**Systemic Regulation of Iron Acquisition by Arabidopsis in Environments with Heterogeneous Iron Distributions**

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Nutrient distribution within the soil is generally heterogeneous. Plants, therefore, have evolved sophisticated systemic processes enabling them to optimize their nutrient acquisition efficiency. By organ-to-organ communication in Arabidopsis thaliana, for instance, iron (Fe) starvation in one part of a root drives the upregulation of a high-affinity Fe-uptake system in other root regions surrounded by sufficient levels of Fe. This compensatory response through Fe-starvation-triggered organ-to-organ communication includes the upregulation of *Iron-regulated transporter 1* (*IRT1*) gene expression on the Fe-sufficient side of the root; however, the molecular basis underlying this long-distance signaling remains unclear. Here, we analyzed gene expression by RNA-seq analysis of Fe-starved split-root cultures. Genome-wide expression analysis showed that localized Fe depletion in roots upregulated several genes involved in Fe uptake and signaling, such as *IRT1*, in a distant part of the root exposed to Fe-sufficient conditions. This result indicates that long-distance signaling for Fe demand alters the expression of a subset of genes responsible for Fe uptake and coumarin biosynthesis to maintain a level of Fe acquisition sufficient for the entire plant. Loss of *IRON MAN/FE-UPTAKE-INDUCING PEPTIDE (IMA/FEP)* leads to the disruption of compensatory upregulation of *IRT1* in the root surrounded by sufficient Fe. In addition, our split-root culture-based analysis provides evidence that the IMA3/FEP1–MYB10/72 pathway mediates long-distance signaling in Fe homeostasis through the regulation of coumarin biosynthesis. These data suggest that the signaling of IMA/FEP, a ubiquitous family of metal-binding peptides, is critical for organ-to-organ communication in response to Fe starvation under heterogeneous Fe conditions in the surrounding environment.

**Keywords:** Arabidopsis thaliana • Coumarin biosynthesis • Iron deficiency response • IRON MAN/FE-UPTAKE-INDUCING PEPTIDE • Iron-regulated transporter 1 • Long-distance signaling • Organ-to-organ communication • Split-root culture

**Introduction**

Iron (Fe) is an essential nutrient for plant growth and development, participating in many significant biological processes including photosynthesis, respiration, redox reactions and nitrogen fixation (Marschner 1995). Although Fe generally is abundant within the soil, its availability to plants is occasionally limited because it exists as insoluble ferric oxides in alkaline pH conditions (Marschner 1995). By contrast, the highly available Fe in acidic soils can be toxic to plants due to the ability of Fe to promote the generation of reactive oxygen species (Becana et al. 1998). Therefore, plants tightly control Fe uptake, translocation and storage to maintain Fe homeostasis at the whole-plant level.

In dicots and nongraminaceous species, plants possess a reduction-based Fe-uptake system called strategy I (Romheld 1987). In the Arabidopsis thaliana system, protons are released by H⁺-ATPases to increase the solubility of ferric chelates in the rhizosphere (Santi and Schmidt 2009). Ferric iron (Fe³⁺) is then reduced by the root surface enzyme *FERRIC REDUCTASE OXIDASE 2* (*FRO2*) (Robinson et al. 1999) and is transported into root cells as ferrous iron (Fe²⁺) by *IRON-REGULATED...* **
TRANSPORTER 1 (IRT1), a high-affinity Fe transporter (Eide et al. 1996, Barberon et al. 2011). The Fe-uptake system is rapidly induced in conditions of Fe deficiency (Kobayashi and Nishizawa 2012). Networks of transcription factors primarily mediate this regulation. The master regulator, FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT), is a basic helix-loop-helix (bHLH) transcription factor that regulates the expression of H⁺-ATPase isofrom 2 (AHAA2), FRO2 and IRT1 (Colangelo and Guerinot 2004, Jakoby et al. 2004). At the protein level, FIT interacts with other bHLH transcription factors, specifically bHLH38/39/100/101 (Wang et al. 2007, Yuan et al. 2008). The heteromeric complex of FIT and bHLH/38/39/100/101 also induces the expression of the Fe-deficiency-inducible MYB10 and MYB72 transcription factors that facilitate Fe translocation (Palmer et al. 2013). Another type of bHLH transcription factor, POPEYE (PYE), also regulates the Fe-deficiency response (Long et al. 2010). PYE is induced in the root pericycle cells in Fe-deficient conditions, and PYE protein negatively regulates genes involved in mobilizing Fe from the roots for translocation into the shoots. The expression of PYE is tightly co-regulated with that of BRUTUS (BTS), a ubiquitin ligase that negatively regulates the Fe-deficiency response (Long et al. 2010, Hindt et al. 2017). Despite progress in understanding the nature of the local Fe-starvation response, the long-distance signaling mechanisms through which a plant regulates root activity according to its Fe status remain largely unknown.

Significant progress has been made in understanding long-distance nitrogen (N) signaling in Arabidopsis in the past decade. Ruffel et al. (2011) revealed that an N-demand long-distance signal conveys information about N starvation in one part of the root, whereas an N-supply long-distance signal conveys information about N availability from one side of the root to the other side (Ruffel et al. 2011). This N-demand signal involves the action of C-terminally encoded peptides (CEPs) (Tabata et al. 2014). Following N deprivation, CEPs are translocated to the shoots through the xylem, where they are recognized by two leucine-rich repeat receptor kinases known as CEP receptors (Tabata et al. 2014). Within the leaf vascular system, this recognition leads to the upregulation of glutaredoxin-like polypeptides that translocate toward the roots and, in combination with local N sensing, participate in controlling the expression of NRT2.1, the primary high-affinity NO₃⁻ transporter in roots (Ohkubo et al. 2017, Ruffel and Gojon 2017).

In the case of long-distance Fe signaling, several reports have demonstrated that the root high-affinity Fe-uptake system is controlled through a long-distance signaling pathway (Vert et al. 2003, Shanmugam et al. 2015). Like long-distance N signaling, Fe starvation on one side of the root system leads to an upregulation of Fe-uptake machinery in roots on the side exposed to the Fe-sufficient medium (Vert et al. 2003). In addition, IRON MAN/FE-UPTAKE-INDUCING PEPTIDES (IMA/FEPS) were recently reported to positively regulate the Fe-deficiency response as mobile signaling candidates (Grillet et al. 2018, Hirayama et al. 2018). IMA1/FEPS3 is predominantly expressed in leaf phloem in leaves. Grafting experiments have shown that IMA1/FEPS3 peptides induced in shoots positively regulate ferric chelate reductase (FCR) activities in roots (Grillet et al. 2018), but their precise function remains unknown.

In this study, we conducted split-root experiments and transcriptomic analyses to investigate the molecular basis for systemic Fe long-distance signaling in A. thaliana. We show that a small subset of genes involved in Fe uptake and coumarin biosynthesis for Fe acquisition were activated in a distal part of the roots exposed to the Fe-sufficient medium in split-root culture conditions. Moreover, the metal-binding peptide IMA1/FEPS-mediated pathways regulate this long-distance signaling to maintain Fe homeostasis.

**Results**

**Change in IRT1 and FRO2 gene expression and root development in response to heterogeneous Fe-starvation conditions**

We used split-root cultures to investigate gene expression changes associated with Fe-related long-distance signaling and root development. Roots were separated into left and right portions exposed to different nutrient conditions to mimic a heterogeneous nutrient distribution in the soil environment (Ruffel et al. 2011). Using a protocol described previously (Ruffel et al. 2011), we created split-root cultures with three different types of Fe conditions: (i) a homogeneous Fe-sufficient control condition (Cont. +Fe: both compartments, 75 µM Fe), (ii) a heterogeneous split condition (Split +Fe/Split −Fe: Split +Fe side, 75 µM Fe; Split −Fe side, 0 µM Fe) or (iii) a homogeneous Fe-deficient condition (−Fe: both compartments, 0 µM Fe) (Fig. 1A). Two-week-old seedlings whose roots had been split were transferred to one split-root Fe conditions for 24 h. The transcript abundance of IRT1 and FRO2, molecular targets of long-distance Fe signaling (Vert et al. 2003), was quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Both IRT1 and FRO2 mRNA levels were higher in the roots placed in the Split +Fe side than the controls and lower in the roots placed in the Split −Fe side than the homogeneous Fe-deficient condition (Fig. 1B; Supplementary Fig. S1A). On the other hand, the compensatory upregulation of AHAA2 was not observed in the split-root Fe-starvation condition (Supplementary Fig. S1B). The upregulation and downregulation of the IRT1 and FRO2 genes in each Split +Fe side and Split −Fe side were maintained for 3 and 5 days, respectively (Supplementary Fig. S2A–B). These results suggest that the expression of IRT1 and FRO2 is regulated by Fe-starvation-triggered organ-to-organ communication and is consistent with previous reports (Vert et al. 2003, Shanmugam et al. 2015).

Thus, the Fe-demand signal from the Split −Fe side may have been conveyed to the Split +Fe side to upregulate the expression of IRT1 and FRO2, whereas the Fe-supply signal from the Split +Fe side may have been conveyed to the Split −Fe side of...
Fig. 1 Compensatory upregulation of IRT1 gene expression and root development in response to a heterogeneous Fe supply. (A) A diagram of the Arabidopsis split-root culture system in which plant roots were separated into left and right regions exposed to different nutrient conditions. (B) Expression levels of the IRT1 gene in split roots of wild-type plants treated with the four Fe conditions shown in (A). All roots were treated with each Fe condition for 24 h (n = 4). (C) Expression levels of the IRT1 gene in roots of wild-type plants treated with +Fe, Split +Fe, Split –Fe, one-half +Fe left (L) and one-half +Fe right (R) conditions. All roots were treated with each Fe condition for 24 h (n = 3). (D) LR elongation in plants treated with each of the four Fe conditions. The bar graph shows the total LR length (mm) normalized by the length of the primary root (mm) (n = 10). Different lowercase letters indicate significant differences by Tukey’s honestly significant difference (HSD) test (P < 0.05). Data represent means ± SD.

The root to suppress the expression of IRT1 and FRO2. Expression analysis in the homogeneous half-Fe condition showed no change in IRT1 expression (Fig. 1C), suggesting that the compensated upregulation of the IRT1 gene was induced only when the remaining local amount of Fe available to the roots was lower than a predetermined threshold value.

Because the lateral root (LR) architecture of Arabidopsis was reported to vary due to split-root N-starvation conditions (Ruffel et al. 2011), we also investigated whether any morphological changes in the root system occurred in response to a heterogeneous Fe environment. Total LR lengths were quantified for each of the three different types of Fe conditions in the split-root cultures. There were no significant differences in the primary root length under any conditions. However, the total LR length was lower in the –Fe condition than the Cont. +Fe condition (Fig. 1D). In the split-root culture conditions, the total LR lengths of Split +Fe and Split –Fe compartments were equivalent to the Cont. +Fe and –Fe conditions, respectively (Fig. 1D). Thus, the total LR lengths of each compartment may be predominantly controlled by local medium conditions in an environment with a heterogeneous Fe supply. These data indicate that long-distance Fe signaling controls the compensatory upregulation of IRT1 and FRO2 gene expression change without changing the root length.

Fe transport activity of split roots placed on the Fe-sufficient side changed in response to Fe starvation of split roots on the other side

Previous reports demonstrated that long-distance Fe signaling leads to changes in IRT1 and FRO2 expression; however, the influence of changes in plant Fe content has not been fully explored (Vert et al. 2003, Shanmugam et al. 2015). To investigate whether the upregulation of IRT1 and FRO2 expression in roots in the Split +Fe side is reflected in the activity of Fe transport, we observed the distribution of fluorescence intensity iron probe RhoNox-4, a Fe2+ specific fluorescent probe (Hirayama et al. 2020), between roots in the Cont. +Fe and Split +Fe sides. Images of the Fe2+ fluorescent probe-treated roots indicated that the cytosolic Fe2+ content of roots on the Split +Fe side was higher than that of those placed in the homogeneous Cont. +Fe condition (Fig. 2A).

Next, we employed an isotopic technique to explore the Fe transport activity from one side of the split root to the shoot. All split roots exposed to Fe-sufficient conditions in split-root culture were transferred to a medium containing 59Fe for 24 h. The 59Fe accumulation in the shoots of the split-root plants supplied with heterogeneous Fe (610.6 ± 155.4 cpm/FW) was approximately four times higher than that of plants homogeneously
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Fig. 2 Upregulated Fe transport activity on one side of the roots in Fe-sufficient conditions in response to Fe starvation of roots on the other side of the culture. (A) The fluorescent pattern of an Fe$^{2+}$ probe in root tips treated with Cont. +Fe and Split +Fe conditions in the presence of 5 µM RhoNox-4 for 3 h. Scale bars = 100 µm. (B) The distribution of $^{59}$Fe in shoots was visualized by radioluminography using an imaging plate. The bar graph shows the amount of $^{59}$Fe in each sample as measured using an NaI scintillation counter ($n = 3$). Student’s t-test was conducted, $P < 0.05$ (*). (C) Fe content of wild-type plant shoots grown in different split-root culture conditions for 7 days (µg/g) ($n = 3$). (D) Chlorophyll a + Chlorophyll b content of wild-type plants grown in different split-root culture conditions for 7 days (nmol/mg) ($n = 6$). (E) Shoot weights of wild-type plants grown in different split-root culture conditions for 7 days (mg) ($n = 6$). Different lowercase letters indicate significant differences by Tukey’s HSD test ($P < 0.05$). All experiments in (A) were conducted with 10 independent biological replicates. All experiments in (B) and (C) were conducted with three independent biological replicates. All experiments in (D) and (E) were conducted with six independent biological replicates. Data represent means ± SD.

supplied with sufficient Fe (156 ± 80.5 cpm/FW) (Fig. 2B). This result suggested that local Fe depletion on one side of the split root elevated the activity of Fe uptake on the other side of the split-root culture and that the Fe was translocated from the root to the shoot.

Furthermore, to investigate the long-term growth effects during split-root Fe-starvation culture, we measured the Fe content of shoots subjected to three split-root Fe conditions.

In the homogeneous −Fe condition, the shoot Fe content was approximately one-half that of the +Fe control condition (Fig. 2C). Although the available Fe content in the medium of the heterogeneous split-root condition was one-half that in the +Fe control condition, plants from both groups maintained the same amount of Fe in their shoots (Fig. 2C). This result indicated that upregulation of the Fe transporter on the Fe-sufficient side of the split-root culture continuously compensated for the Fe...
contents of the shoots. In addition, the chlorophyll content and shoot weights in the split-root condition were similar to those of the +Fe control condition (Fig. 2D, E). Thus, we hypothesize that this compensating Fe-uptake activity might be reflected in the chlorophyll content and shoot weight in heterogeneous split-root conditions.

Transcriptome analysis in response to heterogeneous Fe starvation

To gain additional insight into the molecular processes responsible for long-distance signaling of Fe starvation, we used RNA-seq analysis of roots subjected to the split-root treatment conditions (Fig. 1A; Supplementary Table S1). RNA was extracted at 24 h after split-root treatment from the roots of four different groups: Cont. +Fe grown in a homogeneous Fe-sufficient medium (Cont. +Fe), a heterogeneous Fe-sufficient medium (Split +Fe), a heterogeneous Fe-deficient medium (Split −Fe) or a homogeneous deficient medium (−Fe) (Fig. 1A). In the homogeneous −Fe condition, a common set of 605 genes was differentially expressed (log2 fold change | ≥ 1) and 297 genes were upregulated (log2 fold change ≥ 1) compared to the Cont. +Fe condition (Supplementary Fig. S3; Supplementary Table S2). In the Split −Fe condition, 570 genes were differentially expressed, with an approximately 40% overlap with genes expressed in the homogeneous −Fe group (Supplementary Fig. S3), suggesting that gene expression in the Fe-starvation local-stabilization treatment side was affected by the local Fe concentration and the Fe availability on the distal side. Among 134 genes downregulated in the Split −Fe side compared to the homogeneous −Fe condition, Gene Ontology (GO) terms related to Fe transport and homeostasis were enriched (Supplementary Fig. S4B; Supplementary Table S3). In the Split +Fe condition, the expressions of 56 genes were upregulated compared with those of the Cont. +Fe condition (Supplementary Table S4). This finding indicated that the expression of a small subset of genes in the roots treated with Fe-sufficient medium was affected by the Fe-starvation information emanating from the distal part of the root system through Fe-starvation-triggered organ-to-organ communication (Supplementary Fig. S4A; Supplementary Table S4).

To profile the expression of Fe-related genes, we focused on genes responsible for Fe homeostasis as listed by Kobayashi and Nishizawa (2012) and Connorton et al. (2017) with some modifications. Based on our analysis of the cluster heatmap, some trends emerged (Fig. 3A). Primarily transcription factors involved in the Fe-starvation response, such as BHLHs and BTS, were upregulated in roots treated with Fe-sufficient medium. On the other hand, genes contributing to the Fe-uptake system and coumarin biosynthesis showed similar expression patterns (Fig. 3A). We confirmed the expression response of eight genes using qRT-PCR. Previous studies have shown that the expression of OLIGOPEPTIDE TRANSPORTER 3 (OPT3) and NICOTIANAMINE SYNTHASE 4 (NAS4) increased significantly in Fe-deficient conditions (Schuler et al. 2012, Zhai et al. 2014), Similarly, the expression of OPT3 and NAS4 was upregulated in the Split −Fe side of the heterogeneous split-root culture and homogeneous −Fe condition (Fig. 3B). OPT3 mediates the uptake of Fe into the phloem, and NAS is an enzyme that catalyzes the trimerization of S-adenosylmethionine to synthesize nicotianamine, a facilitator of Fe distribution (Klatte et al. 2009, Schuler et al. 2012). OPT3 and NAS4 may, therefore, function in mediating Fe partitioning to the shoot. qRT-PCR data also showed that BHLH101, one of the transcription regulators of the Fe-starvation response (Wang et al. 2013), is upregulated in roots from the Split −Fe side of the split-root cultures and homogeneous −Fe condition. This result indicated that transcriptional regulation of these genes is controlled primarily by the conditions of local Fe availability. By contrast, MYB10 was upregulated in roots from the Split +Fe side of the split-root cultures and homogeneous −Fe condition. Thus, compensated upregulation was observed in the MYB10 expression patterns. MYB10 and MYB72 have been proposed to work together with the BHLH Ib subfamily (BHLH038, 039, 100, 101) and FIT/FFR transcription factor complexes to positively regulate the expression of Fe-deficiency-inducible genes involved in the biosynthesis of metal chelators (Yuan et al. 2008, Palmer et al. 2013, Wang et al. 2013). Therefore, our result suggested that MYB10 and BHLH101 transcription factors may take on different roles in the Fe-starvation response in heterogeneous Fe conditions. In addition to MYB10, two Fe-metabolism-related genes, SCOPOLETIN 8-HYDROXYLASE (58H) and CYP82C4, were also upregulated in roots from the Split +Fe side of split-root cultures and those subjected to the homogeneous −Fe condition (Fig. 3C) (Murgia et al. 2011, Rodriguez-Celma et al. 2013b). Recent studies demonstrated that coumarin secretion is essential for Fe acquisition (Schmid et al. 2014, Rajniak et al. 2018). 58H and CYP82C4 are required for phenylpropanoid metabolism, specifically coumarin biosynthesis (Schmid et al. 2014, Rajniak et al. 2018). Therefore, in Arabidopsis, Fe-uptake genes and coumarin biosynthesis genes are regulated by long-distance signaling to acquire Fe effectively at the Fe-rich side. Altogether, expressions of both Fe-uptake and coumarin biosynthesis genes were induced through systemic signaling for Fe demand. We also confirmed the expression level of two genes, VACUOLAR IRON TRANSPORTER-LIKE 1 (VTL1) and FERRITIN 1 (FER1), which were previously shown to be involved in Fe homeostasis (storage) (Briat et al. 2010, Gollhofer et al. 2014). VTL1 and FER1 were highly expressed in roots in the +Fe control condition and the Split +Fe side of split-root cultures (Fig. 3D), indicating increased Fe storage.

IMA/FeP peptides are essential for long-distance signaling of Fe homeostasis

To determine how long-distance signaling for Fe demand is accomplished, we conducted RNA-seq analysis at several time points (0, 6, 12 and 24 h) of shoots and roots subjected to the split-root culture treatment using three different Fe conditions (Supplementary Fig. S5). Given that compensatory
upregulation of IRT1 in roots is initiated 24 h after split-root Fe-starvation treatment, the organ-to-organ communication pathway should induce shoot-derived factors before 24 h. Therefore, we searched for candidate gene groups from the list of genes whose expression in shoot under Fe-starved split-root cultures at 6 and 12 h is upregulated compared with the control conditions. RNA-seq data analysis revealed that 41 genes were commonly upregulated in the four conditions: (i) in the shoot 6 h after split-root Fe-starvation treatment, (ii) in the shoot 6 h after treatment in the homogeneous −Fe condition, (iii) in the shoot 12 h after the split-root Fe-starvation treatment and (iv) in the shoot 12 h after treatment in the homogeneous −Fe condition, compared with control conditions for 6 and 12 h (Supplementary Fig. S6). IMA1 and IMA2 were among the upregulated gene groups (Supplementary Tables S5, S6), and expressions of all IMA/FEP genes were induced before 24 h (Supplementary Fig. S7). In particular, the induction of IMA1, IMA2 and IMA3/FEP1 expressions was strong (Supplementary Fig. S7). IMA/FEP was recently discovered as a novel family of metal-binding peptides (Grillet et al. 2018, Hirayama et al. 2018). IMA/FEP peptides harbor a C-terminal consensus sequence conserved across angiosperms. Reciprocal grafting experiments with wild-type Arabidopsis and octuple ima8x mutants revealed that IMA peptides derived from shoots positively regulate FCR activities in roots (Grillet et al. 2018). To clarify the role of IMA peptides in Fe-starvation-triggered organ-to-organ communication for Fe demand, we checked whether compensated upregulation of IRT1 on the Split +Fe side of split-root culture occurred in ima multiple mutants. The octuple ima8x mutants were shown to be unresponsive to Fe starvation in a previous study (Grillet et al. 2018); however, ima8x mutants were extremely chlorotic and too small for the split-root culture analysis. Therefore, we used septuple ima7x mutants (ima8x plant transformed with IMA1pro::YFP-IMA1 constructs) (Grillet et al. 2018), which were responsive to Fe starvation, but their responsivity was significantly lower than the wild type (Fig. 4A). The ima7x mutants showed an Fe-starvation response under homogeneous −Fe conditions in split-root cultures (Supplementary Fig. S8A). However, in the ima7x mutant, the expression level of IRT1 did not change significantly in the Cont. +Fe control condition or in the Split +Fe side of split-root cultures (Fig. 4B). These results revealed that the IMA-mediated mechanism controls long-distance signaling from the Fe-starvation side of roots to the Fe-sufficient side. We also observed that the suppression of IRT1 expression on the Split −Fe side of split-root culture was arrested in the ima7x mutant. This observation indicated that the Fe-supply signal from the Split +Fe side as well as Fe-demand signal was also weakened in the ima7x mutant (Fig. 4B). Similar to the ima7x mutant, derepression of the IRT1 gene was observed on the Split −Fe side of split-root culture in pye mutants (Supplementary Fig. S9A–B), suggesting that PYE might be involved indirectly in the regulation of long-distance signaling for Fe supply. Further analysis is required to elucidate the relationship between PYE-mediated Fe homeostasis and organ-to-organ communication for Fe supply. Next, to investigate the effect of IMA overexpression in this system, we checked

Fig. 3 Transcriptome profile of Fe-related genes expressed in heterogeneous Fe supply conditions. (A) Heatmap of z-score-normalized log-transformed ppm of Fe-related genes (Kobayashi and Nishizawa 2012, Conmorton et al. 2017) in each split-root culture condition. Expression of genes marked with an asterisk was verified by qRT-PCR (as described in Materials and Methods). (B) Expression levels of bHLH101, OPT3 and NAS4 mRNA, which are upregulated in the Fe-starved medium, in the split roots of wild-type plants in four split-root culture conditions. (C) Expression levels of MYB10, SBH and CYP82C4 mRNA, which are upregulated in both the Fe-starved medium and the Fe-rich sides of the split-root cultures, in the split roots of wild-type plants in four split-root culture conditions. (D) Expression levels of VTL1 and FER1 mRNA, which are upregulated in only the Fe-rich side of the split-root cultures, in the split roots of wild-type plants in four split-root culture conditions. Different lowercase letters indicate significant differences by Tukey’s HSD test (P < 0.05). All experiments (B) and (D) were conducted with three independent biological replicates. All experiments (C) were conducted with four independent biological replicates. Data represent means ± SD.
the expression level of IRT1 from CaMV 35S promoter-IMA3 gene OX plants in split-root cultures. IMA3 OX plants showed normal Fe-starvation responses in the homogeneous –Fe condition of split-root cultures (Supplementary Fig. S8B). We found that IRT1 expression was specifically induced in the roots exposed to the Fe-sufficient medium but not in the Fe-starved roots of split-root culture (Fig. 4C), even though IMA3 transcripts were overexpressed. Thus, each root might integrate shoot-derived signals with the local Fe availability in the soil to decide whether or not to upregulate Fe uptake. Furthermore, in the Fe-sufficient roots of IMA3 OX plants, genes that are essential for Fe-starvation-triggered organ-to-organ communication for Fe demand, i.e., IRT1 for Fe uptake, MYB10 and MYB72 transcriptional factors for the regulation of coumarin biosynthesis and S8H and CYP82C4 for coumarin biosynthesis, were strongly induced (Fig. 4D). These results suggested that IMA3/FEP1 pathway mediates long-distance signaling in Fe homeostasis through the regulation of coumarin biosynthesis.

We also conducted reciprocal grafting experiments with wild-type Arabidopsis and ima7x mutants and qRT-PCR analysis of Fe long-distance signaling target genes. Wild-type self-grafting plants displayed a sensitive Fe-starvation response, namely, upregulation of IRT1, S8H and CYP82C4 expression under Fe starvation for 24 h. In comparison, self-grafted ima7x mutants had significantly lower responsiveness than self-grafted wild-type plants (Fig. 5A–C). Grafting ima7x scions onto wild-type rootstocks did not rescue the upregulation of IRT1, S8H and CYP82C4 genes under Fe starvation for 24 h (Fig. 5A–C). By contrast, wild-type scions grafted with ima7x rootstocks induced the expression of the above-mentioned genes to a level similar to that of self-grafted wild-type plants (Fig. 5A–C). Wild-type scions grafted with ima7x rootstocks also rescued the phenotype of primary root length (Supplementary Fig. S10A–B). These data support the concept that IMA/IFP upregulate Fe uptake and the expression of coumarin biosynthesis genes for the long-distance signaling of Fe starvation in the roots as a shoot-derived descending signal. On the other hand, IMA/IFP genes are also expressed in the roots, although at lower levels than in the shoot (Rodriguez-Celma et al. 2013a, Gautam et al. 2021). In split-root heterogeneous Fe conditions, IMA/IFP genes were upregulated in the Split –Fe side of the heterogeneous split-root culture system and homogeneous –Fe condition, similar to bHLH101, OPT3 and NAS4 (Fig. 3A–B; Supplementary Tables S1, S2). However, the significance of induction of IMA/IFPs root expression in long-distance Fe signaling is not yet known. Further analysis is required to elucidate the biological roles of IMA/IFP genes in roots.

**Coumarin biosynthesis on the Fe-sufficient side of split-root cultures changed in response to Fe starvation on the other side through the MYB10/72 pathway**

Finally, to investigate whether MYB10 and MYB72 mediate systemic Fe-demand signaling, we conducted the split-root Fe-starvation experiments using myb10 myb72 mutants (Palmer et al. 2013). In comparing wild-type plants and myb10 myb72 mutants, we observed upregulation of IRT1 in the roots exposed to the Fe-sufficient medium of split-root cultures after 24 h in both types of plants (Fig. 6A). By contrast, no such systemic upregulation of S8H and CYP82C4 was detected in the myb10 myb72 mutant roots (Fig. 6B, C). Both wild-type plants and myb10 myb72 mutants displayed upregulation of IRT1, S8H and CYP82C4 in the roots exposed to the homogeneous –Fe condition of split-root culture after 24 h (Supplementary Fig. S11A–C). These results indicate that MYB10 and MYB72 are critical components for systemic Fe-demand signaling, specifically for regulating coumarin biosynthesis.

**Discussion**

**Fe-uptake activity and transport from roots on the Fe-sufficient side of split-root cultures changed in response to Fe starvation on the other side**

Recent studies in plant science have focused on the systemic regulation of nutrient acquisition (Ruffel et al. 2011, Tabata et al. 2014, Sinclair et al. 2018, Kawai et al. 2022). Here, we combined a genomic approach with the split-root culture system to dissect the molecular basis for long-distance signaling in a heterogeneous Fe soil environment. First, we established the relationships between Fe-related long-distance signaling controlling gene expression and root development, especially LR growth. When N is locally supplied to the root systems, plants preferentially proliferate roots in the N-rich zone by stimulating LR elongation and/or branching (Zhang et al. 1999). In* Arabidopsis*, detailed analyses of a split-root culture system revealed that the adaptive responses of LR on the N-sufficient side involved long-distance signaling (Ruffel et al. 2011, 2016). Giehl et al. (2012) also revealed that a localized Fe supply triggers LR elongation in* Arabidopsis* by altering auxin distribution (Giehl et al. 2012). Our experiment documented that IRT1 and FRO2 mRNA levels were higher in roots on the Split +Fe side and lower in roots on the Split −Fe side of the roots (Fig. 1B; Supplementary Fig. S1A). Thus, two-way communication by Fe demand and satiety signals may be associated with long-distance Fe signaling from one side of the root to the opposite side. However, we observed no significant difference in total LR growth between homogeneous Fe-sufficient controls and the split-root heterogeneous Fe-sufficient condition (Fig. 1D). Therefore, the total LR growth of each compartment may be predominantly controlled by local Fe conditions in environments having a heterogeneous Fe supply. Alternatively, morphological changes in LRs may occur later during this heterogeneous experimental condition, following gene expression changes 24 h after initiating the split-root treatment.

We also identified the physiological significance of long-distance Fe regulation in* Arabidopsis* shoot growth. The Fe-uptake and transport activities of roots on the Fe-sufficient side in split-root heterogeneous conditions were compensatorily upregulated in response to Fe starvation on the other side (Fig. 2A, B). Thus, plants maintained the same Fe content and biomass in their shoots when experiencing a heterogeneous
Fig. 4 IMA/FEP peptides are required for Fe long-distance signaling. (A) Expression levels of IRT1 in roots of 2-week-old wild-type plants and ima7x mutants in response to Fe-starvation treatment for 24 h. Statistical significance was assessed by Student’s t-test, \( P < 0.01 \) (**). (B) Expression levels of IRT1 in split roots of wild-type plants and ima7x mutants in three split-root culture conditions. (C) Expression levels of IRT1 in split roots of wild-type plants and IMA3 OX plants in three split-root culture conditions. Different lowercase letters indicate significant differences by Tukey’s HSD test (\( P < 0.05 \)). (D) Expression levels of IRT1, MYB10, MYB72, S8H and CYP82C4 in roots of 2-week-old wild-type plants and IMA3 OX plants. Statistical significance was assessed by Student’s t-test, \( P < 0.01 \) (**). All experiments in (A) and (D) were conducted with three independent biological replicates. All experiments (B) and (C) were conducted with four independent biological replicates. Data represent means ± SD.

Fe supply in the split-root condition, compared with that of the +Fe control condition (Fig. 2C, D, E). Our observations were also supported by a previous report in which the levels of IRT1 and FRO2 proteins were maintained for at least one week in split-root heterogeneous Fe conditions; however, the high accumulation of IRT1 and FRO2 mRNA declined over the week (Vert et al. 2003). Thus, this compensated regulation supports Fe-uptake activity and Fe translocation from the roots to the shoot over a long period. Expression analysis in homogeneous one-half-Fe conditions showed no upregulation of IRT1 (Fig. 1C), suggesting that the compensated upregulation of IRT1 was induced only when the remaining local amount of Fe in the roots was lower than a predetermined threshold value. Recently, Ota et al. (2020) proposed that Arabidopsis possesses two long-distance pathways for N acquisition (Ota et al. 2020). In N-replete or N-moderate conditions, the CEPD-like 2 (CEPD1/2) pathway works from shoot to root, dependent on the shoot N status. When roots are subjected to N-limited conditions, the CEPDL2 and CEP downstream 1/2 (CEPD1/2) pathways are upregulated (Ota et al. 2020). With respect to systemic Fe signaling, the concentration of Fe in the phloem regulated by OPT3 plays a role in the shoot–root communication of Fe demand (Maas et al. 1988). Therefore, as with long-distance N signaling, two pathways, the shoot–root or the root–shoot–root pathway, may be balanced by the shoot Fe status and rhizosphere Fe availability.
Systemic regulation of gene expression in response to heterogeneous Fe-starvation conditions

To further refine our model for long-distance signaling of Fe starvation, we conducted genome-wide expression analyses using the split-root treatment conditions. In response to a heterogeneous N supply, roots display a wide range of responses, including the transcriptional regulation of hundreds of genes (Gansel et al. 2001, Remans et al. 2006, Ruffe et al. 2008, Ruffel et al. 2011, Mounier et al. 2014). In heterogeneous Fe-starvation conditions, however, we observed that only a small subset of genes involved in the Fe-uptake system for Fe acquisition was activated in the distant parts of the roots exposed to Fe-sufficient medium in split-root cultures (Supplementary Fig. S4A; Supplementary Table S4). This result suggests that compensatory upregulation of long-distance Fe signaling can be targeted to several specific pathways (Fig. 3). We showed that S8H and CYP82C4, two genes required for coumarin biosynthesis, were upregulated on the Split +Fe side of split-root cultures (Fig. 3C). Coumarins are released in Fe-deficient conditions and are essential for efficiently mobilizing the acquisition of Fe in alkaline conditions (Schmidt et al. 2014). Previous studies demonstrated that the MYB10/72 pathway positively regulates the expression of Fe-deficiency-inducible genes involved in the biosynthesis of coumarins (Palmer et al. 2013, Stringlis et al. 2018). In the multiple mutants of IMA/FEP, the compensatory upregulation of long-distance Fe signaling on the Split +Fe side of split-root cultures was abolished (Fig. 4B). In addition, overexpression of IMA3 enhanced the expression of genes for Fe uptake and coumarin biosynthesis regulation, including those of MYB10 and 72 transcription factors (Fig. 4D). Reciprocal grafting experiments with wild-type Arabidopsis and ima7x mutants revealed that IMA peptides derived from shoots positively regulate expression levels of coumarin biosynthesis genes in roots (Fig. 5). Furthermore, the loss of MYB10/72 led to a reduction in systemic upregulation of coumarin biosynthesis under Fe-starved split-root cultures. Therefore, IMA3/FEP1-mediated MYB10/72 signaling pathway could be a key process in systemic long-distance Fe signaling to regulate the Fe-uptake system on the Fe-sufficient side of the root in a heterogeneous Fe environment. Recently, Gautam et al. (2021) and Robe et al. (2021) showed that IMA1 and IMA2 improve fraxetin via increased expression of coumarin biosynthesis genes under alkaline pH conditions (Gautam et al. 2021, 2021). Arabinodips shifts Fe–fraxetin complexes via an IRT1-independent strategy (Robe et al. 2021). Soil pH and the distribution of components that form the soil ecosystem, including nutrients, are spatially heterogeneous (Xue et al. 2019). Therefore, IMA-mediated long-distance signaling that activates coumarin biosynthesis might be a pathway that plants have acquired to adapt to heterogeneous soil environments, although the universality of this hypothesis should be verified further. In mammals, Fe homeostasis in the body is regulated by the metal-binding peptide TRANSFERRIN (Anderson and Vulpe 2009). Each TRANSFERRIN molecule can carry two Fe ions. TRANSFERRIN moves through the blood and is recognized by the transferrin receptors on the surface of the target cells. The TRANSFERRIN–receptor complex is endocytosed by the cell, and Fe is dissociated and released (Anderson and Vulpe 2009). Analogous to the mammalian system, IMA peptides could be recognized by some receptors in the target cell in

Fig. 5 IMA/FEP peptides induced in shoots positively regulate target genes for Fe long-distance signaling in roots. (A) Expression levels of IRT1 in roots of reciprocally grafted plants between wild type and ima7x mutants under Fe-starvation treatment for 24 h. (B) Expression levels of S8H in roots of reciprocally grafted plants under Fe-starvation treatment for 24 h. (C) Expression levels of CYP82C4 in roots of reciprocally grafted plants under Fe-starvation treatment for 24 h. Statistical significance was assessed by Student’s t-test, P < 0.05 (*). All experiments were conducted with four independent biological replicates. Data represent means ± SD.
plants. IMA peptides have been shown recently to interact with the ubiquitin ligase BTS, and this interaction is essential for the Fe-deficiency response (Li et al. 2021). The unloading process of IMA peptides at the target root cell is still unclear, but BTS is one of the receptor candidates for mobile molecules in Fe long-distance signaling.

We also documented alterations in the expression patterns of IRT1 mRNA in loss-of-function mutants of FIT in heterogeneous Fe conditions. FIT functions upstream of MYB72 as a master transcription factor for the Fe-starvation response (Sivitz et al. 2012). In the fit mutant, the expression level of IRT1 remained very low in the Cont. +Fe control condition and in split-root cultures (Supplementary Fig. S9A, C). This result suggests that the fundamental mechanism by which FIT mediates the local Fe-starvation response is important for initiating long-distance signaling from the Fe-starvation side of roots to the Fe-sufficient side to upregulate IRT1.

Compared to long-distance signaling for Fe demand, Fe-supply signaling is not entirely understood. The best-understood mechanism of satiety signaling through root–shoot–root communication is nodule formation (Okamoto et al. 2013, 2016, Okuma et al. 2020). Secreted CLE (CLAVATA3/Endosperm surrounding region-related) peptides mediate this long-distance signaling for supply. CLE-RS1/2 peptides are induced by rhizobial inoculation and are transmitted to the shoots where the HAR1 receptor recognizes the peptides. This interaction then sends microRNA miR2111 to roots to suppress further nodule formation (Okamoto et al. 2013, 2016, Okuma et al. 2020). So far, the two-way communication by demand and satiety signals, which is associated with long-distance signaling from one side of the root to the opposite side, is reported in only N and Fe starvation conditions. The two conditions have the following in common: First, both N and Fe are distributed heterogeneously in the soil. Secondly, both nutrients are essential for plant development, but in excess, they are harmful (Becana et al. 1998, Hachiya et al. 2021). However, other two-way communication pathways could respond to heterogeneous soil environments. Further investigation is required to analyze whether other nutrients also possess two-way communication by demand and satiety signals through root–shoot–root communication or not.

**Materials and Methods**

**Plant materials and growth conditions**

*A. thaliana* (Arabidopsis) ecotype Columbia (Col) was used as the wild-type plant for these experiments. Arabidopsis seeds were surface-sterilized with 5% (w/v) bleach for 10 min, washed five times with sterile water, incubated at 4 °C for three days, and planted on a medium containing KNO$_3$ (9.4 mM), NH$_4$NO$_3$ (1 mM), MgSO$_4$ (0.75 mM), CaCl$_2$ (1.5 mM), KH$_2$PO$_4$ (0.625 mM), H$_3$BO$_3$ (50 µM), MnCl$_2$ (50 µM), ZnCl$_2$ (15 µM), CuCl$_2$ (0.053 µM), CoCl$_2$ (0.055 µM), Na$_2$MoO$_4$ (0.52 µM) and Fe-EDTA (75 µM); 0.5% (w/v) sucrose; and 1.5% (w/v) agar. The pH was adjusted to 5.7 with 1 M KOH. Seedlings were grown at 22°C with a photoperiod of 16-h light and 8-h darkness at a light intensity of 100 µmol m$^{-2}$ s$^{-1}$. For gene expression analysis between wild-type and IMA3 OX plants, plants were incubated for 2 weeks, and their roots were sampled. For Fe-starvation treatment, 2-week-old Arabidopsis plants were transferred to an Fe-deficient medium for 24 h and roots were sampled.

![Fig. 6](https://example.com/fig6.png) The MYB10/72 pathway is required for systemic regulation of coumarin biosynthesis under heterogeneous Fe conditions. (A) Expression levels of IRT1 in split roots of wild-type plants and myb10/72 mutants in three split-root culture conditions. (B) Expression levels of S8H in split roots of wild-type plants and myb10/72 mutants in three split-root culture conditions. (C) Expression levels of CYP82C4 in split roots of wild-type plants and myb10/72 mutants in three split-root culture conditions. Different lowercase letters indicate significant differences by Tukey’s HSD test (P < 0.05). All experiments were conducted with four independent biological replicates. Data represent means ± SD.
**Split-root culture of Arabidopsis**

Seven days after sowing, primary roots were split below two LRs and allowed to grow on a plate for additional three days. Plants with two main roots were transferred to the center of split Petri dish plates divided into two compartments that contained solid medium. After four days, plants with similar-sized roots on both sides of the divide were selected for gene expression analysis and were transferred to one of three types of split-culture conditions for 24 h. To examine root morphology, 10-day-old plants in which the primary root had been split into two roots were transferred to one of three types of split culture conditions for one week. Root length was measured in 10 independent samples.

**Micrografting of Arabidopsis**

Micrografting of 4-day-old Arabidopsis plants was performed using a supportive micrografting chip and method described previously (Tsutsui et al. 2020). After a 2-week incubation of grafted plants, the primary root length was measured, and the Fe-starvation treatment for 24 h was conducted.

**Quantitative reverse-transcriptase polymerase chain reaction**

Total RNA was extracted from plant samples using an RNeasy MiniElute Clean up Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The RNA concentration and quality were determined using a BioSpec-nano spectrophotometer (SHIMADZU, Kyoto, Japan). CDNA synthesis from the RNA sample was carried out using the SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific) following the manufacturer’s instructions. An equal concentration of CDNA from each sample was used in qRT-PCR with TaqMan Real-time PCR Master Mixes (Thermo Fisher Scientific, Waltham, Massachusetts, USA) or KAPA SYBR Fast qPCR Kit (Sigma-Aldrich, St. Louis, Missouri, USA). For expression analyses, total RNA was extracted from the roots of Arabidopsis plants. PCR conditions were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. *AtFea1* was used as the internal standard. All experiments were conducted with three independent biological replicates. Primer sequences were as follows: *AtFea1*-F: 5′-CTTGGTCTCAAGCAGAGATTT-3′; *AtFea1*-R: 5′-TGCTTCTGGCGGTGTATC-3′; *IR1*-F: 5′-ACCCATAAAGAATTCTTTCTATATT-3′; *IR1*-R: 5′-CACCGGAAAACTGTACAC-3′; *IR2*-F: 5′-AGACACCTTGTTCATCCC-3′; *IR2*-R: 5′-TCACACCTTGCACTTACAC-3′; *BHLH110*-F: 5′-CCAACGAGAAAGTTCTACAC-3′; *BHLH110*-R: 5′-TCCCCAAGGACATACATTGAC-3′; *OPT3*-F: 5′-CACAACACCTGTGTGACC-3′; *OPT3*-R: 5′-ATGCTCACCTGGGTCGTT-3′; *NAS4*-F: 5′-CCGGTTTCTTCCCA-3′; *NAS4*-R: 5′-CGCGAACAGTAAGAGGAGA-3′; *MYB10*-F: 5′-GTCTTTAACCTTGCTGGA-3′; *MYB10*-R: 5′-GGTTAAGGTTGGATGGTG-3′; *MYB72*-F: 5′-GACTCGAGAGTTACAAAGGC-3′; *MYB72*-R: 5′-GTTGACACCACTGTCATC-3′; *BH150F*-F: 5′-GGGAGGACTCCAGCC-3′; *BH150F*-R: 5′-GTCGTTTAGGACGCCAGC-3′; *BH150R*-F: 5′-CCCACTGCTGGGTGGATC-3′; *BH150R*-R: 5′-GGTTAAGGTTGGATGGTG-3′; *CYP26C4*-F: 5′-GCAAATCACCCTGC-3′; *CYP26C4*-R: 5′-CCACCTGGTTCGAGGCAGC-3′; *VTL1*-F: 5′-GGTGAAGTTGTCGAAGC-3′; *VTL1*-R: 5′-GGTTAAGGTTGGATGGTG-3′; *FRO2*-F: 5′-AAGAGCATACCATTGA-3′; *FRO2*-R: 5′-ACCACTCGTCGTACTC-3′.

**Synthesis of the RhoNox-4 iron (Fe(2+) fluorescent probe and its use**

The RhoNox-4 iron (Fe(2+)) fluorescent probe was synthesized as previously described (Hirayama et al. 2020). Briefly, a solution of N-boc-piperazinyl-rodhamine (Grimm and Lavis 2011) (76 mg, 0.11 mmol) and m-chloroperbenzoic acid (22 mg, 0.13 mmol) was stirred at 0°C for 5 min and then at room temperature for 30 min. The solution was evaporated, and the residue was purified by silica gel column chromatography (chloroform:methanol = 100:1 to 20:1), producing RhoNox-4 as a white powder after drying (38 mg, 48%). The purity and identity were confirmed by 1H-NMR (JEOL ECA-500 spectrometer). 1H-NMR (CD3OD, 500 MHz) δ: 8.12 (d, J = 2.3 Hz, 1H), 8.04 (d, J = 7.4 Hz, 1H), 7.82–7.68 (m, 2H), 7.65 (dd, J = 8.9, 2.6 Hz, 1H), 6.97 (d, J = 9.2 Hz, 1H), 6.86 (d, J = 2.3 Hz, 1H), 6.76 (dd, J = 9.2, 2.3 Hz, 1H), 6.65 (d, J = 8.6 Hz, 1H), 4.27–4.08 (m, 2H), 3.94–3.67 (m, 2H), 3.67–3.44 (m, 4H), 3.24 (t, J = 5.2 Hz, 6H), 1.49 (s, 9H), 1.48 (s, 9H).

After split-root culture treatment for 24 h, 5 µM RhoNox-4 was added to the roots in the homogenous Fe-sufficient control condition and Fe-sufficient side of heterogeneous split condition and incubated for 3 h.

**Microscopic analyses**

Laser scanning confocal microscopy was performed with Leica SP8 system (Leica Camera AG, Wetzlar, Hessen, Germany) on Fe(2+) fluorescent probe-treated roots at objectives of 20X. Roots were stained with RhoNox-4 in a 5 µM solution for 3 h and viewed with 552 nm excitation and with 567–763 nm emission.

**Iron transport assay**

After 24-h Fe starvation, split-root-cultured Arabidopsis plants were subjected to the Fe transport assay. Roots from the left side that were grown in a medium containing sufficient Fe were transferred to a medium with 75 µM Fe labeled with 59Fe (10 kBq). After 24-h incubation, the distribution of 59Fe in the shoot was visualized by radioluminescopy using an imaging plate (Bas-IP MS 2040, Fujifilm, Tokyo, Japan) and an FLA-5000 image reader (Fujifilm). The amount of 59Fe in each sample was measured using an NaI scintillation counter (ARC-370, Hitachi Aloka Medical, Ltd., Tokyo, Japan) for 10 min. All experiments were conducted with three independent biological replicates.

**Measurement of chlorophyll content**

Chlorophyll was extracted from the shoots using NN-dimethylformamide in the dark at 4°C overnight. Chlorophyll concentrations were measured according to the method of Porra et al. (1989). All experiments were conducted with six independent biological replicates.

**Measurement of Fe concentration**

Shoots were harvested, dried at 60°C for 48 h, and digested with HNO3 and H2O2 at 100°C. Digested samples were dissolved and diluted with 0.08 M HNO3 and analyzed using inductively coupled plasma-mass spectrometry (Agilent 7800 ICP-MS; Agilent Technologies, Santa Clara, California, USA). All experiments were conducted with three independent biological replicates.

**RNA-seq analysis**

Extracted RNA was converted into a CDNA library using a NEBNext Ultra RNA Library Prep Kit for Illumina, a NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Multiplex Oligos for Illumina (New England BioLabs, Ipswich, Massachusetts, USA, https://www.neb.jp/), according to the manufacturer’s protocols. Libraries were applied to the NextSeq5000 sequencer (Illumina); nearly 10 million reads were obtained for each library. For the time-series RNA-seq analysis, sequence analysis of 40 million reads was performed with a NovaSeq 6,000 Sequencing System (Illumina). Library preparation and sequencing were conducted by Macrogen Japan, Inc. (Tokyo, Japan). The adapter sequences in short reads were trimmed by bc2fastq (Illumina), and low-quality nucleotides (base quality < 25) were masked by N with the original script. Reads shorter than 50 bp were removed, and the rest were mapped to the CDNA reference by Bowtie (Langmead et al. 2009) with the following options: “--all--best--strata” (Langmead et al. 2009). Reads were counted by transcript models.

**Statistical analyses**

All statistical analyses were conducted using Microsoft Excel or R programs. Details of the analyses are provided in the figure legends. GO analysis was performed with the web-based tool agriGO (http://bioinfo.cau.edu.cn/agriGO/) (Du et al. 2010).
Supplementary Data

Supplementary data are available at PCP online.

Data Availability

RNA-seq data have been deposited to the DNA Data Bank of Japan database under the accession codes DRA013130 and DRA013693.

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Author Contributions

R.T., T.K. and H.S. conceived the research and designed the experiments. R.T., T.K., S.I., H.T., K.I., H.T., H.T., K.T., T.S., T.H. and S.I. performed experiments and analyzed the data. R.T., T.K., T.H. and H.S. wrote the paper.

Disclosures

The authors have no conflicts of interest to declare.

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