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Time course analysis of gene expression over 24 hours in Fe-deficient barley roots

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Abstract Typical for a graminaceous plant, barley secretes mugineic acid-family phytosiderophores (MAs) to acquire iron (Fe). Under Fe-deficient conditions, MAs secretion from barley roots increases markedly. Secretion shows a diurnal pattern, with a clear peak 2–3 h after sunrise and cessation within a few hours. Microarray analyses were performed to profile the Fe deficiency-inducible genes in barley roots and diurnal changes in the expression of these genes. Genes encoding enzymes involved in MAs biosynthesis, the methionine cycle, and methionine biosynthesis were highly induced by Fe deficiency. The expression of sulfate transporters was also upregulated by Fe deficiency. Therefore, all of the genes participating in the MAs pathway from sulfur uptake and assimilation to the biosynthesis of MAs were upregulated in Fe-deficient barley roots. In contrast to MAs secretion, the transcript levels of these genes did not show diurnal changes. The amount of endogenous MAs gradually increased during the day after MAs secretion ceased, and was highest before secretion began. These results show that MAs biosynthesis, including the supply of the substrate methionine, occurs throughout the day, and biosynthesized MAs likely accumulate in barley roots until their secretion into the rhizosphere. In contrast, the levels of transcripts encoding an Fe(III)–MAs complex transporter, two putative metal–MAs complex transporters, and HvYS1 were also increased in Fe-deficient barley roots, and the levels of two of these transcripts showed diurnal rhythms. The Fe(III)–MAs complex transporters may absorb Fe(III)–MAs diurnally, synchronous with the diurnal secretion of MAs.

Keywords Barley · Fe-deficiency · Microarray · Mugineic acid

Abbreviation
MAs Mugineic acid family phytosiderophores

Introduction

Iron (Fe) is an essential nutrient for plant growth and crop productivity. Although it is abundant in soil, it is not very soluble and not easily available to plants under aerobic conditions in the physiological pH range. Higher plants have evolved two major strategies for Fe acquisition (Marschner et al. 1986). Graminaceous plants secrete mugineic acid family phytosiderophores (MAs) from their roots to chelate and solubilize Fe in the rhizosphere (Takagi 1976); Fe(III)–MAs complexes are then absorbed through Fe(III)–MAs complex transporters in the cell membrane (Curie et al. 2001; Murata et al. 2006). The production and secretion of MAs increases markedly in response to Fe deficiency (Takagi et al. 1984). The biosynthetic pathway to produce MAs from L-methionine has been clarified through intensive physiological and biochemical studies (Mori and Nishizawa 1987; Shojima et al. 1990; Ma et al. 1999; Bashir et al. 2006). The genes encoding enzymes that participate in the MAs biosynthetic pathway are highly induced during Fe deficiency (Higuchi et al. 1999; Takahashi et al. 1999; Nakanishi et al. 2000; Kobayashi et al. 2001). The genes of the methionine cycle are also upregulated in Fe-deficient

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rice and barley plants (Kobayashi et al. 2005; Fig. 1). Barley secretes a larger amount of MAs, especially mugineic acid and 3-epihydroxymugineic acid, than other graminaceous plants, and is therefore more tolerant of low Fe availability. The secretion of MAs by barley roots follows a distinct diurnal rhythm (Takagi et al. 1984; Marschner et al. 1986). A peak in secretion occurs just after the onset of illumination and ceases within 2–3 h. This diurnal secretion may help prevent the microbial degradation of MAs in the rhizosphere (Römheld and Marschner 1990).

To dissect the molecular mechanisms of MAs secretion, microarray analysis was performed in barley plants grown under Fe-deficient conditions. The first investigation of comprehensive gene expression in Fe-deficient plants was performed in barley roots using a rice cDNA array containing 8,987 (9 K array) rice expressed sequence tags (Negishi et al. 2002). This experiment showed that many genes involved in MAs biosynthesis and the methionine cycle are induced by Fe deficiency in barley roots. The rice 9 K array has also been used to study expression in rice roots, and it has been shown that the genes involved in MAs biosynthesis and the methionine cycle are also upregulated in Fe-deficient rice roots (Kobayashi et al. 2005). Several researchers have used microarray analysis to examine gene expression in non-graminaceous plants subjected to Fe deficiency (Thimm et al. 2001; Wang et al. 2002; Wintz et al. 2003). Several regulatory, signaling, and transporter genes are induced by Fe deficiency in tomato (*Lycopersicon esculentum* L.) roots (Wang et al. 2002), while in *Arabidopsis*, several genes involved in respiration and metal transport are induced in the roots and shoots (Thimm et al. 2001; Wintz et al. 2003).

Recently, microarray chips containing barley genes have become commercially available. In this study, we conducted a time-course microarray analysis using a barley DNA chip to elucidate changes in expression that occur over a 24-h period in Fe-deficient barley roots.

Materials and methods

Plant material

Seeds of barley (*Hordeum vulgare* L. cv Ehimehadaka no. 1) were germinated, and the seedlings were grown hydroponically as described previously (Higuchi et al. 1996) under a daily light regime of 14 h of light (22°C) and 10 h of dark (17°C). To induce Fe deficiency, plants were transferred to culture solutions lacking Fe 2 weeks after germination. After 2 weeks of Fe-deficiency treatment (i.e., when symptoms of severe Fe deficiency appeared), the roots of the Fe-sufficient and -deficient plants were harvested every 3 h from the onset of illumination. Three plants were harvested at each time point and stored at −80°C until RNA and endogenous MAs extraction. We

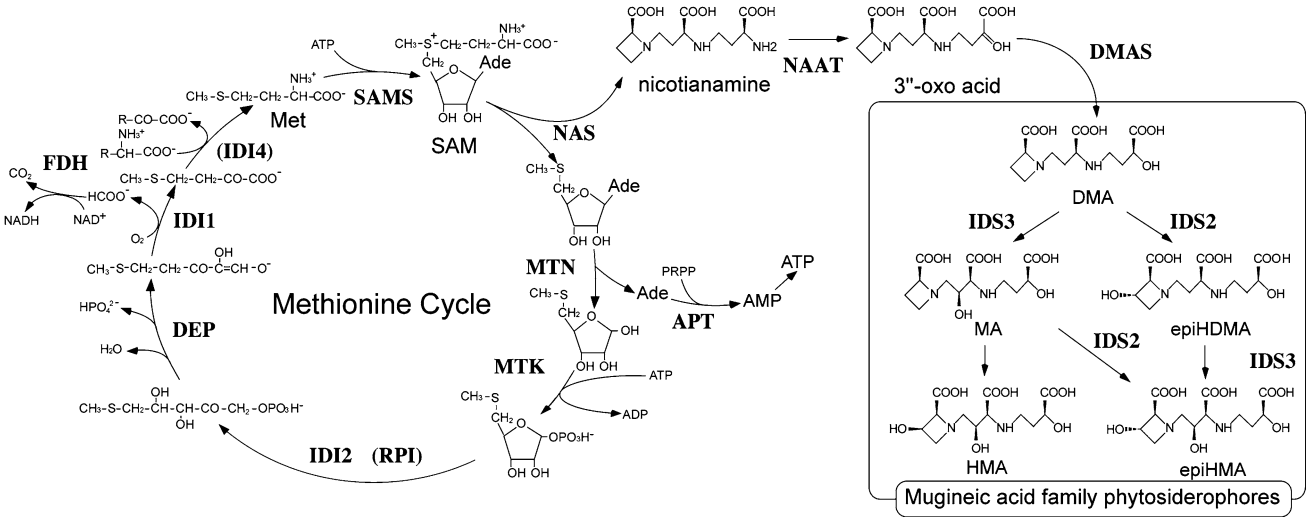

Fig. 1 The methionine cycle and the MAs biosynthetic pathway in graminaceous plants. All of the genes except *SAMS* were induced by Fe deficiency and showed no diurnal expression rhythm. *SAMS* *S*-adenosyl-Met synthase; *NAS* nicotianamine synthase; *NAAT* nicotianamine aminotransferase; *DMAS* 2′-deoxymugineic acid synthase; *IDS2* and *IDS3* deoxygenases catalyzing the hydroxylation of MAs; *DMA* 2′-deoxymugineic acid; *MA* mugineic acid; *epiHDMA* 3-epihydroxy-2′-deoxymugineic acid; *epiHMA* 3-epihydroxymugineic acid; *MTN* methylthioadenosine/*S*-adenosyl homocysteine nucleosidase; *MTK* methylthioribose kinase; *IDI2* eukaryotic initiation factor 2B-like methylthioribose-1-phosphate isomerase; *RPI* putative ribose-5-phosphate isomerase; *DEP* methylthioribulose-1-phosphate dehydratase-enolase-phosphatase; *IDI1* 2-keto-methylthiobutyric-acid-forming enzyme; *IDI4* putative aminotransferase catalyzing the synthesis of Met from 2-keto-methylthiobutyric acid; *FDH* formate dehydrogenase; *APT* Ade phosphoribosyltransferase; *PRPP* phosphoribosyl pyrophosphate. *Hordeum vulgare* L. cv Ehimehadaka no. 1 secretes three types of MAs: DMA, MA, and epiHMA

duplicated the plant preparation and harvest, and different plant materials were used for microarray analysis and Northern blotting.

Microarray analysis

Total RNA was extracted from Fe-deficient roots harvested 0, 6, 12, and 18 h after the onset of illumination and from Fe-sufficient roots harvested 0 and 12 h after the onset of illumination. The RNAs were labeled, fragmented using a One Cycle Target Labeling Kit (Affymetrix, Santa Clara, California, USA), and used for microarray analysis (single replicates). The prepared probes were hybridized to Affymetrix GeneChip® Barley Genome Arrays, and the array chips were washed, stained, and scanned on a GeneChip Scanner 3000 (Affymetrix) according to the manufacturer's recommendations. The six data sets were analyzed using Affymetrix GCOS software. Each data set was linearly normalized using a scaling method to a mean signal intensity of 500 units. Those genes with a signal intensity of <200 were excluded from the subsequent analysis. To select for Fe deficiency-inducible genes, gene expression in the Fe-deficient roots at the start of illumination (0 h) and after 12 h of illumination (12 h) was compared with that in the Fe-sufficient roots at 0 and 12 h, respectively. Those genes with an induction ratio ≥2.0 in the Fe-deficient roots at 0 or 12 h were recognized as Fe deficiency-inducible genes. For the time-course analysis of gene expression in the Fe-deficient roots, the microarray data for the Fe-deficient roots at 6, 12, and 18 h were compared with those for the Fe-deficient roots at 0 h. Those genes showing diurnal changes similar to the pattern of MAs secretion from Fe-deficient barley roots were identified among the Fe deficiency-inducible genes. Changes in gene expression were determined using GCOS software. Genes whose expression was lower at two time points or more than at 0 h were designated as diurnally regulated.

Northern blot analysis of *HvYS1* and putative Fe–MAs complex transporter genes

Northern blot analysis was conducted with ^{32}P-labeled probes specific for *HvYS1* (AB214183), two putative Fe–MAs complex transporter genes (UniGenes 18532 and 12454; HarvEST ver. 1.50, http://www.harvest.ucr.edu), and genes encoding enzymes that participate in the methionine cycle (*IDI1*, *IDI2*, and *IDI4*; Yamaguchi et al. 2000a, b; Kobayashi et al. 2005). The primers were designed based on the 3′-UTR regions of *HvYS1* and two other genes: *HvYS1*, 5′-AATAGGCATACTTGATTAAAG TTAT-3′ and 5′-GTGTTTTGTTTTACTGGAGATGCTT-3′; UniGene 18532, 5′-TACTTCAAGCCATCTCTTGC CTAGGGCGTAA-3′ and 5′-AACAGAGCTGCTGCTAT TTTTGCAA-3′; and UniGene 12454, 5′-ACTAGATGAG TGTCATGCCTGATA-3′ and 5′-ATCTTCACACCCAGT CTTATACTCAT-3′. Using these primers, fragments were amplified by polymerase chain reaction (PCR) with a cDNA library synthesized from the mRNAs of Fe-deficient barley roots. The fragments were ligated into a vector (pCR®-Blunt II; Invitrogen, Carlsbad, California, USA) and sequenced. The vector was then digested with *Eco*RI and each fragment was purified. Specific probes were synthesized from each purified fragment using a Random Primer DNA Labeling Kit (Takara, Tokyo, Japan). For *IDI1*, *IDI2*, and *IDI4*, excised ORFs were used as templates to synthesize specific probes. A CsCl-gradient ultracentrifugation method (Glisin et al. 1974) was used to extract total RNA from barley roots harvested every 3 h. The materials were prepared at different times and were not the same plant materials used for our microarray analysis. Total RNA (10 μg) was separated, blotted, and hybridized with the probes as described previously (Higuchi et al. 1999). Radioactivity was detected using an FLA-5000 image analyzer (Fuji Film, Tokyo, Japan).

Extraction and detection of endogenous MAs

Endogenous MAs were purified from the time-course samples of Fe-deficient and -sufficient barley roots described above. Endogenous MAs were extracted as described previously (Higuchi et al. 2001). The extracted MAs were concentrated and detected by HPLC using a method developed for detecting MAs secreted from roots (Mori and Nishizawa 1987). *Hordeum vulgare* L. cv Ehimehadaka no. 1 secretes three types of MAs: DMA, MA, and epiHMA.

Results

Fe deficiency-inducible genes in barley roots

A barley GeneChip was used to analyze the gene expression profile in barley roots under Fe-deficiency stress. At 0 or 12 h, 82 genes were identified as induced by Fe deficiency (Table 1). Many of these genes had already been reported to be Fe deficiency-inducible, and almost all of them were involved in MAs biosynthesis. Seven genes encoding isozymes of nicotianamine (NA) synthase (NAS), which catalyzes the first step in MAs biosynthesis, were induced under Fe deficiency. Two NA aminotransferase genes (*HvNAAT-A* and *HvNAAT-B*), a deoxymugineic acid synthase gene (*HvDMAS1*), and *IDS2* and *IDS3* were also found to be inducible by Fe

Table 1 List of genes upregulated in Fe-deficient barley roots 0 and 12 h after the start of illumination

Spot no.	Unigene no.	GenBank hit	Putative gene identification	Induction ratio	
				0 h	12 h
MAs biosynthesis					
Contig21662_at	20753	AB022688	*Houdeum vulgare*, HvNAS1	2.5	3.0
Contig10740_at	10925*	AB011265	*Hordeum vulgare*, HvNAS2	3.2	6.1
AB011264_at	26828*	AB011264	*Hordeum vulgare*, HvNAS3	3.7	11.3
AB011266_at	26829*	AB011266	*Hordeum vulgare*, HvNAS4	4.9	21.1
Contig23870_s_at	23520	AB011268	*Hordeum vulgare*, HvNAS5	4.9	10.6
Contig10741_at	8246	AF136941	*Hordeum vulgare*, HvNAS6	3.7	13.0
AB019525_at	23984	AB019525	*Hordeum vulgare*, HvNAS7	4.9	24.3
Contig6722_at	4946	AF108438	*Hordeum vulgare,* HvDMAS1	3.2	4.6
Contig7288_at	5430	D88273	*Hordeum vulgare*, HvNAAT-A	7.0	13.0
Contig7287_at	5429	AB005788	*Hordeum vulgare*, HvNAAT-B	2.1	1.9
D10057_at	25375	D10057	*Hordeum vulgare*, IDS2	13.9	12.1
D37796_at	25377	AB024058	*Hordeum vulgare*, IDS3	4.6	12.1
Methionine cycle					
HF18A22r_s_at	983	D88272	*Hordeum vulgare*, formate dehydrogenase	7.5	18.4
Contig5085_at	3613	AF536565	Saccharum hybrid cultivar, phosphoribosyl pyrophosphate	6.1	7.5
Contig7835_at	5675	AY142637	*Arabidopsis thaliana*, putative ribose 5-phosphate isomerase (RPI)	5.7	9.8
HS07O05u_s_at	4389	AB206815	*Horderum vulgare*, IDI4	5.7	10.6
Contig5719_at	4121	AY593959	*Oryza sativa*, methylthioribose kinase (MTK1)	5.3	6.1
Contig2725_at	1849	AB038775	*Hordeum vulgare*, IDI2	3.5	4.0
Contig3205_s_at	2194	AB025597	*Hordeum vulgare*, IDI1	3.2	4.0
Contig17201_at	14374	AF113125	*Homo sapiens*, E-1 enzyme (MASA)	2.8	3.5
Contig7611_at	5593	AF458088	*Oryza sativa*, methylthioadenosine/*S*-adenosyl homocysteine nucleosidase	2.1	2.8
Fe–MAs complex transporter					
Contig16464_at	13343	AB214183	*Hordeum vulgare*, HvYS1	4.3	4.6
Contig21611_at	18532	AT5G24380	*Arabidopsis thaliana* metal-nicotianamine transporter YSL2	4.3	6.1
Contig16506_at	12454	AY515564	*Arabidopsis thaliana* metal-nicotianamine transporter YSL6	2.0	1.1
Fe-deficiency inducible gene					
AY013246_CDS-5_at	24057	AB063580	*Hordeum vulgare*, tonoplast ABC transporter IDI7	9.2	8.6
Contig13800_at	9987	AB022764	*Hordeum vulgare*, D-protein	3.0	5.3
Contig2421_at	1643	X58540	*Horderum vulgare*, IDS1	2.0	2.5
Sulfur metabolism					
Contig12465_at	9445	AF297044	*Zea mays*, homocysteine *S*-methyltransferase-1	7.0	5.7
Contig8619_at	6398	AK176573	*Arabidopsis thaliana*, serine acetyltransferase (Sat-106)	2.6	3.0
Contig20387_at	17003	X82454	*Stylosanthes hamata*, low affinity sulphate transporter	2.3	1.7
Contig23272_at	19207	AF453838	*Zea mays*, satase(serine acetyl transferase) isoform II	2.3	3.7
Contig18260_at	14849	AAA97952	*Hordeum vulgare*, High affinity sulfate transporter HVST1	2.1	1.3
Contig2776_at	1863	AK099593	*Zea mays*, ATP sulfurylase	1.7	2.0

Table 1 continued

Spot no.	Unigene no.	GenBank hit	Putative gene identification	Induction ratio	
				0 h	12 h
Transporters					
Contig7895_at	5937	AK175547	*Arabidopsis thaliana*, putative peptide transporter (PTR2-B)	3.5	2.8
Contig16199_at	13330	AK176369	*Arabidopsis thaliana*, putative tetracycline transporter protein	3.0	2.8
Contig24221_at	22333	BT005105	*Arabidopsis thaliana*, putative ABC transporter protein	2.5	4.0
Components of translation					
HS08O16u_s_at	261	AY049041	*Triticum aestivum*, 28S ribosomal RNA	4.9	6.5
Contig377_s_at	257	AF168852	*Hordeum jubatum*, 18S small subunit ribosomal RNA	4.6	1.5
Contig376_at	244	AY049041	*Triticum aestivum*, 28S ribosomal RNA	4.0	3.2
HVSMEc0009K16f_s_at		Y082604	Saccharum hybrid cultivar, 23S ribosomal RNA	2.5	1.4
MitoContig7_at		P41096	*Horderm vulgare*, chloroplast 50S ribosomal protein L2	2.0	0.6
Regulation of transcription					
Contig16361_at	12290	AF532781	*Zea mays*, putative zinc finger protein	3.5	2.3
Contig13717_at	10850	AB028132	*Oryza sativa*, Dof zinc finger protein	2.8	3.5
Contig24365_at	23806	AF532781	*Zea mays*, putative zinc finger protein	2.8	3.0
Contig19502_at	16447	S59852	*Zea mays*, Dof2 zinc finger protein	2.0	1.5
Others					
Contig5311_at	3821	AB086416	*Hordeum vulgare*, *O*-methyltransferase	13.0	2.6
Contig17933_at	14786	AX653652	*Oryza sativa*, plant genes involved in defense against pathogens	4.6	4.6
Contig6208_at	4462	AY035393	*Arabidopsis thaliana*, putative sterol 4-alpha-methyl-oxidase	3.2	2.1
HVSMEf0021O16r2_s_at	3006	AY093159	*Arabidopsis thaliana*, putative aspartate aminotransferase	3.0	4.3
Contig14427_at	12096	AY451241	*Cynodon dactylon*, FAD-linked oxidoreductase BG60	2.8	4.0
Contig6435_at	4646	E12730	*Spartina anglica*, phosphoenolpyruvate carboxykinase	2.3	2.3
rbags22p06_s_at	8706	X56877	*Zea mays*, transposable element Bg sequence	2.3	1.3
Contig23510_at	22877	BAC80125.1	*Oryza sativa*, putative beta-1,3-glucanase	2.3	2.3
Contig18717_at	16481	AF289633	*Arabidopsis thaliana*, inositol polyphosphate 5-phosphatase I	2.1	1.5
Contig19861_at	16354	AY691949	*Zea mays*, alcohol dehydrogenase 1 (adh1A)	2.1	4.6
Contig6358_at	4609	DQ234265	*Glycine max*, salt-tolerance protein	2.0	1.0
Contig15634_at	13306	AF190634	*Nicotiana tabacum*, UDP-glucose:salicylic acid glucosyltransferase	2.0	2.3
Contig6509_at	4745	AK059981	*Xenopus tropicalis,* nitrilase family member 2	1.9	3.2
Contig2424_at	20901	AK065481	*Hordeum vulgare*, putative integral membrane protein	1.9	2.5
Contig3640_at	2510	AK103315	*Triticum aestivum,* gamma-glutamylcysteine synthetase (GSH1) mRNA	1.7	2.0
Contig8727_s_at	6330	AK066328	*Arabidopsis thaliana,* putative formamidase	1.7	2.3
Contig12803_at	12615	AP005311	*Ananas comosus,* class-1 LMW heat shock protein	1.7	2.1
Unknown					
HK04A15r_at	35194*		Unknown	52.0	13.9
Contig11420_at	9109	L27809	*Actinidia deliciosa* var. deliciosa, unknown protein	24.3	13.0

Table 1 continued

Spot no.	Unigene no.	GenBank hit	Putative gene identification	Induction ratio	
				0 h	12 h
Contig6033_at	6179*		Unknown	9.2	6.1
Contig10522_at	8107	BD340740	Newly found single nucleotide polymorphisms (SNPs)	4.0	1.9
Contig21245_at	16627	BT002001	*Arabidopsis thaliana*, putative protein	3.7	2.6
Contig23467_at	21412	AY114725	*Arabidopsis thaliana*, unknown protein	3.2	3.7
HB20B10r_at	32977*		Unknown	3.2	0.5
HVSMEf0012L17f_at	35243		Unknown	3.0	2.6
Contig9283_at	7127	AK111009	*Oryza sativa*, putative protein	2.6	2.5
EBro08_SQ009_G15_at	31575*		Unknown	2.6	1.4
Contig24328_at	23373	AY062857	*Arabidopsis thaliana*, unknown protein	2.5	1.0
Contig17966_s_at	11986		Unknown	2.5	1.1
Contig5871_at	4312		Unknown	2.3	2.3
Contig25290_at	22766		Unknown	2.3	1.5
Contig4992_at	5094*		Unknown	2.1	1.4
Contig19563_at	17173		Unknown	2.0	1.7
EBro04_SQ004_C02a_at	27230		Unknown	2.0	1.9
Contig24105_at	14023	AK066068	*Oryza sativa*, putative protein	1.7	2.1
Contig19320_at	16660	AK063130	*Oryza sativa*, putative protein	1.4	2.1
Contig15697_at	12613		Unknown	0.7	2.6

UniGene no. indicates genes designated by HarvEST version 1.50; *asterisks* indicate the UniGene number from HarvEST version 1.35. Genes were considered upregulated if the induction ratio was $\geq$2.0

deficiency. In addition to genes involved in MAs biosynthesis, Fe deficiency also upregulated genes encoding all enzymes of the methionine cycle (Fig. 1) with the exception of the SAM synthase gene (*SAMS*). Because MAs are synthesized from *S*-adenosyl-L-methionine (SAM), methionine recycling is essential for MAs synthesis. Furthermore, genes encoding sulfate transporters and enzymes involved in the methionine biosynthetic pathway were induced. In total, 21 of the 82 Fe deficiency-inducible genes were related to MAs biosynthesis. In addition to these genes and those involved in sulfur assimilation, the Fe(III)-MAs transporter gene *HvYS1* was found to be upregulated in Fe-deficient barley roots. *HvYS1* transports Fe–MAs complexes from the rhizosphere to barley roots, and the expression of *HvYS1* is increased in Fe-deficient barley roots (Murata et al. 2006). Two genes homologous to *HvYS1* were also induced by Fe deficiency. Because the genes expressed in the Fe-sufficient roots changed between 0 and 12 h, the induction ratio of several genes also changed. In particular, the expression of *NAS2-7* in the Fe-sufficient plants decreased at 12 h compared to at 0 h; therefore, the induction of these genes by Fe deficiency was significantly increased at 12 h (data not shown).

Fe deficiency-inducible genes showing diurnal rhythms

To examine the diurnal rhythm in the expression of Fe deficiency-inducible genes under Fe-deficient conditions, additional microarray analyses were performed. Expression in the Fe-deficient roots at sunrise (0 h) was compared with that in the Fe-deficient roots at 6, 12, and 18 h after sunrise. The expression of 14 of the Fe deficiency-inducible genes shown in Table 1 showed diurnal changes in the Fe-deficient roots, similar to the MAs secretion pattern (Table 2). However, the levels of transcripts encoding enzymes involved in MAs biosynthesis and the methionine cycle did not change diurnally. For example, the transcript levels of *IDI1*, *IDI2*, and *IDI4*, which participate in the methionine cycle, were confirmed by Northern blot analysis (Figs. 1, 2a). The expression of these genes showed no diurnal changes, remaining high in the Fe-deficient roots throughout the 24-h period monitored. These expression patterns were in line with our microarray data.

In contrast, the expression of *HvYS1* and a putative metal–MAs complex transporter gene (UniGene 12454, which shows similarity to *AtYSL6*) showed diurnal rhythms, and both genes were downregulated at 6 and 12 h (Table 2). Northern blot analysis confirmed that the levels

Table 2 Diurnally regulated Fe deficiency-inducible genes in Fe-deficient barley roots

Spot no.	Unigene no.	Induction ratio by Fe-deficiency	Fold of changes to 0 h				Description by GCOS			Gene identification
			0 h	6 h	12 h	18 h	6 h	12 h	18 h	
Contig16464_at	13343	4.3	1.0	0.8	0.7	1.0	D	D	NC	*Hordeum vulgare*, HvYS1
Contig16506_at	12454	2.0	1.0	0.7	0.4	0.7	D	D	D	*Arabidopsis thaliana*, metal-nicotianamine transporter YSL6
Contig5311_at	3821	13.0	1.0	0.3	0.2	1.2	D	D	NC	*Hordeum vulgare*, *O*-methyltransferase,
HS08O16u_s_at	261	4.9	1.0	0.5	0.5	0.8	D	D	NC	*Triticum aestivum*, 28S ribosomal RNA gene
Contig376_at	244	4.0	1.0	0.9	0.5	0.8	NC	D	D	*Triticum aestivum*, 28S ribosomal RNA gene
Contig16361_at	12290	3.5	1.0	0.4	0.6	1.1	D	D	NC	*Zea mays*, putative zinc finger protein
Contig12465_at	9445	7.0	1.0	0.7	0.7	0.9	D	D	NC	*Zea mays*, homocysteine *S*-methyltransferase-1
Contig6208_at	4462	3.2	1.0	0.7	0.8	1.1	D	D	NC	*Arabidopsis thaliana*, putative sterol 4-alpha-methyl-oxidase
Contig6435_at	4646	2.3	1.0	0.6	0.7	1.1	D	D	NC	*Spartina anglica*, phosphoenolpyruvate carboxykinase.
Contig6358_at	4609	2.0	1.0	1.1	0.5	0.3	NC	D	D	*Glycine max*, salt-tolerance protein
HB20B10r_at	32977*	3.2	1.0	0.4	0.1	0.8	D	D	NC	Unknown
HVSMEf0012L17f_at	35243	3.0	1.0	0.7	0.7	0.9	D	D	NC	Unknown
EBro08_SQ009_G15_at	31575*	2.6	1.0	0.5	0.6	0.8	D	D	NC	Unknown
Contig18260_at	14849	2.1	1.0	0.8	0.4	0.8	NC	D	D	Unknown

UniGene no. indicates genes designated by HarvEST version 1.50; *asterisks* indicate the UniGene number from HarvEST version 1.35. Gene expression in the Fe-deficient roots at sunrise (0 h) was compared to that in the Fe-deficient roots at 6, 12, and 18 h after sunrise. Genes designated 'D' (decrease) and 'NC' (no change) were detected using GCOS software

of these transcripts increased under Fe deficiency (Fig. 2b). The expression of *HvYS1* and UniGene 12454 was high at the start of illumination, gradually decreased during illumination, and increased again at the start of darkness. However, the transcript levels of another gene that encodes a putative metal–MAs complex transporter (UniGene 18532, homologous to *AtYSL2*) did not change during the day.

Degree of endogenous MAs change during the day in Fe-deficient barley roots

The amount of endogenous MAs in the roots was increased in the Fe-deficient barley, reaching 1,000 times the level in the Fe-sufficient roots at the start of illumination (Fig. 3). Furthermore, the amount of endogenous MAs showed a diurnal rhythm in the Fe-deficient roots, peaking at the start of illumination then decreasing to its lowest level 6 h later. The amount of endogenous MAs then gradually increased again until the start of illumination.

Discussion

We used a DNA microarray designed from barley genes to examine the gene expression profile of Fe-deficient barley roots over 24 h. Among the 25,500 genes on the array, 82 were identified as Fe deficiency-inducible. Twenty of these genes had already been reported to be inducible by Fe deficiency, indicating the reliability of the plant material and the method for expression profiling in Fe-deficient barley plants.

The methionine cycle, which recycles methionine, is required for MAs biosynthesis in Fe-deficient barley roots (Ma et al. 1995; Fig. 1), and a number of genes related to the methionine cycle are Fe deficiency-inducible in barley and rice roots (Negishi et al. 2002; Kobayashi et al. 2005). Our microarray analysis showed that all of the genes involved in the methionine cycle, with the exception of *SAMS*, were induced by Fe deficiency in barley roots. The intensity of the *SAMS* signal was high, and its level of expression was among the top 50 on the array. Because SAM plays various roles in plants, *SAMS* expression is likely to occur at high levels. Therefore, the induction ratio of *SAMS* during Fe deficiency might be lower than that of other genes involved in the methionine cycle. In addition to the genes involved in this process, genes involved in MAs biosynthesis were highly upregulated in the Fe-deficient barley roots.

The transport of sulfate into the cell is a major regulatory step in sulfur metabolism (Vauclare et al. 2002). There is clear evidence that the levels of transcripts encoding transporters involved in the initial steps of sulfur uptake from the rhizosphere and in the vascular transport of sulfate

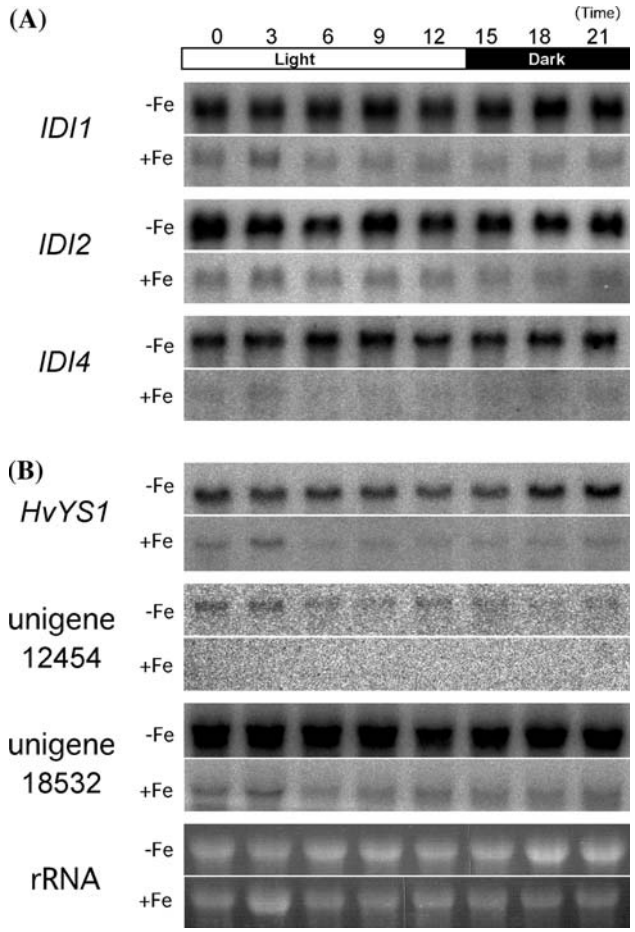

Fig. 2 Northern blot analysis reveals diurnal changes in the transcript levels of barley genes encoding iron-phytosiderophore transporters and enzymes participating in the methionine cycle. UniGenes 12454 and 18532 encode putative transporter genes. *IDI1*, *IDI2*, and *IDI4* encode enzymes that participate in the methionine cycle. Time (0) means the start of illumination

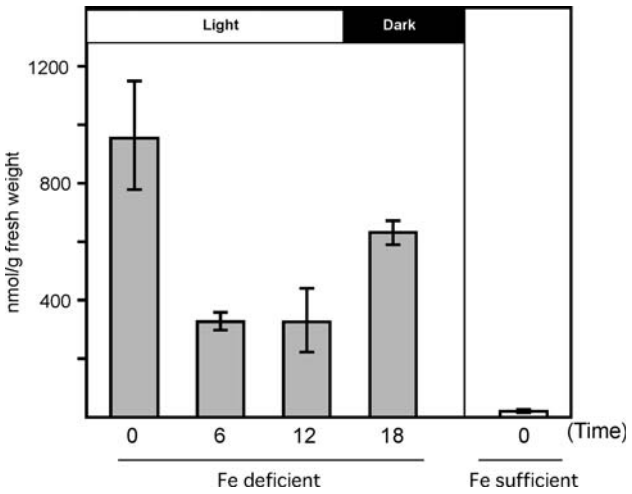

Fig. 3 Diurnal changes in the amount of endogenous MAs in Fe-deficient and -sufficient barley roots. MAs include DMA, MA, and epiHMA. Time (0) means the start of illumination. *Error bars* represent the standard deviation ($n = 3$)

are controlled by the sulfur supply (Buchner et al. 2004). Genes encoding a high-affinity sulfate transporter and a putative low-affinity sulfate transporter were upregulated in the Fe-deficient barley roots (Table 1). Sulfate transporters have been classified into groups based on their localization, kinetic properties, and sequence similarity (Grossman and Takahashi 2001). High-affinity transporters are expressed primarily in the roots of plants undergoing sulfate starvation; they contribute to sulfate uptake but not to the transport of sulfur to the shoots (Shibagaki et al. 2002; Yoshimoto et al. 2002). Low-affinity transporters are expressed in vascular tissues, and may be involved in the transport of sulfate within the plant. The induction of high- and low-affinity sulfate transporter genes during Fe deficiency suggests that sulfur uptake and translocation increase to meet the increased demand for methionine to support MAs production. In addition, several genes involved in the methionine biosynthetic pathway were upregulated by Fe deficiency. An increase in sulfur assimilation is crucial for the response of barley roots to Fe deficiency. As MAs constitute 1–2% of the root dry weight in Fe-deficient barley, increased methionine synthesis seems to be essential for the proper functioning of the methionine cycle under Fe-deficient conditions.

One-fourth of the genes upregulated by Fe deficiency were related to MAs biosynthesis, indicating that MAs biosynthesis is a major event in barley roots under Fe deficiency. Our time-course microarray analysis of Fe-deficient roots revealed that the level of transcription of all of the genes involved in the methionine cycle, MAs biosynthesis, and sulfate transport was continuously high over a 24-h period. The expression of *IDI1*, *IDI2*, and *IDI4*, which participate in the methionine cycle, was confirmed by Northern blot analysis. In accordance with our microarray data, the expression of these genes was highly induced by Fe deficiency but did not change diurnally (Fig. 2a). We previously reported that NAS and NAAT activity is induced by Fe deficiency, and that the activity of these proteins is unchanged during the day (Kanazawa et al. 1995). *NAS* and *NAAT* transcription in the Fe-deficient barley roots was strongly correlated with NAS and NAAT activity. This suggests that MAs biosynthesis occurs throughout the day, whereas Fe-deficient barley roots secrete MAs diurnally (Takagi et al. 1984; Marschner et al. 1986).

The change in the endogenous amount of MAs in Fe-deficient barley is opposite to the pattern of MAs secretion. MAs secretion peaks 2–3 h after sunrise (Takagi et al. 1984; Marschner et al. 1986). In contrast, the levels of endogenous MAs were highest at the start of illumination and then decreased, reaching their lowest levels 6 h after sunrise. The time points corresponding to the highest and

lowest levels of endogenous MAs fell before and after MAs secretion, respectively. Thus, the amount of endogenous MAs increased gradually throughout the day (Fig. 3). Our data indicate that MAs are biosynthesized continuously throughout the day and accumulate in the roots until secretion.

In contrast to the expression of genes involved in MAs biosynthesis, the transcript levels of *HvYS1* and UniGene 12454, a putative metal–MAs complex transporter gene, were not only induced by Fe deficiency but also showed diurnal changes (Table 2, Fig. 2). Their expression patterns paralleled the secretion of MAs from barley roots. Peak expression was observed at the start of illumination (0 h), after which it decreased during the day and increased during the night. HvYS1 has been reported to be responsible for the uptake of Fe–MAs complexes by barley roots because of its localization and substrate specificity (Murata et al. 2006). The change in the expression of *HvYS1* suggests that the uptake of Fe–MAs complexes occurs mainly during the period of MAs secretion. Diurnal MAs secretion may help avoid MAs degradation by microorganisms (Römheld and Marschner 1990), and the high transport activity of Fe–MAs complexes may be required just after MAs secretion.

Recent studies have shown that plants have many *YS1*-like genes, each with a different expression pattern and function in plant cells (Curie et al. 2001; DiDonato et al. 2004; Koike et al. 2004; Jean et al. 2005; Murata et al. 2006). Our microarray analysis revealed that genes encoding two candidate metal–MAs complex transporters, UniGenes 12454 and 18532, are upregulated in Fe-deficient barley roots, like *HvYS1*. Barley roots secrete mainly MA and epiHMA under Fe-deficient conditions. HvYS1 transports Fe–MAs complexes but not other metal–MAs complexes (Murata et al. 2006). However, the transporters that take up Fe-epiHMA or other metal–MAs complexes have not yet been reported. As the expression pattern of UniGene 12454 was similar to that of *HvYS1*, 12454 is a promising candidate for the uptake of other metal–MAs complexes from the rhizosphere. UniGene 18532 is homologous to *Arabidopsis YSL2* (*AtYSL2*). AtYSL2 transports Fe(II) and copper in complexes with NA (DiDonato et al. 2004), and plays a role in intracellular metal transport (Schaaf et al. 2005). The level of UniGene 18532 transcription was maintained throughout the day in the Fe-deficient barley roots, unlike the expression of *HvYS1* and UniGene 12454 (Fig. 2). The protein encoded by this gene may be involved in the intracellular transport of metals and in Fe homeostasis.

In contrast to the barley genes, the expression of genes involved in MAs biosynthesis in rice, *OsNAS1*, *OsNAS2*, *OsNAAT1*, and *OsDMAS1*, is induced by Fe deficiency and shows diurnal changes in Fe-deficient roots (Nozoye et al. 2004). The Fe deficiency-responsive *cis*-acting elements IDE1 and IDE2 were identified in the promoter region of *HvIDS2* (Kobayashi et al. 2003). Sequences homologous to IDE1 are also present in Fe deficiency-inducible genes in *Arabidopsis*, barley, and rice, including the above genes, and these elements act in rice and barley roots under conditions of Fe deficiency (Kobayashi et al. 2003). Recently, the rice transcription factor IDEF1, which specifically binds IDE1, was identified (Kobayashi et al. 2007). IDEF1 regulates the response to Fe deficiency in plants. In addition, promoter elements conferring diurnal expression have been identified in the promoter regions of the above four rice genes, and these elements are thought to contribute to their diurnal expression in Fe-deficient rice roots (Nozoye et al. 2004). The evening element (EE; AAATATCT) is a motif for evening-specific expression in *Arabidopsis* (Harmer et al. 2000), and a mutation in this element could cause a shift in rhythm from evening- to dawn-specific expression (Harmer and Kay 2005). The CBS element (CIRCADIAN CLOCK ASSOCIATED 1 binding site; AAAAATCT) is another motif for the diurnal regulation of gene expression, and this element differs from the EE element by only one nucleotide (Michael and McClung 2002). In addition, some clock-controlled genes predicted by enhancer trapping analysis do not have an exact EE sequence or CBS elements but contain a single mismatched sequence (Michael and McClung 2003). The expression of rice genes, which contain EE- or CBS-like elements, is regulated diurnally (Nozoye et al. 2004). We previously reported the genomic fragments of *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, *IDS2*, and *IDS3*, which participate in MAs biosynthesis (Higuchi et al. 1999; Takahashi et al. 1999; Nakanishi et al. 2000; Kobayashi et al. 2001). To examine the differences in the diurnal expression of these genes in rice and barley roots under Fe-deficient conditions, we searched for CBS and EE elements in the promoter regions of the barley genes (Table 3). The promoter regions of these genes contained EE-like sequences, a CBS element, and a CBS-like element, similar to the rice genes. Interestingly, more of these elements were present

Table 3 Number of CBS and EE elements in the promoter regions of barley genes involved in MAs synthesis

Gene name	Promoter regeon	CBS	EE
HvNAS1	1957	1 (9)	0 (7)
HvNAATA	2405	0 (14)	0 (4)
HvNAATB	660	0 (1)	0 (0)
IDS2	1702	0 (8)	0 (1)
IDS3	2243	1 (10)	0 (4)

CBS CCA1 binding site (AAAAATCT); *EE* evening element (AAAATATCT). The numbers of exact matches and one mismatch are shown in *parentheses* in the promoter regions

in the promoter region of the barley genes than the rice genes, although the expression of these genes in the Fe-deficient barley roots showed no diurnal pattern. These results suggest that Fe deficiency is dominant over diurnal changes in the expression of MAs biosynthesis-related genes in barley roots. However, these results do not mean that there is no diurnal regulation of Fe deficiency-inducible genes in barley roots. For example, the level of transcription of *HvYS1* and UniGene 12454, a putative metal–MAs complex transporter gene, showed diurnal changes in addition to induction by Fe deficiency. Because the sequences of the promoter regions of these transporter genes have not been reported, it is unknown whether they contain the *cis* elements described above. The transcript levels of these transporter genes are regulated by two factors, Fe deficiency and the diurnal cycle, which is different from the expression of genes involved in MAs biosynthesis. The biosynthesis of large amounts of MAs probably requires the expression of genes involved in MAs biosynthesis to remain high throughout the day. In contrast, MAs are secreted diurnally from Fe-deficient barley roots (Takagi et al. 1984; Marschner et al. 1986), and the expression of genes encoding metal–MAs complex transporters may be synchronized with MAs secretion to effectively take up Fe- and metal–MAs complexes from the rhizosphere. This regulation of the expression of genes involved in MAs biosynthesis and Fe–MAs metal complex transporters probably plays a key role in the ability of barley to tolerate low Fe availability.

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