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Title
The novel transcription factor IDEF1 regulates iron-deficiency response and tolerance

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Introduction

Most living organisms require iron for growth and reproduction, and the iron absorbed by plants constitutes a major source of iron for animals and humans. Although abundant in mineral soils, iron is only sparingly soluble under aerobic conditions at high pH. Consequently, in calcareous soils, which constitute about 30% of the world's cultivated soils, plants often exhibit iron-deficiency symptoms that manifest as chlorosis (yellowing caused by chlorophyll deficiency), reducing crop yield and quality. Higher plants use two major iron uptake strategies under conditions of low iron supply: reduction (Strategy I) and chelation (Strategy II). The Strategy-II mechanism is specific to graminaceous plants and is mediated by the mugineic acid family phytosiderophores (MAs), which are natural iron chelators, (Mori 1999). The expression of genes participating in these Fe-acquisition mechanisms is coordinately upregulated in response to iron deficiency (Mori 1999; Kobayashi et al. 2005), but the molecular mechanisms regulating iron-deficiency-induced genes are largely unknown.

Recent studies have demonstrated that in rice, an iron-deficiency-induced basic helix–loop–helix (bHLH) transcription factor, OsIRO2, regulates the Strategy II-based iron-deficiency response (Ogo et al. 2006, 2007). The core sequence to which OsIRO2 binds (CACGTGG) is overrepresented in iron-deficiency-inducible gene promoters in rice (Ogo et al. 2006), but its actual function in a given promoter has not been identified. In non-graminaceous plants, the involvement of several bHLH transcription factors in the iron-deficiency response, including tomato FER and the *Arabidopsis* FIT (formerly FIT1/FRU/AtbHLH29), AtbHLH38, and AtbHLH39, has been reported (Colangelo and Guerinot 2004; Walker and Connolly 2008), but their functional *cis* sequences have not been determined.

We previously analyzed the promoter region of the barley iron-deficiency-inducible *IDS2* gene using transgenic tobacco plants and identified the novel iron-deficiency-responsive *cis*-acting elements IDE1 and IDE2 (iron-deficiency-responsive element 1 and 2; Kobayashi et al. 2003). IDE1 and IDE2 synergistically induce iron-deficiency-specific gene expression in tobacco roots and in rice roots and leaves (Kobayashi et al. 2003, 2004). The promoter regions of many iron-deficiency-inducible genes in barley, rice, and *Arabidopsis* possess IDE-like sequences (Kobayashi et al. 2003, 2005). This suggests that gene regulation mechanisms involving IDEs are not only conserved among graminaceous (Strategy-II) plants but also are functional in non-graminaceous (Strategy-I) plant species. In the present study, we identified a novel rice transcription factor, IDEF1, which specifically binds to IDE1. We provide evidence that IDEF1 functions as a key component regulating the response to and tolerance of iron deficiency.

Materials and Methods

Most of the materials and methods in the present report have been described previously in Kobayashi et al. (2007), with exception for the information found below.

Construction of IDEF1 Knockdown Rice

To suppress *IDEF1* expression by RNA interference, a 300-bp fragment corresponding to

the 3' UTR of the *IDEF1* gene was amplified by PCR with the primers 5'-CACCATGCATTTGGGGTTAATTGC-3' and 5'-CCAACATAACTGAATTAAATAAAC-3'. The amplified fragment was inserted into the Gateway pENTR/D-TOPO vector (Invitrogen; http://www.invitrogen.com/), and the sequence was verified. Using an LR Clonase reaction (Invitrogen), the fragment was transferred into the destination vector pIG121-RNAi-DEST (Ogo et al. 2007) to construct the *IDEF1*–RNAi vector. This plasmid contains two inverted repeats of the *IDEF1* gene fragment in opposite directions connected by the *GUS* linker sequence. Transformation of rice (cv. Tsukinohikari) was performed by an *Agrobacterium*-mediated method.

Plant Materials and Growth Conditions

Non-transgenic (NT) and transgenic rice (cv. Tsukinohikari) lines were used for growth and expression assays. T_1 or T_2 seeds of *IDS2* promoter–*IDEF1* transformants (*I2*pro-*IDEF1*; *I2p-IDEF1,* in Kobayashi et al. 2007) and *IDEF1*–RNAi transformants were germinated on Murashige and Skoog (MS) medium containing hygromycin B (50 mg L^{-1}). NT seeds were germinated on MS medium lacking hygromycin. After 16 days of culture followed by an acclimation period of 3 d, the plantlets were transferred to nutrient solution (Kobayashi et al. 2005) in a greenhouse with a 30°C light/25°C dark cycle under natural light conditions. Fe-deficiency treatment was imposed on 26-day-old plantlets by transfer into culture solution without Fe(III)–EDTA or pH adjustment. The chlorophyll content of the youngest leaf was measured using a SPAD-502 chlorophyll meter (Konica–Minolta; http://www.konicaminolta.com). The nutrient solution was renewed on day 4.

Results and Discussion

Identification of IDEF1 as a Transcription Factor Recognizing the CATGC Sequence within IDE1

To identify candidates for IDE1-binding transcription factors, we first searched for known recognition sequences of transcription factors in IDE1 and IDE1-like sequences. IDE1 was found to possess a CATGC sequence similar to the Sph motif (TCCATGCAT)/RY element (CATGCA), which is recognized by the plant-specific B3 DNA binding domain of the ABI3/VP1 family of transcription factors (Suzuki et al. 1997; Reidt et al. 2000; Mönke et al. 2004). Furthermore, the IDE1-like sequences present within proximal regions of the *OsNAAT1* and *OsNAS2* promoters (Kobayashi et al. 2003, 2005) possess a canonical Sph motif/RY element. ABI3/VP1-family transcription factors transmit the abscisic acid signal and transactivate various genes during seed maturation (Suzuki and McCarty, 2008). A database search revealed five rice genes containing the B3 domain homologous to the ABI3/VP1 family of transcription factors (OsVP1, OsLFL1/OsFUS3, AK107456, AK072874, and AK101356; Fig. 1*A*), among which only OsVP1 and OsLFL1/OsFUS3 have been functionally characterized (Hattori et al. 1994; Peng et al. 2007; Moreno-Risueno et al. 2008). AK107456, AK072874, and AK101356 showed no pronounced homology to OsVP1 or OsLFL1/OsFUS3 outside of the B3 domain. A yeast assay system and an electrophoretic mobility shift assay (EMSA) were used to confirm the binding activity of rice ABI3/VP1 members to IDE1 *in vivo* and *in vitro*. In yeast cells carrying the *lacZ* gene under the control of three tandem repeats of IDE1, only AK107456

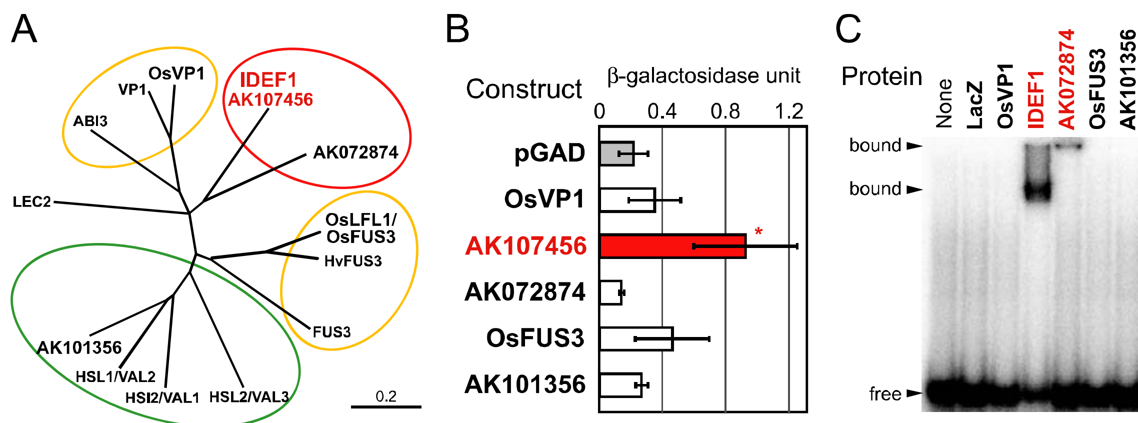

Figure 1. Identification and characterization of IDEF1. **(A)** Phylogenic tree of the ABI3/VP1 family of transcription factors. Deduced subgroups are indicated by circles: red, IDE1/Sph/RY-binding factors; orange, Sph/RY-binding factors transmitting the abscisic acid signal during seed maturation (ABI3/VP1 subgroup and FUS3 subgroup); and green, repressors of seed maturation-related genes during germination (VAL subgroup). **(B)** Binding assay of yeast GAL4 activation domain-fused rice ABI3/VP1 family members for IDE1 binding in yeast carrying IDE1 x 3-*lacZ*. Shown are β-galactosidase activity units (means ± s.d.; n = 5) and significant differences as compared to a vector control (pGAD). Data were analyzed using a *t*-test (*$p < 0.05$). **(C)** EMSA of rice ABI3/VP1 family members for IDE1 binding. An IDE1 probe was incubated with ABI3/VP1-family proteins fused to maltose binding protein (MBP); the LacZ–MBP fusion protein (LacZ) was used as a control.

induced substantial LacZ activity (Fig. 1*B*); this was designated as IDEF1 (IDE binding factor 1). Consistent with the yeast results, an electrophoretic mobility shift assay (EMSA) showed that IDEF1 specifically binds to IDE1 and Sph/RY, but not to IDE2 (Fig. 1*C*; data not shown). The AK072874 protein also bound specifically to IDE1 and Sph/RY to form an immobilized band on the plate, whereas OsVP1, OsLFL1/OsFUS3, and AK101356 exhibited no specific binding to IDE1 (Fig. 1*C*). The precise recognition sequence of IDEF1 and AK072874 was determined by competition experiments using mutated IDE1 sequences. IDEF1 and AK072874 specifically recognized the CATGC sequence present within IDE1, which is shorter than the previously reported minimal recognition sequence (CATGCA) of ABI3/VP1 transcription factors (Reidt et al. 2000; Mönke et al. 2004).

Northern blot analysis was conducted to detect the expression of the *ABI3/VP1* family of genes in rice roots and leaves during the vegetative stage. The *IDEF1* transcript was expressed constitutively in roots and leaves, with no obvious regulation by iron deficiency. AK072874 expression was not detected in these organs. Because of the substantial expression of *IDEF1* among the subgroup of IDE1/Sph/RY-binding factors (Fig. 1*A*), we focused on further characterizing IDEF1 in relation to the plant iron-deficiency response.

A transiently expressed IDEF1–green fluorescent protein (GFP) localized to the nucleus in onion epidermal cells. A database search of expressed sequence tags (ESTs) revealed the presence of *IDEF1* homologs in several graminaceous species, but no obvious homologs belonging to the *IDEF1* subgroup were found in non-graminaceous plants.

Transactivation Analysis of IDEF1 in Tobacco Plants

To characterize the *in planta* function of IDEF1, we first produced transgenic tobacco plants containing two copies of IDE1 fused to the β-glucuronidase (*GUS*) gene as a reporter, and then introduced either the *IDEF1* gene under the control of the constitutive 35S promoter (35S–*IDEF1*) or a vector control (VC) as an effector. 35S–*IDEF1* tobacco plants had slightly higher chlorophyll contents under conditions of iron deficiency than VC plants, but no other phenotypic differences were observed. Constitutive expression of the *IDEF1* transgene was detected in roots and leaves of 35S–*IDEF1* plants. These transformants did not show substantial GUS activity in iron-sufficient roots or iron-sufficient or -deficient leaves. In iron-deficient roots, however, strong GUS activity driven by the duplicated IDE1 was observed in VC plants and was even more evident in 35S–*IDEF1* plants. This iron-deficiency-induced and root-specific transactivation of GUS expression suggests that other factors are required for IDE-based activation *in planta*.

The IDEF1 Expression Level in Rice Affects Iron-Deficiency Tolerance and Iron-Deficiency-Induced Gene Expression

To investigate the function of IDEF1 in rice, we produced transgenic rice plants with induced or repressed *IDEF1* expression. For *IDEF1* induction, we introduced the *IDEF1* gene under the control of the iron-deficiency-inducible *IDS2* promoter (*I2*pro–*IDEF1* lines). For *IDEF1* repression, we used an RNA interference technique (*IDEF1*–RNAi lines). With an adequate iron supply, the *I2*pro–*IDEF1* and *IDEF1*–RNAi lines grew similarly to the non-transformants (NT). Time-course observations of these lines and NT grown in hydroponic medium lacking iron revealed that the decline in leaf chlorophyll was faster in *IDEF1*–RNAi lines and slower in *I2*pro–*IDEF1* lines compared to NT plants (Figs. 2*A, B*; data not shown). *I2*pro–*IDEF1* lines also grew better than NT seedlings when germinated in calcareous soil without additional micronutrient fertilizer, as confirmed by higher leaf chlorophyll contents and greater shoot lengths (Fig. 2*C*). These results demonstrate that *IDEF1* expression levels positively affect tolerance to Fe deficiency at the early stage.

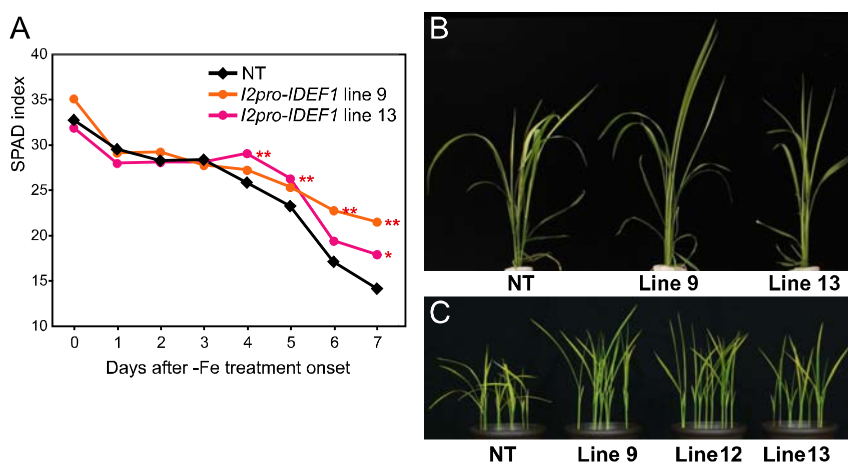

Figure 2. Tolerance of iron deficiency conferred by the expression of IDEF1 in rice. **(A)** Mean chlorophyll content (SPAD index) in the youngest leaves of *I2*pro–*IDEF1* transformants (lines 9 and 13) and NT during Fe-deficiency treatment in hydroponic culture. Data were analyzed using a *t*-test (*$p < 0.05$; **$p < 0.01$); n = 3–18. **(B)** Iron-deficiency-tolerant phenotype of *I2*pro–*IDEF1* transformants (lines 9 and 13) after five days of hydroponic iron-deficiency treatment. **(C)** Iron-deficiency-tolerant phenotype of *I2*pro–*IDEF1* transformants (lines 9, 12, and 13) 17 days after sowing in a calcareous soil.

Expression analysis of these transgenic plants revealed that alteration of *IDEF1* transcript levels affect the expression of many iron-deficiency-induced genes involved in iron uptake and utilization. The most pronounced was transactivation of *OsIRT1*, a ferrous transporter gene (Ishimaru et al. 2006), and *OsIRO2*, an iron-deficiency-induced bHLH transcription factor gene (Ogo et al. 2006, 2007). Because *OsIRT1* and *OsIRO2* possess IDEF1 binding core sequence (CATGC) in proximal regions of their promoter, IDEF1 is thought to regulate *OsIRT1* and *OsIRO2* directly through binding of IDE1-like elements.

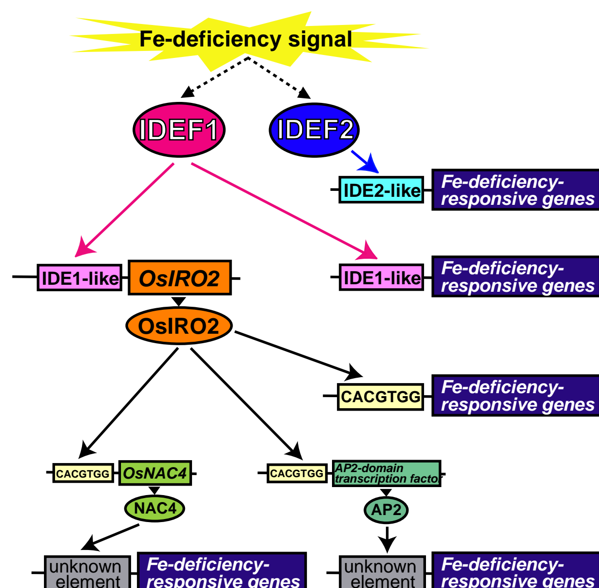

Figure 3. Proposed regulatory network for the induction of iron-deficiency-responsive genes *via* IDEF1, IDEF2, and OsIRO2. OsIRO2, which is induced by iron deficiency and is positively regulated by IDEF1, binds to the CACGTGG element to activate another subset of iron-deficiency-responsive genes, including two transcription factor genes: *OsNAC4* and the AP2 domain-containing gene. These transcription factors may then regulate iron-deficiency-responsive genes lacking IDEs and CACGTGG in their promoter regions (Ogo et al. 2007).

Gene Regulation Network of Iron-deficiency Responses in Rice

In another study, we identified another IDE-binding factor, IDEF2, which specifically recognizes IDE2 but not IDE1 (Ogo et al. 2008). IDEF2 belongs to the NAC transcription factor family, with no sequence homology to IDEF1. In contrast to previously reported transcription factors related to plant nutrition, IDEF1 and IDEF2 bind specifically to the functionally identified *cis*-acting elements, IDE1 and IDE2, respectively. Transcripts of *IDEF1* and *IDEF2* are constitutively expressed and their levels do not increase under conditions of iron deficiency (Ogo et al. 2008). Thus, IDEF1 and IDEF2 may constitute key components that transmit the iron-deficiency signal at an early stage. Furthermore, IDEF1 positively regulates the iron-deficiency-induced expression of OsIRO2, which plays a central role in regulating the iron-deficiency-induced genes involved in Strategy II iron acquisition (Ogo et al. 2006, 2007). Based on these results, we suggest the presence of a sequential gene regulatory network in response to iron deficiency (Fig. 3). In this regulatory network, *IDEF1* regulates tolerance to iron deficiency through the regulation of iron-acquisition-related genes. This regulation occurs directly through the binding of IDEF1 to IDE1-like elements and also indirectly through the induction of *OsIRO2*.

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