

ORIGINAL ARTICLE

Contribution of iron associated with high-molecular-weight substances to the maintenance of the SPAD value of young leaves of barley under iron-deficient conditions

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Abstract

In higher plants, it is well known that the retranslocation of iron from old leaves to young leaves is difficult; as a result, iron deficiency leads to interveinal chlorosis, particularly in the young leaves. However, in the case of barley, young chlorotic leaves can grow under conditions of long-term iron deficiency. Previously, we have reported that the distribution and retranslocation characteristics of iron in barley may be better adapted to iron deficiency than those in rice. Furthermore, barley maintained a relatively high chlorophyll index (SPAD value) even when its iron content was not higher than that of rice. In this study, we aimed to predict the chemical form of iron that contributes to the physiologically available iron in barley leaves. To examine the correlation between plant growth and the SPAD value with the amount of fractionated iron, we cultured plant materials in a culture solution containing various iron concentrations. We compared these correlations among barley, rice and sorghum and among three barley cultivars. To compensate for the amount of mugineic acid phytosiderophores (MAs) in the culture solution, we cultured different plant species in the same container. The results revealed that the amount of soluble iron associated with the high-molecular-weight substances (MW >10,000) correlated with the SPAD value of the young barley leaves and the R^2 value (determination coefficient) of barley was higher than the values of rice and sorghum.

Key words: barley, rice, soluble iron, sorghum, SPAD.

INTRODUCTION

Iron is an essential trace element found in plants that is required for chlorophyll synthesis. It is well known that the retranslocation of iron from old leaves to young leaves is difficult; as a result, iron deficiency leads to interveinal chlorosis, particularly in young leaves. Although the mechanism for iron absorption via roots has been effectively clarified at the molecular biological level (Schmidt 2003), experimental evidence regarding iron utilization in shoots remains limited and unsystematic.

It has been observed that barley has acquired a strong tolerance to iron deficiency. Strategy II plants, including

graminaceous plants, typically excrete mugineic acid phytosiderophores (MAs) and absorb Fe^{3+} -MA conjugates via a specific transporter present on their plasma membrane (Curie *et al.* 2001; Marschner 1995a; Mori 2001; Römheld and Marschner 1986; Takagi *et al.* 1976). It is well known that the tolerance of strategy II plants to iron deficiency correlates with the amount of MAs secreted from their roots (Marschner 1995b; Takagi *et al.* 1976).

Without considering the differences in the ability of barley and rice to secrete MAs, we demonstrated the differential availability of iron to the shoots of these plant species by coculturing, that is, barley and rice were grown in the same container (Maruyama *et al.* 2005). In rice, iron deficiency resulted in severe chlorosis of the young leaves, whereas the old leaves remained green. In barley, iron deficiency decreased the chlorophyll index (SPAD values) of the old leaves, whereas the young chlorotic leaves continued to grow. As the iron content in the rice leaves was not lower than that in the barley leaves (Maruyama *et al.* 2005), we hypothesized

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that the mechanisms for the utilization of limited iron in the leaf cells were well developed in barley.

In this study, we aimed to predict the chemical form of iron that constitutes physiologically available iron in barley. The amount of soluble iron associated with high-molecular-weight substances (MW >10,000) correlated with the SPAD value, and the R^2 value (determination coefficient) for the young barley leaves was higher than the values for rice and sorghum leaves.

MATERIALS AND METHODS

Plant materials and chlorophyll index of leaves

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were soaked in tap water for 1 day and then germinated on wet paper placed in the dark at $24 \pm 3^\circ\text{C}$ for 3 days. Barley seeds (*Hordeum vulgare* L. cv. Ehimehadaka No. 1, cv. Shirohadaka and cv. Kairyo-ohgara) and sorghum seeds (*Sorghum bicolor* L. Moench cv. Keller [sweet sorghum]) were also germinated on wet paper placed in the dark at $24 \pm 3^\circ\text{C}$ for 3 days. The seedlings were transferred to culture solutions containing all the ingredients at half the concentration of those in a standard culture solution. The composition of the standard culture solution used in the present study has been described by Maruyama *et al.* (2005). When the first true leaf appeared, the plants were transferred to the standard culture solution. Twelve plants were grown in a 13-L container. The culture solution was adjusted to pH 5.5 with $0.5 \text{ mol L}^{-1} \text{ H}_2\text{SO}_4$ or $1 \text{ mol L}^{-1} \text{ NaOH}$, and a fresh solution was prepared every 3 days. The plants were grown with $100 \mu\text{mol L}^{-1}$ Fe-ethylenediaminetetraacetic acid (EDTA) for the first 6 days and then with various concentrations of Fe-EDTA for a subsequent 18 days in a temperature-controlled greenhouse at $24 \pm 3^\circ\text{C}$ under natural light conditions. The leaves were numbered as follows: the cotyledon, 1; the first true leaf, 2; and the second true leaf, 3. The young leaves represented the youngest and second youngest leaves, and the old leaves represented the oldest, second oldest and third oldest true leaves. The chlorophyll index of the leaves was measured using a SPAD chlorophyll meter (SPAD-502; Minolta, Tokyo, Japan). We measured the SPAD value at the middle of the long axis of each leaf obtained from three individual plants and calculated the mean values. After 24 days the plants were harvested and the leaves were separated.

Measurement of the fractionated iron

For estimation of the total iron content, the leaves of the three plants were dried for 3–4 days at 60°C , and the dried leaves were digested for 12 h in a solution of concentrated HNO_3 at 120°C and then dissolved in 2 mL of 1N HCl (1 mol L^{-1}). The fractionation procedure for

iron has been described by Maruyama *et al.* (2005). All procedures were carried out on ice in a dimly lit room to prevent a change in the chemical form of iron. The leaves of the three plants were homogenized in liquid N_2 using a mortar and pestle, and the resultant powder was suspended in 10 mL water and mixed vigorously; this solution was then centrifuged for 30 min at 4°C and 20,000 g. The supernatant liquids were passed through a filter (DISMIC-25 cs, $0.45 \mu\text{m}$; Toyo Roshi, Tokyo, Japan) to remove cell debris. The filtrate was designated “water-soluble iron”. Part of the water-soluble iron was passed through an ultrafiltration membrane (Amicon Ultra-4 10000 MWCO; Millipore, Billerica, MA, USA). This filtrate was designated “under-10,000-MW iron”. The iron content of each solution was immediately measured using an atomic absorption spectrophotometer (AA-680 or AA-670; Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

SPAD value of the youngest leaves and the total iron content in each sorghum leaf

To predict the chemical form of iron that contributes to the high iron availability in barley shoots, we compared barley with sorghum, another graminaceous crop plant, in addition to rice. First, we cocultured barley and sorghum in the same container to compensate for the amount of MAs secreted by the plants as previously described (Maruyama *et al.* 2005) and we compared the SPAD values of the youngest leaves and the total iron content in each leaf of barley and sorghum (Fig. 1).

The SPAD values of the youngest barley leaves fluctuated during the growth period under iron-deficient conditions (Fig. 1A). This finding was consistent with our previous data (Maruyama *et al.* 2005). The SPAD values of the youngest sorghum leaves displayed a pattern similar to that of the barley leaves under iron-deficient conditions (Fig. 1B). The fluctuations in the SPAD values under control conditions were fewer than those observed under iron-deficient conditions in both barley and sorghum (Fig. 1A,B).

The measurement of the total iron content in the barley and sorghum leaves revealed that the pattern of iron distribution for each leaf was different in barley and sorghum (Fig. 1C,D). Similar to rice, sorghum accumulated more iron in the old leaves compared with barley. In contrast, the total iron content in the youngest barley and sorghum leaves was higher than that in the middle leaves of both plants. In barley and sorghum, no alteration was observed in the total iron content and the patterns of iron distribution as a result of iron deficiency.

These results demonstrated that, compared to rice, barley and sorghum possessed a greater ability to maintain

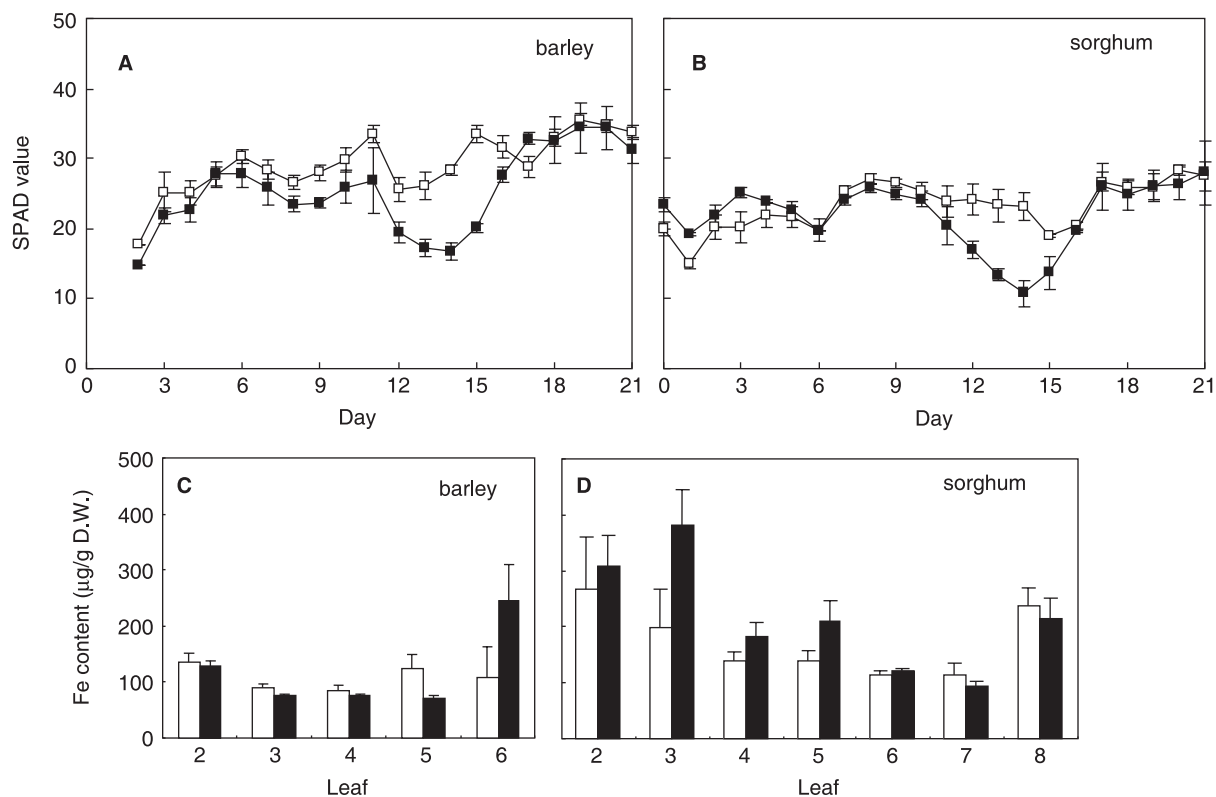


Figure 1 Chlorophyll index (SPAD value) and total iron content in cocultured barley and sorghum. Changes in the SPAD values of the youngest leaf in (A) barley (Ehimehadaka No. 1) and (B) sorghum, □, control; ■, without iron. Each marker denotes mean \pm standard error ($n = 3$). Total iron content in each leaf, □, control; ■, without iron in (C) barley (Ehimehadaka No. 1) and (D) sorghum. Each bar denotes mean \pm standard error ($n = 3$). D.W., dry weight.

a high SPAD value of the young leaves and that the pattern of iron distribution for each sorghum leaf was intermediate between that of barley and rice.

Comparison of the demand and availability of iron among graminaceous species and barley cultivars

The patterns of iron distribution for each leaf and the iron demand needed to maintain the SPAD value of the young leaves differed among the graminaceous species, barley, rice and sorghum. To predict the chemical form of iron that contributes to the high iron availability in a barley shoot, we attempted to correlate the amount of fractionated iron and the growth or SPAD value under moderate iron-deficient conditions. To focus on the iron metabolism in the shoot, any differences in the ability to acquire iron from the culture solution should be compensated as much as possible. We demonstrated that the iron content of rice increased by coculturing with barley under iron-deficient conditions (Maruyama *et al.* 2005). Therefore, we cocultured a combination of barley and rice or a combination of barley and sorghum in the same container with culture solutions containing

various concentrations of iron, that is, 0, 0.3, 0.6, 1, 3, 10, 30 and 100 $\mu\text{mol L}^{-1}$ Fe-EDTA.

Figure 2 illustrates the results for barley (Ehimehadaka No. 1) and rice and barley (Ehimehadaka No. 1) and sorghum. When the iron concentration ranged from 0 to 10 $\mu\text{mol L}^{-1}$, the reduction rates for plant height and fresh weight of barley were lower than those observed for rice and sorghum. These results revealed that in contrast to rice and sorghum, barley exerted an ability to maintain growth not only under conditions of severe iron deficiency, but also under moderate conditions of iron deficiency.

Next, we measured the content of fractionated iron in the young and old leaves using the same plant materials shown in Fig. 2. The iron present in the leaves was fractionated into total iron, water-soluble iron and low-molecular-weight iron (lower than 10,000 MW) as described previously (Maruyama *et al.* 2005). The correlations between the content of each type of iron fraction in the young and old leaves and the relative fresh weight and plant height of the aerial parts were compared among the three species. The patterns of correlation between the iron content and the relative

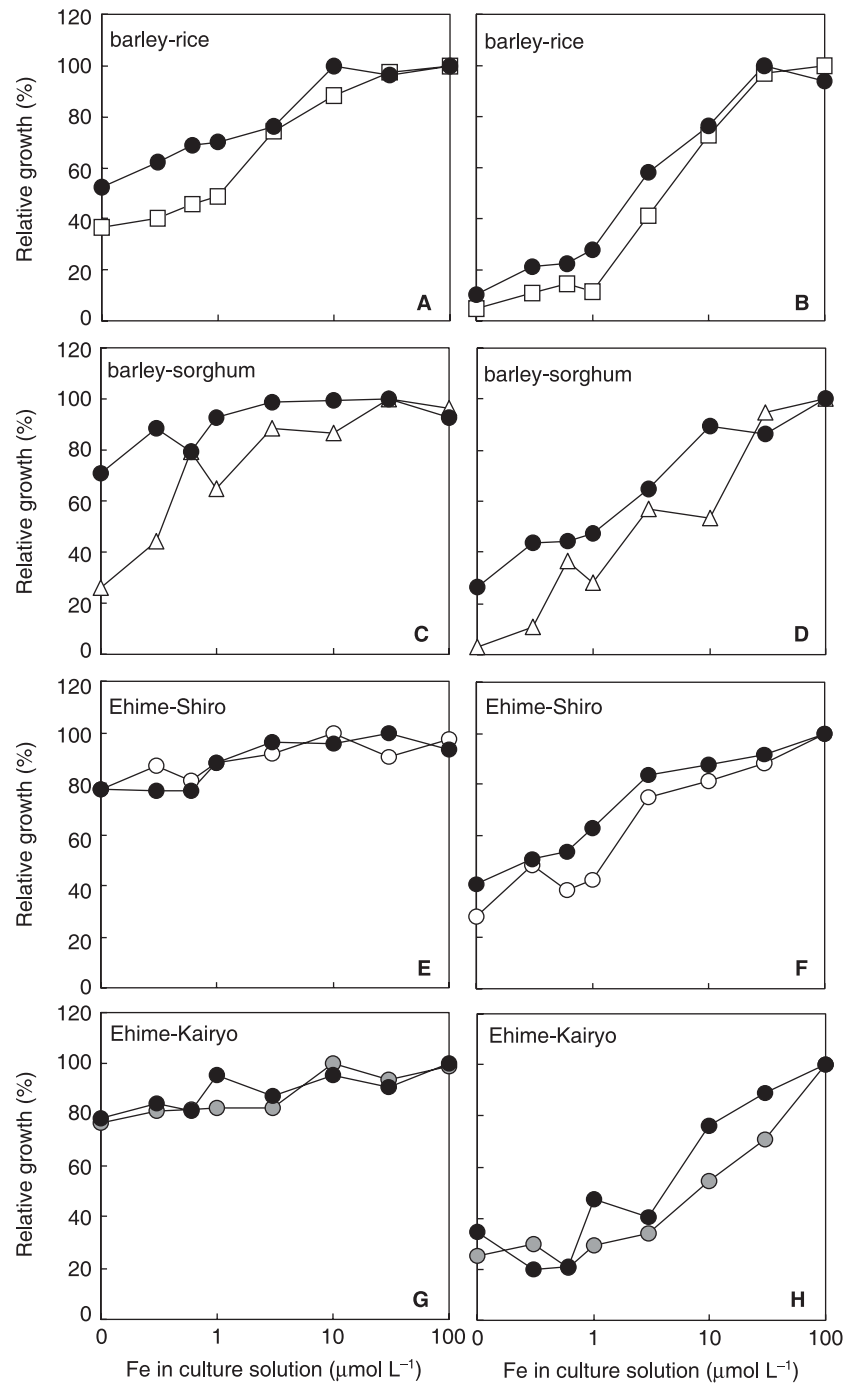


Figure 2 Relative growth of the cocultured plants under various iron-deficient conditions. Plants were cultured for 24 days in culture solutions containing 0, 0.3, 0.6, 1, 3, 10, 30 or 100 $\mu\text{mol L}^{-1}$ iron. The iron concentration of the culture solution was represented as a logarithmic value. The plant height and fresh weight of the aerial parts were represented as a relative growth percentage that was normalized to the highest value observed for each species. Each marker denotes the mean ($n=3$). A, C, E and G represent the plant height and graphs B, D, F and H represent the fresh weight of the aerial part. (A,B) Cocultured barley and rice. ●, barley (Ehimehadaka No. 1); □, rice. (C,D) Cocultured barley and sorghum. ●, barley (Ehimehadaka No. 1); △, sorghum. (E,F) Cocultured Ehimehadaka No. 1 and Shirohadaka. ●, Ehimehadaka No. 1; ○, Shirohadaka. (G,H) Cocultured Ehimehadaka No. 1 and Kairyohgara. ●, Ehimehadaka No. 1; ●, Kairyohgara.

fresh weight were similar to those between the iron content and the relative plant height and showed more clear correlations; the correlations between the iron content and relative fresh weight are shown in Fig. 3. The regression lines represent cases with comparatively high R^2 values. The content of the under-10,000-MW iron fraction was very low, and no correlation was observed between the iron content of this fraction and

the relative fresh weight of the aerial part (data not shown).

With regard to all three species, a correlation was observed between the water-soluble iron content in the young leaves and the relative fresh weight of the aerial part (Fig. 3A, $R^2=0.74$ and $R^2=0.53$, respectively; Fig. 3E, $R^2=0.85$ and $R^2=0.63$, respectively). In general, the water-soluble iron content in the young leaves

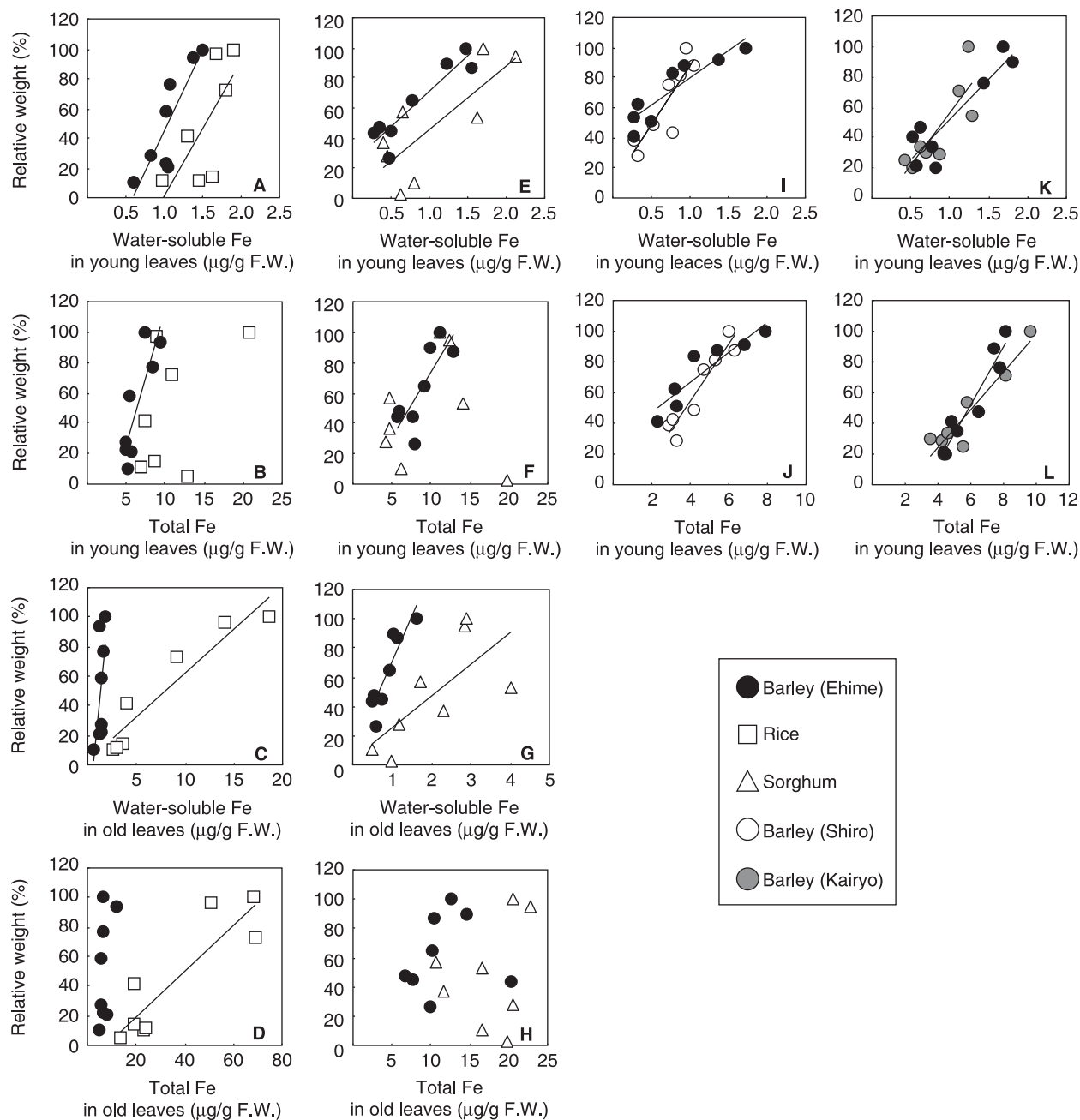


Figure 3 Correlation between the iron content and relative fresh weight (F.W.) of the aerial part. (A–D) Comparison of barley (Ehimehadaka No. 1) with rice. (E–H) Comparison of barley (Ehimehadaka No. 1) with sorghum. (I, J) Comparison of Ehimehadaka No. 1 with Shirohadaka. (K, L) Comparison of Ehimehadaka No. 1 with Kairyō-ohgara. (A, E, I, K) Correlation between the water-soluble iron content in the young leaves and the relative fresh weight of the aerial parts. (B, F, J, L) Correlation between the total iron content in the young leaves and the relative fresh weight of the aerial parts. (C, G) Correlation between the water-soluble iron content in the old leaves and the relative fresh weight of the aerial parts. (D, H) Correlation between the total iron content in the old leaves and the relative fresh weight of the aerial part.

contributes to plant growth. Barley exhibited higher R^2 values than rice and sorghum. Moreover, even though the water-soluble iron content of barley was lower than that of rice and sorghum, barley maintained the fresh weight of the aerial part. Thus, the young barley leaves

utilized iron more efficiently and contributed to its growth. Only barley exhibited a correlation between total iron content in the young leaves and relative fresh weight of the aerial part (Fig. 3B, $R^2 = 0.72$; Fig. 3F, $R^2 = 0.66$). These results also suggest that the availability

of iron in the young barley leaves is higher than that in rice and sorghum.

A correlation was also observed between the water-soluble iron content in the old leaves and the relative fresh weight of the aerial parts (Fig. 3C, $R^2 = 0.43$ and $R^2 = 0.90$, respectively; Fig. 3G, $R^2 = 0.81$ and $R^2 = 0.51$, respectively). In particular, with regard to rice, a large amount of iron in the old leaves demonstrated a high correlation with the relative fresh weight of the aerial part (Fig. 3C, $R^2 = 0.90$; Fig. 3D, $R^2 = 0.79$). Considering that rice possesses a lower ability to retranslocate iron compared with barley (Maruyama *et al.* 2005), iron translocated from the roots via the xylem may be unloaded to each leaf and accumulated in a manner similar to that in the old rice leaves. Therefore, the amount of iron accumulated in the old leaves may reflect the amount supplied to the young leaves, thus, exhibiting a high correlation with plant growth.

To evaluate whether the abovementioned results are characteristic of barley, we carried out identical experiments using three barley cultivars – Ehimehadaka No. 1, Shirohadaka and Kairyohagara. Ehimehadaka No. 1, which has been used in previous studies, exhibits a strong tolerance to iron deficiency; the other two cultivars are not as tolerant as Ehimehadaka No. 1. The patterns of iron distribution to each leaf and iron content were almost identical in the three cultivars under both iron-deficient and iron-sufficient conditions (data not shown).

We cultured a combination of Ehimehadaka No. 1 and Kairyohagara or a combination of Ehimehadaka No. 1 and Shirohadaka in the same container with culture solutions containing various iron concentrations. The reduction rates for plant height were identical in the three cultivars (Fig. 2E,G). In contrast, the reduction rate for the fresh weight of Ehimehadaka No. 1 was lower than the rates for Kairyohagara and Shirohadaka. These results suggest that under iron-deficient conditions, the three cultivars possess identical abilities with regard to elongation of young leaves; however, their photosynthetic productivity differs.

The patterns of correlation between the content of each type of iron fraction in the young and old leaves and the relative fresh weight of the aerial part were similar among the three barley cultivars (Fig. 3I–L). The iron content in the old leaves did not correlate with the plant growth in the three cultivars (data not shown). Both, the correlation between the water-soluble iron content and the relative fresh weight of the aerial parts and that between the total iron content in the young leaves and the relative fresh weight of the aerial parts were high in the three cultivars ($R^2 = 0.69$ – 0.89). The fresh weight of Shirohadaka was reduced more than that of Ehimehadaka No. 1, although the iron content in both cultivars was identical under iron-deficient

conditions (Fig. 3I,J). With regard to the correlation between the water-soluble iron content in the young leaves and the relative fresh weight of the aerial part, Ehimehadaka No. 1 exhibited a higher R^2 value than Shirohadaka and Kairyohagara (Fig. 3I, $R^2 = 0.82$ and $R^2 = 0.75$, respectively; Fig. 3K, $R^2 = 0.82$ and $R^2 = 0.69$, respectively). These results suggest that the iron availability in the young leaves of Ehimehadaka No. 1 is higher than that in the young leaves of Shirohadaka and Kairyohagara.

Correlation between the contents of fractionated iron and the SPAD values

We demonstrated that the water-soluble iron content correlated with the relative fresh weight of the aerial parts. The decrease in the SPAD values is a typical indication of iron deficiency and results in reduced productivity. Accordingly, the correlations between the content of each type of iron fraction and the SPAD values of the young and old leaves were compared among the three species – barley, rice and sorghum – and among the three barley cultivars – Ehimehadaka No. 1, Kairyohagara and Shirohadaka. The plant materials used for these analyses were identical to those used for the experiment illustrated in Figs 2 and 3. Taking the results shown in Fig. 2 into consideration, data from the plants cultured under conditions of moderate iron deficiency (0 – $10 \mu\text{mol L}^{-1}$ iron) were used for the analyses. The under- $10,000$ -MW iron fraction had a very low iron content, and no correlation was observed between the iron content of this fraction and the SPAD values (data not shown). The correlations between the total iron content and SPAD values and between the water-soluble iron content and SPAD values are shown in Fig. 4. The regression lines represent cases of comparatively high R^2 values.

Barley, particularly the Ehimehadaka No. 1 cultivar, maintained high SPAD values with low total iron and water-soluble iron contents in the young leaves. The total iron content correlated with the SPAD values only in the young barley leaves (Fig. 4A, $R^2 = 0.76$). This finding is consistent with the correlation with fresh weight (Fig. 3B,F). Correlations were observed between the water-soluble iron content and SPAD values of the old leaves for all three species (Fig. 4D, $R^2 = 0.90$ and $R^2 = 0.80$; Fig. 4D, $R^2 = 0.82$ and $R^2 = 0.72$) and in the young barley leaves (Fig. 4C, $R^2 = 0.65$). The correlations regarding the water-soluble iron in the old leaves were consistent with those regarding fresh weight (Fig. 3C,G). Low correlation between the water-soluble iron content and SPAD values and low SPAD values of the young rice and sorghum leaves may be consistent with the low R^2 values indicated in Fig. 3A,E.

Taken together, a small amount of iron contributed to the increase in the SPAD value of young barley

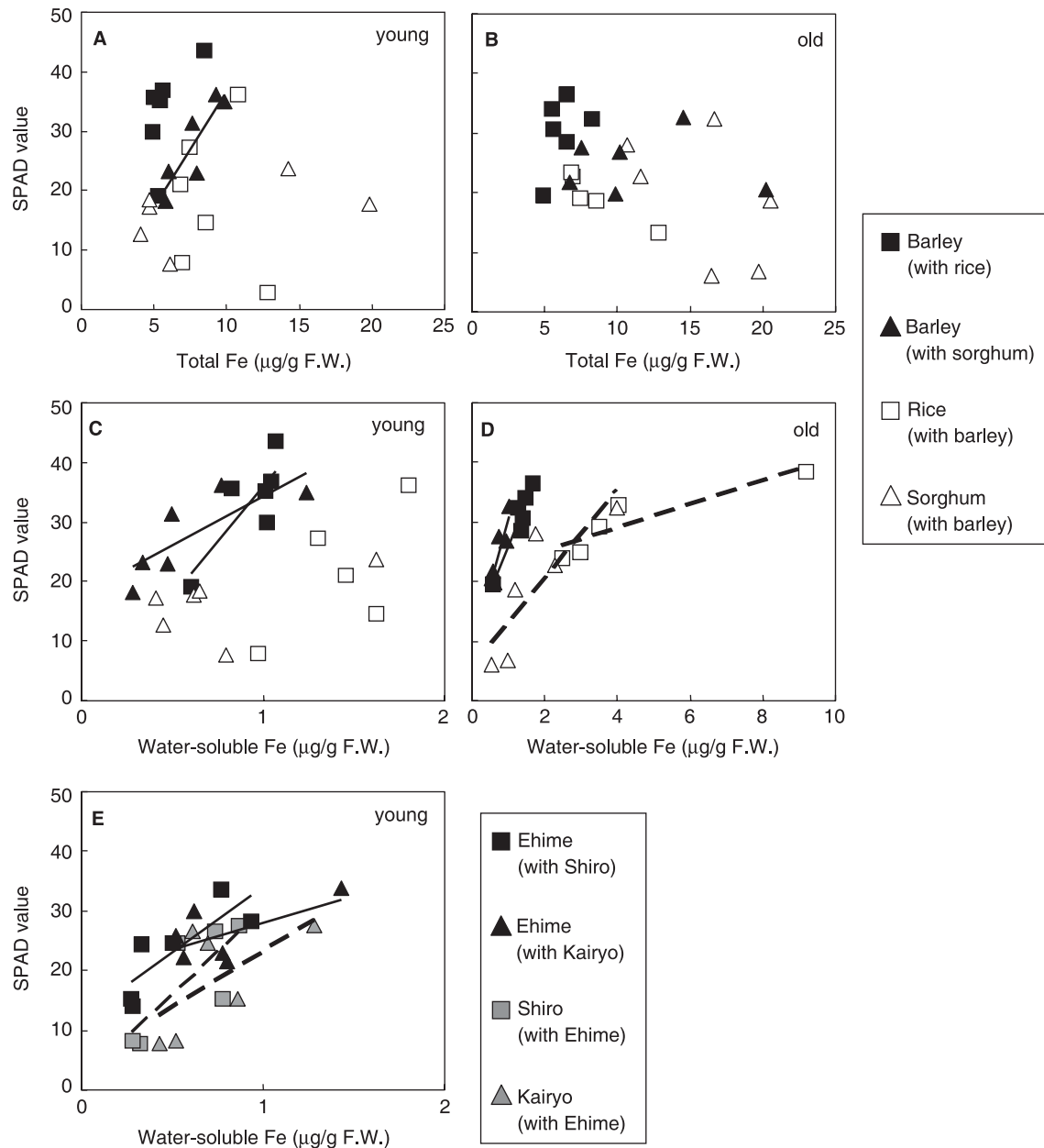


Figure 4 Correlation between the iron content and the SPAD values. (A,B) Correlation between the total iron content and the SPAD value. (C,D,E) Correlation between the water-soluble iron content and the SPAD value. (A,C,E) The young leaf. (B,D) The old leaf. (A,B,C,D) Cocultured barley (Ehimehadaka No. 1), rice and sorghum. ■, barley with rice; ▲, barley with sorghum; □, rice with barley; △, sorghum with barley. (E) Cocultured barley cultivars Ehimehadaka No. 1, Shirohadaka and Kairyō-ohgara. ■, Ehimehadaka No. 1 with Shirohadaka; ▲, Ehimehadaka No. 1 with Kairyō-ohgara; ■, Shirohadaka with Ehimehadaka No. 1; ▲, Kairyō-ohgara with Ehimehadaka No. 1.

leaves and the subsequent increase in the growth of the aerial parts of barley; that is, the availability of water-soluble iron in barley may be higher than that in rice and sorghum. The water-soluble iron contributed to the increase in the SPAD values of old leaves and the subsequent increase in plant growth in all three species. This is reasonable because mature leaves

generally contribute to productivity. However, young rice and sorghum leaves are more susceptible to iron deficiency than young barley leaves; furthermore, the elongation of rice and sorghum leaves is prevented (Fig. 2A,C). Thus, based on the above findings, rice and sorghum may be more sensitive to iron deficiency than barley.

Correlations between the content of water-soluble iron and the SPAD values were observed in the young leaves of the three barley cultivars (Fig. 4E, $R^2 = 0.67$, $R^2 = 0.39$, $R^2 = 0.60$ and $R^2 = 0.39$). These may be characteristic of barley compared with rice and sorghum. Ehimehadaka No. 1 maintained higher SPAD values than the other two cultivars, whereas the water-soluble iron content was identical among the three cultivars (Fig. 4E). This may be reflected in the correlation of the fresh weight in the case of Shirohadaka (Fig. 3I).

Considering that the under-10,000-MW iron content was negligible, these results suggest that the water-soluble iron associated with high-molecular-weight substances contributes to the maintenance of the SPAD value of young barley leaves. This high-molecular-weight iron may mainly be associated with proteins because the SPAD value represents the chlorophyll index, and under iron-deficient conditions more than 60% of the leaf iron is believed to be localized in the lamella (Terry and Low 1982). Many thylakoid membrane proteins require iron to function as a cofactor for redox reactions. Thus, it is reasonable that the amount of iron associated with the proteins contributes to the SPAD value and plant growth.

Conclusion

The study results suggest that in addition to a discussion on the total iron content and the strategies for iron acquisition from the rhizosphere, it is necessary to consider iron availability in leaf cells to investigate the adaptation of plants to iron deficiency. Further investigations are required regarding not only the acquisition and transport of iron at the tissue level, but also the metabolism of iron in cells under iron-deficient conditions.

To date, damage to the chloroplast (Marschner 1995c) and an imbalance in heavy metals (Kobayashi *et al.* 2003) as a result of iron deficiency have been reported. However, the ability of plant cells to compensate for the damage caused by iron deficiency is not well known. Recently, remodeling of the photosynthetic apparatus under iron-deficient conditions has been reported in an alga (Michel and Pistorius 2004; Moseley *et al.* 2002). In our study, the water-soluble iron fraction may have included these photosynthesis-related proteins. The changes in the photosynthesis-related proteins and proteins related to the redox state of iron in higher plants should be investigated under iron-deficient conditions in the context of the mechanism for iron deficiency tolerance.

Previously, we reported that barley has a greater ability to relocate iron than rice (Maruyama *et al.* 2005). However, the source and translocation form of this mobile iron in barley remain to be identified. It is

unclear whether the water-soluble iron reported in our study is related to the mobile iron present in barley. It is predicted that nicotianamine is a carrier of iron in the phloem (von Wirén *et al.* 1999); however, nicotianamine is present in trace amounts in the barley shoot (Higuchi *et al.* 2001). Ferritin is well known as a storage form of iron, and it can release iron (Briat 1996). Thus, ferritin may be the source of mobile iron in barley. Ferritin may be included in the water-soluble iron fraction. The role of ferritin in response to excess iron or oxidative stress has been investigated; however, the function of ferritin under conditions of moderate iron deficiency remains to be clarified.

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