

⁵²Fe Translocation in Barley as Monitored by a Positron-Emitting Tracer Imaging System (PETIS): Evidence for the Direct Translocation of Fe from Roots to Young Leaves via Phloem

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The real-time translocation of iron (Fe) in barley (*Hordeum vulgare* L. cv. Ehimehadaka no. 1) was visualized using the positron-emitting tracer ⁵²Fe and a positron-emitting tracer imaging system (PETIS). PETIS allowed us to monitor Fe translocation in barley non-destructively under various conditions. In all cases, ⁵²Fe first accumulated at the basal part of the shoot, suggesting that this region may play an important role in Fe distribution in graminaceous plants. Fe-deficient barley showed greater translocation of ⁵²Fe from roots to shoots than did Fe-sufficient barley, demonstrating that Fe deficiency causes enhanced ⁵²Fe uptake and translocation to shoots. In the dark, translocation of ⁵²Fe to the youngest leaf was equivalent to or higher than that under the light condition, while the translocation of ⁵²Fe to the older leaves was decreased, in both Fe-deficient and Fe-sufficient barley. This suggests the possibility that the mechanism and/or pathway of Fe translocation to the youngest leaf may be different from that to the older leaves. When phloem transport in the leaf was blocked by steam treatment, ⁵²Fe translocation from the roots to older leaves was not affected, while ⁵²Fe translocation to the youngest leaf was reduced, indicating that Fe is translocated to the youngest leaf via phloem in addition to xylem. We propose a novel model in which root-absorbed Fe is translocated from the basal part of the shoots and/or roots to the youngest leaf via phloem in graminaceous plants.

Keywords: Barley • Fe translocation • Phloem • Positron-emitting tracer • Real-time imaging • Xylem.

Abbreviations: BAS, bio-imaging analyzer system; DC, discrimination center; DMA, 2'-deoxymugineic acid; epiHMA, 3-epihydroxymugineic acid; MAs, mugineic acid family phytosiderophores; NA, nicotianamine; PETIS, positron-emitting tracer imaging system; PMPS, positron multiprobe system.

Introduction

Iron (Fe) is an essential nutrient for plants because it is required for various cellular functions, including heme and chlorophyll biosynthesis, photosynthesis, and as a component of Fe-S cluster-containing enzymes. Although abundant in soils, Fe often forms insoluble ferric hydroxide precipitates that limit its availability for plants. Therefore, plants have evolved two distinct strategies to solubilize and efficiently take up Fe, i.e. Strategy I and Strategy II (Römheld and Marschner 1986). Strategy I plants (dicots and non-graminaceous monocots) rely on acidification of the rhizosphere by H⁺-ATPase activity, reduction of Fe³⁺-chelate to Fe²⁺ by reductase, and Fe²⁺ transport across the root cell membrane (Marschner et al. 1986). Genes encoding the Fe³⁺-chelate reductases, *FRO2* (Robinson et al. 1999) and *FRO1* (Waters et al. 2002), and the Fe²⁺ transporters, *IRT1* (Eide et al. 1996) and *IRT2* (Vert et al. 2001), have been isolated from *Arabidopsis*. Strategy II plants (graminaceous monocots) secrete

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mugineic acid family phytosiderophores (MAs), such as 2'-deoxymugineic acid (DMA) and 3-epihydroxymugineic acid (epiHMA), into the rhizosphere, where MAs chelate Fe^{3+} , and the resultant Fe^{3+} -MAs complex is taken up into roots through an Fe^{3+} -MAs complex transporter in the plasma membrane of the root cells (Takagi 1976). The *YS1* gene, which encodes an Fe^{3+} -MA transporter, has been isolated using the maize mutant *yellow stripe 1* (*ys1*; Curie et al. 2001).

To date, little is known about how Fe is translocated in intact plants after absorption by the roots. It is assumed that Fe is transported to the shoot via the xylem, driven by the transpiration stream and root pressure, and unloaded from xylem to older leaves. Mori (1998) reported that ^{59}Fe was not transported to aluminum foil-covered leaves of intact barley plants supplied with $^{59}\text{Fe}^{3+}$ -DMA, suggesting that Fe translocation from roots to leaves depends on the transpiration stream in barley. Zhang et al. (1995) reported that the majority of ^{59}Fe translocated to the shoot was present in mature leaves 24 h after the supply of $^{59}\text{Fe}^{3+}$ -EDTA to Fe-sufficient and Fe-deficient French bean. The study suggested that Fe transported via the xylem does not directly reach the shoot apex, which is the site of highest Fe demand, but does so only after remobilization from older leaves. In the case of nitrogen, xylem to phloem transfer was suggested by the enrichment of amino compounds relative to sugar in phloem sap collected from terminal stem tissues, inflorescences or fruit stalks compared with that from petioles in white lupin (*Lupinus albus*) (Layzell et al. 1981, Pate 1986). Recently, Tanaka et al. (2008) also showed that xylem-phloem transfer mediated by NIP6;1 is required for boric acid transport to the young leaves. However, little is known about Fe translocation in intact plants after absorption by the roots.

We previously examined the transport of ^{59}Fe in barley (*Hordeum vulgare* L. cv. Ehimehadaka no. 1) by supplying $^{59}\text{Fe}(\text{III})$ -epiHMA to roots, and detection by autoradiography (Mori 1998). Recently, positron-emitting nuclides have been used in plants to study the distribution and translocation of ^{18}F (Kume et al. 1997), ^{11}C (Fujikake et al. 2003, Matsuhashi et al. 2005), $^{13}\text{NO}_3^-$ (Hayashi et al. 1997, Matsunami et al. 1999, Ohtake et al. 2001), ^{13}N (Kiyomiya et al. 2001b), H_2^{15}O (Mori et al. 2000, Kiyomiya et al. 2001a, Nakanishi et al. 2002, Tsukamoto et al. 2004), ^{52}Fe (Ishimaru et al. 2006, Suzuki et al. 2006, Ishimaru et al. 2007), ^{52}Mn (Tsukamoto et al. 2006) and ^{62}Zn (Watanabe et al. 2001, Suzuki et al. 2006, Suzuki et al. 2008) using a positron-emitting tracer imaging system (PETIS) (Fujimaki 2007). With this technique, γ -rays produced by positron-emitting nuclides are detected by scintillation detectors, allowing the real-time investigation of the movement of elements in intact plants. In this study, we used PETIS to monitor the real-time translocation of ^{52}Fe in intact barley plants under Fe-deficient or dark conditions

and showed that phloem is involved in ^{52}Fe transport to the young leaves.

Results

Fe^{3+} -DMA absorption and translocation in Fe-deficient and Fe-sufficient barley

To examine the effect of Fe deficiency on the absorption and translocation of Fe in intact plants, $^{52}\text{Fe}^{3+}$ -DMA was supplied to the roots of both Fe-deficient and Fe-sufficient barley plants, and the translocation of ^{52}Fe was monitored using PETIS and autoradiography. Experiments were independently repeated in triplicate: the results of one experiment are shown in Fig. 1 and the others are shown in Supplementary Figs. S1 and S2. Autoradiography images showed that the shoots of Fe-deficient plants were more strongly labeled than those of Fe-sufficient barley, and the discrimination center (DC), i.e. the basal part of the shoot, was strongly labeled in both Fe-deficient and Fe-sufficient plants (Fig. 1A, B). ^{52}Fe accumulation in the DC was also observed by PETIS (Fig. 1C, D). Fig. 1E shows a montage of PETIS images. In Fe-deficient barley, the signal in the DC (Fig. 1E, left arrowhead) appeared within 30 min, and an image of the leaf sheath appeared within 90 min (Fig. 1E). In contrast, in Fe-sufficient barley, the signal in the DC appeared within 90 min (Fig. 1E, right arrowhead). The radioactivity observed in images of shoots of Fe-deficient barley was higher than that of Fe-sufficient barley shoots in a 6 h experiment, showing that more ^{52}Fe was translocated to shoots in Fe-deficient barley than in Fe-sufficient barley (Fig. 1E, Supplementary Movie 1). Fig. 1F shows the time course change of radioactivity in the DC (indicated in Fig. 1C, D, squares) calculated from the PETIS data. In triplicate experiments, Fe-deficient DCs accumulated ^{52}Fe to >10 times greater levels compared with Fe-sufficient DCs at the end of the experiments. We have established an ^{52}Fe translocation analysis method by PETIS in barley.

Effect of light on Fe absorption and translocation

The secretion of MAs from barley follows a distinct diurnal rhythm (Takagi et al. 1984). A peak in secretion occurs just after daybreak. We speculated that light may influence Fe uptake and translocation. The effect of light on ^{52}Fe translocation was examined by performing the same experiments in light and in darkness. In the dark, ^{52}Fe translocation to the DC and leaves was observed (Fig. 2A, C). In the light, the second and the third leaves of Fe-deficient barley accumulated ^{52}Fe to almost the same level (Fig. 2B). However, in the dark, the accumulation of ^{52}Fe in the second leaf was less than in the third leaf, and the youngest leaf (we defined the 'youngest leaf' as the newest leaf visible in an intact plant)

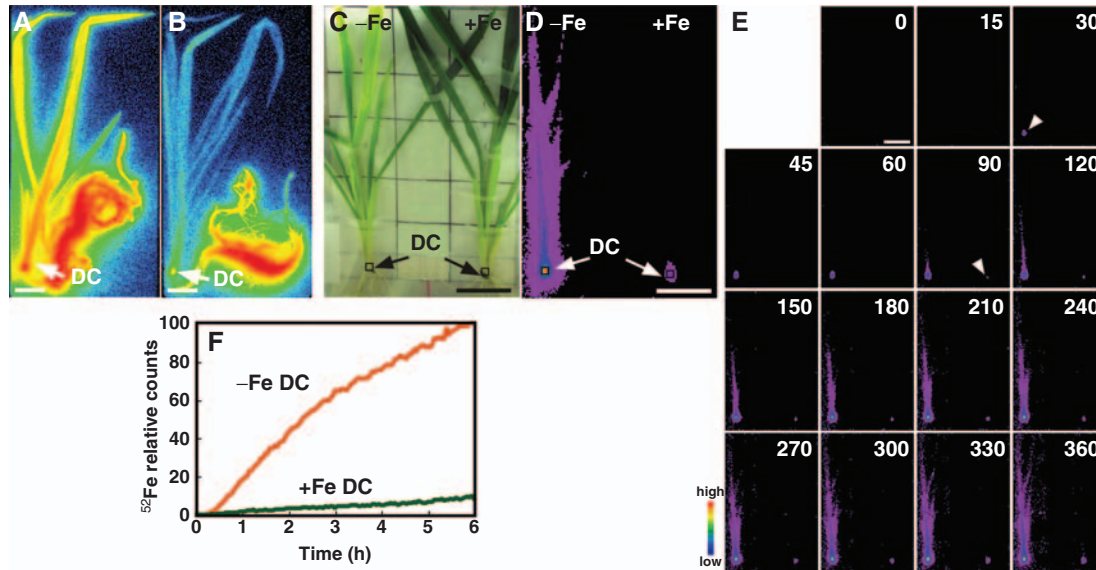


Fig. 1 ^{52}Fe translocation from roots to shoots in Fe-deficient ($-\text{Fe}$) and Fe-sufficient ($+\text{Fe}$) barley (Experiment 1). (A and B) Images of ^{52}Fe translocation in Fe-deficient (A) and Fe-sufficient (B) barley using BAS-1500. (C) Gross image of Fe-deficient (left) and Fe-sufficient (right) barley analyzed using PETIS. The same frame was used for D and E. (D) PETIS images of ^{52}Fe accumulation after 6 h. (E) Time course of radioactivity accumulation analyzed using PETIS. The images are shown at 15 and 30 min intervals (0–60 and 60–360 min, respectively). Data were scored every 3 min. Arrowheads indicate the first detection of DC (left arrowhead $-\text{Fe}$, right arrowhead $+\text{Fe}$). (F) Time course of radioactivity accumulation in the DC of Fe-deficient and Fe-sufficient barley (squares in C and D). The maximum measured radioactivity was defined as 100. Activities are shown as percentages of the maximum value. Scale bar = 4 cm. Experiments 2 and 3 are shown in **Supplementary Figs. S1 and S2**, respectively.

accumulated more ^{52}Fe than the older leaves (**Fig. 2A**, **Supplementary Movie 2**).

The time course of ^{52}Fe radioactivity in the DC, leaf sheath and the youngest leaf (**Fig. 2A, B**, squares) was obtained from PETIS and the positron multiprobe system (PMPS) data (**Fig. 2E, F**). In the dark, radioactivity in the DC (**Fig. 2E**) was higher than that in the light, while radioactivity in the leaf sheath (point I in **Fig. 2A, F**) was lower than that in the light (point III in **Fig. 2B, F**) at the end of the 6 h experiment. Moreover, ^{52}Fe accumulation in the youngest leaf (the fourth leaf) did not change between in the dark (point II in **Fig. 2A, F**) and in the light (point IV in **Fig. 2B, F**). The ^{52}Fe content of the youngest leaf after 6 h of absorption was higher (or equal in **Supplementary Fig. S3**) in darkness, but the ^{52}Fe content of older leaves (third, second and first leaves) was considerably lower (**Fig. 2G**). The experiments were repeated in triplicate, and similar results were obtained, as shown in **Supplementary Figs. S3 and S4**.

Effect of steam treatment on Fe translocation

The ^{52}Fe accumulation pattern in the youngest leaf in the dark was different from that in the older leaves. This suggests the possibility that the mechanism and/or pathway of Fe translocation to the youngest leaf may be different from that to the other leaves. Therefore, we used the heat-girdling

method (Fisher 2000, Jeannette *et al.* 2000), which enabled us to distinguish phloem transport from xylem transport. Phloem transport can be blocked without inhibiting xylem transport by appropriate steam treatment. We examined if the heat-girdling method also blocked the phloem transport without affecting the xylem transport in barley under our experimental conditions. First, we checked whether this method blocked phloem transport by supplying ^{11}C via a leaf; ^{11}C signal was detected only in the leaf above the steam-treated position and it was not detected in the other parts of the steam-treated plants (**Fig. 3**, **Supplementary Fig. S5**), whereas ^{11}C was detected in leaf sheaths, roots and the youngest leaf in addition to the ^{11}C -fed leaf in the untreated plant (**Fig. 3**). Secondly, to confirm that our steam treatment did not affect the xylem transport, the effect of steam treatment on the relative water content after 5 h incubation in the growth chamber was examined (**Table 1**). Relative water contents of both steam-treated youngest and older leaves were not significantly different from those of untreated leaves. However, relative water contents of detached youngest or older leaves under the same condition decreased to 24.8 and 49.8%, respectively. These results showed that our heat-girdling effectively inhibited phloem transport, but xylem transport was not significantly affected.

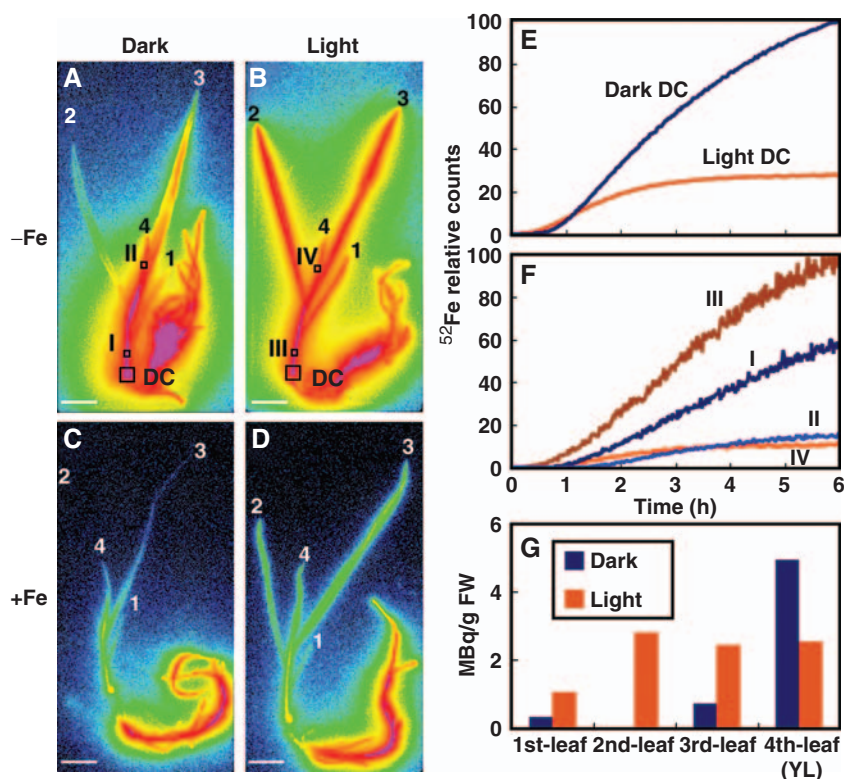


Fig. 2 ^{52}Fe translocation in Fe-deficient (-Fe) and Fe-sufficient (+Fe) barley in the dark and in the light (Experiment 1). (A–D) Images of ^{52}Fe translocation in Fe-deficient (A, B) and Fe-sufficient (C, D) barley in the dark (A, C) and in the light (B, D) using BAS-1500. Leaf numbering: 4, youngest leaf (YL); 3, third leaf; 2, second leaf; 1, first leaf. (E and F) Time course of ^{52}Fe translocation in the DC (E) and at the points indicated in A and B (F) of Fe-deficient barley measured by PMPS and PETIS, respectively. The maximum measured radioactivity in PMPS and PETIS was defined as 100. Activities are shown as percentages of the maximum value. (G) ^{52}Fe content in the leaves of Fe-deficient barley after 6h.

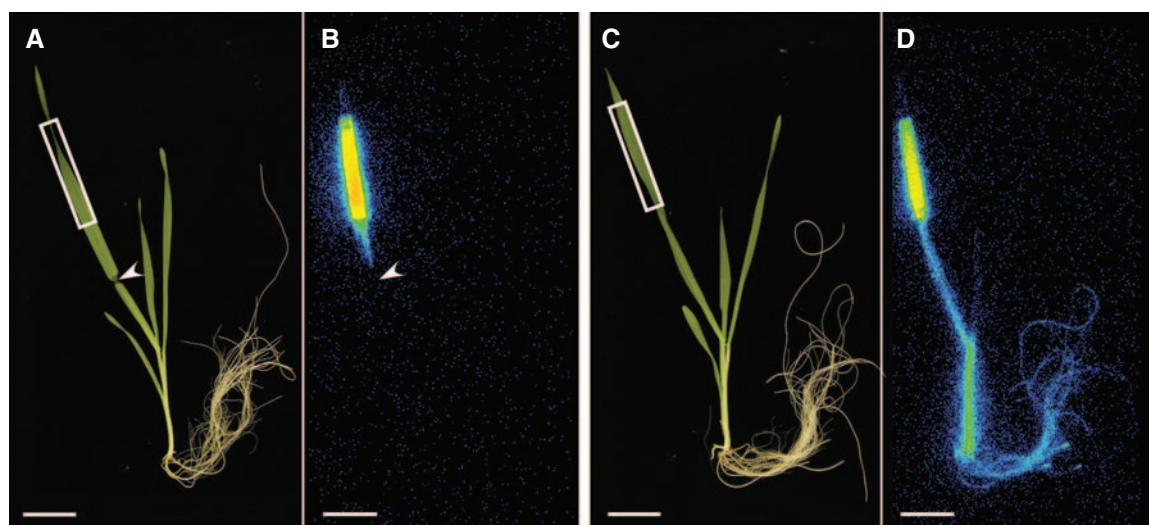


Fig. 3 Effect of heat-girdling on ^{11}C -labelled photoassimilate translocation from the leaf in barley (Experiment 1). (A and C) Gross images of steam-treated (A) and untreated (C) barley. (B and D) Images of the distribution of ^{11}C in steam-treated (B) and untreated (D) barley detected by a BAS-1500. The arrowhead indicates the position of steam treatment. Rectangles indicate the $^{11}\text{CO}_2$ -fed region. Scale bar = 4 cm.

Table 1 Relative water content (%) of the steam-treated, untreated or detached youngest and older leaves after 5 h of the treatment

Leaves	Steam treated	Untreated	Detached
Youngest	84.6 ± 11.3a	92.8 ± 8.6a	24.8 ± 7.0c
Older	88.2 ± 6.5a	94.5 ± 2.9a	49.8 ± 8.6b

Values are the average ± SD (*n* = 5–8).

Values followed by different letters were significantly different (*P* < 0.01).

Parts of the youngest leaf (fourth leaf, Fig. 4A) and the most recently expanded leaf (third leaf, Fig. 4A) of an Fe-deficient barley plant were treated with steam, and $^{52}\text{Fe}^{3+}$ -DMA was supplied to the roots for 4 h. In the treated plant, ^{52}Fe translocation above the steam-treated regions of the youngest leaf was severely suppressed, but it was not affected in the expanded third leaf (Fig. 4B). In the untreated plant, ^{52}Fe was translocated to all parts of the plant, including the youngest leaf (Fig. 4C, D). In the youngest leaf, steam treatment reduced the proportion of accumulated ^{52}Fe in the steam-treated region compared with that in the lower region by 75% in this experiment (Fig. 4E). In contrast, the proportion of accumulated ^{52}Fe was not affected in the expanded leaf (Fig. 4E). We repeated this experiment in triplicate and, in all the experiments, steam treatment reduced the ^{52}Fe translocation to the youngest leaves, whereas it did not reduce the ^{52}Fe translocation to the older leaves (Supplementary Figs. S6, S7). In our triplicate experiments, the size of the youngest leaves varied to some extent. We noticed that the ratio of the phloem transport, i.e. the ratio of the inhibition of ^{52}Fe translocation by steam treatment, was higher in small leaves than that in larger leaves; (75, 91 and 61% inhibition of ^{52}Fe translocation by the steam treatment in Fig. 4, Supplementary Figs. S6 and S7, respectively).

Discussion

We traced the time course of ^{52}Fe translocation in intact barley using PETIS and produced dynamic animations of the PETIS images (Supplementary Movies 1 and 2).

Role of the DC in mineral and metabolite transport in graminaceous plants

In all experiments, ^{52}Fe primarily accumulated in the DC, a region that contains the shoot meristem, node and internode (Mori 1998, Itoh *et al.* 2005). In the node, the structure of the vascular bundles is complicated and they are enlarged (Kawahara *et al.* 1974, Kawahara *et al.* 1975). In addition, transfer cells, which play an important role in distributing minerals, are present in the nodes (Zee 1972). Mori (1998) reported that the supply of $^{59}\text{Fe}^{3+}$ -epiHMA or $[^{32}\text{P}]\text{PO}_4^{3-}$ to roots or leaves of barley resulted in strong labeling of this

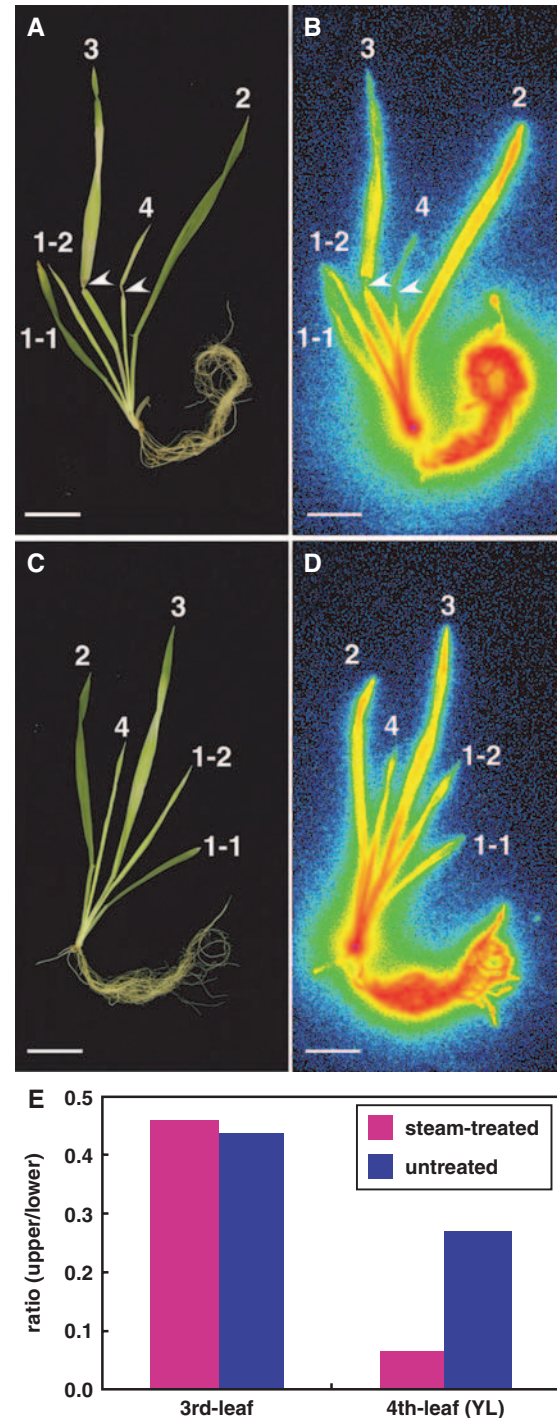


Fig. 4 Effect of heat-girdling on ^{52}Fe translocation from the roots to the shoots in Fe-deficient barley (Experiment 1). (A and C) Gross images of steam-treated (A) and untreated (C) barley. (B and D) Images of the distribution of ^{52}Fe in steam-treated (B) and untreated (D) barley detected by a BAS-1500. Arrowheads indicate the position of steam treatment. Leaf numbering: 4, youngest leaf (YL); 3, third leaf; 2, second leaf; 1-2, youngest leaf of the first tiller; 1-1, first leaf. Scale bar = 4 cm. (E) The ratio of the ^{52}Fe radioactivity of the upper region to that of the lower region after heat-girdling the third and fourth youngest leaves (YL).

region and termed it the DC. Similarly, ^{52}Fe first accumulated in the DC and was then distributed to all plant tissues (**Fig. 1E, Supplementary Movie 1**). We have also reported that radioactivity accumulated in the DCs of barley and rice, and was then distributed to shoots, in studies using [^{11}C]methionine (Nakanishi et al. 1999, Bughio et al. 2001), $^{13}\text{NH}_4^+$ (Kiyomiya et al. 2001b) and H_2^{15}O (Mori et al. 2000, Kiyomiya et al. 2001a, Nakanishi et al. 2002, Tsukamoto et al. 2004). Therefore, it is likely that not only Fe but also other minerals and metabolites accumulate in the DC after absorption from the roots in graminaceous plants. Our present study further supports the important role of the DC in distributing minerals and metabolites in graminaceous plants.

Effect of Fe status on Fe absorption and translocation from the roots

More ^{52}Fe was translocated to shoots in Fe-deficient barley than in Fe-sufficient barley (**Fig. 1D, E, Supplementary Figs. S1, S2, Supplementary Movie 1**), suggesting that the demand for Fe affects its translocation. Previous research has shown that Fe starvation increases the $^{59}\text{Fe}^{3+}$ -MAs influx in roots of barley (Römheld and Marschner 1986, Mihashi and Mori 1989, Mori 1998). In the present study, an image of the DC in Fe-deficient barley appeared within 30 min, whereas an image of the DC in Fe-sufficient barley appeared within 90 min (**Fig. 1E**). This result shows that Fe deficiency enhanced the translocation of ^{52}Fe from the roots to the DC. In roots, the expression of many transporter genes is induced by Fe deficiency. For example, Fe deficiency induces the expression of the Fe^{3+} -MAs transporter gene (*YS1*) in roots of maize (Curie et al. 2001). Similarly, Fe deficiency induced the expression of the Fe^{3+} -MAs transporter gene in barley (*HvYS1*; Murata et al. 2006) and rice (*OsYSL15*; Inoue et al. 2009). Not only the transporters involved in Fe uptake, but also the transporters involved in Fe translocation may be induced under Fe deficiency.

^{52}Fe was preferentially translocated to younger leaves (**Fig. 1A, B, Supplementary Figs. S1, S2**). Brown et al. (1965) and Mori (1998) reported that Fe accumulation depended on leaf age. Because Fe is required for chlorophyll biosynthesis and photosynthesis, younger leaves function as strong sinks. Indeed, the Fe deficiency symptom, chlorosis, first appears in the youngest leaf. Therefore, it is conceivable that Fe is translocated preferentially to the sink. The DC may play a critical role in this regulation of Fe distribution to the sink organs. In shoots, some Fe and Fe-chelate transporter genes, such as *YS1* (Curie et al. 2001), *AtNRAMP3* (Thomine et al. 2003), *AtNRAMP4* (Thomine et al. 2000) and *OsYSL2* (Koike et al. 2004), are up-regulated under Fe deficiency. These transporter genes may be expressed at the DC and regulate the distribution of Fe in plants.

Role of phloem transport in Fe translocation

To investigate the details of Fe translocation to the youngest leaf, phloem transport in the leaf was blocked by steam treatment (Fisher 2000, Jeannette et al. 2000) to distinguish xylem transport from phloem transport. We confirmed that the translocation of ^{11}C -labeled photoassimilates from the treated leaf to other plant parts was inhibited, although the water flow was still active following steam treatment (**Fig. 3, Supplementary Fig. S5, Table 1**). After steam treatment, ^{52}Fe translocation from the roots to the upper regions of the youngest leaf in Fe-deficient barley was strongly reduced, but that to the fully expanded leaf was not affected (**Fig. 4**). In addition, translocation to the second leaf was also unaffected by steam treatment (data not shown). These results showed that Fe is mainly translocated to the youngest leaf via phloem and partly via xylem, whereas it is translocated to older leaves mainly via xylem in Fe-deficient barley. As the leaf grows, the pathway of Fe translocation to the leaf appears to change from phloem to xylem. ^{52}Fe translocation to the youngest leaf in the dark was also observed in Fe-sufficient barley (**Fig. 2C, D**). Therefore, Fe may be translocated to the youngest leaf via phloem, regardless of Fe status. In the case of nitrogen, xylem to phloem transfer occurred in white lupin (Layzell et al. 1981, Pate 1986). In *Arabidopsis*, xylem-phloem transfer may be involved in boric acid transport to the young leaves (Tanaka et al. 2008). Similarly, xylem to phloem transfer of Fe may occur in barley plants.

The location of Fe transfer from xylem to phloem for translocation to the youngest leaf remains unknown. As described earlier, the DC contains a node and internode (Itoh et al. 2005), in which the vascular bundles are complicated and enlarged (Kawahara et al. 1974, Kawahara et al. 1975). *OsIRT1*, which encodes the Fe^{2+} transporter in rice, was expressed in the phloem of the DC (Ishimaru et al. 2006). Therefore, it is conceivable that Fe may be transferred from xylem to phloem in the DC.

OsNAS1, *OsNAS2* and *OsNAS3*, which encode nicotianamine (NA) synthase in rice, were constitutively expressed in the companion cells of the roots of both Fe-sufficient and Fe-deficient plants (Inoue et al. 2003). This implies that NA is synthesized in the companion cells of the roots and functions in phloem loading or unloading of Fe. Furthermore, genes that participate in DMA synthesis, such as *OsNAAT1* (NA aminotransferase gene in rice) and *OsDMAS1* (DMA synthase gene in rice), were expressed in these cells, suggesting that DMA in addition to NA is synthesized in these cells (Bashir et al. 2006, Inoue et al. 2008). Therefore, it is possible that Fe may be transferred from xylem to phloem in the roots. Zhang et al. (1995) reported that Fe supplied via the xylem in French bean did not directly reach the apex, which has the highest Fe demand, but did so only after remobilization from older leaves. However, in our experiments ^{52}Fe arrived at the

youngest leaf in the light after 1 h (Fig. 2F), which is too short a time for Fe to have been re-translocated from older leaves. Fe transfer from xylem to phloem in older leaves may not contribute to short-term Fe translocation from the roots to the youngest leaf. These results suggest that Fe may be transferred from xylem to phloem in the DC and/or the roots.

Based on our results and previous knowledge, we propose a novel model in which root-absorbed Fe is translocated from the DC and/or the roots to the youngest leaf via phloem in graminaceous plants (Fig. 5). In graminaceous plants, YS1 and its homologs (e.g. HvYS1 and OsYSL15) are responsible for Fe^{3+} -MAs uptake from the soil (Curie et al. 2001, Murata et al. 2006, Inoue et al. 2009). Fe is then transported from the epidermis of the root to the xylem symplastically, although the form of Fe is still unknown. In xylem, Fe may be transported as an Fe^{3+} -citrate complex ($[\text{FeCitrateOH}]^{-1}$ and $[\text{FeCitrate}_2]^{-3}$; Tiffin 1966, López-Millán et al. 2000) and Fe^{3+} -DMA (Mori et al. 1991). Fe may be transferred from xylem to phloem in the DC and/or the roots. In phloem,

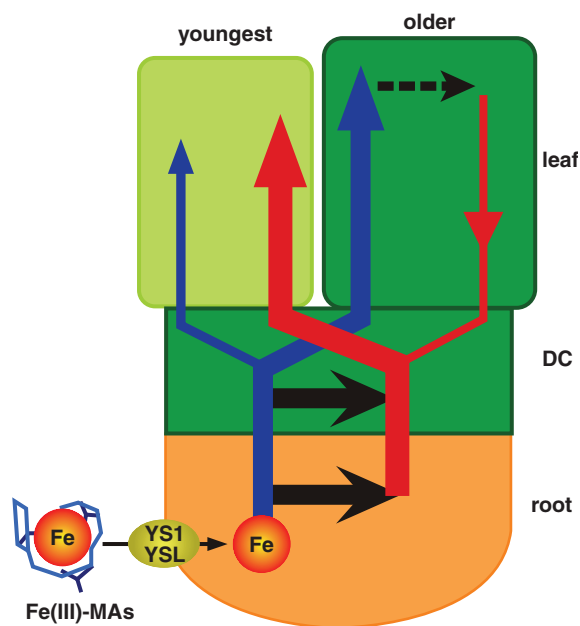


Fig. 5 A schematic model of root-absorbed Fe transport in graminaceous plants based on our results and previous knowledge. In graminaceous plants, YS1 and its homologs (YSL) are responsible for Fe^{3+} -MAs uptake from the soil. Fe is then transported to the xylem, and then the phloem. Although the exact location of Fe transfer from xylem to phloem remains unknown, Fe transfer may occur in the DC and/or the roots (thick black arrows). Fe is mainly translocated to the youngest leaf via phloem (thick red arrow) and partly via xylem (thin blue arrow), whereas Fe is mostly translocated to older leaves via xylem (thick blue arrow). Fe translocation to younger leaves occurs in preference to older leaves. Our study does not exclude the possibility that re-translocation of Fe from the older leaves has some contribution to the long-term Fe translocation to the youngest leaf (dotted black line and thin red arrow).

Fe may be transported mainly as an Fe-NA complex (Koike et al. 2004). The Fe ligand exchange from citrate or DMA to NA may occur during Fe transfer from xylem to phloem in the vascular tissues. Fe is mainly translocated to the youngest leaf via phloem and partly via xylem, whereas Fe is translocated to older leaves mainly via xylem. Fe translocation to younger leaves occurs preferentially compared with translocation to older leaves.

Materials and Methods

Plant materials

Barley (*Hordeum vulgare* L. cv. Ehimehadaka no. 1) seeds were grown hydroponically in modified Kasugai's medium: 0.7 mM K_2SO_4 , 0.1 mM KCl, 0.1 mM KH_2PO_4 , 2.0 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM MgSO_4 , 10 μM H_3BO_3 , 0.5 μM MnSO_4 , 0.2 μM CuSO_4 , 0.5 μM ZnSO_4 , 0.01 μM $(\text{NH}_4)_2\text{MoO}_4$ and 0.1 mM Fe-EDTA, as described previously (Mori and Nishizawa 1987). Fe-deficient plants were generated by culturing in medium lacking Fe for 1 week prior to beginning ^{52}Fe absorption experiments. All absorption experiments were conducted at about 3 weeks after germination, and the sizes and root weights of plants within each experimental set-up were approximately the same.

Production of ^{52}Fe

^{52}Fe (half-life 8.27 h) was produced by the $^{nat}\text{Cr}(\alpha, xn)^{52}\text{Fe}$ reaction, in which a 1.5 mm thick chromium foil (natural isotopic composition, 99.9% purity; Goodfellow Metals Ltd., Cambridge, UK) was bombarded with a 100 MeV α beam [generated by the Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) AVF cyclotron; Gunma, Japan; Watanabe et al. 2001]. About 1 MBq of ^{52}Fe was produced using a beam current of 3 μA for 2 h. ^{52}Fe was radiochemically separated from the target using the method described by Watanabe et al. (2001). After the pH of the $^{52}\text{Fe}^{3+}$ solution without cold Fe was adjusted to about pH 3 using 1 M KOH, the $^{52}\text{Fe}^{3+}$ was chelated with 1.12 μmol of DMA in the dark for 1 h.

Production of ^{11}C

The positron-emitting radioisotope ^{11}C (half-life 20.39 min) was produced using a $^{14}\text{N}(p, \alpha)^{11}\text{C}$ reaction, in which nitrogen gas was bombarded with a 10 MeV proton beam from the TIARA AVF cyclotron. About 50 MBq of ^{11}C were produced using a beam current of 1 μA for 2 min. $^{11}\text{CO}_2$ was produced from the ^{11}C and oxygen present in the target chamber.

PETIS and PMPS

Plant samples were placed between two opposed two-dimensional block detectors consisting of $\text{Bi}_4\text{Ge}_3\text{O}_{12}$

scintillator arrays. The ^{52}Fe and $^{11}\text{CO}_2$ produced as above were used as positron-emitting tracers. Two annihilation γ -rays emitted from the decaying positrons were detected simultaneously (Kume et al. 1997, Uchida et al. 2004). The original position of the annihilation was localized at the intersection of the object plane determined by a line connecting the two detection points on the detectors. The field of view was 143×215.6 mm, and the spatial resolution was 2.4 mm. After automatic correction for decay and relative detection efficiencies within the field of view, the resulting image was displayed on a monitor. After the imaging procedure was completed, the regions of interest (DC in Fig. 1, I–IV in Fig. 2 and Supplementary Figs. S3, S4) within the data obtained were set and the radioactivity behavior over time within each region was extracted from the data. Two pairs of PMPS detectors were used for time course tracer analysis of ^{52}Fe (DC in Fig. 2, Supplementary Figs. S1–S4). Although the principles of PMPS are almost the same as those of PETIS, PMPS detectors directly measure the radioactivity time course but bypass the imaging procedure.

Heat-girdling and relative water content of the leaves

Treatment with steam and calculation of relative water contents of leaves were carried out following Jeannette et al. (2000). Steam ($\sim 100^\circ\text{C}$) was produced using a soldering iron and wet paper towels, and was directed for 5 s towards the leaf at a distance of 1 cm. The tops of the steam-treated leaves were excised, weighed (fresh weight) and placed in distilled water in a 15 ml tube in the dark (4°C) overnight. Rehydrated weight was measured and the sample was then dried. The relative water content was calculated from $[(\text{fresh weight} - \text{dry weight})/(\text{rehydrated fresh weight} - \text{dry weight})] \times 100$.

Absorption and translocation of ^{52}Fe in plants

To study the absorption of ^{52}Fe by roots, the roots of a single plant were placed in a polyethylene bag containing 15 ml of $5\times$ modified Kasugai's solution lacking Fe. To maintain the geometry, the plant and the bag were placed between two acrylic boards centered between the PETIS detectors in a chamber at 25°C , 65% humidity and a photon flux density of $320 \mu\text{mol m}^{-2} \text{ s}^{-1}$. $^{52}\text{Fe}^{3+}$ -DMA (0.28–1.14 MBq) in 1 ml of water was added to the culture with gentle aeration for immediate mixing. The PETIS detectors were focused on the shoot. After a 4–6 h trace analysis, the plant was removed from the polyethylene bag and the roots were gently washed for 1 min in 50 ml of 0.01 mM EDTA solution. Next, the plant was exposed to a bio-imaging analyzer system (BAS) imaging plate (Imaging Plate BAS-MP2040S, Fujifilm, Tokyo, Japan) to obtain an autoradiographic image. After 30 min, the plate was scanned using a BAS-1500 (Fujifilm, Tokyo, Japan). In

the dark treatment and the steam treatment experiments, the plants were cut into parts after the PETIS and autoradiography analyses. The radioactivity of each part was then determined by γ -ray spectrometry using an HPGe detector (crystal diameter 58.0 mm, length 67.3 mm) coupled to an MCA-7700 multichannel analyzer (Seiko EG & G Co., Ltd., Matsudo, Japan). The peak analysis was performed using Gamma Studio 2nd Edition SP2 (Seiko EG & G Co., Ltd.). The radioactivity of the ^{52}Fe was determined from the peak area at 168 keV. The effects of Fe deficiency (7 d pre-treatment), dark (13 h pre-treatment) and steam treatment of leaves on ^{52}Fe absorption and translocation were examined. In the steam treatment experiment, Fe-deficient barley was used and the experiments were performed under the light condition.

Each experiment was repeated at least three times, and the results of one of the experiments are shown in Figs. 1–4 and those of the other two are shown in the Supplementary figures. Since the absolute radioactivity of ^{52}Fe varied between the experiments, detected signal values cannot be compared between different experiments. The maximum measured radioactivity in PETIS and PMPS was defined as 100 in each experiment. Activities are shown as percentages of the maximum value in each experiment.

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