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Formate Dehydrogenase, an Enzyme of Anaerobic Metabolism, Is Induced by Iron Deficiency in Barley Roots[1]

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To identify the proteins induced by Fe deficiency, we have compared the proteins of Fe-sufficient and Fe-deficient barley (*Hordeum vulgare* L.) roots by two-dimensional polyacrylamide gel electrophoresis. Peptide sequence analysis of induced proteins revealed that formate dehydrogenase (FDH), adenine phosphoribosyltransferase, and the *Ids3* gene product (for Fe deficiency-specific) increased in Fe-deficient roots. FDH enzyme activity was detected in Fe-deficient roots but not in Fe-sufficient roots. A cDNA encoding FDH (*Fdh*) was cloned and sequenced. *Fdh* expression was induced by Fe deficiency. *Fdh* was also expressed under anaerobic stress and its expression was more rapid than that induced by Fe deficiency. Thus, the expression of *Fdh* observed in Fe-deficient barley roots appeared to be a secondary effect caused by oxygen deficiency in Fe-deficient plants.

In Fe-deficient calcareous soils graminaceous plants secrete mugineic acid family phytosiderophores, which are natural Fe chelators, from the roots (Takagi, 1976) to solubilize Fe required for plant growth. This Fe-acquisition mechanism in graminaceous plants is called strategy II and in nongraminaceous plants it is called strategy I (Takagi et al., 1984; Marschner et al., 1986). The pathway of the biosynthesis of mugineic acid family phytosiderophores has been established (Mori and Nishizawa, 1987, 1989; Shojima et al., 1989, 1990; Mori et al., 1990; Ma and Nomoto, 1993). Among the enzymes involved in this biosynthetic pathway, Higuchi et al. (1994, 1996) purified nicotianamine synthase and Kanazawa et al. (1994) purified nicotianamine aminotransferase. Comparison of 2D profiles of proteins in barley (*Hordeum vulgare* L.) roots under Fe-sufficient and Fe-deficient conditions (Suzuki et al., 1995, 1997) allowed us to identify a 36-kD protein that was specifically induced by Fe deficiency. In addition, several genes related to the Fe-deficiency response have been reported: *Ids1* (Okumura et al., 1991), *Ids2* (Okumura et al., 1994), and *Ids3* (Nakanishi et al., 1993). In this study, we characterized several other proteins induced by Fe-deficiency stress in barley roots, one of which was identified as FDH. FDH was induced not only by Fe deficiency but also by anaerobic stress. The relationship between Fe deficiency and anaerobic stress in barley roots is discussed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of barley (*Hordeum vulgare* L. cv Ehimehadaka no. 1) were germinated at room temperature on paper towels soaked with distilled water. Plants were transferred 4 d after germination to a plastic net floating on tap water at pH 5.5 in a greenhouse under natural light. On d 10, plants were transferred to a continuously aerated nutrient solution of the following composition: 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 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 $(NH_4)_6Mo_7O_{24}$, and 0.1 mM Fe-EDTA. The pH of the culture solution was adjusted to 5.5 daily with 1 N HCl. Fe deficiency was started on d 20 using the same solution, but without Fe-EDTA. The nutrient solution was changed every 7 d. Plant roots were harvested 40 d after germination. Anaerobiosis was achieved by bubbling nitrogen gas through the nutrient solution overnight to purge oxygen gas in the solution, followed by a continuous flow of nitrogen gas throughout the anaerobic experiment.

Protein Extraction for 2D PAGE

The procedure for extraction of proteins was as described by Damerval et al. (1986) with slight modifications. The roots were homogenized in liquid nitrogen with a mortar and pestle, and the powder was resuspended in a cold solution of 10% (w/v) TCA in acetone with 0.1% (v/v) 2-ME. Proteins were allowed to precipitate for 60 min at −20°C and were then centrifuged at 16,000*g* for 30 min at 4°C. The supernatant solution was discarded and the pellet was rinsed with cold acetone containing 0.1% (v/v) 2-ME for 60 min at −20°C and then centrifuged at 16,000*g* for 30 min at 4°C. The supernatant solution was discarded and

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Abbreviations: CNBr, cyanogen bromide; FDH, formate dehydrogenase; 2D, two-dimensional; 2-ME, 2-mercaptoethanol.

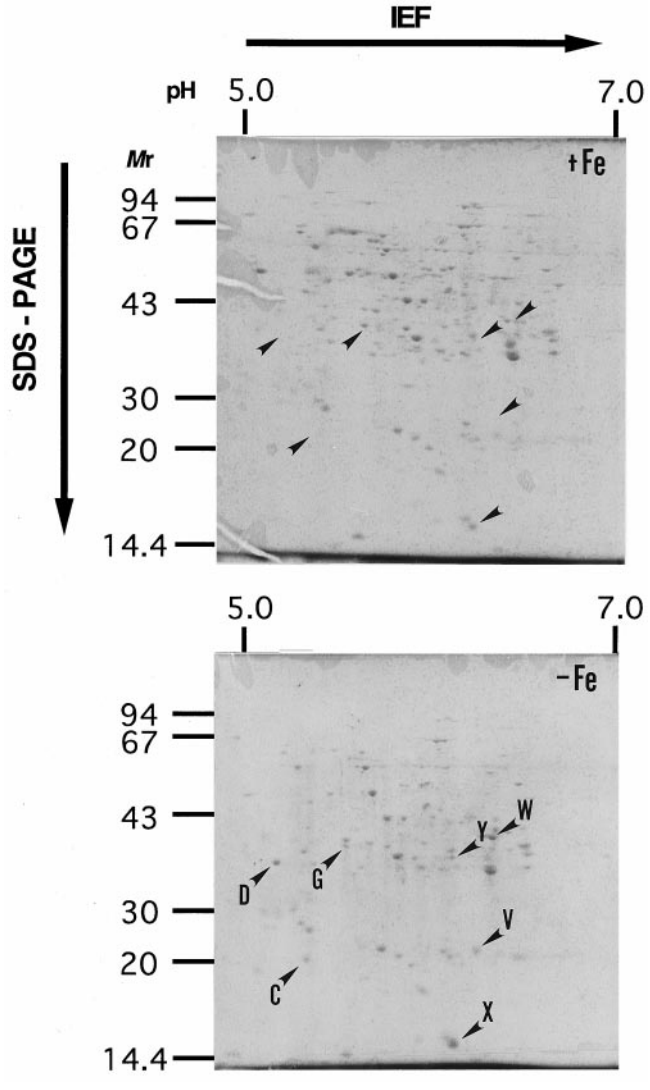

Figure 1. 2D PAGE gel from barley roots grown under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. Each gel was loaded with 200 μg of root proteins. Several spots of proteins that increased under Fe deficiency are indicated with arrowheads. C, Adenine phosphoribosyltransferase; Y, IDS3; and W, FDH. Other spots are unknown (Table I). M_r is reported in thousands.

the pellet was dried under reduced pressure, dissolved (50 μL mg^{-1} dry weight) in sample buffer (9.5 M urea, 2% [w/v] Triton X-100, and 5% [v/v] 2-ME), and centrifuged at 16,000*g* for 10 min at room temperature. The supernatant solution was used for 2D PAGE. Protein concentrations were estimated by the method of Bradford (1976).

2D PAGE

2D PAGE was performed following the method of O'Farrell (1975). Gel length in the column (2.5 × 130 mm) was 100 mm. To cover the pI range from 5.0 to 8.0, the gel contained 1.6% (v/v) pH 5.0 to 8.0 ampholines and 0.4% (v/v) pH 3.0 to 10.0 ampholines. Protein extracts (200 μg) were subjected to IEF at 400 V for 15 h and at 800 V for 1 h, and the gels were equilibrated for 15 min in the SDS-PAGE sample buffer (2.3% [w/v] SDS, 10% [w/v] glycerol, 5% [v/v] 2-ME, 62.5 mM Tris-HCl, pH 6.8, and 0.1% [w/v] bromphenol blue), before loading onto slab gels for 12.5% (w/v) SDS-PAGE in the second dimension. The gel was stained with 0.25% (w/v) Coomassie brilliant blue R-250 in a mixture of 50% (v/v) methanol and 10% (v/v) acetate and destained in a solution of 50% (v/v) methanol and 10% (v/v) acetate.

Chemical and Enzymatic Digestion of Proteins and Amino Acid Sequence Analysis

Chemical or enzymatic digestion was used to determine the internal sequence of the proteins. Chemical digestion with CNBr was according to the method of Gross (1967) with the following modifications. Isolated protein spots from 50 2D PAGE gels were pooled by electroblotting onto a PVDF membrane according to the method of Towbin et al. (1979). Proteins were eluted from the membrane by soaking in a 10-fold volume of 70% (v/v) formic acid containing 1% (w/v) CNBr in a 1.5-mL microtube and incubating overnight at 4°C. The supernatant was collected, dried under reduced pressure, resuspended in the SDS-PAGE sample buffer, and incubated overnight at room temperature. Enzymatic digestion was performed according to the method of Cleveland et al. (1977) or Aebersold et al. (1987). After digestion of proteins, the peptides were separated by electrophoresis using Tricine/SDS-PAGE (Schägger and von Jagow, 1987) in 16.5% (w/v) acrylamide gels. Peptides were transferred by electroblotting onto a PVDF membrane and stained with Coomassie brilliant blue. Each band on the PVDF membrane was cut out and the amino acid sequence was determined by automated Edman degradation on a gas-phase sequencer (model 477A protein sequencer and model 120A PTH analyzer, Applied Biosystems).

FDH Assay

For the assay of FDH activity, the roots were homogenized in liquid nitrogen with a mortar and pestle, and the

Table I. *Sequences of CNBr digestion fragments*

Spot	Fragment	Sequence
W	N terminus	AHT?AGLKKI
	WCN-7	DTQAVADA?SRGHIA?YGS?
	WCN-6	FVLITGPFHAPYVTHGERIK
	WCN-5	AHTSAGSKKIVGVFYQAGEY
	WCN-4	RILKLLRN
	WCN-3	RILFKLRNFLPGYQQVMKGE
Y	N terminus	Blocked
	YCN-4	ENILHATPAPV
	YCN-3	?EQFFHLPA?DKA?LY?E
	YCN-2	GIQADYFEGD L?G?NVIL?I
C	N terminus	Blocked
	CLP-1	GKPGEVISEEYSLEYG?DKI
	CLP-2	RIPGEVIP
	CCNS-2	HVGHVSPNDRDLIVDDLIHQ
	CCNM-1	HDGAVAKLASRLGAKVVEIA

powder was resuspended in the following buffer: 100 mM Tris-HCl, pH 8.0, 1 mM PMSF, 1 mM EDTA, and 0.2% (w/v) Triton X-100. FDH activity on nondenaturing polyacrylamide gels was visualized according to the method of Uotila and Koivusalo (1979) as follows. A 7% (w/v) native acrylamide gel was used with the discontinuous buffer system of Laemmli (1970). One-hundred micrograms of soluble protein from roots or leaves was fractionated at 100 V for 2 h at room temperature and then incubated in darkness for 30 min at room temperature in the following solution: 100 mM sodium phosphate buffer, pH 7.0, 50 mM sodium formate, 0.8 mM NAD^+, 0.03 mg mL^{-1} phenazine methosulfate, and 0.4 mg mL^{-1} nitroblue tetrazolium.

Cloning of cDNA Encoding FDH

A pYH23 cDNA library prepared from poly(A^+) RNA of Fe-deficient barley roots (kindly provided by Hirotaka Yamaguchi, The University of Tokyo) was screened with an FDH PCR product corresponding to the partial amino acid sequences of FDH: GGIGTITTYTAYCARGCIGGIGARTAY and GCRTCIGCIACIGCYTGIGTRTCCAT (shaded sequences in Fig. 3). The PCR probe was labeled with a random-primer-labeling kit (version 2, Takara Biomedicals, Gennevilliers, France) in the presence of [α-^{32}P]dATP. The cloned cDNA was sequenced according to the protocol of a sequencing kit (Dye Terminator Cycle Sequencing Ready Reaction kit, Perkin-Elmer) using a DNA sequencer (model A373, Applied Biosystems). Hybridization probes for Southern blotting were prepared by digesting the cloned cDNA corresponding to the FDH gene (*Fdh*) with *Sac*I, and the smaller fragment (Fig. 3, underlined) was radiolabeled as described above. The labeled DNA was purified on a Nick column (Pharmacia) and used as a probe for both Southern and northern hybridization analyses.

Genomic Southern Hybridization

Barley genomic DNA was prepared from leaves by the method of Murray and Thompson (1980) using cetyltrimethylammonium bromide. The DNA was digested with *Bam*HI, *Eco*RI, or *Hin*dIII, separated on a 0.8% (w/v) agarose gel (30 μg per lane), and alkali-transferred onto a nylon membrane (Hybond-N^+, Amersham). The membrane was hybridized with the labeled *Sac*I fragment of *Fdh* with 5× SSPE, 4× Denhardt's solution, and 100 μg mL^{-1} salmon-sperm DNA at 65°C overnight. The washing conditions were three times with 2× SSPE plus 0.1% (w/v) SDS at 65°C for 30 min.

RNA Isolation and Northern Hybridization

Total RNA was isolated from roots or leaves according to the procedure of Logmann et al. (1987). RNA (10 μg per lane) was separated on 1.2% agarose gels containing 5% (v/v) formaldehyde and blotted onto nylon membranes (Hybond-N^+, Amersham). The membrane was hybridized with the *Sac*I fragment of *Fdh* under the same conditions described above. The washing conditions were: 6× SSPE at 65°C for 10 min and then twice with 2× SSPE plus 0.1% (w/v) SDS at 65°C for 10 min. The radioactivity was detected and quantified using an image analyzer (BAS-2000, Fuji, Tokyo, Japan).

RESULTS

2D Electrophoresis of Barley Root Proteins

Proteins prepared from Fe-sufficient and Fe-deficient roots and analyzed on 2D PAGE gels showed different protein patterns after Coomassie brilliant blue staining (Fig. 1). In the roots of Fe-deficient plants, the protein spots named C, D, G, V, W, X, and Y were present at higher concentrations than in Fe-sufficient plants (Fig. 1). Protein spots C, D, G, and V had previously been observed to increase during Fe deficiency (Mori et al., 1988; Suzuki et al., 1995), but the W, X, and Y spots were newly identified in this study.

Amino Acid Sequences of Each Protein

Among the seven proteins with increased concentrations in Fe-deficient roots, the D protein has been previously identified as a 36-kD peptide (Suzuki et al., 1995). Three proteins (C, W, and Y) were successfully sequenced. The N-terminal sequence of protein W was sequenced by Edman degradation, but the N termini of C and Y appeared to be blocked. The W protein appeared to be FDH (EC 1.2.1.2) based on homology of the sequences of internal peptides from CNBr digestion fragments (WCN-3, -4, -5, -6, and -7 in Table I) to other FDH sequences (the SwissProt and GenBank databases were used for the homology search). FDH was previously reported to be expressed in Fe-deficient roots of tomato and in the roots of the nicotianamine-free mutant *chloronerva* by Herbik et al. (1996). The C protein was transferred onto a PVDF membrane and digested with CNBr (CCNS-2 and CCNM-1 in Table I) or lysilendopeptidase (CLP-1 and CLP-2 in Table I). The sequences of peptides from C obtained by chemical or enzymatic digestion suggested that it is adenine phosphoribosyltransferase (EC 2.4.2.7). The sequences of CNBr digestion fragments

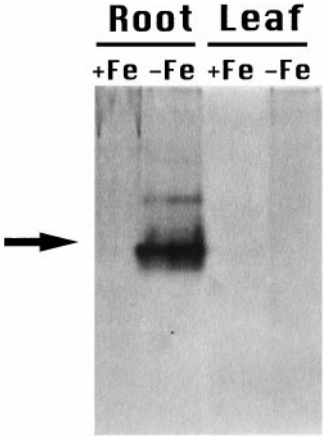

Figure 2. FDH assay on nondenaturing polyacrylamide gel. Each lane was loaded with 100 μg of soluble proteins from barley roots or leaves of Fe-sufficient (+Fe) or Fe-deficient (−Fe) plants.

from the Y protein (YCN-2, -3, and -4 in Table I) completely matched the translated products from the cDNA sequence of the *Ids3* gene (Nakanishi et al., 1993, accession nos. D37796 and D10058). Thus, FDH, adenine phosphoribosyltransferase, and the *Ids3* gene product showed increased accumulation in Fe-deficient barley roots.

FDH Assay

An increase in FDH activity (Fig. 2) was also detected in Fe-deficient roots, confirming the accumulation of FDH observed by 2D PAGE. FDH activity was not detected in Fe-deficient leaves, Fe-sufficient roots, or Fe-sufficient leaves at the protein concentration tested (100 μg).

Isolation of Barley cDNA Encoding FDH

A cDNA library prepared from poly(A^+) RNA of Fe-deficient barley roots was screened with a PCR probe designed to encode FDH (Fig. 3). Several clones were thus identified. The cloned cDNA encoded 377 amino acids of an open reading frame corresponding to a protein with a molecular mass of 41.5 kD and a pI of 6.7. The deduced molecular mass and pI were those of W in 2D PAGE. A sequence consisting of 21 amino acids at the N terminus might be a transit peptide targeted to mitochondria, based on comparison with potato (*Solanum tuberosum*) tuber FDH (Colas des Francs-Small et al., 1993). Comparison of barley FDH with FDH from other plants or bacteria is shown in Figure 4. The NAD^+-binding site from Lys-193 to Asn-228

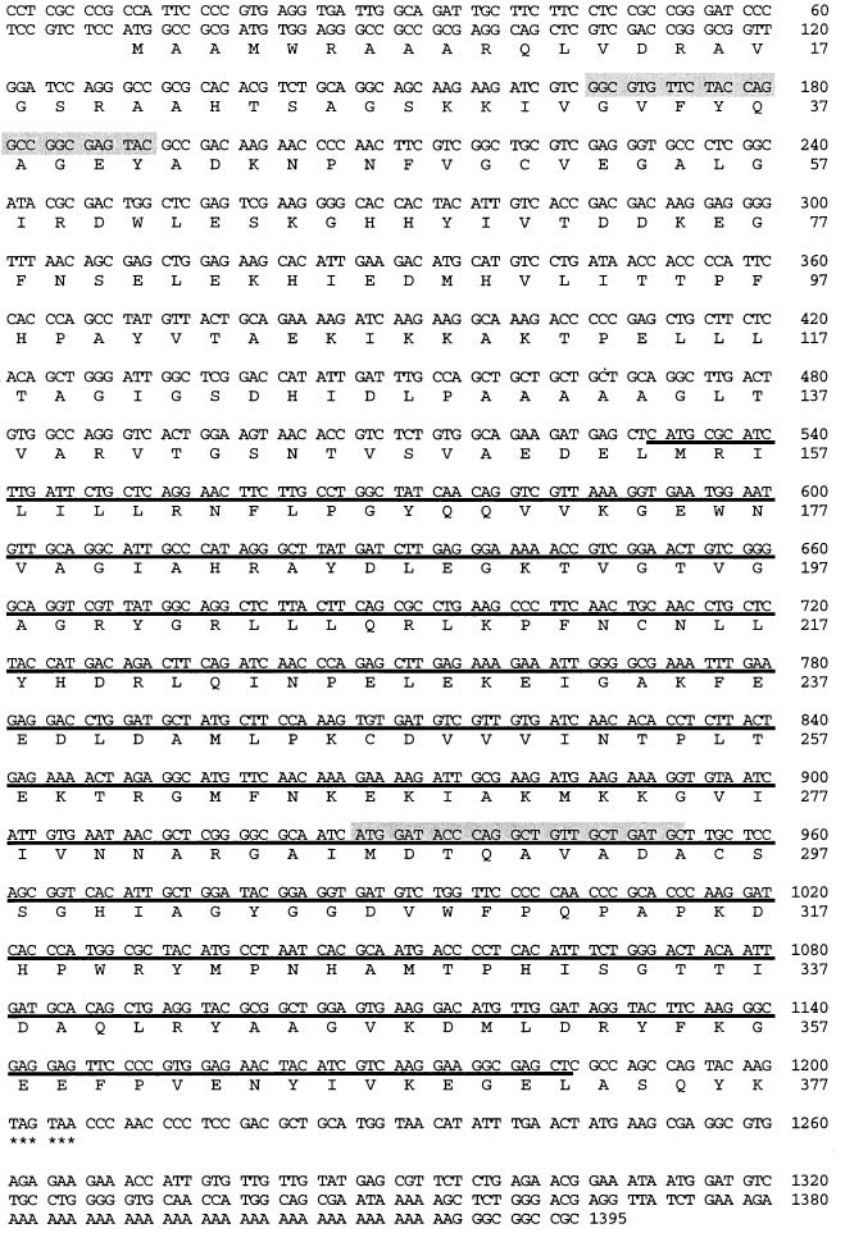

Figure 3. DNA and deduced amino acid sequence of barley FDH. Position of primers for PCR are shaded and the *Sac*I fragment is underlined.

Figure 4. Comparison of amino acid sequences from barley FDH with FDH from other organisms. The partial amino acid sequences from the W protein are underlined. The probable NAD^+-binding site is boxed, and the formate-binding site is shaded (Lamzin et al., 1992). The homology between barley FDH and other FDHs was 82.7% (*S. tuberosum*; Colas des Francs-Small et al., 1993), 53.0% (*N. crassa*; Chow and RajBhandary, 1993), 50.9% (*Pseudomonas* sp.; Tishkov et al., 1991), 50.0% (*H. polymorpha*; sequence A06214 was submitted to EMBL data bank by C.P. Hollenberg and Z. Janowicz in 1989), and 49.6% (*C. methylica*; Allen and Holbrook, 1995).

(Lamzin et al., 1992) and the formate-binding site from Arg-285 are indicated. The sequences were highly conserved between barley and potato in contrast to the sequences at the N terminus, which displayed very low sequence homology.

Southern Hybridization Analysis

The copy number of *Fdh* in barley was assessed by Southern hybridization analysis (Fig. 5). One fragment was observed in the *Bam*HI- and *Hin*dIII-digested DNA. Three fragments were detected in the *Eco*RI lane but the largest (10 kb) may represent an incomplete digestion product, since the sum of the molecular masses of the other two fragments (5.5 and 4.2 kb) is approximately 10 kb. Since there were no restriction enzyme sites for *Bam*HI, *Eco*RI, and *Hin*dIII in the cloned *Fdh* cDNA, we conclude that the *Fdh* gene is a single copy in the barley genome and that an *Eco*RI site is probably present within an intron.

Northern Hybridization Analysis

To investigate the expression of *Fdh*, northern hybridization analysis was performed (Fig. 6). In the control (Fe-sufficient) plants, no *Fdh* mRNA was detected in either the leaves or the roots (Fig. 6, compare +Fe leaf and +Fe root). In contrast, *Fdh* was strongly expressed in the roots of Fe-deficient plants but not in the leaves (Fig. 6, compare −Fe leaf and −Fe root).

The induction of *Fdh* expression required 1 d of Fe deficiency, with the amount of *Fdh* mRNA increasing gradually day by day (Fig. 7). After 14 d, *Fdh* expression reached a maximum and remained constant for 28 d. When Fe was resupplied in the form of Fe-EDTA to the culture solution of Fe-deficient plants, *Fdh* mRNA quickly diminished and was barely detectable on d 7 after the addition of Fe.

In bacteria and unicellular algae, formate is produced in large quantities under anaerobic conditions (Kreuzberg, 1984; Ferry, 1990). We therefore examined the expression of barley *Fdh* under anaerobic conditions. As in prokaryotes, *Fdh* expression increased in barley under anaerobic conditions (Fig. 8); the increase in *Fdh* mRNA began at 12 h compared with 1 d for Fe deficiency. The amount of *Fdh* mRNA present after 48 h of anaerobiosis was approximately equivalent to 10 to 14 d of Fe deficiency (quantification by the image analyzer is not shown).

DISCUSSION

Based on peptide sequencing of protein spot W, FDH was among the proteins induced by Fe deficiency in barley roots. FDH activity and mRNA were detected in Fe-deficient barley roots but were undetectable in Fe-deficient barley leaves (Figs. 2 and 6). In *Pseudomonas* sp. FDH,

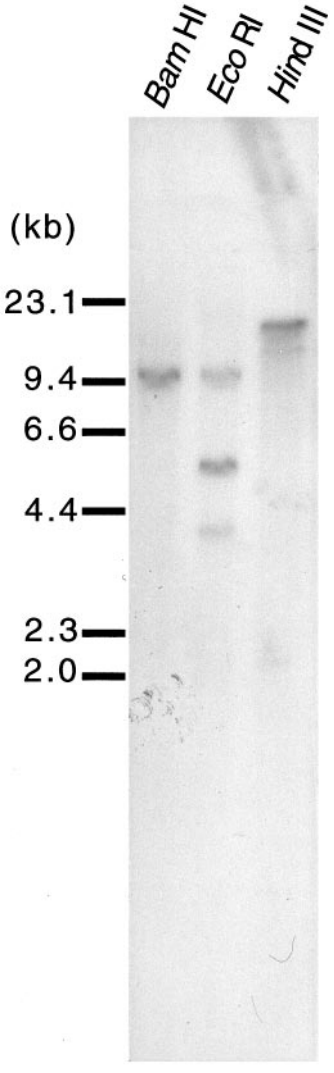

Figure 5. Southern hybridization analysis of *Fdh*. Genomic DNA from barley was digested with *Bam*HI, *Eco*RI, and *Hin*dIII and then blotted onto a nylon membrane and hybridized with a ^{32}P-labeled *Sac*I fragment of *Fdh*.

Arg-288 has been proposed to be a formate-binding site (Lamzin et al., 1994; Popov and Lamzin, 1994), and the His-341–Gln-317 pair is necessary for the binding of formate (Tishkov et al., 1996). These amino acids are conserved in all plant FDHs, including that of barley (Fig. 4). Yeast (*Hansenula polymorpha* and *Candida methylica*) FDH and *Neurospora crassa* FDH have two inserted regions that are absent in barley, potato, and *Pseudomonas* sp. (Fig. 3, residues 134–135 and 331–335).

Colas des Francs-Small et al. (1993) reported that FDH in potato tubers was located in the mitochondria. The N-terminal sequence of barley FDH shows little homology to potato FDH, but its hydropathy plot is similar to that of transit peptides for mitochondria targeting. Therefore, FDH activity detected in barley roots might be derived from mitochondria.

FDH mRNA was detectable after 1 d of Fe deficiency, reaching the maximal level after 2 weeks. A few days after

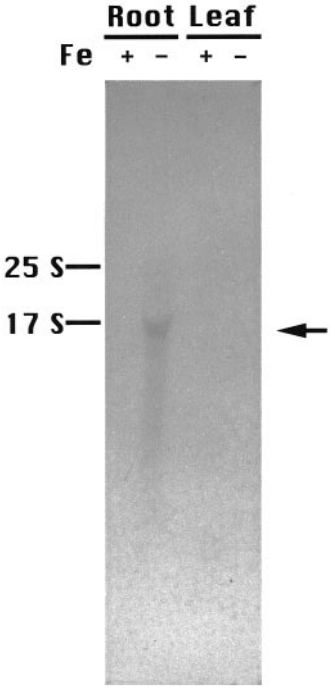

Figure 6. Northern hybridization analysis of *Fdh*. RNA was extracted from Fe-deficient (−) or Fe-sufficient (+) roots or leaves. Each lane was loaded with 10 μg of RNA. Total RNA was extracted after 14 d of Fe deficiency.

the addition of Fe into the Fe-deficient solution, *Fdh* expression decreased and on d 7 was undetectable. Although this inductive response pattern to Fe deficiency is very similar to that of *Ids3*, the response to the Fe resupply is slower (Nakanishi et al., 1993). *Ids3* is one of the clones we have isolated in barley roots by the differential hybridization method, and it supposedly encodes a putative mugineic acid synthase. We observed that the transcript of *Ids3* gene is increased by Fe deficiency and we have confirmed in this experiment that *Ids3* is actually translated and the *Ids3* protein is accumulated in Fe-deficient barley roots (Fig. 1; Table I).

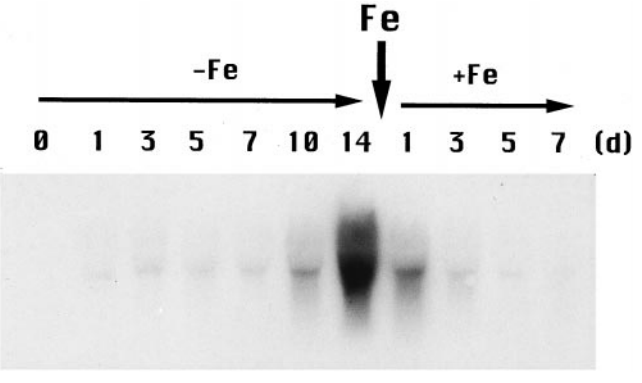

Figure 7. Expression of *Fdh* during Fe deficiency in barley roots. Each lane was loaded with 10 μg of RNA. Total RNA was isolated after 0, 1, 3, 5, 7, 10, and 14 d of Fe deficiency and 1, 3, 5, and 7 d after Fe resupply.

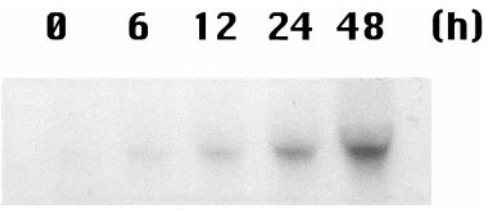

Figure 8. Expression of *Fdh* under anaerobic conditions. Each lane was loaded with 10 μg of RNA. Total RNA was isolated after 0, 6, 12, 24, and 48 h of anaerobic treatment.

In addition to Fe-deficient conditions, the barley *Fdh* was also expressed under anaerobic conditions (Fig. 8). Moreover, this inductive response to anaerobic stress began 12 h after treatment and was more rapid than the response to Fe deficiency. Formate is reported to be produced in large quantities in bacteria (Ferry, 1990) and unicellular algae (Kreuzberg, 1984) under anaerobic conditions. Colas des Francs-Small et al. (1993) suggested that a major, uncharacterized metabolic pathway exists in the mitochondria of nonphotosynthetic tissues, which produces large quantities of formate. Therefore, induction of FDH by anaerobic stress in barley roots (Figs. 7 and 8) is conceivable in light of the above-mentioned results in bacteria and algae.

The more rapid response of *Fdh* transcript to anaerobic stress than that to Fe deficiency indicates that the expression of *Fdh* is primarily induced by anaerobic stress. The expression of *Fdh* in Fe-deficient barley roots suggests that Fe deficiency caused changes similar to the ones caused by anoxia through changes in heme biosynthesis. For example, Fe regulates the biosynthesis of δ-aminolevulinic acid (by δ-aminolevulinic acid synthase; Pushnik and Miller, 1989) and protoporphyrinogen IX (by co-proporphyrinogen III oxidase). These are the common precursors of protoporphyrin IX, from which chlorophyll *a* in the chloroplasts and heme in the mitochondria are synthesized. Moreover, Fe regulates the biosynthesis of divinyl protochlorophyllide (by Mg-protoporphyrin IX monomethylester cyclase), which is the precursor of chlorophyll in the chloroplasts (von Wettstein et al., 1995).

Fe is also incorporated into protoporphyrin IX to become heme in the mitochondria. Therefore, Fe deficiency not only lowers the amount of chlorophyll in the chloroplasts of shoots but also the amount of heme in the mitochondria of both shoots and roots (Marschner, 1995). Since large amounts of heme are needed for energy production by the respiratory chain, the inhibition of energy production by decreasing respiration in mitochondria could be caused by Fe deficiency. In addition, we previously reported that Fe deficiency in rice roots caused morphological malformation of mitochondria and a decrease in the energy charge from 0.748 to 0.520 (Mori et al., 1991). Therefore, anaerobiosis-like changes may be produced in mitochondria of Fe-deficient barley roots.

Anaerobic stress causes acute damage to the plant by reducing available energy, which may result in its turning to formate metabolism to produce NADH by FDH. On the other hand, Fe deficiency may cause anoxia by the depletion of Fe from heme and, secondarily, by the depletion of heme protein as the result of inhibition of heme biosynthesis. If the metabolism of formate in plant roots is similar to that of bacteria, as was suggested by Colas des Francs-Small et al. (1993), the formate pathway would be induced either by the decrease of heme (Fe deficiency) or by reduced electron transport in the respiratory chain of mitochondria (anaerobiosis). In conclusion, FDH induction might be caused by the anoxia induced by Fe deficiency in spite of the presence of oxygen.

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