loci in the grafted sample had a sequencing depth > 2.0. An overview of the methods used in our study is showed in Fig. 1c, d.

qRT-PCR. First strand cDNA was synthesized using HiScript ® II Q RT SuperMix for qPCR (Vazyme, R232-01). qRT-PCR was performed using an ABI QuantStudio™ 6 Flex system (ThermoFisher Scientific) with a SensiFAST™ SYBR Lo-ROX Kit (Bioline, BIO-94005) For qRT-PCR the thermocycler protocol was as follows: pre-denaturation, 1 min at 95°C, 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 45 sec. ACTIN (*M. domestica*) was used as reference gene, and the relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method⁵⁹. Primers used are listed in Supplementary Table 3.

In situ hybridization of *OPT3*. In situ hybridization was performed as previously described⁶⁰. A DIG RNA Labeling Kit (SP6/T7) (Roche, Cat. No. 11175025910) was used to label the antisense and sense RNA probes according to the manufacturer's protocol. RNA hybrids were detected by immunohistochemistry staining using an alkaline phosphatase (AP)-conjugated primary antibody⁶¹. Probe sequences are listed in Supplementary Table 3.

Perls staining. $K_4[Fe(CN)_6]:HCl=1:1$ (Perls) was used to visualize Fe^{3+} localization as previously described⁶². The roots and leaves of transgenic *M. domestica* were incubated in the Perls solution for 1 h then washed with water. A methanol solution containing 10 mM sodium azide and 0.3% (v/v) H_2O_2 was used to clear the roots and leaves then the samples were washed in 0.1M phosphate buffered saline solution (composed of NaH_2PO_4 and Na_2HPO_4 , pH 7.4).

Phylogenetic analysis. Phylogenetic analysis was conducted using MEGA7⁶³ and the Neighbor-Joining method⁶⁴. Evolutionary distances were computed according to the JTT matrix-based method⁶⁵, with a gamma shape parameter of 0.9. The phylogenetic tree was subject to 1,000 bootstrap replicates.

Cloning and sequencing of *OPT3*. A 5' and 3' Rapid Amplification of cDNA Ends (RACE) procedure was used to confirm the full-length mRNA sequence of *MdOPT3*, *MxOPT3* and *MbOPT3* as previously described^{66,67}. In order to confirm the stable SNPs, the full-length coding sequence (CDS) of *MdOPT3*, *MxOPT3* and *MbOPT3* were cloned from cDNA reverse transcribed from total mRNA using the pTOPO-Blunt Cloning vector (AidLab, CV15). All primers are listed in Supplementary Table 3.

Transient expression. Using the BamHI site, the mRNA sequence of *MdOPT3* and *AtOPT3* were cloned using the NEBuilder Assembly Tool (NEB, #E5520S) into the pRI101-AN vector, respectively. We used virus-induced gene silencing to suppress *MdOPT3* expression in ‘Golden Delicious’. The *MdOPT3* fragment (Supplemental Table S3) was cloned into the pTRV2 vector⁶⁸.

All constructs were transformed into GV3101 Chemically Competent *Agrobacterium tumefaciens* cells (Weidi Biotechnology, AC1001). Positive clones were mixed to give equal concentrations of pTRV⁶⁹ and pTRV2, or its derivatives, and introduced into *M. domestica*, *Populus tremula*, *Solanum lycopersicum* and *A. thaliana* seedlings using vacuum infiltration and injection, respectively⁵⁸. The transformation procedure used a pressure of 0.8 MPa, which was applied twice for 5 min time. Distilled water was used to wash away the bacteria on the surface of the seedlings.

RNA-protein pull down and MS assay. The *MdOPT3* 3' UTR fragment was transcribed as probe using the In vitro Transcription T7 Kit (TaKaRa,6140) and the target RNA was labeled using the PierceTM RNA 3' Desthiobiotinylation Kit (ThermoFisher, 20163). Proteins were extracted from apple plant seedlings using the Plant Total Protein Extraction Kit (Sangon, C500053). RNA-protein pull-down was performed using the PierceTM Magnetic RNA-Protein Pull-Down Kit (ThermoFisher, 20164). Mass spectrometry (Cloud-seq Biotechnology Co., Ltd.) was used to identify the target proteins that bind to *MdOPT3* 3' UTR and subsequent bioinformatics analysis was performed to functionally annotate the target proteins.

SPR. The 3' UTR RNA probes of *MdOPT3*, *MxOPT3* and *AtOPT3* were transcribed using the In vitro Transcription T7 Kit (Takara, 6140). The GST-tagged RNA-binding proteins were expressed using the pGEX4T-1 prokaryotic expression vector (cwbiotech, CW2198) in *Escherichia coli* BL21 Chemically Competent Cells (Transgen, CD901-02). SPR measurements were performed using the GST Capture Kit on the Biacore X100 platform (GE Healthcare). RNA and RNA-binding protein binding levels were tested using a Biacore binding test program (GE Healthcare) with purified RNA probes as the mobile phase, and GST-tagged RNA-binding proteins as the stationary phase.

Declarations

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Conflict of interests

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

T.W. and Z.H. conceived and designed the research. Y.S. X.L., P.H., M.F. and Z.X conducted the experiments. J.T., Y.W., X.X. and X.Z. contributed reagents and analytical tools. T.W. and X.L. wrote the manuscript. C.Z gave advice and edited the manuscript. All authors read and approved the manuscript.

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Figures

Figure 1

Detection of transmissible mRNAs associated with responses to Fe stress in *Malus*. a, Model of Fe stress signals transported from root to leaf (red arrow) and leaf to root (blue arrow). b, Samples collected from the grafted rootstocks and scions at 0 h, 24 h and 3 d $\pm$ Fe treatment. The non-grafted control samples were collected from Mx/Mb roots and Md leaves. c, Design of the micro-graft experiments and experimental strategy. HISAT2 is software used for read mapping. The blue and gray boxes indicate the mobile RNAs in both directions, and yellow box indicates the RNAs that are mobile specifically in response to Fe deficiency. d, Specific screening methodology. Single nucleotide polymorphisms (SNPs) between different samples were used to identify transmissible mRNAs. I.II.III and IV means sufficient and insufficient Fe for 24 h and 3 d. The different colors indicate total SNPs after comparison with the *Malus* reference genome.

Using the transient transformation of heterologous OPT3 in Malus plant and Arabidopsis to identify the mobility difference of OPT3 between Malus plant and Arabidopsis. The expression of heterologous OPT3 were detected by RT-PCR in the roots of transgenic plants, and the untransformed plants were used as controls.

Figure 5

a Analysis of the secondary structure of MdOPT3 3' UTR sequence. b GO enrichment analysis of proteins interacting with MdOPT3 3'UTR sequence. c Candidate RNA-binding proteins relatively expression level in roots and leaves of Md/Mx and Md/Mb graft complexes under Fe-sufficient and Fe-deficient treatments. d RNA-binding protein domain analysis.

Figure 6

SPR analysis of the binding of candidate RNA-binding proteins to MdOPT3, MxOPT3 and AtOPT3 3' UTR. The binding of candidate RNA-binding proteins with the MdOPT3, MxOPT3 and AtOPT3 3'UTR sequences was tested and the response curves of three RNA-binding proteins, b MD12G1223200, c MD04G1208800 and d AT3G26420.1. to the MdOPT3, MxOPT3 and AtOPT3 3'UTR sequences are shown.

Figure 7

OPT3 in both *Malus* and *Arabidopsis thaliana* responds to iron deficiency stress. Relative expression of a MdOPT3, b MdIRT1 and c MdFIT1 in roots and leaves of MdOPT3-suppressed *Malus* plants., d Fe concentration in roots and leaves of MdOPT3-suppressed *Malus* plants. Relative expression of AtOPT3 e, f AtIRT1, g AtFRO2 and h AtFIT1 in roots and leaves of *A. thaliana* wild type (WT) and opt3-2 seedlings.

Figure 8

OPT3 moves from shoots to regulate Fe deficiency response in roots of *Malus* plants. The relative expression of a *OPT3*, b *IRT1*, c *FRO2* and d *FIT* in reverse graft complex (Mx/Md, Mb/Md) leaf and root under Fe-sufficient and Fe-deficient treatments. The relative expression of e *AtIRT1*, f *AtFRO2* and g *AtFIT* in *Arabidopsis* graft complex (WT/WT, opt3-2/opt3-2, WT/opt3-2, opt3-2/WT) shoot and root under Fe-sufficient and Fe-deficient treatments. * Statistically significant difference ($P < 0.05$), ** highly significant as determined by a Student's t-test.

Figure 9

Schematic overview of OPT3 mobility from shoot to root in *Malus* plants and *Arabidopsis thaliana*. OPT3 mRNA mobility lead to differences in iron redistribution between *Malus* plants and *A. thaliana* roots. a, OPT3 mRNAs in *Malus* shoots are transported to roots by binding to RNA-binding proteins. b, OPT3 mRNAs detected in *Malus* roots include the endogenous sequences of the root and as well as those of OPT3 mRNAs transferred from the shoot. The OPT3 from shoot and OPT3 in root jointly participate in the

Fe redistribution of root from xylem to phloem. c, d, *A. thaliana* OPT3 mRNAs are not transported from the shoot to the root.

Supplementary Files

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