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Characterization of mugineic-acid–Fe transporter in Fe-deficient barley roots using the multi-compartment transport box method

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Summary. We have investigated the mugineic-acid–Fe transport activity of Fe-deficient barley roots, using the multi-compartment transport box system. The roots maintained Fe transport activity for 20 h after excision. The following results were obtained. (1) In Fe-deficient roots, mugineic acid addition enhanced the transport of Fe by 32.2 times over that of the control (with $FeCl_3$ addition). (2) The mugineic-acid–^{55}Fe transport activity of Fe-deficient roots was 18.4-fold higher than that of the Fe-sufficient roots. (3) The mugineic-acid–^{55}Fe transport activity was decreased (7.13% based on the control) by treatment with 5 μM carbonylcyanide *m*-chlorophenyl hydrazone (CCCP). Pretreatment with 0.1 mM dicyclohexyl carbodiimide (DCCD) lowered the transport activity (10.7% based on the control) and 1 mM *N*-ethylmaleimide (NEM) pretreatment reduced the transport activity to a value equivalent to 2.41% of that in the control. It is concluded that mugineic-acid–Fe transporter is induced in its activity and/or amount by Fe-deficiency treatment and has an SH residue at its active site, and that the transporter needs the proton motive force produced by ATPase. We detected three polypeptides (14, 28 and 40 kDa) in the root plasma membrane that were induced under Fe-deficiency treatment.

Key words: Barley — Fe-deficiency — Phytosiderophore — Plasma membrane — Transporter

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Abbreviations. *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; MA, mugineic acid; NEM, *N*-ethylmaleimide.

Introduction

Under Fe-deficient conditions, barley roots secrete mugineic-acid-family phytosiderophores (MAs). The secreted mugineic acid, for instance, forms the mugineic-acid–Fe chelate, thus solubilizing the Fe from insoluble Fe-hydroxides in the rhizosphere. Then, barley absorbs the mugineic-acid–Fe complex and utilizes the Fe (Takagi et al. 1984). Using the whole plant, it has been suggested that barley absorbs Fe as mugineic-acid–Fe complex (Mori et al. 1987) and that this absorbing system is energy-dependent (Takagi 1984). Marschner et al. (1987) proposed that Fe-deficiency treatment enhanced the phytosiderophore-^{59}Fe absorption by whole-plant barley root. However, no further information about the phytosiderophore-Fe-absorbing system of barley has been obtained. The mugineic-acid–Fe absorption experiments using whole plant could not exclude the additive effect of MAs which were secreted by the plant roots. Using excised roots instead of whole plant roots to lower the additive effect of secreted MAs, we analyzed in more detail the mugineic-acid–Fe uptake system by the multi-compartment transport box method (Kawasaki et al. 1983).

Materials and methods

Plant culture. Seeds of barley (*Hordeum vulgare* cv. Ehimehadaka no. 1) were germinated on tap water (pH 5.5) in the phytotron. Room temperature was maintained at 20° C/14-h light and 15° C/10-h dark. After about ten days, the seedlings were transplanted and Fe-deficiency treatment was started as described previously (Mori and Nishizawa 1987). After 2–3 weeks of Fe-deficiency treatment, the apical 5-cm portion of roots was excised, washed with fresh culture medium, and used for transport experiments. The standard culture medium has the following composition (ppm): N, 32 (NO_3N=24, NH_4N=8); P_2O_5, 16; K_2O, 32; CaO 32; MgO, 32; B, 0.4; Mo, 0.04; Zn, 0.04; and Cu 0.02.

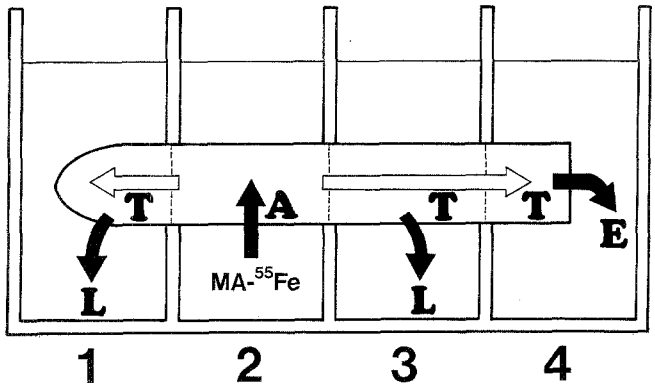

Fig. 1. Diagram of multi-compartment transport box experiment. *Arrows* show the flow of mugineic-acid-^{55}Fe complex supplied to compartment 2. *1, 2, 3* and *4* are compartment numbers; *A:* accumulation of ^{55}Fe in root segment in compartment 2; *T:* translocated ^{55}Fe into root segments in compartments 1, 3 and 4; *L:* leaked ^{55}Fe from root into the media in compartments 1 and 3; *E:* exuded ^{55}Fe from the basal cut end of the root into the medium in compartment 4

Transport experiment. Transport experiments using the multi-compartment transport box system were carried out following the method described by Kawasaki et al. (1983). To minimize the remaining phytosiderophores in the roots, the roots were excised after at least 5 h of secretion of MAs under light condition. As illustrated in Fig. 1, excised barley roots were laid horizontally with the apical part of the root in compartment 1 and the basal cut end in compartment 4. Ten roots were used for each treatment in all experiments, unless otherwise mentioned. Each compartment was filled with 6 ml standard culture medium. The medium was adjusted to pH 6.0. Then mugineic-acid-$^{55}Fe^{3+}$ (1:1) was supplied to the medium in compartment 2, unless otherwise mentioned. The standard condition of absorption treatment was 20 h at 25° C in the dark. After that, the medium in each compartment was completely transferred to a test tube, and 1 ml of each medium was mixed with 10 ml liquid scintillator (Clea-sol I, Nacalai tesque, Kyoto, Japan) and was analyzed by scintillation counter (Packard). Then, the roots were cut out at the border point between each compartment. Each segment was put into a liquid scintillation vial and then mixed with 10 ml liquid scintillator. After standing for about 10 h, the radioactivity was measured.

Preparation of mugineic-acid-$^{55}Fe^{3+}$ complex. Mugineic acid was obtained from Dr Takagi and Dr Kawai of Iwate University, Japan. $^{55}FeCl_3$ (specific activity = 22.45 mCi/mg) was purchased from DuPont/NEN Research Products. Mugineic-acid-$^{55}Fe^{3+}$ complex was prepared as follows: $^{55}FeCl_3$ (4.22 μmol) in 0.1 ml 0.5 M HCl was neutralized with 0.1 ml 0.5 M NaOH, and 1.3 mg (4.2 μmol) mugineic acid powder was added to the $^{55}FeCl_3$ solution. The solution was incubated for 180 min at 50° C. This mixture was loaded onto a Sephadex G-10 column (10 mm diameter × 300 mm length). The column was pretreated with 1 ml 10% bovine serum albumin (Sigma) to cover the adsorbent group, washed with 10 ml 10% EDTA-Na_2 to remove the contaminating Fe, and washed with an adequate amount of deionized redistilled water. Then the column was eluted with deionized redistilled water and the eluate (1 ml each of mugineic-acid-$^{55}Fe^{3+}$ solution) was collected using a fraction collector. The mugineic-acid-$^{55}Fe^{3+}$ solution contained in fractions 10–15 was used for the transport experiments.

Relationship between 'transport' and 'exudate'. As mentioned earlier, the apical part of the root was laid out in compartment 1 and the basal cut end in compartment 4. Mugineic-acid-Fe was generally supplied to the medium in compartment 2 (Fig. 1). The amount of mugineic-acid-^{55}Fe distributed to the root portion in compartment 2 represents 'accumulation', whereas the amount in compartments 1, 3 or 4 represents 'translocation'. On the other hand, the amount of mugineic-acid-^{55}Fe distributed in the medium in compartments 1 or 3 represents the 'leakage' while that in compartment 4 represents the 'exudate'. The quantity of mugineic-acid-^{55}Fe in 'transport' refers to the summation of mugineic-acid-^{55}Fe in 'translocation', 'leakage' and 'exudate'. Note that 'accumulation' includes both mugineic-acid-^{55}Fe taken up into the cell and adsorbed to the root surface, and that there is a problem in measuring the root radioactivity by liquid scintillation counter, i.e., physical quenching (self-absorption). Therefore, in order to obtain a reliable value, it is better to measure the radioactivity of the liquid medium rather than that of the root. Correlation analysis was performed in order to determine the relationship between the 'exudate' and 'transport'. As revealed in Fig. 2, 'exudate' and 'transport' had a very good correlation ($r = 0.938$, $P < 0.01$) suggesting that 'transport' can be predicted with a high degree of accuracy by using the 'exudate' data. Considering this, the amount of mugineic-acid-Fe in the 'exudate' will be used to discuss the differences in the 'transport' activity among some treatments in this paper.

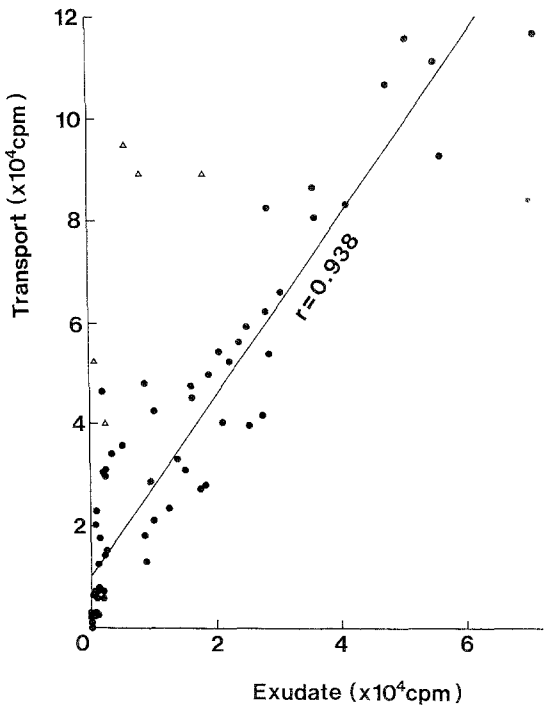

Fig. 2. Relationship between 'transport' and 'exudate'. All the data obtained through this series of transport experiment were plotted (see Materials and methods). (△) The data obtained in Experiment 10; these data were excluded in calculating the correlation coefficient (see Results)

Preparation of plasma membrane fraction from barley roots. Using the aqueous two-phase partition method (Larsson 1985), plasma membrane fractions were prepared from barley roots grown under either Fe-deficient or Fe-sufficient conditions. In brief, the frozen roots were crushed into pieces in liquid nitrogen and homogenized in a buffer described by Yoshida et al. (1983). The homogenate was filtered through four layers of gauze and centrifuged at 10000 *g* for 15 min. The supernatant was centrifuged at 156000 *g* for 20 min and the microsomal fraction was sedimented. From the microsomal fraction, the plasma membrane fraction was purified as U5 fraction by aqueous two-phase partition.

Electrophoresis of plasma membrane proteins. Plasma membrane proteins of Fe-deficient and Fe-sufficient barley roots were analyzed with SDS polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of acrylamide was 10%. The plasma membrane proteins were extracted with phenol following the method of Hurkman and Tanaka (1986), then solubilized with SDS and loaded onto an SDS-PAGE gel. The gel was stained with silver by following the procedure given in the manual for the Sil-Best stain kit (Nacalai Tesque, Kyoto, Japan).

Results

Experiment 1: Effect of mugineic acid on Fe transport in the roots

First, we examined the role of mugineic acid in Fe transport in the Fe-deficient roots. As shown in Table 1, mugineic acid enhanced the Fe transport by 32.2 times over that of the control.

Table 1. Effect of mugeneic acid on ^{55}Fe transport in Fe-deficient roots

^{55}Fe form	Accumulation (cpm/24 h for 8 roots)	Translocation	Leakage	Exudate	Transport
$FeCl_3$	52924	13704	5348	901	19953
MA-Fe	70135	46590	6767	28991	82348

The words heading the columns are defined in Materials and methods and in the legend of Fig. 1

Experiment 2: Effect of Fe-deficiency treatment on Fe transport in roots

The roots excised from barley which had been grown under Fe-deficient and Fe-sufficient conditions were tested to examine whether Fe nutrient condition affects the Fe 'transport' activity of roots. Fe supplied as $^{55}FeCl_3$ was transported by the Fe-deficient roots but not by the Fe-sufficient roots (Table 2). On the other hand, when Fe was supplied as mugineic-acid-^{55}Fe, even the Fe-sufficient roots could transport the element, although only in trace amounts (Table 3). Among the above four treatments, Fe transport activity was highest in the Fe-deficient roots supplied with mugineic-acid-^{55}Fe. As shown in Table 3 and Fig. 3, Fe transport in the Fe-deficient roots was 18.4 times higher than that in the Fe-sufficient roots, when

Table.2. Effect of Fe-deficiency treatment on $^{55}FeCl_3$ transport in roots

Treatment	Accumulation (cpm/21 h for 10 roots)	Translocation	Leakage	Exudate	Transport
+Fe	14388	142	0	0	142
−Fe	36795	5467	488	1332	7287

+Fe and −Fe mean Fe-sufficient and Fe-deficient growth of the plant, respectively

Table 3. Effect of Fe-deficiency treatment on mugineic-acid-^{55}Fe transport in roots

Treatment	Accumulation (cpm/22 h for 10 roots)	Translocation	Leakage	Exudate	Transport
+Fe	61924	2222	83	274	2579
−Fe	62181	29721	1306	5047	36074

+Fe and −Fe mean Fe-sufficient and Fe-deficient growth of the plant, respectively

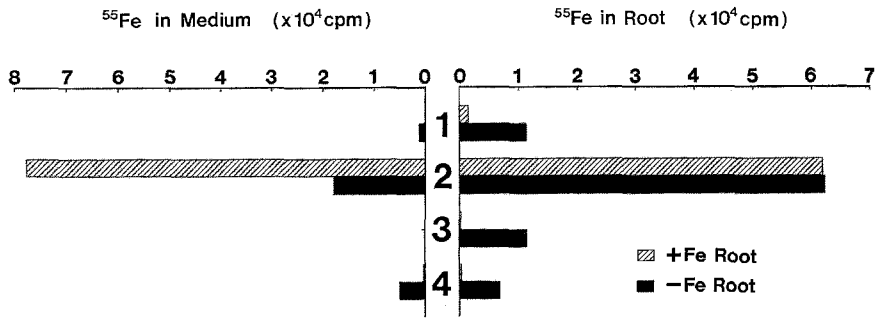

Fig. 3. Effect of Fe-deficiency treatment on mugineic-acid-^{55}Fe transport in roots. *1, 2, 3* and *4:* compartment numbers (see Fig. 1). Mugineic-acid-^{55}Fe was supplied to compartment 2. Fe transport in Fe-deficient (−Fe) roots was 18.4 times higher than that in Fe-sufficient (+Fe) roots

Fe was supplied as mugineic-acid-^{55}Fe. Therefore, we can say that Fe-deficient conditions induce Fe transport activity in the roots.

Experiment 3: Site of mugineic-acid-^{55}Fe uptake in roots

To determine the site of Fe uptake in the roots, mugineic-acid-^{55}Fe was separately supplied to compartment 1, 2 and 3. The data in Table 4 show that the transport of Fe supplied to the compartment 2 and 3 was higher by approximately 20.9 and 22.7 times, respectively, over that of the Fe supplied in compartment 1. This implies that the dominant site for Fe uptake is about 5-40 mm from the root tip. Thus, in the succeeding experiments, Fe was supplied to compartment 2.

Experiment 4: Time dependency of mugineic-acid-^{55}Fe transport in the excised roots

We also examined the time course change of mugineic-acid-^{55}Fe transporting rate (exudate ^{55}Fe, cpm/h). As reflected in Fig. 4, the rate was almost constant over 2-10 h after the supply of mugineic-acid-^{55}Fe. The average amount of Fe in the exudate was 0.109 nmol/h for 10 roots when 4.43 nmol mugineic-acid-^{55}Fe was added. After 23 h, the excised roots still possessed the transport activity, even though the activity was 26.3% of the average over 2-10 h. To avoid any error that might be derived from time shift, all the data of the transport experiment were obtained at 20 h after mugineic-acid-^{55}Fe supply, unless otherwise mentioned.

Experiment 5: Effect of sucrose on mugineic-acid-^{55}Fe transport in the roots

To elucidate whether the mugineic-acid-Fe transport system is energy-dependent and what is the direct energy source of the transport, we conducted experiments 5, 6 and 7. First, sucrose was supplied to compartment 1 (apical part of the root) and mugineic-acid-^{55}Fe was supplied to the next upper compartment (2). As shown in Table 5, the supply of 1 μmol sucrose to 6 ml medium (0.17 mM) increased the transport by 2.05 times over that of the control. But at concentrations higher than 0.17 mM, the transport was depressed. It is interesting that the ^{55}Fe accumulation in the root at compartment 2 (to which mugineic-acid-^{55}Fe was supplied) showed the opposite trend: namely, sucrose increased the accumulation of ^{55}Fe near the point of its supply and did not facilitate the translocation of ^{55}Fe to the upper part of the root (compartment 3 and 4). It seems that sucrose facilitated Fe accumulation and utilization just at the site of Fe supply but did not facilitate translocation to the other parts of the root.

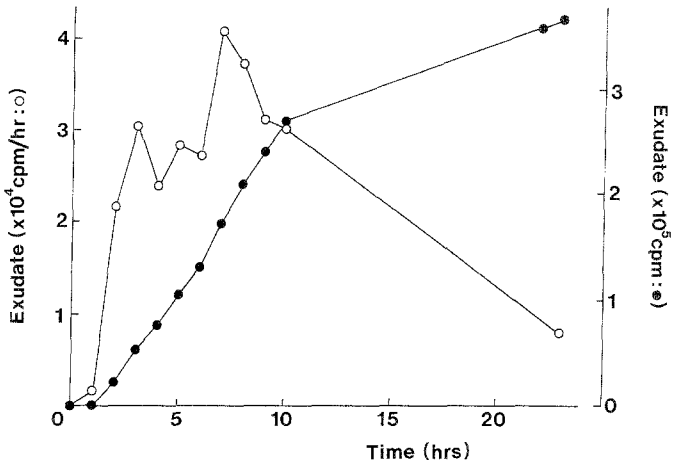

Fig. 4. Time dependency of mugineic-acid-^{55}Fe transport in excised roots. The average amount of exudate from 2-10 h was 0.109 nmol Fe/h for 10 roots

Table 4. Site of mugineic-acid-^{55}Fe uptake in Fe-deficient roots

Compartment	Accumulation (cpm/20 h for 10 roots)	Translocation	Leakage	Exudate	Transport
1	108068	10521	2462	2454	15437
2	67190	51631	12550	51318	115499
3	58758	53322	2544	55674	111540

The compartment number refers to the site where mugineic-acid-^{55}Fe was supplied (see Fig. 1)

Table 5. Effect of sucrose supply on mugineic-acid-^{55}Fe transport in Fe-deficient roots

Sucrose (μmol/6 ml)	Accumulation (cpm/7.5 h for 10 roots)	Translocation	Leakage	Exudate	Transport
0	72782	12608	1863	27491	41962
1	62533	32400	3936	56353	92689
10	89862	18715	13600	24054	56369
100	104153	18477	414	14042	32933

Sucrose was supplied at compartment 1

Table 6. Effect of ATPase inhibitor (DCCD) pretreatment on mugineic-acid-^{55}Fe transport in Fe-deficient roots

DCCD (μM)	Accumulation (cpm/20 h for 10 roots)	Translocation	Leakage	Exudate	Transport
0 (control)	30147	9714	135	18528	28377
1	34835	14027	17	25667	39711
10	37737	9742	281	17490	27513
100	31393	3895	75	1978	5948
1000	20902	1552	22	1112	2686

Both mugineic-acid-^{55}Fe and reagent were supplied in compartment 2. Absorption was conducted at 15° C. DCCD was supplied as an ethanol solution, the control receiving 1% ethanol

Experiment 6: Effect of DCCD on mugineic-acid-^{55}Fe transport in the roots

To examine the involvement of endogenous ATP on mugineic-acid-^{55}Fe transport in the roots, the roots were pretreated with DCCD, an ATPase inhibitor. Note that pretreatment with the reagent was only in compartment 2 (where the mugineic-acid-^{55}Fe was supplied) in the following similar experiments. As shown in Table 6, pretreatment with 100 μM DCCD strongly inhibited the mugineic-acid-^{55}Fe transport (only 10.7% based on the control). This means that the mugineic-acid-Fe transport process involves an endogenous ATP-consuming step.

Experiment 7: Is mugineic-acid-Fe transport dependent on proton motive force?

As presented in Table 7, treatment with 5 μM CCCP, a proton conductor, strongly inhibited the mugineic-acid-^{55}Fe transport to as low as 7.13% based on the control. This suggests that mugineic-acid-Fe transport is closely related to the proton motive force across the plasma membrane (Reinhold and Kaplan 1984). Treatment with 5 μM nigericin, an ionophore for both H^+ and K^+, also inhibited the mugineic-acid-^{55}Fe transport (Table 8) but the inhibition rate was less than that of CCCP (49.7% based on the control). The difference between the inhibition effects caused by CCCP and nigericin is probably due to the difference in the reaction mechanisms between these two reagents. CCCP acts as a proton conductor and eliminates the proton gradient across the plasma membrane. On the other hand, nigericin acts as an exchanger of H^+ and K^+ and does not completely remove the proton gradient. In the next experiment, the involvement of the K^+ ion in mugineic-acid-Fe transport in the roots was studied.

Table 7. Effect of proton conductor (CCCP) on mugineic-acid-^{55}Fe transport in Fe-deficient roots

CCCP (μM)	Accumulation (cpm/24 h for 10 roots)	Translocation	Leakage	Exudate	Transport
0 (control)	47471	52987	6264	47952	107203
5	44456	17340	13745	3421	34506

Absorption was conducted at 20° C.
CCCP was supplied as a DMSO solution, the control receiving 0.05% DMSO

Table 8. Effect of H^+ and K^+ ionophore (nigericin) on mugineic-acid-^{55}Fe transport in Fe-deficient roots

Nigericin (μM)	Accumulation (cpm/23 h for 8 roots)	Translocation	Leakage	Exudate	Transport
0 (control)	70627	23243	7590	19068	49901
5	47302	11485	7925	9468	28878

Nigericin was supplied as an ethanol solution, the control receiving 0.5% ethanol

Experiment 8: Effect of K^+ ion and valinomycin on mugineic-acid-Fe transport in the roots

The results in Fig. 5 revealed that the mugineic-acid-^{55}Fe transport in the control was enhanced by 2.52 times in the presence of 10 mM KCl. However, under the same KCl concentration, this K^+ effect was almost completely eliminated when 5 μM valinomycin, a K^+ ionophore, was added. The elimination of the K^+ gradient by valinomycin diminished the enhancing effect of K^+. Too much KCl (50 mM) also decreased the stimulating effect of KCl. From these results, it seems that not only H^+ but also the K^+ ion plays an important role in the mugineic-acid-Fe transport. A K^+ gradient may be coupled with the transport of mugineic-acid-Fe.

Experiment 9: Identification of the amino acid residues at the active center of mugineic-acid-Fe transporter protein

To identify the amino acid residues at the active center of mugineic-acid-Fe transporter protein,

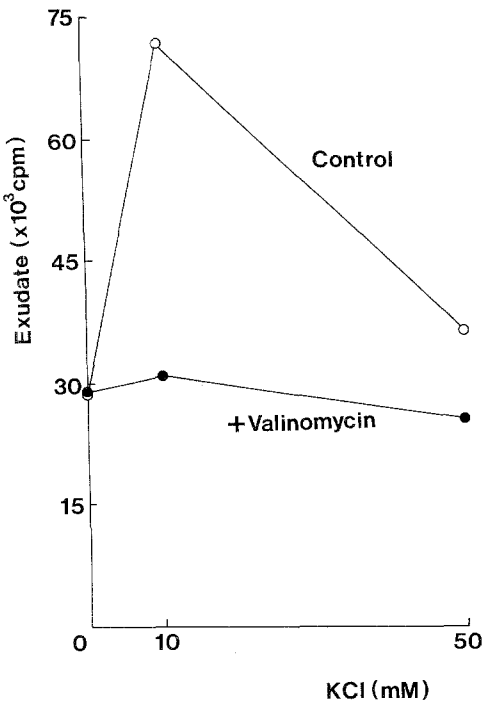

Fig. 5. Effect of K^+ ion and valinomycin on mugineic-acid–Fe transport in roots. The enhancing effect of KCl was completely eliminated by coexistence with 5 μM valinomycin, a K^+ ionophore

the mugineic-acid–Fe transport activity was measured after modifying the amino acid residues. NEM and *p*-APMSF were used to modify the SH group of Cys residues and the OH group of Ser residues, respectively. The modified part of the roots was just in the compartment to which mugineic-acid–^{55}Fe was supplied. As shown by the data in Table 9, pretreatment with 1 mM NEM for 60 min almost completely reduced the transport activity of mugineic-acid–^{55}Fe (2.41% based on the control). It is remarkable that NEM pretreatment also reduced the 'accumulation' at the root part in compartment 2. Thus, the mugineic-acid–Fe transporter protein was thought to have a Cys residue at the active site. Pretreatment with 1 mM *p*-APMSF for 60 min also reduced the transport of mugineic-acid–^{55}Fe (13.3% based on the control; Table 10). But at concentrations lower than 1 mM, reduction of the transport was not so clear.

Table 9. Effect on NEM pretreatment on mugineic-acid–^{55}Fe transport in Fe-deficient roots

NEM (mM)	Accumulation (cpm/23 h for 8 roots)	Translocation	Leakage	Exudate	Transport
0 (control)	59555	50087	93	36181	86361
0.01	68052	29996	216	22301	52513
0.1	71969	26999	1823	16224	45046
1	13094	4547	564	872	5983
10	6113	3562	2621	601	6784

The roots in compartment 2 were treated with NEM for 1 h at 25° C, after which the medium was displaced with standard culture medium and absorption was conducted. NEM was supplied as an ethanol solution, the control receiving 1% ethanol

Table 10. Effect of *p*-APMSF pretreatment on mugineic-acid–^{55}Fe transport in Fe-deficient roots

p-APMSF (μM)	Accumulation (cpm/20 h for 10 roots)	Translocation	Leakage	Exudate	Transport
0 (control)	53843	15484	373	15395	31252
1	55054	10550	476	9923	20949
5	48589	17470	1347	21290	40107
10	44848	9175	4	8854	18033
100	36708	10619	666	12344	23629
1000	53252	4807	560	2054	7421

The roots in compartment 2 were treated with *p*-APMSF for 1 h at 15° C, after which the medium was displaced with standard culture medium and absorption was conducted at 15° C. The control received distilled water

Experiment 10: Effect of coexistence of amino acids on mugineic-acid–^{55}Fe transport in roots

We examined the possibility that the mugineic-acid–Fe complex and some amino acids share the same transporter protein for their transport into the cell. For this purpose, we studied the effects of the coexistence of some amino acids on the mugineic-acid–^{55}Fe transport. Among the amino acids studied, Arg and Lys most strongly inhibited the mugineic-acid–^{55}Fe transport (up to 10.7% and 30.3% based on the control, respective-

Table 11. Effect of amino acid coexistence on mugineic-acid–^{55}Fe transport in Fe-deficient roots

Amino acid (10 mM)	Accumulation (cpm/23 h for 10 roots)	Translocation	Leakage	Exudate	Transport
– (control)	67921	52179	28392	8496	89067
Pro	64834	58888	30108	5898	94894
Arg	94761	37705	13668	906	52279
Trp	44360	61713	8766	18588	89067
Lys	94658	29868	7956	2574	40398

The control received culture medium only

ly) while in contrast, they increased the accumulation (up to 139.5% and 139.4% based on the control, respectively; Table 11). These results suggest that mugineic-acid–Fe, Arg and Lys may share the same transporter for their transport in the roots. It is interesting to note that Trp increased the mugineic-acid–^{55}Fe transport by about 2.19-fold over the control (Table 11; see Discussion). In this experiment, the correlation between the 'exudate' and 'transport' was low ($r=0.66$); thus, the data obtained were excluded in calculating the correlation coefficient given in the Materials and methods (Fig. 2). The low correlation in this experiment is probably due to the fact that, in some treatments, 'leakage' was so high such that the measured value for 'transport' became larger than that expected from the 'exudate'.

Electrophoresis of plasma membrane proteins

From 400 g roots, about 4 mg U5 fraction was obtained. The U5 fraction was stained by silicotungstic acid/CrO_3, a specific stain for plasma membrane (Nagahashi et al. 1978), and observed under the electron microscope. Since almost all of the membrane vesicles were stained (Mihashi et al. 1989), U5 must be a highly plasma-membrane-enriched fraction. The electrophoretic patterns of plasma membrane proteins from the Fe-deficient and Fe-sufficient barley roots were compared. Three polypeptide bands (14, 28 and 40 kDa) seemed to be induced under Fe-deficiency condition (Fig. 6).

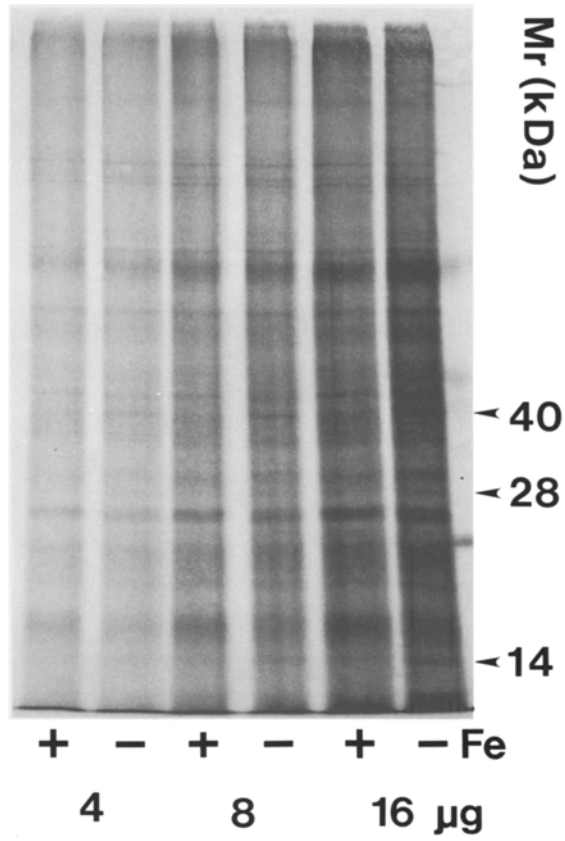

Fig. 6. Electrophoretic pattern of Fe-sufficient (+Fe) and Fe-deficient (−Fe) barley root plasma membrane proteins. Protein (4, 8 or 16 µg) was loaded per lane as indicated. Three polypeptides (14, 28 and 40 kDa) seemed to be induced under Fe-deficiency treatment

Discussion

Mugineic acid enhanced ^{55}Fe 'transport' in the Fe-deficient roots (Table 1). To examine whether this effect is specific to mugineic acid or other chelators, Takagi et al. (1984) have already shown, by using the whole plant, that some natural and synthetic chelators do not enhance Fe uptake but inhibit the mugineic-acid-mediated Fe uptake.

Fe-deficiency treatment strongly induced ^{55}Fe tansport when ^{55}Fe was supplied as either $^{55}FeCl_3$ or mugineic-acid–^{55}Fe (Tables 2 and 3). This enhancing effect on the Fe uptake by Fe-deficient intact barley roots was reported by Clarkson and Sanderson (1978) and Marschner et al. (1987). But by using the excised roots, we were able to show more clearly the differences between the Fe-deficient and Fe-sufficient roots. 'Mugineic-acid–Fe transporter protein' increased in amount and/or activity under Fe-deficiency treatment. 'Mugineic-acid–Fe transporter protein' is a hypothetical protein that may be related to the mugineic-acid–Fe transport activity across the plasma membrane. We detected three polypeptides (14, 28 and 40 kDa) induced under Fe-deficiency in the root plasma membrane (Fig. 6). These polypeptides may be related to the 'mugineic-acid–Fe transporter protein'. Our final goal is to characterize and purify this transporter protein and to clone the gene responsible for it.

The site of mugineic-acid–^{55}Fe uptake was about 5–40 mm from the root tip (Table 4). Using intact roots, it was also reported that the Fe uptake rate was high at the 10–50-mm zone (Clarkson and Sanderson 1978) or at 5–15-mm zone (Marschner et al. 1987) from the root tip.

The excised Fe-deficient roots maintained mugineic-acid–^{55}Fe 'transport' activity for 23 h but the activity decreased gradually after 10 h of the

absorption period (Fig. 4). The decrease in activity might have been due to shortage of the energy source. Utilizing sucrose as an energy source increased the transport about twofold over the control at 0.17 mM sucrose concentration (Table 5). On the other hand, sucrose concentrations higher than 0.17 mM depressed the transport. This depressive effect might be partly due to the severe difference in osmotic pressures between the apical root cell sap and the outer medium in compartment 1. The direction of the water flow might be from cell to medium and mugineic-acid-^{55}Fe complex was forced to move in the same direction.

Pretreatment with the ATPase inhibitor DCCD lowered the mugineic-acid-^{55}Fe transport. The data in Table 6 show that endogenous ATP is needed for the mugineic-acid-^{55}Fe transport in the root. The proton conductor CCCP also strongly inhibited mugineic-acid-^{55}Fe transport (Table 7). This means that this transport across the plasma membrane is coupled to proton motive force. Treatment with the H^+ and K^+ ionophore nigericin produced the same trend (Table 8). This strongly supports the idea that the mugineic-acid-^{55}Fe transport is coupled with the proton gradient across the plasma membrane and that the K^+ ion is also involved in it. In fact, K^+ ion at 10 mM concentration enhanced the transport and this effect was completely abolished by valinomycin treatment to decrease the K^+ gradient across the plasma membrane (Fig. 5). Involvement of K^+ ions in Fe uptake has been discussed in the dicotyledonous (strategy I plant) root (Jolley et al. 1988). K^+ ions may also play an important role in Fe uptake in strategy II plants.

In order to characterize the mugineic-acid-^{55}Fe transporter protein on plasma membranes, we searched for the amino acid residue at its active site. As shown in Table 9, the Cys modifier NEM strongly depressed mugineic-acid-^{55}Fe transport. The tansporter thus seems to have a Cys residue at its active site. However, we cannot be certain because, under some conditions, NEM can also act as a modifier of the NH_2 group and we cannot control the conditions suitable for modifying only the SH group. The Ser modifier *p*-APMSF did not show any clear inhibiting tendency on the transport (Table 10). Moreover, the coexistence of *p*-APMSF at concentrations lower than 1 mM increased the transport (data not shown). This proton motive effect on mugineic-acid-^{55}Fe transport might have occurred indirectly, because *p*-APMSF acted as a protease inhibitor and protected the transporter from digestion by the endogenous protease.

From Experiment 10, it is suggested that the mugineic-acid-Fe complex, Arg and Lys may share the same transporter during their uptake in the roots (Table 11). But this assumption needs further detailed experiments since we cannot exclude the possibility that metabolites of these amino acids may have affected the mugineic-acid-Fe transport through an indirect route. On the other hand, the enhanced transport effect of Trp (Table 11) might be that of the plant hormone, IAA, as a metabolic product from Trp. From this result, there emerged a possibility that some plant hormones may have an effect on the mugineic-acid-Fe transport in the roots. Experiments concerning this must be done in the future.

In conclusion, it is suggested that the Fe is absorbed as mugineic-acid-Fe in barley roots, and there is a protein in barley roots which is involved in mugineic-acid-Fe transport. The transporter is enhanced in activity and/or in amount by Fe-deficiency treatment. There are at least three polypeptides (14, 28 and 40 kDa) in the root plasma membrane induced under Fe-deficiency treatment. The mugineic-acid-Fe transport is ATP-dependent and is coupled with proton motive force. K^+ ions may be involved in mugineic-acid-Fe transport. The transporter possibly has a Cys residue at its active site. Arg and Lys may share the same active site of the transporter.

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