Expression of iron-acquisition-related genes in iron-deficient rice is co-ordinately induced by partially conserved iron-deficiency-responsive elements

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Abstract

Rice plants (Oryza sativa L.) utilize the iron chelators known as mugineic acid family phytosiderophores (MAs) to acquire iron from the rhizosphere. Synthesis of MAs and uptake of MA-chelated iron are strongly induced under conditions of iron deficiency. Microarray analysis was used to characterize the expression profile of rice in response to iron deficiency at the genomic level. mRNA extracted from iron-deficient or iron-sufficient rice roots or leaves was hybridized to a rice array containing 8987 cDNA clones. An induction ratio of greater than 2.0 in roots was observed for 57 genes, many of which are involved in iron-uptake mechanisms, including every identified or predicted step in the methionine cycle and the biosynthesis of MAs from methionine. Northern analysis confirmed that the expression of genes encoding every step in the methionine cycle is thoroughly induced by iron deficiency in roots, and almost thoroughly induced in leaves. A promoter search revealed that the iron-deficiency-induced genes related to iron uptake possessed sequences homologous to the iron-deficiency-responsive cis-acting elements IDE1 and IDE2 in their promoter regions, at a higher rate than that showing no induction under Fe deficiency. These results suggest that rice genes involved in iron acquisition are co-ordinately regulated by conserved mechanisms in response to iron deficiency, in which IDE-mediated regulation plays a significant role.

Key words: Iron deficiency-inducible expression, iron-deficiency-responsive elements, methionine cycle, microarray analysis, mugineic acid family phytosiderophores, Oryza sativa.

Introduction

Plant growth and reproduction can be severely affected by various biotic and abiotic stresses. Among the abiotic stresses, iron (Fe) deficiency constitutes a major factor leading to a reduction in crop yield, especially in calcareous soils in which the solubility of Fe is extremely low, owing to the high pH. Higher plants have evolved two major strategies for Fe acquisition (Römheld and Marschner, 1986). Non-graminaceous plants use Strategy I, which involves proton excretion from the roots to the rhizosphere, reduction of Fe(III) chelates at the root surface to reduce Fe(III) to the more soluble Fe(II) form, and transport of the generated Fe(II) ions across the root plasma membrane. In contrast, graminaceous plants use Strategy II, which is mediated by the synthesis and secretion of natural Fe chelators, the mugineic acid family phytosiderophores (MAs). The secreted MAs solubilize Fe(III) in the rhizosphere, and the resulting Fe(III)–MA complexes are reabsorbed into the root cells (Takagi, 1976). Synthesis and secretion of MAs are dramatically enhanced under Fe-deficient conditions. The biosynthetic pathway of MAs from methionine (Met) has been identified in intensive physiological and biochemical studies (Mori and Nishizawa, 1987; Shojima et al., 1990; Ma et al., 1999).

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Abbreviations: EST, expressed sequence tag; IDE, iron-deficiency-responsive element; Met, methionine; MAs, mugineic acid family phytosiderophores.

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The Met cycle is highly active in roots, enabling these organs to meet the increased demand of Met required for the synthesis of MAs (Ma et al., 1995; Fig. 1).

Efforts to elucidate the mechanisms of Fe acquisition at the molecular level have led to the identification of many graminaceous genes that respond to Fe deficiency. Barley (Hordeum vulgare L.) genes have been isolated that encode all of the enzymes in the biosynthetic pathway of MAs from Met (HvSAMS, Takizawa et al., 1996; HvNAS, Higuchi et al., 1999; HvNAAT, Takahashi et al., 1999; HvDMAS, Negishi et al., 2002b; HvIDS3, Nakanishi et al., 1993, 2000; HvIDS2, Okumura et al., 1994). Rice (Oryza sativa L.) orthologues of the NAS and NAAT genes have also been isolated (OsNAS, Higuchi et al., 2001a; OsNAAT, Inoue et al., 2004). Expression of most of these genes in roots is strongly induced by Fe deficiency. Several Fe-deficiency-inducible barley genes that are closely related to the Met cycle have also been isolated, including the genes that encode Fe-deficiency-induced protein 1 (HvID1; Yamaguchi et al., 2000a), formate dehydrogenase (HvFDH; Suzuki et al., 1998), and adenine phosphoribosyltransferase (HvAPT; Itai et al., 2000; Fig. 1).

**Fig. 1.** The methionine cycle and the biosynthetic pathway of MAs in graminaceous plants. The expression of the depicted enzymes was found in previous studies, or the present study, to be induced by Fe deficiency. Promoters of rice genes corresponding to the underlined enzymes contain IDE1-like (straight line) or IDE2-like (wavy line) sequences, as confirmed in Table 2. Rice lacks the IDS2 and IDS3 dioxygenases and only produces 2'-deoxymugineic acid (DMA). Met, methionine; Ade, adenine; SAM, S-adenosyl-Met; SAMS, S-adenosyl-Met synthetase; NAS, nicotianamine synthase; NAAT, nicotianamine aminotransferase; DMAS, 2'-deoxymugineic acid synthase; IDS2 and IDS3, dioxygenases catalysing the hydroxylation of MAs; DMA, 2'-deoxymugineic acid; MA, mugineic acid; epiHDMA, 3-epihydroxy-2'-deoxymugineic acid; epiHMA, 3-epihydroxymugineic acid; MTN, methylthioadenosine/5'-adenosyl homocysteine nucleosidase; MTK, methylthioborate kinase; IDI2, eukaryotic initiation factor 2B-like methylthioborate-1-phosphate isomerase; RPI, putative ribose-5-phosphate isomerase; DEP, methylthiobululose-1-phosphate dehydratase-endorphosphatase; IDI1, 2-keto-methylthiobutyric-acid-forming enzyme; IDI4, putative aminotransferase catalysing the synthesis of Met from 2-keto-methylthiobutyric acid; FDH, formate dehydrogenase; APT, Ade phosphoribosyltransferase; PRPP, phosphoribosyl pyrophosphate.
addition to these genes involved in MA biosynthesis, other Fe-deficiency-inducible genes from barley have been isolated, including a metallothionein-like gene (HvIIS1; Okumura et al., 1991), an eIF2B2-like gene (HvIID2; Yamaguchi et al., 2000b), and a tonoplastic-located ABC-type transporter gene (HvIIT1; Yamaguchi et al., 2002). All of the Fe-deficiency-inducible genes isolated from barley are expressed almost exclusively in the roots. Recently, genes involved in Fe transport have also been isolated. Curie et al. (2001) isolated the maize (Zea mays L.) gene encoding the Fe(III)-MA transporter YS1, whose expression is induced by Fe deficiency both in roots and shoots. Of the 18 putative YS1 homologues of rice (OYSILs), OYSIL2 is expressed in Fe-deficient leaves, and has been shown to encode a Fe(II)-nicotianamine and manganese(II)-nicotianamine transporter (Koike et al., 2004). Expression of OYSIL15 and OYSIL16 is induced in Fe-deficient roots. Buglio et al. (2002) isolated the rice Fe(II) transporter gene, OsIRTI1, which is homologous to the Arabidopsis thaliana Fe(II) transporter gene AtIRTI1 (Eide et al., 1996). Many of the identified rice Fe-deficiency-inducible genes are expressed in leaves as well as roots.

Despite these important findings, the responses of plants to Fe nutritional status have not yet been fully characterized. Microarray techniques have recently emerged as a comprehensive approach for analysing the expression profile of genes at the genomic level. Several researchers have used these techniques with Strategy I plants subjected to Fe-deficiency treatment. Several regulatory, signalling, and transporter genes are induced, similarly, by phosphorus, potassium, and Fe deficiencies in tomato (Lycopersicon esculentum L.) roots (Wang et al., 2002). In Fe-deficient Arabidopsis plants, several genes involved in respiration and metal transport are induced in roots and shoots (Thimm et al., 2001; Wintz et al., 2003). The expression of many of Fe-deficiency-induced genes in Arabidopsis roots is deregulated in the mutant of the AbHHLH29 gene (FIT1; Colangelo and Guerinot, 2004), which is homologous to the tomato gene FER encoding a putative basic helix-loop-helix (bHLH) transcription factor indispensable for the Strategy I response (Ling et al., 2002; Bauer et al., 2004). To analyse changes in the expression profiles of Fe-deficient Strategy II plants, mRNA extracted from Fe-deficient or Fe-sufficient barley roots was hybridized with a rice ‘9k microarray’ containing 9897 expressed sequence tags (ESTs; Negishi et al., 2002a). Many genes involved in MA synthesis, together with a number of genes possibly involved in the diurnal secretion of MAs, are induced by Fe deficiency in barley roots.

In the present study, microarray analysis was carried out using the rice 9k microarray and rice mRNA extracted from Fe-deficient or Fe-sufficient roots. The genes induced by Fe deficiency in rice roots included genes involved in every identified or predicted step of MA biosynthesis from Met as well as the Met cycle. The response of these genes to Fe deficiency was confirmed by northern analysis. Furthermore, these Fe-acquisition-related genes induced by Fe deficiency contained sequences homologous to the recently identified iron-deficiency-responsive cis-acting elements IDE1 and IDE2 (Kobayashi et al., 2003) in the promoter regions, at a higher rate than genes whose expression was not affected by Fe deficiency. These results suggest that rice genes related to Fe acquisition are co-ordinately regulated by conserved mechanisms in response to Fe deficiency.

### Materials and methods

**Plant material and growth conditions**

Rice seeds (Oryza sativa L. cv. Nipponbare) were germinated on paper soaked with distilled water. After germination, the seedlings were transferred to a Saran net floating on nutrient solution in a greenhouse, with a 14/10 h, 30/25 °C, light/dark cycle. The composition of the nutrient solution was as follows: 0.70 mM K₂SO₄, 0.10 mM KCl, 0.10 mM KH₂PO₄, 2.0 mM Ca(NO₃)₂, 0.50 mM MgSO₄, 10 μM H₂B₄O₁₂, 0.50 μM MnSO₄, 0.50 μM ZnSO₄, 0.20 μM CuSO₄, 0.01 μM (NH₄)₂MoO₄, and 100 μM Fe(III)-EDTA. The pH of the nutrient solution was adjusted daily to 5.5 with 1 M HCl, and the nutrient solution was renewed weekly. When the fourth leaf appeared, the seedlings were transferred to a 20 l plastic container containing nutrient solution with or without Fe(III)-EDTA and grown for 2 weeks.

**Microarray analysis**

Total RNA was extracted from roots and leaves of Fe-deficient or Fe-sufficient rice plants using the caesium chloride/guanidine method. Microarray analysis was performed as described on the Rice Microarray Opening Site (http://cdna01.dna.affrc.go.jp/RMOS/pdf/array_protocol_e.pdf). The hybridized rice array (‘9k microarray’) contained 8987 individual ESTs with lengths of approximately 0.5–2.0 kb. Hybridization analysis was performed twice, and the mean induction ratio of genes in Fe-deficient roots or leaves to Fe-sufficient roots or leaves was calculated.

**Northern blot analysis**

Total RNA was extracted from roots or leaves using the method of Prescott and Martin (1987), and 10 μg per lane were electrophoresed in 1.2% (w/v) agarose gels containing 0.66 M formaldehyde and transferred to Hybond-N⁺ membrane (Amersham, USA). Full-length rice cDNA clones were acquired from the Rice Full-Length cDNA Database (KOME; http://cdna01.dna.affrc.go.jp/cDNA), as described below, for use as probes. The accession numbers of these cDNAs are as follows: AK065872 for 9k array element no. 8150, AK120288 for no. 5925, AK065321 for no. 8935, AK067649 for no. 7070, AK060326 for no. 8217, AK103443 for no. 3187, AK066172 for no. 7033, AK071516 for no. 7043, AK099947 for no. 3251, AK073627 for no. 3328, AK068661 for no. 7260, AK065890 for no. 7330, AK067869 for no. 8170, AK068387 for no. 5629, AK070275 for no. 7315, and AK103445 for no. 2581. To detect the expression of OsSAMS1 (element no. 7778), full-length cDNA of accession number AK072051 was digested using BamHI and the 3'-fragment, composed mainly of the 3'-uncoding region, was used as a specific probe. For 9k array element no. 8336, the EST itself was used to prepare a probe because no full-length cDNA clone was available. Full-length cDNA fragments of AK120288, AK060326, AK071516, AK066172, AK068387, AK070275, 3'-fragment of AK072051, and the EST fragment of clone no. 8336 were 32P-labelled and hybridized at 65 °C
as described by Kobayashi et al. (2001). The other full-length cDNA fragments were labelled with digoxigenin (DIG) and hybridized at 68 °C as described by Engler-Blum et al. (1993) and Yoshihara et al. (2003).

**Promoter analysis**

The sequences of the ESTs were matched to full-length rice cDNA clones by searching the KOME site with BLAST. All full-length cDNA clones with an E-value of less than e-50 were considered to be close homologues that would cross-hybridize in microarray and northern hybridization analyses. The promoter regions within 1.5 kb upstream of the putative translation start sites of the full-length cDNA clones were obtained. The promoter sequences of ESTs with no corresponding full-length cDNAs were estimated to begin 1.5 kb upstream of the first ATG in the EST sequence. Arabidopsis gene promoters within 1.5 kb upstream of the putative translation start sites were obtained from The Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org/).

Those promoters were divided into each category and searched for IDE1- and IDE2-like sequences, as well as other cis-acting elements. IDE1 and IDE2 consist of two and three modules of nine bases, respectively (Kobayashi et al., 2003). Mutations of any of the nine-base modules lead to an almost complete loss of function. Therefore, sequences at least 66% identical to IDE1 (i.e. six or more identical bases out of nine bases) at both of the two nine-base modules were defined as homologous to IDE1. Since this definition led to the identification of very few sequences homologous to IDE2, a less strict definition permitting some discrepancy at the outside borders was used to identify sequences homologous to the putative IDE2 core. Among the sequences with at least 14 identical bases to the 27-base overall sequence of IDE2, those bearing six or more bases identical to IDE2 out of the nine bases in the central nine-base module, and at least 66% identical in both of the two flanking modules, allowing up to six bases located at the outside borders to be disregarded, were assumed to be IDE2-like. The search also looked for promoters possessing authentic TGACG, G-box (CACGTG), or AFT1 consensus binding sites (PyPuCACCCPu). Sequences with at least 12 identical bases to the consensus IDRS sequence between the ZmFer1 and AtFer1 promoters (CACGAGNCCNCCAC) were assumed to be IDRS-like.

**Results**

**Up-regulation of all genes involved in the Met cycle, as well as in the biosynthesis of MAs from S-adenosyl-Met in Fe-deficient rice roots**

A microarray containing 8987 rice ESTs (9k microarray) was used to analyse the gene expression profile of rice roots and leaves under conditions of Fe deficiency. The induction ratio was calculated as the relative increase in expression under conditions of Fe deficiency, compared with the expression under Fe-sufficient conditions. In total, 57 genes (71 clones) showed an induction ratio of greater than 2.0 in rice roots. Genes encoding enzymes involved in the biosynthetic pathway of MAs were dominant among the clones whose expression was strongly induced in roots (Table 1). These genes covered all of the identified or predicted steps of the production of MAs from S-adenosyl-Met, and of the Met cycle (Fig. 1). Expression of a gene encoding formate dehydrogenase (OsFDH), which is closely linked to the Met cycle, was also strongly induced in Fe-deficient roots. OsNAS2, OsNAAT1, OsFDH, and a gene encoding methythioribulose-1-phosphate dehydratase- 

**Higher occurrence of sequences homologous to Fe-deficiency-responsive cis-acting elements in the promoter regions of the Fe-deficiency-induced genes**

The novel Fe-deficiency-responsive cis-acting elements IDE1 and IDE2, which synergistically mediate Fe-deficiency-specific gene expression in tobacco (Nicotiana tabacum L.) roots (Kobayashi et al., 2003), have previously...
been identified. Seventy-two promoter sequences of the genes that showed an induction ratio of more than 2.0 in roots were identified in rice genomic databases and examined for sequences homologous to IDE1 and IDE2. Twenty-two of the Fe-deficiency-induced genes possessed sequences highly homologous to the 18-base overall sequence of IDE1 within 1.5 kb upstream of the putative translation start site (Table 2). Interestingly, the promoter regions of six of the ten genes with the highest induction ratios, and all four genes with the highest ratios, possessed sequences homologous to IDE1.

In contrast to IDE1, sequences homologous to the entire 27-base sequence of IDE2 were only rarely found in the promoter regions of the genes analysed, regardless of the induction ratio. However, a modified search method for putative core IDE2 sequences (see Materials and methods) identified IDE2-like sequences in the promoter region of 14 of the Fe-deficiency-induced genes (Table 2). Notably, some of the induced genes, especially those involved in the biosynthesis of MAs, possessed multiple IDE-like sequences in their promoter regions. For example, the promoters of OsNAS2, the closest homologue of OsFDH, as well as the closest homologue of OsID1, contained two IDE1-like sequences and one IDE2-like sequence. The promoter of the methylthioribose kinase gene (OsMTK1) also possessed both IDE1-like and IDE2-like sequences. In addition, a metallothionein gene, OsIDS1, whose barley homologue exhibits strong Fe-deficiency-inducible expression (Okumura et al., 1991), carried three IDE1-like sequences within its 1.5 kb promoter region, one of which was also homologous to IDE2, and showed a 1.8-fold induction ratio in roots (Table 2).

Northern blot analysis was carried out to confirm the induction pattern of some of the genes with promoters containing IDE1-like sequences (Fig. 2B). Gene elements 8336 and 7260 increased expression in response to Fe

Table 1. Clones related to the synthesis of MAs, the Met cycle, or Fe uptake showing an induction ratio greater than 2.0 in Fe-deficient roots

<table>
<thead>
<tr>
<th>Element no.</th>
<th>Element no.</th>
<th>Accession no.</th>
<th>Putative gene identification</th>
<th>Induction ratio</th>
<th>Fe-deficiency-induced expression confirmed by northern analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6254</td>
<td>AU176507</td>
<td>Nicotianamine synthase OsNAS2</td>
<td>23.3 7.6 Root, leaf (Inoue et al., 2003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6307</td>
<td>D25143</td>
<td>Nicotianamine aminotransferase OsNAAT1</td>
<td>21.2 9.4 Root, leaf (Inoue et al., 2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7783</td>
<td>AU173091</td>
<td>Nicotianamine aminotransferase OsNAAT1</td>
<td>7.4 3.4 Root, leaf (Inoue et al., 2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8956</td>
<td>D24418</td>
<td>Nicotianamine synthase OsNAS1</td>
<td>6.0 1.8 Root, leaf (Higuchi et al., 2001a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5949</td>
<td>AU173810</td>
<td>Deoxymugineic acid synthase OsDMAS1</td>
<td>2.1 1.3 Root (Nozoye et al., 2004)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Related to Met cycle**

8150 D23770 Formate dehydrogenase OsFDH 9.6 2.6 Root, leaf (this report)
925 AU164691 Aminotransferase OsID4a 9.1 1.2 Root, leaf (this report)
8935 D23987 Dehydratase-enzyme-phosphatase DEP 7.5 2.0 Root, leaf (this report)
7070 D25025 Methylthioribose kinase OsMTK1 6.2 1.3 Root, leaf (this report)
6258 AU031747 Methylthioribose kinase OsMTK1 5.1 1.3 Root, leaf (this report)
6332 AU030270 Methylthioribose kinase OsMTK1 4.7 1.1 Root, leaf (this report)
7386 D23753 Methylthioribose kinase OsMTK1 2.9 1.0 Root, leaf (this report)
8217 D24651 elf2B-like methylthioribose-1-phosphate isomerase OsID1a 3.9 1.0 Root, (leaf) (this report)
972 C71966 elf2B-like methylthioribose-1-phosphate isomerase OsID2b 2.8 0.9 Root, (leaf) (this report)
3187 C26897 ribose-5-phosphate isomerase RPIb 3.1 1.5 Root, leaf (this report)
7033 AU031688 Methylthioadenosine/3'-adenosyl homocysteine nucleosidase MTN 3.1 1.0 Root (this report)
7043 D24436 S-adenosylmethionine synthetase OsSAMS2 2.6 0.9 Root, (leaf) (this report)
3251 C28663 OxID1a; submergence induced protein sip2 2.5 1.5 Root, leaf (this report)
5328 AU1172608 Adenine phosphoribosyltransferase OsAPTlc 1.6 1.7 Root, leaf (this report)
7778 D24100 S-adenosylmethionine synthetase OsSAMS1c 1.4 0.7 (Root, leaf) (this report)
5061 AU174654 S-adenosylmethionine synthetase OsSAMS1c 1.3 0.7 (Root, leaf) (this report)
7859 AU063547 S-adenosylmethionine synthetase OsSAMS1c 1.2 0.8 (Root, leaf) (this report)

**Fe(II)-MAs transport**

7453 D24682 Fe(III)-MAs transporter OsYSL15 10.2 1.0 Root (Koike et al., 2004)

**Fe(II) transport**

7958 D49213 Fe(II) transporter OsIRT1 3.1 0.9 Root (Buglio et al., 2002)

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a Putatively catalyses the transamination step in the synthesis of Met from 2-keto-methylthiobutyric acid in the Met cycle.
b The isomerization from methylthioribose-1-phosphate to methylthiouribulose-1-phosphate is deduced to be catalysed by an eukaryotic initiation factor 2B a-subunit (elf2Ba-like protein OsID2), and also possibly by a putative ribose-5-phosphate isomerase (RPI).
c Clones with an induction ratio of less than 2.0 but confirmed by northern analysis to be Fe-deficiency-induced.
deficiency in roots and, more strongly, in leaves. Gene element 7315 was confirmed to be up-regulated in Fe-deficient roots. By contrast, no clear induction by Fe deficiency in roots was observed for gene elements 7330, 8170, and 5629. OsIDS1 (gene element 2581) was confirmed to be strongly up-regulated in Fe-deficient roots and moderately up-regulated in Fe-deficient leaves. Strong transcriptional induction of gene element 2798 encoding a putative bHLH transcription factor has been confirmed previously in roots and leaves (Ogo et al., 2004).

In order to investigate whether IDE-like sequences are overrepresented among the Fe-deficiency-inducible promoters analysed, the promoters of genes showing either no alteration or suppression in Fe-deficient roots were also searched for IDE-like sequences (Table 3). The rate of the presence of IDE1-like sequences among promoters with an induction ratio greater than 2.0 in Fe-deficient roots (Ratio >2.0; Total) was 30.6%, which was moderately higher than that among uninduced (Ratio 1.0) or suppressed (Ratio <0.6) sets of promoters, while the occurrence of IDE2-like promoters was found to be similar among induced, uninduced, and suppressed promoters. When the inducible promoters were divided into the subgroups of promoters related to Fe uptake and others, Fe-uptake-related promoters had a distinctly higher occurrence of IDE1-like (44.4%) and IDE2-like (33.3%) sequences, compared with other sets of promoters. A search was also made for the presence of other known cis-acting elements, including TGACG (component of as-1 element; Lam et al., 1989), G-box (CACGTG), IDRS (CACGAGNCCGCCAC)-like (suppressor of phytoferritin expression under low Fe availability; Petit et al., 2001), and AFT1 binding sites (PyPuCACCCPu, Fe-deficiency-responsive element of yeast genes regulating Fe and Cu utilization; Yamaguchi-Iwai et al., 1996). These cis-acting elements were either similarly distributed among the groups, or less frequent in Fe-uptake-related promoters.

In addition, promoters of Arabidopsis genes whose transcriptional responses to Fe-deficiency in roots have been reported (Eide et al., 1996; Robinson et al., 1999; Thomine et al., 2000, 2003; Wintz et al., 2003; Bauer et al., 2004; Colangelo and Guerinot, 2004) were also acquired from the database, and searched for the cis-sequences. IDE1-like sequences were present at a higher rate in Fe-deficiency-induced promoters, especially among Fe-uptake-related gene promoters, while IDE2-like sequences and other cis-sequences were not.

Discussion

Due to the high tolerance of barley to Fe deficiency, most of the graminaceous genes responding to Fe deficiency were first isolated from this plant. Recently, the induction of these genes has been detected again through microarray analysis (Negishi et al., 2002a). In the present study, microarray analysis was used with rice, a very important graminaceous crop that is highly susceptible to Fe deficiency.

Co-ordinated induction of genes involved in the synthesis of MAs and the Met cycle is triggered by Fe deficiency in rice roots and leaves

In Fe-deficient barley roots, the expression of the genes corresponding to all of the steps in the biosynthesis of MAs from Met is induced, as confirmed by northern and microarray analyses (Higuchi et al., 1999; Takahashi et al., 1999; Nakanishi et al., 2000; Negishi et al., 2002a; T Negishi et al., unpublished results). Several genes related to the Met cycle, including HvIDII, HvFDH, and HvAPT, are also
Table 2. Genes with IDE1- and/or IDE2-like sequences within their 1.5 kb promoter regions, and that have an induction ratio of greater than 2.0 in Fe-deficient roots

Full-length cDNA clones from the Rice Full-length cDNA Database (KOME: http://cdna01.dna.aflrc.go.jp/cDNA/) were identified based on the array clones, and the promoter sequences within 1.5 kb upstream of the putative translation start sites were examined for IDE1- and IDE2-like sequences, as described in the Materials and methods. The IDE-like sequences identified are indicated as positions from the putative translation start sites. Confirmation of the induction by northern analysis in either previous studies or in this report is indicated. Only one representative clone is shown for each gene with multiple spots on the array slide.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Putative gene identification</th>
<th>Induction ratio Root</th>
<th>Induction ratio Leaf</th>
<th>Accession no.</th>
<th>IDE1-like sequences</th>
<th>IDE2-like sequences</th>
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<tr>
<td>6254</td>
<td>Nicotianamine synthase OsNAS2</td>
<td>23.3</td>
<td>7.6</td>
<td>AK112011</td>
<td>−250/−267, −309/−292</td>
<td>−1033/−1007</td>
</tr>
<tr>
<td>6307</td>
<td>Nicotianamine aminotransferase</td>
<td>21.2</td>
<td>9.4</td>
<td>No hit</td>
<td>−290/−273</td>
<td>Root, leaf</td>
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<tr>
<td>8336</td>
<td>Unknown</td>
<td>12.0</td>
<td>45.8</td>
<td>No hit</td>
<td>−1197/−1180</td>
<td>Root, leaf</td>
</tr>
<tr>
<td>7260</td>
<td>Unknown</td>
<td>11.8</td>
<td>78.7</td>
<td>No hit</td>
<td>−1255/−1238</td>
<td>Root, leaf</td>
</tr>
<tr>
<td>8150</td>
<td>Formate dehydrogenase OsFDH</td>
<td>9.6</td>
<td>2.6</td>
<td>AK065872a</td>
<td>−432/−449</td>
<td>Root, leaf</td>
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<tr>
<td>8150</td>
<td>Formate dehydrogenase OsFDH</td>
<td>9.6</td>
<td>2.6</td>
<td>AK064610b</td>
<td>−386/−403, −1060/−1077</td>
<td>Root, leaf</td>
</tr>
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<td>7070</td>
<td>Methylthioribose kinase OsMTK1</td>
<td>6.2</td>
<td>1.3</td>
<td>AK067649</td>
<td>−1136/−1153</td>
<td>Root, leaf</td>
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<td>8956</td>
<td>Nicotianamine synthase OsNAS1</td>
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<td>1.8</td>
<td>AK112069</td>
<td>−240/−266</td>
<td>Root, leaf</td>
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<td>2798</td>
<td>bHLH transcription factor</td>
<td>5.2</td>
<td>15.2</td>
<td>AK073335</td>
<td>−1148/−1174</td>
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<td>7001</td>
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<td>3.2</td>
<td>AK068484</td>
<td>−933/−959</td>
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<td>Fe(II) transporter OsIRT1</td>
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<td>AK0710681</td>
<td>−1100/−1083</td>
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<td>Ribose-5-phosphate isomerase RPI</td>
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<td>−651/−634</td>
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<td>AK062219</td>
<td>−117/−100, −1331/−1314</td>
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<td>Serine/threonine protein kinase YK11</td>
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<td>2.6</td>
<td>AK121335</td>
<td>−256/−239</td>
<td>Co-ordinated induction of Fe-acquisition-related genes</td>
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<tr>
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<td>3.4</td>
<td>AK120491</td>
<td>−428/−411</td>
<td>Co-ordinated induction of Fe-acquisition-related genes</td>
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<tr>
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<td>AK065890</td>
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<tr>
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<td>1.5</td>
<td>AK099497b</td>
<td>−1200/−1217, −1255/−1233, −238/−264</td>
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<td>AK072051</td>
<td>−1000/−983</td>
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a The full-length cDNA of accession number AK065872 is identical to the 9k array element 8150, and that of accession number AK064610 is highly homologous.

b The full-length cDNA of accession number AK103834, whose promoter contains no IDE-like sequences, is identical to the 9k array element 3251, and that of accession number AK099497 is highly homologous.

c The full-length cDNA of accession number AK065927, whose promoter contains no IDE-like sequences, is identical to the 9k array element 3280, and that of accession number AK066568 is highly homologous.

d Clones with an induction ratio of less than 2.0 but confirmed by northern analysis to be Fe-deficiency-induced.
induced in barley roots by Fe deficiency (Suzuki et al., 1998; Itai et al., 2000; Yamaguchi et al., 2000a; Negishi et al., 2002a). The present microarray study clearly demonstrated that all of the genes involved in the synthesis of MAs are induced in Fe-deficient rice roots (Table 1; Fig. 2A). Among the rice genes involved in the biosynthesis of MAs, all of the genes participating in the steps from S-adenosyl-Met to MAs have already been shown by northern analysis to be up-regulated by Fe deficiency in roots (Higuchi et al., 2001a; Inoue et al., 2003, 2004; Nozoye et al., 2004). Microarray analysis re-identified these genes as having high induction ratios (Table 1), confirming the validity of this technique. The rice homologues of genes involved in the Met cycle, OsFDH and OsIDI1, were also up-regulated in Fe-deficient rice roots (Table 1; Fig. 2A).

Furthermore, the genes with high induction ratios detected in the microarray analysis covered every predicted step of the Met cycle. Recently, the OsMTK1 gene was isolated from rice and the enzyme activity of the encoding protein was demonstrated (Sauter et al., 2004). OsMTK expression was strongly induced under sulphur deficiency, indicating its link to sulphur metabolism. Sauter et al. (2004) also reported that OsMTK expression was unaffected by Fe deficiency, possibly due to the difference in culture condition from the experiments conducted in this work. The other genes encoding enzymes in the Met cycle were deduced by their homology to the ykr genes of the Met salvage pathway in Bacillus subtilis (Murphy et al., 2002; Sekowska and Danchin, 2002; Ashida et al., 2003; Sekowska et al., 2004). A previous microarray analysis revealed that the expression of many genes related to the Met cycle is also up-regulated by Zn deficiency in barley, but not consistently in rice (M Suzuki et al., unpublished data). In addition, the genes induced in Fe-deficient Arabidopsis roots, reported by Colangelo and Guerinot (2004), included the closest homologue of IDI2 in Arabidopsis (At2g05830), as well as the AtMTK gene (At1g49820) and a putative ATP sulphurylase gene (At4g14680). Although Strategy I plants do not synthesize MAs, Fe-deficient Arabidopsis may activate the Met cycle to synthesize nicotianamine, which serves as a common metal chelator in all higher plants (Hell and Stephan, 2003; Takahashi et al., 2003). Arabidopsis NAS genes are induced by Fe deficiency (Wintz et al., 2003; Bauer et al., 2004; Colangelo and Guerinot, 2004; Y Ushio et al., unpublished data).

Northern blot analysis confirmed that in rice, the expression of genes involved in every predicted step of the Met cycle is induced (Fig. 2A), as well as every step in the biosynthesis of MAs from Met in response to Fe deficiency. Although a previous microarray analysis of barley transcripts detected the up-regulation of some of the genes related to the Met cycle and sulphur assimilation pathways (Negishi et al., 2002a), this is the first report to identify the overall induction of the Met cycle by Fe deficiency. This expression pattern implies the presence of certain coordinated mechanisms, similar to the expression of the ykr genes in B. subtilis grown under conditions of Met deprivation (Murphy et al., 2002). Northern analysis also clearly demonstrated that the expression of all of the related genes, except for MTN, is also induced in rice leaves (Fig. 2A). Previous studies have shown that OsNAS1, OsNAS2, and OsNAAT1 were similarly induced in rice leaves (Higuchi et al., 2001a; Inoue et al., 2003, 2004). By contrast, in barley, Fe-uptake-related genes are expressed almost specifically in roots. Higuchi et al. (2001a) proposed that in barley, root-specific induction of NAS genes has developed, allowing the plant to tolerate severe Fe deficiency. The results of the present study suggest that
a regulation mechanism has been conserved for the expression of Fe-uptake related genes that are up-regulated in both roots and leaves of Fe-deficient rice, but are up-regulated in barley only in Fe-deficient roots.

The up-regulated genes in the microarray analysis were notably concentrated in Fe-uptake functions. In particular, 14 of the 35 genes with induction ratios greater than 2.5 were related to Fe uptake (Table 1). This implies the importance of Strategy II responses in graminaceous species, and the possible importance of other characterized Fe-deficiency-induced genes, including those encoding putative transporters and transcription factors.

**Sequences homologous to IDE are overrepresented among the promoter regions of the Fe-deficiency-induced genes involved in Fe uptake**

Although a large number of Fe-deficiency-inducible genes have been isolated, the molecular mechanisms of plant responses to Fe deficiency are largely unknown. To provide novel tools to clarify these mechanisms, the promoter region of the Fe-deficiency-inducible HvIDS2 gene was recently analysed, identifying the novel iron deficiency-responsive cis-acting elements IDE1 and IDE2 which synergistically stimulate Fe-deficiency-induced expression in roots of transgenic tobacco (Kobayashi et al., 2003). Sequences homologous to IDE1 were also identified in several other Fe-deficiency-inducible promoters of barley, rice, and Arabidopsis genes related to Fe uptake and translocation, suggesting the conservation of these cis-acting elements in various genes and species.

The microarray analysis from this study and rice genome database information has enabled further assessment of the distribution of sequences homologous to IDE1 or IDE2 in Fe-deficiency-responsive gene promoters. The presence of IDE1-like and IDE2-like sequences in the upstream regions was observed in 22 and 14, respectively, of the 72 promoters that showed the greatest induction ratio in roots (Table 2). Many of those promoters with IDE-like sequences are also involved in the biosynthesis of MAs or Fe transport (Table 1), covering the majority of the steps for the production of MAs, including the Met cycle (Fig. 1, underlined). This suggests that the overall induction of the expression of Met-cycle-related genes is not simply due to Met deprivation by the synthesis of MAs, but is directly mediated, at least in part, by Fe deficiency through IDE-like cis-acting elements. Furthermore, the expression patterns of the Met-cycle-related genes (Fig. 2A), as well as the genes for the production of MAs from S-adenosyl-Met (Higuchi et al., 2001a; Inoue et al., 2003, 2004; Nozoye et al., 2004), are similar to that observed when the barley promoter fragments containing IDE1 and IDE2 were introduced into rice (Kobayashi et al., 2004). On the other hand, the expression patterns of some of the genes with other functions, with promoter regions containing IDE1-like sequences (Fig. 2B), differed somewhat. For instance, gene elements 8336 and 7260, whose functions are unknown, were more strongly induced in leaves than in roots. The promoters of these genes may contain other unknown cis-acting elements that confer strong induction in Fe-deficient leaves.

The rate of occurrence of IDE-like sequences in the promoter regions of the gene sets showing induction ratios >2.0, 1.0, and <0.6 in Fe-deficient roots were then compared (Table 3). Subdivision of the genes with induction ratios >2.0 into Fe-uptake-related and other genes revealed the overrepresentation of IDE-like sequences in Fe-uptake-related promoters versus any other set of promoters. The pronounced, but incomplete, existence of IDE-like sequences in Fe-uptake-related promoters supports the importance of IDE-mediated regulation in the mechanism of Fe acquisition in rice plants, and implies the presence of other regulatory components. The network regulating plant responses to cold, drought, high-salinity, and abscisic acid has been characterized extensively through the dissection of various cis/trans interactions (Shinozaki et al., 2003). Promoters of Arabidopsis and rice genes induced by cold, drought, high-salinity, or abscisic acid possess relevant cis-elements, including the dehydration-responsive element (DRE), the abscisic acid-responsive element (ABRE), and the myc recognition sequence, at distinguishable, but not overwhelming, rates (Seki et al., 2001; Chen et al., 2002; Simpson et al., 2003; Rabbani et al., 2004), reflecting the presence of multiple induction pathways (Shinozaki et al., 2003).

A search was also made for other known cis-acting sequences, including TGACG, G-box, IDRS-like sequences, and AFT binding sites, among the sets of promoters. None of these sequences were overrepresented among the Fe-uptake-related promoters searched (Table 3). A tandem repeat of TGACG composes the enhancer-like element as-1 (Lam et al., 1989), which is also present within the 47 fragment of the cauliflower mosaic virus 35S promoter that supports the function of IDE2 synergistically (Kobayashi et al., 2003). However, canonical as-1 sequences were rarely found in each set of promoters (data not shown). These results suggest the presence of other kinds of cis-acting elements responsible for Fe-deficiency-induced expression in rice roots. Conversely, some kinds of generally present cis-acting elements that might not be overrepresented could also have a certain function for specific expression, depending on the neighbouring promoter contexts.

A promoter search of Arabidopsis genes whose transcriptional response to Fe deficiency in roots has been reported (Eide et al., 1996; Robinson et al., 1999; Thomine et al., 2000, 2003; Wintz et al., 2003; Bauer et al., 2004; Colangelo and Guerinot, 2004) revealed that IDE1-like sequences, but not IDE2-like sequences, were enriched in the Fe-deficiency-inducible promoters (Table 3). Promoters of AtFRO (ferric-chelate reductase genes) and AtNAS were
especially abundant with IDE1-like sequences (Kobayashi et al., 2003; data not shown), which resulted in the overrepresentation of IDE1-like sequences among the Arabidopsis promoters related to Fe uptake (Table 3). Recent reports have shown significant roles for a putative bHLH transcription factor (AtbHLH29/FIT1/FRU) in Fe-deficiency-induced expression of various genes, including AtFRO2 and AtIRT1 in Arabidopsis roots (Colangelo and Guerinot, 2004; Jakoby et al., 2004). The results from this study suggest that IDE1-like sequences might also regulate the expression of some of the genes involved in Fe utilization in Strategy I plants. It has been reported that barley HvNAS1 and HvIDS2 promoters, as well as a set of IDE1 and IDE2, are able to induce Fe-deficiency-responsive expression in a Strategy I plant, tobacco (Higuchi et al., 2001b; Yoshihara et al., 2003; Kobayashi et al., 2003).

In conclusion, the overall response of rice genes involved in the synthesis of MAs to Fe deficiency, which is most likely regulated by IDE-mediated and other unknown pathways has been revealed. The identification of the trans-acting factors responsible for the pathways will contribute greatly to an understanding of the regulatory mechanism. Further characterization of IDE1 and IDE2, including confirmation of the essential bases, will also prove beneficial to a more precise clarification of IDE-mediated regulation under Fe deficiency.

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Co-ordinated induction of Fe-acquisition-related genes


