Title
A novel NAC transcription factor, IDEF2, which recognizes the iron deficiency-responsive element 2, regulates genes involved in iron homeostasis

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Introduction

Iron (Fe) is an essential element for most living organisms. Fe deficiency, a major abiotic stress, reduces crop yield, especially in calcareous soils in which the solubility of Fe is extremely low due to the high soil pH. Graminaceous plants take up Fe from soil using natural Fe chelators, the mugineic acid family phytosiderophores (MAs). MAs and its precursor nicotianamine (NA) are also involved in metal translocation in plants. The genes that encode key enzymes for MAs biosynthesis have been isolated from graminaceous plants such as barley and rice. In addition, several transporters involved in uptake and translocation of Fe have been characterized. Expression of these biosynthetic genes and transporter genes is up-regulated in response to Fe deficiency, but the molecular mechanisms regulating these Fe deficiency-induced genes are largely unknown. We previously analyzed the promoter region of the barley Fe deficiency-inducible gene *IDS2,* which encodes a dioxygenase catalyzing hydroxylation of MAs, and identified the novel Fe deficiency-responsive *cis*-acting elements IDE1 and IDE2. Recently, we identified a novel ABI3/VP1 family transcription factor that specifically recognizes IDE1; this we designated IDEF1 (IDE-binding factor 1) (Kobayashi et al., 2007). In the present study, we isolated a novel NAC domain transcription factor, IDEF2, which specifically binds to IDE2, using yeast one-hybrid screening. We provide evidence that IDEF2 functions as a key transcription factor in regulating the Fe-deficiency response.

Materials and Methods

Yeast one-hybrid screening—The reporter vectors (Fig. 1A) were constructed using target DNA fragments synthesized by sense oligomers and antisense primers (Ogo et al., 2008). The yeast one-hybrid screening of rice was performed using the BD MATCHMAKER Library Construction & Screening Kits (Clontech) according to the user manual. The cDNA expression library was constructed with mRNA from Fe-deficient rice roots. Screening was performed on medium lacking His, but in the presence of 50 mM 3-aminotriazole. The LacZ assay was performed according to the Yeast Protocols Handbook (Clontech). 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside was used as substrate.

Construction of IDEF2 RNAi rice and CRES-T rice—Three hundred base pairs of the 3’ UTR of *IDEF2* were amplified, and the amplified fragment was cloned into pIG121-RNAi-DEST as described previously (Ogo et al., 2007). To construct the vector for the chimeric repressor gene-silencing technology (CRES-T) system, coding sequences of *IDEF2*, except for the stop codon, were amplified with the primer included in the repression domain-coding region (Hiratsu et al., 2003). The amplified fragment was fused to the rice *Actin1* promoter. The transformation of rice (*Oryza sativa* L. cv. Tsukinohikari) was performed as described previously (Toki et al., 2006). T_1 seed were used for analysis.

Determination of metal concentrations—Rice was grown hydroponically without Fe for 6–8 days before harvesting. The roots were washed serially in 5 mM EDTA and ion-exchanged water. The metal concentrations were measured as previously described (Ishimaru et al., 2006).

Results and Discussion

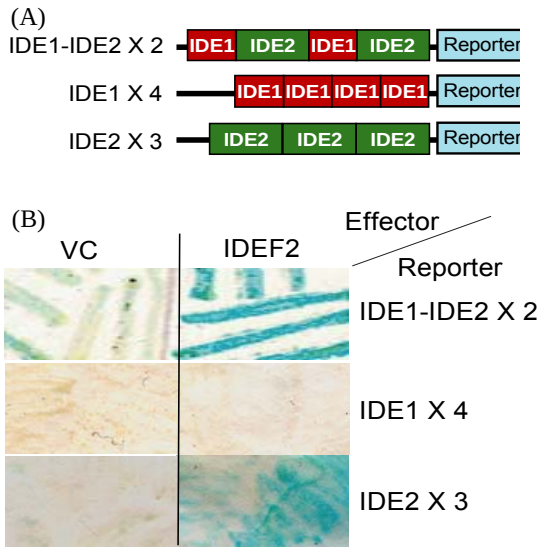

Figure 1. Identification and characterization of IDEF2 in yeast. (A) Schematics of the reporter genes used in the yeast one-hybrid. (B) IDE binding activity of IDEF2. LacZ activity was detected by blue staining.

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 Fe-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 arranged IDE1 and IDE2 (IDE1-IDE2 × 2) (Fig. 1A). Through the screening of 3.2×10^6 cDNA clones, one positive clone was obtained, which encoded a NAC family transcription factor corresponding to the rice full-length cDNA, AK099540. We designated this clone IDEF2 (IDE-binding factor 2). To examine recognition sequences of IDEF2 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 IDEF2, IDE2 × 3 induced substantial LacZ activity, while IDE1 × 4 did not (Fig. 1B). These results revealed that IDEF2 preferentially bound IDE2, but not IDE1. Furthermore, the binding core sequence of IDEF2 protein in IDE2 was determined as CA[A/C]G[T/C][T/C/A][T/C/A] by electrophoretic mobility shift assay and cyclic amplification and selection of targets (CASTing).

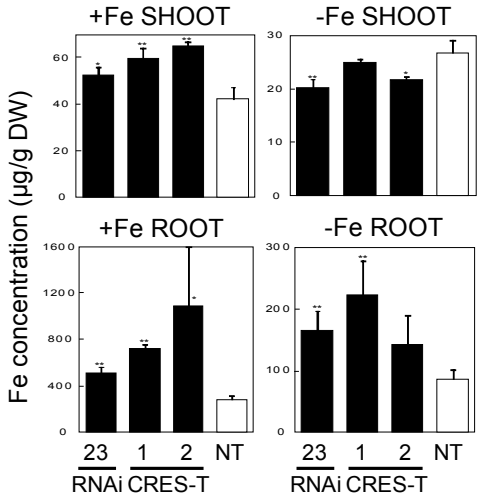

Figure 2. Iron concentration of the transgenic rice and NT under Fe-deficient (-Fe) and Fe-sufficient (+Fe) conditions. Data are presented as means ± SD (n = 3). **, $p < 0.01$; *, $p < 0.05$ (n = 3).

Characterization of IDEF2—To examine the function of IDEF2 *in planta*, we applied the RNAi technique and CRES-T (Hiratsu et al., 2003) to IDEF2 in rice. An RNAi line with strong repression of IDEF2 (RNAi23) and two lines with strong expression of *IDEF2 ORF-repression domain* (CRES-T1, CRES-T2) were selected and further analyzed. RNAi23, CRES-T1, CRES-T2, and non-transgenic rice (NT) were grown hydroponically in Fe-sufficient and Fe-deficient culture. Interestingly, the Fe concentrations of roots and shoots of the RNAi and CRES-T rice under Fe-sufficient conditions were much higher than those of NT (Fig. 2). In contrast, under Fe-deficient conditions, the Fe concentrations of shoots of the RNAi and CRES-T rice were somewhat lower than those of NT, although the Fe concentrations of the

roots were higher than those of NT. These results indicate that the nutritional status of Fe differs between the transgenic plants and NT. In addition, this difference varied between Fe-deficient and Fe-sufficient conditions. Thus, repression of the function of IDEF2 caused aberrant Fe homeostasis in plants.

Conclusion—IDEF2 recognizes the Fe deficiency-responsive element 2 and is involved in Fe distribution between shoots and roots.

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