A Novel NAC Transcription Factor, IDEF2, That Recognizes the Iron Deficiency-responsive Element 2 Regulates the Genes Involved in Iron Homeostasis in Plants*§

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Iron is essential for most living organisms, and thus iron deficiency poses a major abiotic stress in crop production. Plants induce iron utilization systems under conditions of low iron availability, but the molecular mechanisms of gene regulation under iron deficiency remain largely unknown. We identified a novel transcription factor of rice and barley, IDEF2, which specifically binds to the iron deficiency-responsive cis-acting element 2 (IDE2) by yeast one-hybrid screening. IDEF2 belongs to an uncharacterized branch of the NAC transcription factor family and exhibits novel properties of sequence recognition. An electrophoretic mobility shift assay and cyclic amplification and selection of targets (CASTing) caused aberrant iron homeostasis in rice. Several genes up-regulated by iron deficiency, including the Fe(II)-nicotianamine transporter gene OsYSL2, were less induced by iron deficiency in the RNAi rice of IDE2, suggesting that IDE2 is involved in the regulation of these genes. Many genes with repressed expression in IDE2 RNAi rice possessed the IDE2-binding core sites in their promoters, and the flanking sequences were also highly homologous to IDE2. IDE2 bound to OsYSL2 promoter region containing the binding core site, suggesting direct regulation of OsYSL2 expression. These results reveal novel cis-element/trans-factor interactions functionally associated with iron homeostasis.

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank®/EBI Data Bank with accession number(s) AB362160 and AB362161.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3.

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The abbreviations used are: MA, mugineic acid; IDE2, iron deficiency-responsive element 2; CASTing, cyclic amplification and selection of targets; RNAI, RNA interference; ORF, open reading frame; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; UTR, untranslated region; NT, nontransgenic rice; GFP, green fluorescent protein; NA, nicotianamine; bHLH, basic helix-loop-helix; AD, activation domain.

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given promoter has not been confirmed. In non-graminaceous plants, several bHLH transcription factors, including tomato FER, Arabidopsis FIT, AthbHLH38, and AthbHLH39, are believed to be involved in the iron-deficiency response (21–24), although their functional cis sequences have not been identified. We previously analyzed the promoter region of the barley iron deficiency-inducible IDS2 gene and identified the novel iron deficiency-responsive cis-acting elements IDE1 and IDE2. These induce iron-deficiency gene expression in tobacco roots, as well as in rice roots and leaves (25, 26). Many genes up-regulated by iron deficiency possess IDE-like sequences in their promoter regions (18, 25). Recently, we have identified a novel ABI3/VP1 family transcription factor that specifically recognizes IDE1; this we designated as IDEF1 (IDE-binding factor 1) (27). IDEF1 transactivates IDE1-mediated expression under iron-deficient conditions and regulates several genes involved in iron acquisition. In this study, we isolated a novel NAC domain transcription factor, IDEF2, which specifically binds to IDE2, by yeast one-hybrid screening. We provide evidence to support that IDEF2 functions as a key transcription factor regulating the iron-deficiency response.

EXPERIMENTAL PROCEDURES

Construction of Reporter Vectors for the Yeast One-hybrid—The reporter vectors for the yeast one-hybrid were constructed using the target DNA fragments shown in supplemental Table S1. IDE1-IDE2 × 2 indicates the target DNA of a twice-repeated DNA fragment of tandemly arranged IDE1 and IDE2. IDE1 × 4 and IDE2 × 3 indicate four and three times tandemly repeated DNA fragments of IDE1 and IDE2, respectively. The double-stranded DNA fragments of the target DNA were synthesized by sense oligomers and antisense primers, followed by in-filling using DNA polymerase. These fragments were inserted into reporter vectors using the restriction sites shown in supplemental Table S1.

Yeast One-hybrid Screening—We performed the yeast one-hybrid screening of rice using the MATCHMAKER library construction and screening kits (K1617-1; Clontech) according to the user manual (PT3529-1; Clontech). The cDNA expression library was constructed with mRNA from iron-deficient rice roots purified with the NucleoTrap mRNA mini kit (Macherey-Nagel, Easton, PA) and the SMART III™ Oligonucleotide primer (Clontech) and the CDS III/6 primer (Clontech). Screening was performed with pHIS2-IDE1-IDE2 × 2 on medium lacking His, but in the presence of 50 mM 3-aminotriazole. The isolated IDE2 clone included all of the longest open-reading frame (ORF) of AK099540, except for the 80 bp at the 3’ end of the ORF.

The yeast one-hybrid screening of barley was performed using the MATCHMAKER one-hybrid system (K1603-1; Clontech) according to the user manual (PT1031-1; Clontech). The cDNA expression library of iron-deficient barley roots was constructed with the pGAD424 vector (Clontech), modified by inserting restriction sites (BamHI-Sall-HindIII-Xbal-EcoRV-NotI-EcoRI) between the EcoRI and Sall sites. Screening was performed with pHISI-IDE2 × 3 on medium lacking His but in the presence of 80 mM 3-aminotriazole. The isolated HvIDEF2 clone was predicted to include the whole ORF.

\[ \text{LacZ Assay of the Yeast One-hybrid—The LacZ assay was performed according to the Yeast Protocols Handbook (Clontech).} \]

The reporter vectors and effector vectors were introduced into yeast YM4271 (Clontech); 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside for the experiment with IDEF2, and o-nitrophenyl-β-d-galactopyranoside for the experiment with HvIDEF2, respectively, were used as substrates. Yeast cells transformed with the empty pGAD7-Rec2 vector (Clontech) or pGAD424 vector were assayed as a negative control for IDEF2 and HvIDEF2, respectively. For the yeast transcription-activating assay, the ORF of IDEF2 in the pGAD7-Rec2 vector was amplified with the primers 5’-AAGCTTTGATATGCGCTCAAACTTTGCTG GCC-3’ and 5’-TTAATCTGACGGCTACCAGATG-3’ and cloned into pCR-BluntII-TOPO (Invitrogen). The cloned and verified IDEF2 ORF was inserted into the HindIII and the XbaI sites of the yeast expression vector pYH23 (10) and introduced into yeast YM4271.

EMSA and CASTing—The ORF of IDEF2 in the yeast transcription-activation assay vector was inserted into pMAL-c2 (New England Biolabs, Beverly, MA) by EcoRI and XbaI sites (MBP-IDEF2). The NAC domain of IDEF2 was amplified using primers of 5’-CTCGAGTCTAGAATGGCTCATAAATTTGCTG GCC-3’ and 5’-TTAATCTGACGGCTACCAGATG-3’ and the full-length cDNA of IDEF2 (AK099540), provided by the National Institute of Agrobiological Sciences, as a template. The amplified fragment was cloned into pCR-BluntII-TOPO and the sequence verified. The inserted NAC domain was cloned into the pMAL-c2 vector at the XbaI and HindIII sites (MBP-IDEF2 NAC domain). These fusion plasmids, as well as pMAL-c2 itself (which expresses MBP-LacZ fusion), were introduced into Escherichia coli strain XL1-Blue. The maltose-binding fusion proteins were produced and purified according to the manufacturer’s instructions (New England Biolabs). EMSA was carried out using the MBP-IDEF2 protein as described previously (19) with slight modifications. Five hundred nanograms of MBP-IDEF2 were incubated with 15 mM HEPES (pH 7.5), 51 mM KCl, 6% glycerol, 0.05% IGPALCA-630 (Sigma), 200 ng of poly(dG-dC)2 (Amersham Biosciences), and 0.5–1.0 ng of the IDE2 probe, which consists of 27-bp IDE2 and its flanking regions corresponding to −272/−227 of the region of the yeast promoter (25). For competition analysis, the IDE2 sequence was substituted for its derivatives, as shown in Fig. 2a. The −366/−323 region of the OsYSL2 promoter (5’-GACAATGGTTATTTATATCAAAACGTGC CAAATATAAGTTGCC-3’) and the −83/−38 region of the AK065090 promoter (5’-GATGCTACTACAGTATTGCAGACGATGGGACACGTACACTAGCTAG-3’) from the predicted transcriptional initiation sites were also used as competitors in Fig. 8 (IDE2-binding core sites are underlined). The whole sequences of the 355 promoter fragments used are shown in Fig. 2f. CASTing was carried out with the MBP-IDEF2 NAC domain proteins as described previously (19), using amylose resin (New England Biolabs) instead of anti-thioredoxin-agarose.

Northern Blot and Quantitative RT-PCR Analysis—Northern blot analysis was performed as described previously (19). For the IDE2 probe, the 500 bp of the non-NAC domain region in the 3’ end of the ORF was amplified by PCR with the
primers 5'-CACCCAGTCCTGTAATTTAGGGGC-3' and 5'-GTGGGAACCTGCTAGCTGGGTT-3'. To detect the expression of HvIDEF2, the HvIDEF2 insertion in the modified pGAD424 was digested using AflII and SacI sites, and the 800 bp-fragment, composed mainly of the non-NAC domain region and the 3'-UTR, was used as a specific probe. For the OsYSL2 probe, a gene-specific region (16) was amplified by PCR. Quantitative RT-PCR was performed as described previously (15) with slight modification. First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) priming with (dT)$_{30}$. The primers used in the RT-PCR were 5'-AGATCCGCCGCACTGCTGCTC-3' and 5'-CTTATTTAAGGCACGGTGCG-3' for AK065090, 5'-ACCCGCAAGAAGGTTCTGCTA-3' and 5'-CGCTTGGGAATACGGTAAACAAATCTG-3' for AK099523, and 5'-GTGGCCACAGCAGAGCGCTG-3' and 5'-CATTCAAGTGTGATGATAGGGG-3' for AK103890.

Observation of Subcellular Localization—The ORF of IDE2 was PCR amplified with primers of 5'-GGTACCATGGCTCACAACCTTGCTGCCC-3' and 5'-GAGCTCTGACAGTGCTGG-3' and the full-length cDNA of IDE2 as the template. The amplified fragment was cloned into pCRII-TOPO, and the sequence was verified. The inserted ORF was cloned into CaMV35S-Sall-Kpn1-sGFP(S65T)-NOS3 (15), which is a derivative of the sGFP vector kindly provided by Dr. Y. Niwa, University of Shizuoka, Japan, at the KpnI and SacI sites. Transient gene expression in onion (Allium cepa) epidermal cells and fluorescence observation were carried out as described previously (28).

Construction of IDEF2 RNAi Rice and CRES-T Rice—To suppress IDEF2 expression, a binary vector for RNA interference (RNAi), pGI21-RNAi-DEST, was constructed (20) using the Gateway technology by modifying plgI21Hm (29). Three hundred base pairs of the 3'-UTR of IDEF2 was amplified with the primers 5'-CACCTTCTCAGTAGCGCCAGGCG-3' and 5'-TGTCTAACTAAGTGCTCAGGC-3'. The amplified fragment was cloned into pGI21-RNAi-DEST as described previously (20). To construct the vector for the chimeric repressor gene-silencing technology (CRES-T) system, the coding sequences of IDEF2, except for the stop codon, were amplified with the primer 5'-CTCGAGTCTAGATGCTCAAGGGCTCTGCTGCC-3' and 5'-GAGCTCTTAAAGCGAACCGAGAAAAGTGCTGCTGCTGCC-3' and 5'-GAGCTCTTAAAGCGAACCGAGAAAAGTGCTGCTGCTGCC-3'. The latter primer included the repression domain SRDX coding region (30). The amplified fragment was cloned into pCRII-TOPO, and the sequence was verified. The inserted ORF-SRDX was cloned into a construct I2 vector (26) at the Xhol/Sacl sites. Then the rice actin 1 promoter restricted by HindIII and SalI in pUC19 was inserted at HindIII and Xhol sites of this vector. Rice actin 1 promoter restricted by HindIII and SalI in pUC19 was inserted at HindIII and Xhol sites of this vector. Rice actin 1 promoter restricted by HindIII and SalI in pUC19 was inserted at HindIII and Xhol sites of this vector. Rice actin 1 promoter restricted by HindIII and SalI in pUC19 was inserted at HindIII and Xhol sites of this vector. Rice actin 1 promoter restricted by HindIII and SalI in pUC19 was inserted at HindIII and Xhol sites of this vector. Rice actin 1 promoter restricted by HindIII and SalI in pUC19 was inserted at HindIII and Xhol sites of this vector. Rice actin 1 promoter restricted by HindIII and SalI in pUC19 was inserted at HindIII and Xhol sites of this vector.

The transformation of rice (O. sativa L. cv. Nipponbare) as a template and the following sequences, 5'-AAGCTTCTAAGGTTCTATGCTG-3' and 5'-GTGGACCTTTACTACAAAAAGGCT-3', and inserted into pUC19 (Toyobo) at HindIII and SacI sites. The transformation of rice (O. sativa L. cv. Tsukinohikari) was performed by Dr. Toki and H. Onodera at the National Institute of Agrobiological Sciences as described previously (31). Twenty independently transformed RNAi and 20 independently transformed CRES-T rice plants were generated. T$_1$ seeds were used for analysis.

Plant Materials and Growth Conditions—Rice was grown hydroponically as described previously (18) under 14-h/10-h light/dark cycles and at 30/25 °C. For Northern blot analysis (Fig. 3a), iron deficiency was initiated 27 days after germination by omitting Fe(III)-EDTA from the culture medium, and plants were harvested on days 5 and 11. For microarray and Northern blot analysis of IDEF2 RNAi and CRES-T rice, iron deficiency was initiated 29–31 days after germination when plant height reached about 34 cm. Plants were harvested on day 7 of iron deficiency. Barley was grown hydroponically, as described previously (19). Plants were harvested on days 1–3 of iron deficiency for construction of the cDNA library for the yeast one-hybrid screening and days 5 and 9 for Northern blot analysis.

Detection of Metal Concentrations—IDEF2 RNAi rice, CRES-T rice, and nontransgenic rice (NT) were transferred to iron-deficient or iron-sufficient medium when their height reached about 45 cm. Plants were harvested 9 days after transfer. The roots were washed serially in 5 mM EDTA and iron-exchanged water. The plants were then wet-ashed with 11 M HNO$_3$ for 40 min at 230 °C using a MarsXpress oven (CEM Corp., Matthews, NC). The metal concentrations were measured by inductively coupled plasma atomic emission spectrometry as described previously (15).

Oligo-DNA Microarray Analysis—The rice 44 K oligo-DNA microarray (Agilent Technologies, San Jose, CA) contains 43,144 unique 60-mer oligonucleotides that were synthesized based on sequence data from the Rice Full-length cDNA Project. Total RNA from RNAi23 and NT rice was prepared from roots or shoots from four biological replicates using the RNeasy mini kit (Qiagen, Valencia, CA). Microarray hybridization, scanning, and data analysis were performed as described previously (19). The reproducibility of the microarray analysis was assessed by a dye swap in each experiment. Genes showing a signal value of >100 and a significant $t$ test ($p < 0.05$) were further analyzed. The RNAi/NT ratio was calculated as (average signal values of the RNAi rice)/(average signal values of the NT rice). The Fe/Fe in the NT plants was calculated as (average signal values of the NT rice under iron-deficient conditions)/(average signal values under iron-sufficient conditions), and these ratios were used for identifying iron-deficiency induction of the genes.

cis Search—The IDEF2-binding sequence was searched within 500 nucleotides upstream of the predicted transcriptional initiation site of the genes. The rice genome sequences were obtained from the Rice Annotation Project Data base. The IDEF2-binding site and its flanking sequences were compared with IDE2 and their homology was calculated without gaps.

RESULTS

Identification of IDEF2 as a Transcription Factor Recognizing IDE2—We conducted yeast one-hybrid screening to identify transcription factors that bind to IDE1 or IDE2. A yeast GAL4 activation domain (AD) fusion cDNA library was constructed from mRNA prepared from iron-deficient rice roots. The screening was performed with a reporter vector that had HIS3 fused to a twice-repeated 45-bp DNA fragment of tandemly
FIGURE 1. Identification and characterization of IDEF2 in yeast. a, schematic diagrams of the reporter genes used in the yeast one-hybrid. b, IDE binding activity of positive clone 51 (AK099540; IDEF2) obtained from a yeast one-hybrid screening. LacZ activity was detected by blue staining. VC, vector control. c, binding assay of IDEF2 for IDE2 and activation assay of IDEF2 in yeast. The IDEF2 gene without fusing to yeast GAL4 AD (pYH-IDEF2) was expressed in yeast carrying the IDEs-lacZ reporters. pYH, vector control. d, binding assay of AD-fused HvIDEF2 for IDE2 binding in yeast carrying IDE2 × 3-lacZ. Shown are β-galactosidase activity units (means ± S.D.; n = 3), and significant differences against a vector control (pGAD) analyzed with a t test (**, p < 0.01). e, phylogenetic tree for IDEF2, HvIDEF2, the homologous genes to IDEF2 in other plant species, and other representatives of NAC family transcription factors related to metal homeostasis or stress response. Enclosed genes are the homologous genes to IDEF2 as follows: Ta, wheat (*Triticum aestivum*, GI655458); Sb, sorghum (*Sorghum bicolour*, CN135689); So, sugarcane (*Saccharum officinarum*, CA086994); Zo, ginger (*Zingiber officinale*, DY375663); Zm, maize (*Zea mays*, EB158917); Fa, Festuca (*Festuca arundinacea*, DT713352); At, Arabidopsis thaliana (ANAC103, AT5g64060); Nb, tobacco (*Nicotiana benthamiana*, CK292994); Ph, Petunia (*Petunia hybrida*, AF509865); Sl, tomato (*Solanum lycopersicum*, BT013683); Si, sugarcane (*Saccharum officinarum*, CA086994); and AK108080, iron deficiency-inducible NAC transcription factors in rice (19, 20), *TnNAM-B1* (DQ869673), a NAC transcription factor in pasta wheat that regulates senescence and remobilization of metals (36); *HvNAM-I* (DQ869678) and *ONAC010* (NP_911241), homologs of *TnNAM-B1* in barley and rice, respectively; *RD26* (AT1g07240) and *ATAF1* (AT1g01720), drought- and/or salt-responsive NAC transcription factors in *Arabidopsis* (33–35); *ATAF2* (At5g08790), a NAC transcription factor involved in regulation of pathogenesis-related genes (35).

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arranged IDE1 and IDE2 (IDE1-IDE2 × 2) (Fig. 1a). Through the screening of 3.2 × 10⁶ cDNA clones, one positive clone was obtained (clone 51), which encoded a NAC family transcription factor corresponding to the rice full-length cDNA, AK099540.

To examine recognition sequences of clone 51 within IDE1-IDE2 × 2, we constructed a reporter gene that had lacZ fused to four tandemly repeated IDE1 (IDE1 × 4) or three tandemly repeated IDE2 (IDE2 × 3) (Fig. 1a). In yeast cells carrying clone 51, IDE1-IDE2 × 2 or IDE2 × 3 induced substantial LacZ activity, whereas IDE1 × 4 did not (Fig. 1b). These results revealed that clone 51 preferentially bound IDE2 but not IDE1. We designated clone 51 as IDEF2 (IDE-binding factor 2). IDEF2 induced gene expression even in the absence of AD in yeast cells carrying the lacZ gene fused to IDE2-IDE1 × 2 or IDE2 × 3 (Fig. 1c), indicating that IDEF2 works as a transcriptional activator.

As IDE1 and IDE2 were identified from the promoter of the barley gene *IDS2*, which encodes dioxygenase of MAs, we also conducted yeast one-hybrid screening with the cDNA library of barley. Then 5.0 × 10⁶ cDNA clones of iron-deficient barley roots were screened with a reporter gene harboring the *HIS3* gene under IDE2 × 3. A close homolog of IDEF2 was selected as a positive clone and named HvIDEF2 (Fig. 1e). In yeast cells carrying the lacZ gene under the control of IDE2 × 3, AD-fused HvIDEF2 induced strong LacZ activity compared with AD alone (Fig. 1d). These results revealed that HvIDEF2 preferentially bound IDE2.

NAC constitutes a plant-specific transcription factor family with a highly conserved N-terminal DNA-binding domain. Some NAC proteins have been implicated in developmental programs, metal homeostasis, drought stress tolerance, and pathogenesis stress (32–36). In *silico* analysis suggested that IDEF2 shares no pronounced similarity to any characterized NAC transcription factors (Fig. 1e). In *silico* search also revealed that certain graminaceous plants carry highly homologous genes to IDEF2 and HvIDEF2. Expressed sequence tags from wheat (*T. aestivum*), sorghum (*S. bicolor*), sugarcane (*S. officinarum*), and *Festuca* (*F. arundinacea*) appear to contain amino acid sequences highly homologous to IDEF2 (Fig. 1e). In addition, proteins with moderate homology to IDEF2 were found in maize (*Z. mays*) and non-grami-
naceous plants such as ginger (Zo), Arabidopsis (At), Petunia (Ph), tomato (Sl), and tobbaco (Nb).

**IDEF2 Predominantly Recognizes CA(A/C)G(T/C)(T/C/A) (T/C/A)**—Specific binding of IDEF2 protein to IDE2 was further confirmed by EMSA (Fig. 2, a–d). Recombinant IDEF2 protein fused to MBP-IDE2 was incubated with end-labeled IDE2 oligonucleotides. The shifted band that corresponded to the DNA-protein complex was observed when MBP-IDE2 was added (Fig. 2b). Nonlabeled IDE2 competed for the IDE2-IDEF2 complex, whereas the IDE1 did not (Fig. 2b). This result corresponds to the yeast one-hybrid assay (Fig. 1) and suggests IDEF2 protein specifically binds to IDE2.

We then conducted competition experiments using end-labeled IDE2 sequences and mutated IDE2 competitors. The oligonucleotides m4 and m5 showed little or no competition for IDE2-IDEF2 complex formation. Oligonucleotides m1, m3, m6, m8, and m9 also showed reduced competition efficiency as compared with IDE2, whereas m2 and m7 competed efficiently (Fig. 2c). The regions of the mutated sites of m3, m4, and m5 were then examined in further detail. One-base mutated oligonucleotides, m33, m41, and m43, did not compete at all (Fig. 2d). m42 competed moderately, whereas m42G did not compete (Fig. 2d). These results suggest that the binding activity of IDEF2 was substantially retained in the A to C mutation in the m42 position but was lost by the A to G mutation. m31 and m32 efficiently competed for binding, whereas m51, m52, and m53 competed only slightly. These results show that IDEF2 predominantly recognizes the CA(A/C)G(TTT) sequence (boxed region in Fig. 2, a and d) within IDE2.

We also carried out a cyclic amplification and selection of targets (CASTing) experiment with the NAC domain of IDEF2, which is considered to be responsible for DNA binding. MBP-IDEF2 NAC domain proteins and oligonucleotides containing random sequences were incubated. The potential IDEF2-binding sites were then enriched by seven cycles of DNA binding, isolation of nucleoprotein complexes, and re-amplification of selected oligonucleotides. Of the amplified and cloned sequences, 73 were sequenced and revealed to be independent of each other. Of these clones, 41 contained the CA(A/C)G(T/C) sequence, which corresponded to the IDEF2 recognizing site determined by EMSA, CA(A/C)G(TTT). Alignment of the sequences of the clones that contained CA(A/C)G(T/C) revealed the consensus sequence to be CA(A/C)G(T/C)(T/C/A) (Fig. 2e; CA(A/C)G(T/C) corresponds to the 3rd to 7th bases). This consensus sequence was in good agreement with the results of EMSA. Although CASTing revealed no specific nucleotides of the 9th base in Fig. 2e, EMSA showed that IDEF2 did not bind to m53 preferentially (Fig. 2d). Therefore, the 9th base is considered to be compatible for T, C, and A but not for G. From these results, we concluded that IDEF2 recognizes CA(A/C)G(T/C)(T/C/A) as the core sequence.

Several studies have shown that NAC proteins recognize the −90 region of the cauliflower mosaic virus 35S promoter (37–39). Furthermore, our promoter analysis in tobacco (25) indicated that the −90/−47 region of the 35S promoter (35S−(−90/−47)) is able to enhance the iron-deficiency responsiveness of IDE2 and might be recognized by transcription factors related to iron-deficiency responses. Therefore, we conducted EMSA using 35S−(−90/−47) and IDEF2 protein (Fig. 2f). IDEF2 efficiently bound to 35S−(−90/−47) despite the absence of CA(A/C)G(T/C)(T/C/A)(T/C/A). The binding to 35S−(−90/−47) efficiently competed for IDE2 and vice versa. The 35S−(−90/−47) contains an as-I element, which is known to be a binding site for the bZIP transcription factor (40, 41). 35S−(−90/−47)-m3, which was mutated at the 3’-half of the TGACG pair of the as-I element, competed with the IDE2 binding, whereas 35S−(−90/−47)-m5 and 35S−(−90/−47)-m5’ and 3’ did not. These results indicate that IDEF2 recognizes the sequences, including the 5’-half of TGACG in 35S−(−90/−47) in addition to binding to IDE2.

**Expression of IDEF2 Genes**—Northern blot analysis revealed that the IDEF2 and HvIDEF2 transcripts were constitutively expressed in iron-sufficient and iron-deficient shoots and roots (Fig. 3).

**Intracellular Localization of IDEF2 Protein**—The IDEF2-GFP fusion protein localized to the nucleus when transiently expressed in onion epidermal cells, whereas GFP alone was distributed throughout the cytoplasm and nucleus (Fig. 4).

**Generation of IDEF2 RNAi and IDEF2 CRES-T Transgenic Rice**—To examine the function of IDEF2 in planta, we applied the RNAi technique and the CRES-T to IDEF2 in rice. The 300-bp 3’-UTR region of IDEF2, which had no homologous sequences in the rice genome, was used for the trigger RNAi. For CRES-T rice, the strong repression domain SRDX (30) was fused to the IDEF2 ORF to restrain the activation ability of IDEF2. Twenty independent RNAi lines and 20 independent CRES-T lines were generated. Expression levels of IDEF2 and IDEF2 ORF-SRDX in iron-deficient roots of these transgenic rice lines were confirmed by Northern blot analysis (Fig. 5 and data not shown). Among the RNAi rice, two lines with strong repression of IDEF2 (RNAi22 and -23) and two lines with moderate repression (RNAi25 and -27) by RNAi were selected (Fig. S5a). For CRES-T rice, two lines with strong expression of IDEF2 ORF-SRDX (Fig. 5b) were selected and further analyzed.

**Aberrant Iron Concentration in IDEF2 RNAi and CRES-T Rice**—RNAi23, CRES-T1, CRES-T2, and NT rice were hydroponically grown in iron-sufficient and iron-deficient culture. Interestingly, the iron concentrations of roots and shoots of the RNAi and CRES-T rice under iron-sufficient conditions were much higher than those of the NT (Fig. 6). On the other hand, under iron-deficient conditions, the iron concentrations of the shoots of the RNAi and CRES-T rice were inclined to be lower than that of the NT, although the iron concentration of the roots was higher than that of the NT. These results indicate that the nutritional status of iron differs between the transgenic plants and the NT. Additionally, this difference also varied between iron-deficient and iron-sufficient conditions. Thus, repression of the function of IDEF2 caused aberrant iron homeostasis in plants.

**Identification of Genes Regulated by IDEF2 Using Microarray Analysis**—To identify the potential target genes of IDEF2, a rice 44 K oligo-DNA microarray analysis was performed to compare the expression profiles of RNAi to those of NT rice. The line RNAi23 was used in this experiment. The RNAi and NT rice plants were grown under iron-deficient conditions, and their RNA was separately extracted from roots and shoots.
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a) IDE1
   ATCAAGCATGCCTCTGTGC
IDE2
   TTGACGCAATTTTTACGTCTCACT
IDE2-m1
   GGTACGCAATTTTTACGTCTCACT
IDE2-m2
   TTGACGCAATTTTTACGTCTCACT
IDE2-m3
   TTGACGCAATTTTTACGTCTCACT
IDE2-m4
   TTGACGCAATTTTTACGTCTCACT
IDE2-m5
   TTGACGCAATTTTTACGTCTCACT
IDE2-m6
   TTGACGCAATTTTTACGTCTCACT
IDE2-m7
   TTGACGCAATTTTTACGTCTCACT
IDE2-m8
   TTGACGCAATTTTTACGTCTCACT
IDE2-m9
   TTGACGCAATTTTTACGTCTCACT
IDE2-m10
   TTGACGCAATTTTTACGTCTCACT
IDE2-m11
   TTGACGCAATTTTTACGTCTCACT
IDE2-m12
   TTGACGCAATTTTTACGTCTCACT
IDE2-m13
   TTGACGCAATTTTTACGTCTCACT
IDE2-m14
   TTGACGCAATTTTTACGTCTCACT
IDE2-m15
   TTGACGCAATTTTTACGTCTCACT
IDE2-m16
   TTGACGCAATTTTTACGTCTCACT
IDE2-m17
   TTGACGCAATTTTTACGTCTCACT
IDE2-m18
   TTGACGCAATTTTTACGTCTCACT
IDE2-m19
   TTGACGCAATTTTTACGTCTCACT

b) Protein
   None
   IDEF2
   IDE1

Competitor
   None
   IDEF2

bound

free

c) Protein
   None
   IDE1
   IDE2
   IDE2-m1
   IDE2-m2
   IDE2-m3
   IDE2-m4
   IDE2-m5
   IDE2-m6
   IDE2-m7
   IDE2-m8
   IDE2-m9

Competitor
   None
   IDE2
   IDE2-m1
   IDE2-m2
   IDE2-m3
   IDE2-m4
   IDE2-m5
   IDE2-m6
   IDE2-m7
   IDE2-m8
   IDE2-m9

bound

d) Protein
   None
   IDEF2

Competitor
   None
   IDE2

bound

(X30)

bound

(X50)

(e)

(%)

(f)

35S(-90/-47)
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m1
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m2
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m3
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m4
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m5
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m6
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m7
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m8
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m9
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m10
ACCTCTCATGACCTTAAGGGATGAG

35S(-90/-47)-m11
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m12
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m13
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m14
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m15
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m16
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m17
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m18
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m19
ACCTCTCATGACCTTAAGGGATGAG
35S(-90/-47)-m20
ACCTCTCATGACCTTAAGGGATGAG

Protein
   None
   IDE2

Competitor
   None
   IDE2

bound

35S(-90/-47)

IDE2
Gene expressions were compared between RNAi rice and NT rice. The RNAi/NT ratio was calculated as (signal values of RNAi rice)/(signal values of NT rice). The \(-\text{Fe}/+\text{Fe}\) ratio in the NT plants was calculated as (signal values of NT rice under iron-deficient conditions)/(signal values under iron-sufficient conditions), and these ratios were used to identify the iron deficiency induction of the genes.

In roots, 350 genes showed an RNAi/NT ratio < 0.5 under iron-deficient conditions. Among these, 69 genes (19.7%) were up-regulated more than 2-fold in response to iron deficiency in rice roots (Fig. 7 and supplemental Table S2). Included in the 69 genes were Fe(II)-NA and Mn(II)-NA transporter gene OsYSL2 (CI446246) (16), and the gene for metallothionein-like protein OsIDS1 (AK103445) (18) (indicated in boldface in supplemental Table S2). Among all the genes on the 44 K microarray (43,114 genes), 875 genes (2.0%) were up-regulated by iron deficiency in NT roots (Fig. 7).

In shoots, 803 genes showed an RNAi/NT ratio < 0.5 under iron-deficient conditions. Among these, 94 genes (11.7%) were up-regulated more than 2-fold in response to iron deficiency in rice shoots (Fig. 7, supplemental Table S3). OsYSL2 and an iron deficiency-inducible Myb transcription factor (AK101209) (19) were repressed in the iron-deficient RNAi shoots compared with the NT shoots (indicated in boldface in supplemental Table S3). Among all the genes on the 44 K microarray, 731 genes (1.7%) were up-regulated by iron deficiency in NT shoots.

Interestingly, of the genes showing reduced expression in IDE2 RNAi rice roots, several genes (AK062422, AK061197, AK065090, OsIDS1, and AK105331) were predicted to be dependent on OsIRO2 (supplemental Table S2, marked IRO) (20).

We conducted Northern blot and quantitative RT-PCR analysis of OsYSL2, AK065090 (heme peroxidase), AK099523 (unknown), and AK103890 (ubiquitin), whose expression was dramatically repressed in the IDE2 RNAi rice in microarray analysis (supplemental Table S2). In the iron-deficient roots of RNAi rice lines 22–27, the expression level of OsYSL2 was strongly associated with that of IDE2 (Fig. 5a). In the IDE2 CRES-T rice, the expression level OsYSL2 was also dramatically repressed (Fig. 5b). The expression levels of AK065090, AK099523, and AK103890 were lower in RNAi23 and RNAi27 than in NT plants (Fig. 5c).

**Promoter Search for the IDE2-binding Site and Its Flanking Sequences** — We searched putative IDE2-binding sequences within 500 nucleotides upstream of the predicted transcriptional initiation site of the genes on the 44 K microarray. During the linker-scanning analysis to identify IDE2, we previously showed that mutating any of the three 9-base portions of the 27-base IDE2 markedly reduced its responsiveness to iron deficiency (25). This finding, together with the EMSA results (Fig. 2), suggests that not only the IDE2-binding core site but also the flanking sequences within IDE2 are needed for maximum IDE2 recognition to regulate gene expression. Therefore, we compared IDE2 sequences flanking the IDE2-binding core site in the promoters possessing the IDE2-binding core site. First, the presence of the IDE2-binding core sites was searched in 500 bases of the promoters. Then the identity rate between IDE2 and the 27-bp promoter sequences, including the binding core site and its flanking sequences, was calculated. Among the 350 genes with an RNAi/NT ratio < 0.5 in roots, 85.1% of the genes possessed the IDE2-binding core sites in their promoters.

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regions, whereas 81.9% of the genes possessed them among all the genes on the 44 K microarray (Table 1). The rate of genes whose 27-bp promoter sequences, including IDEF2-binding core sites, shared more than 40% identity with IDE2 was 76.6% among the genes with a ratio of RNAi/NT < 0.5 in roots, whereas 73.6% met this criterion among all the genes on the 44 K microarray (Table 1). The rates of genes sharing more than 40, 45, 50, and 55% identity with IDE2 in roots or shoots are also shown in Table 1. The IDEF2-binding core sites and the highly homologous flanking sequences to IDE2 were inclined to be enriched in the promoters of the genes with a ratio of RNAi/NT < 0.5 in both roots and shoots. OsYSL2 (CI446246), OsIDS1 (AK103445), iron deficiency-inducible Myb transcription factor gene (AK101209), AK065090 (Fig. 5c), and AK099523 (Fig. 5c) possessed the IDEF2-binding core sites in their promoters, and their flanking sequences were inclined to be highly homologous to IDE2 (supplemental Tables S2 and S3).

To determine whether IDEF2 actually binds to any of the putative binding sites in the IDEF2 targets, we performed EMSA analysis using competitors containing the IDEF2-binding core sites present within 500 bases of the promoters of OsYSL2 and AK065090 (Fig. 8), whose expression levels are strongly dependent on IDEF2 (Fig. 5 and supplemental Tables S2 and S3).

Northern blot analysis of IDEF2 and OsYSL2 in CRES-T rice. Each lane was loaded with 10 μg of total RNA. In Northern blots of IDEF2, the lower bands observed only in the CRES-T lanes correspond to mRNA for the IDEF2 ORF fused to the SRDX domain. c, quantitative RT-PCR analysis of AK065090, AK099523, and AK103890 in RNAi rice. The values represent the number of copies of transcripts in 1 ng of total RNA in three reactions (means ± S.D.).

FIGURE 5. Northern blot and quantitative RT-PCR analysis of IDEF2 and its target genes in rice roots grown under iron-deficient conditions. NT, nontransgenic rice. a, Northern blot analysis of IDEF2 and OsYSL2 in RNAi rice. b, CRES-T. c, quantitative RT-PCR analysis of AK065090, AK099523, and AK103890 in RNAi rice. The values represent the number of copies of transcripts in 1 ng of total RNA in three reactions (means ± S.D.).
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S2 and S3). OsYSL2 promoter fragment with two IDEF2-binding core sites showed prominent competition efficiency for IDEF2-IDE2 binding (Fig. 8), strongly suggesting that OsYSL2 is directly regulated by IDEF2. Similar competition patterns were observed by using the OsYSL2 promoter fragment as a probe (data not shown). On the other hand, AK065090 promoter fragment with two IDEF2-binding core sites showed little competition.

**DISCUSSION**

We isolated novel NAC transcription factors, IDEF2 and HvIDE2, from rice and barley as IDE2 binding transcription factors using yeast one-hybrid screening (Fig. 1). The genes under regulation of IDEF2 were identified by expression analy-

![Image 324x578 to 552x734]

![Image 60x447 to 288x733]

**TABLE 1**

| Core | 44 K RNAi/NT <0.5 | Root | Shoot |
|------|------------------|------|-------|
| 44%  | 81.9             | 85.1 | 87.0  |
| 45%  | 73.6             | 76.6 | 79.1  |
| 50%  | 48.8             | 55.7 | 52.1  |
| 55%  | 31.3             | 35.1 | 36.9  |

**FIGURE 7. Summary of the microarray experiment for IDEF2 RNAi rice.** The RNAi/NT ratio was calculated as (signal values of the RNAi rice)/(signal values of NT rice) and used to identify genes regulated by IDEF2. The −Fe/+Fe ratio in the NT plants was calculated as (signal values of NT rice under iron-deficient conditions)/(signal values under iron-sufficient conditions), and used to identify iron-deficiency induction of the genes. Among the genes regulated by IDEF2 (RNAi/NT <0.5), or among all genes on the 44 K microarray (44K), the genes up-regulated by iron deficiency (−Fe/+Fe >2) are indicated in black.

**FIGURE 8. Binding assay of IDEF2 with its putative target promoters by EMSA.** The IDE2 probe was incubated with 500 ng of the MBP-IDEF2 fusion protein. Competition experiments were carried out by adding 50-fold excess unlabeled competitors. OsYSL2 and AK065090 competitors were composed of −36/−323 region of the OsYSL2 promoter and the −83/−38 region of the AK065090 promoter, respectively, each containing two IDEF2-binding core sites. The shifted bands are indicated.

sis using IDEF2 RNAi rice. Of the genes up-regulated by iron deficiency, a subset of these genes, such as OsYSL2, AK065090, AK099523, and AK103890, was repressed in IDEF2 RNAi rice (Figs. 5 and 7, supplemental Tables S2 and S3). This result revealed that IDEF2 is involved in gene regulation in plant responses to iron deficiency. Northern blot analysis of IDEF2 and OsYSL2 in IDEF2 RNAi rice showed that their expressions are strongly correlated (Fig. 5a). OsYSL2 expression was also dramatically repressed in the IDEF2 CRES-T rice (Fig. 5b). EMSA showed that IDEF2 actually binds to the OsYSL2 promoter (Fig. 8). These results strongly suggest that IDEF2 directly regulates the expression of OsYSL2.

IDEF2 exhibited novel properties of sequence recognition among the NAC transcription factor family. EMSA showed that IDEF2 predominantly recognized CA(A/C)G(T/C)(T/C/A)(T/C) within IDE2 (Fig. 2). Furthermore, a CASTing experiment was performed using MBP-IDEF2 NAC domain protein. The binding properties of IDEF2 determined by the EMSA and CASTing experiment were almost the same. These results suggest that the NAC domain of IDEF2 is mostly responsible for binding to DNA.

Several graminaceous plants other than rice and barley also carry NAC transcription factors highly homologous to IDEF2, and non-graminaceous plants and maize carry moderately homologous genes (Fig. 1e). This suggests that IDEF2 is widely conserved in plants. IDEF2 belongs to a distinct subfamily of NAC transcription factors from iron deficiency-inducible NAC transcription factors (AK108080, AK063703, and AK063399) (19, 20); wheat TtNAM-B1, which is related to senescence and metal remobilization (36); RD26/ANAC072 and ATAF1, which are involved in the regulation of drought and cold stress-inducible genes (32–34); and ATAF2, which is involved in the regulation of pathogenesis-related genes (35) (Fig. 1e). Interestingly, the IDEF2-binding core site in IDE2 is similar to the RD26/ANAC072-binding site (33). In addition to binding to the core-binding site, IDEF2 also exhibits weaker recognition of the flanking sequences within IDE2, as observed by slightly decreased competition activity.
in IDE2-m1, -m6, -m8, and -m9 (Fig. 1c) and also by dissimilar competition activity between promoter fragments of OsYSL2 and AK065090 (Fig. 8). This is consistent with our previous results on IDE2 identification (25), in which the 27-bp IDE2 was divided into three parts of nine bases, where we found that any of the three nine-base portions were needed for the response to iron-deficiency stress. Although the IDE2-binding core site identified in this study is considered to be essential for IDE2 to bind DNA, the flanking sequences within IDE2 are also considered to be important for IDE2 to activate transcription or distinguish which genes to regulate. Many genes with repressed expression in the RNAi rice possessed IDE2-binding core sites in their promoter regions, and their flanking sequences are highly homologous to IDE2 (Table 1). These genes are considered to be candidates for direct targets of IDE2. The IDE2-binding core site and its flanking sequences highly homologous to IDE2 were inclined to be enriched in genes under the regulation of IDE2.

Several genes with repressed expression in the RNAi rice were regulated by iron deficiency-inducible bHLH transcription factor, OsiRO2 (supplemental Table S2). Transcriptional co-activators are suggested to be required for OsiRO2 to regulate gene expression (20). Therefore, IDE2 and OsiRO2 may cooperatively regulate some of the iron deficiency-inducible genes.

We previously demonstrated that the pair of IDE1 and IDE2 synergistically confers iron deficiency-inducible expression of the barley ID2 promoter (25). In the absence of IDE1, IDE2 alone produces little response to iron deficiency in the minimal promoter context, whereas connecting to the −90/−47 region of the 35S promoter enhances the function of IDE2 for iron deficiency-inducible expression (25). Our EMSA results revealed that IDE2 is able to recognize the 35S−(−90/−47) despite the absence of the IDE2-binding core sequence of IDE2 (Fig. 2f). IDE2 preferentially recognized sequences, including the TGACG sequence of the 5′-half of 35S−(−90/−47) (Fig. 2f). This DNA recognition property of IDE2 was not fully in accordance with that of previously characterized NAC proteins that recognize the 35S promoter (37–39). These results indicate that the IDE2 recognition sequence within the 35S−(−90/−47) may function as a novel cis-acting element involved in response to iron deficiency with dual recognition of IDE2 in addition to IDE2. To our knowledge, specific binding of a single DNA-binding site to two dissimilar DNA sequences is unusual among characterized transcription factors, and thus might be a unique character of IDE2. Our CASTing experiment indicated that IDE2 prefers IDE2-like binding sequences (Fig. 2e), suggesting that IDE2 predominantly functions via IDE2-like binding sequences. We recently identified that a pair of IDE1 and IDE1 is able to induce strong iron deficiency response in tobacco roots (27) in a similar fashion to prominent synergy conferred by the pair of IDE1 and IDE2 (25). These facts indicate that various combinations of cis-acting elements would be responsible for modulating the iron deficiency response, including IDE2 recognition sequences similar to either IDE2 or 35S−(−90/−47).

In the IDE2 RNAi rice and the CRES-T rice, gene expression of the Fe(II)-NA and Mn(II)-NA transporter gene OsYSL2 was dramatically repressed under iron-deficient conditions. NA, the precursor of MAs, is involved in translocation of metals not only in graminaceous plants but also in non-graminaceous plants (5, 6, 42, 43). Several reports have described the abnormal distribution of iron in plants in the absence of NA or mutation of related transporters (6, 42–45). The NA-free tomato mutant chloronerva shows interveinal chlorosis but overaccumulates iron in shoots and roots. Because of a deficiency of NA in chloronerva, iron is not chelated by NA in soluble and available forms within the cells (43). Accordingly, the chloronerva mutant constitutively expresses genes involved in iron uptake and accumulates more iron in all tissues. In a similar example, an Arabidopsis ferric reductase defective3 (frd3) mutant has been described (46). Recently, Durrett et al. (47) reported that FRD3 is responsible for loading citrate into the vacuature, a process necessary for the correct localization of iron throughout the plant. In the frd3 mutant, genes involved in iron uptake are also constitutively up-regulated (46, 47). Consequently, frd3 mutant plants overaccumulate iron as well as other metals, such as manganese and zinc, in roots. The IDE2 RNAi rice and the CRES-T rice were inclined to contain lower amounts of iron in shoots and higher amounts of iron in roots than the NT under iron-deficient conditions. In contrast, they contained higher amounts of iron both in shoots and roots under iron-sufficient conditions. These alterations in iron concentrations indicate that repression of the expression or activation ability of IDE2 caused impaired absorption or translocation of iron. Furthermore, the pattern of the abnormality in iron nutrition was different between iron-deficient and iron-sufficient conditions. OsYSL2, whose expression was dramatically repressed in the RNAi and CRES-T rice of IDE2, is suggested to be responsible for translocation of Fe(II)-NA and Mn(II)-NA in rice (16). Under iron-deficient conditions, repressed induction of OsYSL2 might have interrupted iron transport to shoots in the IDE2 RNAi rice and the CRES-T rice, leading to lower concentrations of iron in shoots and higher concentrations of iron in roots. In addition to repression of OsYSL2, several iron deficiency-inducible genes related to signaling and metabolism were also repressed in the IDE2 RNAi rice (supplemental Tables S2 and S3). These composite effects were considered to cause the abnormality in iron concentrations. We need to further examine this interesting phenotype of the RNAi and the CRES-T rice of IDE2 with detailed physiological and molecular biological experiments.

Transcripts of IDE2 and HvIDE2 are constitutively expressed and do not increase under iron deficiency (Fig. 3). Similar lack of transcriptional regulation in response to iron deficiency has also been observed for IDEF1 (27), which specifically recognizes IDE1 and might function in coordination with IDE2. Further examination will be required to determine how IDEF1 and IDEF2 activate the expression of genes under iron-deficient conditions. IDEF proteins may be regulated at the post-transcriptional level and may sense iron
levels and transmit the iron-deficiency signal to downstream targets.

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