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The spatial expression and regulation of transcription factors IDEF1 and IDEF2

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• *Background and Aims* Under conditions of low iron availability, rice plants induce genes involved in iron uptake and utilization. The iron deficiency-responsive *cis*-acting element binding factors 1 and 2 (IDEF1 and IDEF2) regulate transcriptional response to iron deficiency in rice roots. Clarification of the functions of IDEF1 and IDEF2 could uncover the gene regulation mechanism.

• *Methods* Spatial patterns of *IDEF1* and *IDEF2* expression were analysed by histochemical staining of *IDEF1* and *IDEF2* promoter-*GUS* transgenic rice lines. Expression patterns of the target genes of IDEF1 and IDEF2 were analysed using transformants with induced or repressed expression of *IDEF1* or *IDEF2* grown in iron-rich or in iron-deficient solutions for 1 d.

• *Key Results IDEF1* and *IDEF2* were highly expressed in the basal parts of the lateral roots and vascular bundles. *IDEF1* and *IDEF2* expression was dominant in leaf mesophyll and vascular cells, respectively. These expression patterns were similar under both iron-deficient and iron-sufficient conditions. *IDEF1* was strongly expressed in pollen, ovaries, the aleurone layer and embryo. *IDEF2* was expressed in pollen, ovaries and the dorsal vascular region of the endosperm. During seed germination, *IDEF1* and *IDEF2* were expressed in the endosperm and embryo. Expression of IDEF1 target genes was regulated in iron-rich roots similar to early iron-deficiency stages. In addition, the expression patterns of IDEF2 target genes were similar between iron-rich conditions and early or subsequent iron deficiency.

• *Conclusions IDEF1* and *IDEF2* are constitutively expressed during both vegetative and reproductive stages. The spatial expression patterns of *IDEF1* and *IDEF2* overlap with their target genes in restricted cell types, but not in all cells. The spatial expression patterns and gene regulation of IDEF1 and IDEF2 in roots are generally conserved under conditions of iron sufficiency and deficiency, suggesting complicated interactions with unknown factors for sensing and transmitting iron-deficiency signals.

Key words: IDE, IDEF, iron deficiency, *Oryza sativa*, reproductive organs, seeds, transcriptional regulation, vegetative organs.

INTRODUCTION

All plants require iron (Fe) for their growth and reproduction. Although abundant in mineral soils, Fe is only slightly soluble under aerobic conditions at high soil pH, especially in calcareous soils, which account for about 30 % of the world's cultivated soils. Fe deficiency is a widespread agricultural problem that reduces plant growth and crop yield (Marschner, 1995). Because plants are the primary food source for humans, the nutritional state of plants is of central importance to human health (Grusak and Dellapenna, 1999). Because excess free Fe is toxic to living cells (Guerinot and Yi, 1994), plants tightly control the level of Fe uptake, utilization and storage in response to Fe availability in the environment.

To take up and utilize Fe from the rhizosphere, higher plants have evolved two major strategies (Marschner *et al.*, 1986): reduction (Strategy I) and chelation (Strategy II). The Strategy II mechanism, which is specific to graminaceous plants, is mediated by natural Fe chelators, the mugineic acid family phytosiderophores (MAs). Graminaceous plants synthesize and secrete MAs from their roots to solubilize Fe(III) in the rhizosphere (Takagi, 1976), and the resulting Fe(III)–MAs complexes are taken up by the roots through Yellow Stripe 1 (YS1)/YS1-Like (YSl) transporters in the plasma membrane (Curie *et al.*, 2001, 2008). MAs are biosynthesized from methionine (Mori and Nishizawa, 1987) via four sequential enzymatic reaction steps (Shojima *et al.*, 1990; Bashir *et al.*, 2006). Rice (*Oryza sativa*) synthesizes 2′-deoxymugineic acid (DMA) and takes up Fe from the rhizosphere using an Fe(III)–DMA transporter such as OsYSL15 (Inoue *et al.*, 2009). Rice also takes up ferrous Fe using the OsIRT1 transporter (Ishimaru *et al.*, 2006).

Under conditions of low Fe availability, rice induces various genes involved in Fe acquisition and utilization (Kobayashi *et al.*, 2005; Kobayashi and Nishizawa, 2008). The genes showing induction under Fe deficiency include those responsible for DMA biosynthesis (nicotianamine synthase genes

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OsNAS1–3, nicotianamine aminotransferase gene *OsNAAT1*, DMA synthase gene *OsDMAS1*; Higuchi *et al.*, 2001; Inoue *et al.*, 2003, 2008; Bashir *et al.*, 2006); the Fe(III)–DMA transporter gene *OsYSL15* (Inoue *et al.*, 2009); the ferrous Fe transporter gene *OsIRT1* (Bughio *et al.*, 2002; Ishimaru *et al.*, 2006); the Fe(II)- and manganese(II)-nicotianamine transporter gene *OsYSL2*, which is thought to be involved in the internal transport of Fe within the plant body (Koike *et al.*, 2004); and the basic helix–loop–helix-type transcription factor gene *OsIRO2* (Ogo *et al.*, 2006). OsIRO2 positively regulates various Fe deficiency-induced genes related to DMA-based Fe acquisition, including *OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1* and *OsYSL15* (Ogo *et al.*, 2007). Over-expression of OsIRO2, using the cauliflower mosaic virus 35S promoter, results in higher induction of these target genes and enhanced tolerance to prolonged low Fe availability (Ogo *et al.*, 2007, 2009; Y. Ogo *et al.*, unpubl.res.)

Previous study used a promoter analysis of the barley MAs biosynthetic gene *IDS2* to identify Fe deficiency-responsive *cis*-acting elements 1 and 2 (IDE1 and IDE2; Kobayashi *et al.*, 2003). IDE1 and IDE2 are also functional in rice roots and leaves (Kobayashi *et al.*, 2004). Two rice transcription factors, IDEF1 (IDE-binding factor 1) and IDEF2, which specifically bind to IDE1 and IDE2, respectively, were recently identified (Kobayashi *et al.*, 2007; Ogo *et al.*, 2008). IDEF1 and IDEF2 belong to the plant-specific transcription factor families ABI3/VP1 and NAC, respectively. IDEF1 recognizes the CATGC sequence within IDE1, whereas IDEF2 predominantly recognizes CA[A/C]G[T/C][T/C/A][T/C/A] within IDE2 as the core binding site. Both *IDEF1* and *IDEF2* transcripts are constitutively expressed in rice roots and leaves. Transgenic rice plants with induced expression of *IDEF1* under the control of the barley *IDS2* promoter exhibited slower progression of leaf chlorosis in Fe-free hydroponic culture and also showed better early growth when germinated on calcareous soil (Kobayashi *et al.*, 2007). Conversely, *IDEF1*-knockdown lines generated by the RNA interference (RNAi) technique exhibited hypersensitivity in Fe-free hydroponic culture (Kobayashi *et al.*, 2009). Expression levels of *IDEF1* also positively affect the expression of various Fe deficiency-responsive genes. On day 7 of Fe deficiency treatment in hydroponic culture, *IDEF1* positively regulates *OsIRO2*, *OsIRT1* and *OsNAS3*, and genes encoding late embryogenesis-abundant proteins (Kobayashi *et al.*, 2007, 2009). Moreover, a majority of the known Fe acquisition/utilization-related genes, including *OsIRO2*, *OsIRT1*, *OsYSL15*, *OsYSL2*, *OsNAS1*, *OsNAS2*, *OsNAS3* and *OsDMAS1*, are positively regulated by IDEF1 during the earlier stages of Fe deficiency (Kobayashi *et al.*, 2009). On the other hand, IDEF2 also regulates Fe homeostasis by inducing another subset of Fe deficiency-responsive genes (Ogo *et al.*, 2008). RNAi-mediated IDEF2-knockdown lines exhibit aberrant Fe distribution between the roots and shoots and are defective in inducing Fe deficiency-responsive genes, including *OsYSL2*, on day 7 of Fe deficiency treatment in hydroponic culture (Ogo *et al.*, 2008). Earlier responses to Fe deficiency in the IDEF2-knockdown lines have not been investigated.

The present report aims to clarify the basal mechanism and properties of gene regulation mediated by IDEF1 and IDEF2. The spatial patterns of *IDEF1* and *IDEF2* expression were analysed by histochemical observation of promoter-β-glucuronidase (*GUS*) transformants. Expression of the target genes of *IDEF1* and *IDEF2* was also analysed by using transformants with altered expression of *IDEF1* or *IDEF2* under Fe-rich conditions or during an early stage of Fe deficiency. The results indicate that both *IDEF1* and *IDEF2* are constitutively expressed during both the vegetative and the reproductive stages and that both function under conditions of Fe sufficiency and deficiency.

MATERIALS AND METHODS

*Production of promoter-*GUS *rice lines*

To construct the *IDEF1* promoter-*GUS* vector, PCR was used to amplify the 1994-bp 5′-upstream region of the *IDEF1* gene from the translation start site (containing 1832 bp upstream from the transcription start site), using genomic DNA as a template. The primers used were 5′-CTCGAGTTAACCAGGAG ACTGACTGG-3′ (forward) and 5′-TCTAGAGTTGCCCTG TTCGCTCGCT-3′ (reverse). The amplified and verified fragment was excised using *Xho*I and *Xba*I (underlined) and placed in the pIG121Hm-Xho vector (Kobayashi *et al.*, 2004). For the *IDEF2* promoter-*GUS* vector, the 3464-bp 5′-upstream region of the *IDEF2* gene from the translation start site (containing 2000 bp upstream from the transcription start site) was amplified by PCR using genomic DNA and the primers 5′-TCTAGACCTAGGTACAACTTGAGGTAG CGGGACA-3′ (forward) and 5′-TCTAGACACTGCAGTG TATATCTTGCC-3′ (reverse). The amplified and verified fragment was excised using *Xba*I (underlined) and placed in the pIG121Hm-Xho vector.

The resultant *IDEF1* promoter-*GUS* or *IDEF2* promoter-*GUS* vector was transformed into rice (*Oryza sativa* L. 'Tsukinohikari') using an *Agrobacterium*-mediated method, as previously described (Hiei *et al.*, 1994; Higuchi *et al.*, 2001). T_1, T_2 or T_3 seeds were used in the analysis. For the *IDS2* promoter-*GUS* experiments, T_1 lines of the previously obtained transformants (I2 lines; Kobayashi *et al.*, 2004) were used.

*Growth conditions and histochemical observation of promoter-*GUS *rice lines*

For experiments in the vegetative stage, T_1, T_2 or T_3 seeds were germinated on Murashige and Skoog (MS) medium with hygromycin B ($50\,mg\,L^{-1}$) and transferred to hydroponic culture (Kobayashi *et al.*, 2005). For Fe deficiency treatments, plants that were 25–31 d old were transferred to a nutrient solution without Fe(III)-EDTA for 1–7 d. For analysis during the flowering and maturing periods, the promoter-*GUS* lines were transplanted to Fe-replete soil with fertilizer, and developing seeds were progressively sampled. Whole roots, transverse sections of roots and leaves, and longitudinally divided flowers and seeds were subjected to histochemical staining as described by Inoue *et al.* (2003) and Nozoye *et al.* (2007). Similar staining patterns were observed in at least three independent lines.

Plant materials and growth conditions for expression analysis of downstream genes of IDEF1 and/or IDEF2

Non-transgenic (NT) and transgenic rice ('Tsukinohikari') lines with induced or repressed expression of *IDEF1* or *IDEF2*

were used for the expression assays. T_1, T_2 or T_3 seeds of *IDS2* promoter-*IDEF1* transformants (*I2*pro-*IDEF1*; *I2p-IDEF1* in Kobayashi *et al.*, 2007), *IDEF1*-RNAi transformants (Kobayashi *et al.*, 2009) and *IDEF2*-RNAi transformants (Ogo *et al.*, 2008) were germinated on MS medium with hygromycin B (50 mg L^{-1}) and transferred to hydroponic culture (Kobayashi *et al.*, 2005). NT seeds were germinated on MS medium lacking hygromycin. For the Fe-rich treatment, plants that were 22–25 d old were transferred to a nutrient solution [containing 100 μM Fe(III)-EDTA] supplemented with 15·8 mg L^{-1} Tetsuriki-TypeX fertilizer (containing approx. 19 μM Fe^{2+}; Aichi Steel, Aichi, Japan; Matsuyama *et al.*, 2008). For Fe-deficiency treatments, plants that were 22–23 d old were transferred to a nutrient solution lacking Fe(III)-EDTA. After 24 h of treatment, roots were harvested from at least three plants of each line to reduce biological variance and were used for expression analysis.

Quantitative RT-PCR analysis

Total RNA was extracted from rice roots using the NucleoSpin RNA Plant Mini Kit (Macherey-Nagel, Düren, Germany). First-strand cDNA was synthesized using ReverTra Ace reverse transcriptase (TOYOBO, Tokyo, Japan) by priming with oligo-d(T)$_{17}$. Quantitative RT-PCR was performed with a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex Taq™ (Perfect Real Time) reagent (TaKaRa, Tokyo, Japan). The gene-specific primers used for PCR are listed in Supplementary Data Table S1 (available online). To exclude the detection of overexpressed RNAi trigger transcripts, the DNA-binding B3 region for *IDEF1* and the non-NAC region for *IDEF2* were quantified. Transcript abundance was normalized against rice *Actin* transcript levels and is expressed as a ratio relative to the levels in NT under Fe-rich conditions.

RESULTS

Spatial patterns of IDEF1 *expression*

Cell-type specificity of *IDEF1* expression was investigated by histochemical observation of transgenic rice plants with the introduced *IDEF1* promoter (2·0 kb)–*GUS* construct (Fig. 1). Expression of *IDEF1* was observed in every organ investigated. In roots, *IDEF1* expression was relatively dominant in the basal part of the lateral roots and near the root tips (Fig. 1A, B). Observation of transverse sections revealed that *IDEF1* expression in the primary roots was mainly limited to the phloem cells (Fig. 1C–F). In leaf blades, *IDEF1* expression was dominant in mesophyll cells and weaker in small vascular bundles (Fig. 1G–I). The phloem companion cells showed the most staining in the vascular bundles (Fig. 1I), whereas the main vascular bundle was not stained (data not shown). In the basal part of the leaf sheath, mesophyll and small vascular cells of the inner layers showed high *IDEF1* expression (Fig. 1J, K), whereas the outer layers showed little expression (Fig. 1J). These expression patterns in vegetative tissues were similar for Fe sufficiency and early or subsequent Fe deficiency (Fig. 1A–K; data not shown).

IDEF1 expression was also present in flowers and developing seeds throughout all stages (Fig. 1L–R). Prior to anthesis, *IDEF1* expression was dominant in the anthers (Fig. 1L, S), among which extremely strong staining was observed in pollen (Fig. 1T, U). Strong expression was also observed in the ovaries and the vascular bundles of the lemma and palea (Fig. 1L). After fertilization, the ovary showed high *IDEF1* expression (Fig. 1M). The vascular bundles of the lemma and palea maintained high expression during the flowering and grain-filling stages (Fig. 1M–P). There was strong staining of the embryo and the aleurone layer in the late stages of maturation (Fig. 1P–R). In embryos, the scutellum, seminal root and coleorhiza were densely stained (Fig. 1V).

In germinating seeds, strong *IDEF1* expression was observed in both the endosperm and the embryo (Fig. 1W–Z). Expression in the endosperm was especially strong on the outer surface, including the aleurone layer and dorsal and ventral vascular bundles, whereas expression in the embryo was especially strong in the epithelium, scutellum, and vascular bundles of the leaf primordium and seminal roots.

Spatial patterns of IDEF2 *expression*

Cell-type specificity of *IDEF2* expression was similarly investigated using *IDEF2* promoter (3·5 kb)–*GUS* transformants (Fig. 2). In roots, *IDEF2* expression was dominant in the vascular bundles of the lateral roots (Fig. 2A–D). Expression of *IDEF2* in the primary roots was mainly restricted to the epidermis, exodermis and vascular cells (Fig. 2E–G). In the leaf blades, *IDEF2* expression was prominent in small and large vascular bundles but was very weak in mesophyll cells (Fig. 2H–J). Similar to *IDEF1*, the expression patterns in vegetative tissues were not altered by Fe availability (data not shown).

IDEF2 was also expressed in flowers and developing seeds (Fig. 2K–S). Before anthesis, *IDEF2* expression was observed in anthers, including pollen, ovaries, and vascular bundles of the lemma and palea (Fig. 2K, P–R). After fertilization, the ovaries were still stained (Fig. 2L, M). In the late maturation stages, *IDEF2* expression was dominant in the dorsal vascular tissues (Fig. 2N, O). In embryos, the epithelium and leaf primordium were mainly stained (Fig. 2S).

In germinating seeds, *IDEF2* expression was strong in both the endosperm and the embryo (Fig. 2T–W). Strong expression was observed in the whole endosperm. In embryos, expression was dominant in the leaf primordium, scutellum, epithelium, and vascular bundles of the scutellum, bud scales and coleorhiza.

Spatial patterns of barley IDS2 *promoter-*GUS *expression in germinating seeds*

The spatial expression patterns of *IDEF1* and *IDEF2* were compared with their main target genes (Supplementary Data Table S2, available online). Among them, the barley *IDS2* gene promoter is assumed to be a putative heterologous target of IDEF1 and IDEF2 when introduced into rice, because it contains the canonical IDE1 and IDE2 elements (Kobayashi *et al.*, 2003, 2004). As the cell-type expression pattern of the *IDS2* promoter in rice has been investigated only in vegetative tissues (Kobayashi *et al.*, 2004), histochemical analysis of germinating

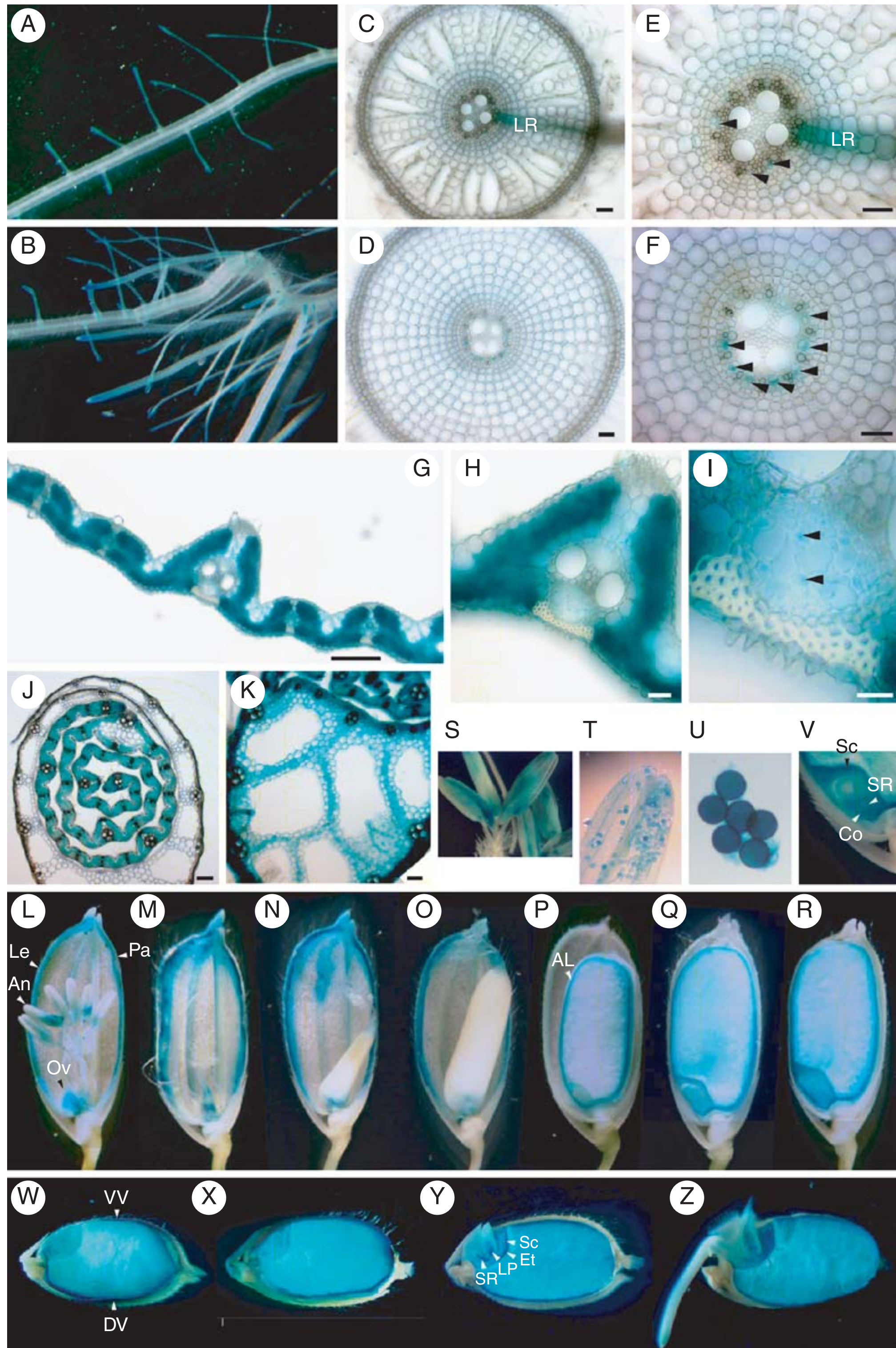

FIG. 1. Cellular localization of *IDEF1* expression as observed by histochemical staining of *IDEF1* promoter-*GUS* expression in transgenic rice plants. (A) Fe-sufficient roots. (B) Fe-deficient roots. (C) Transverse section of Fe-sufficient roots. (D) Transverse section of Fe-deficient roots. (E, F) Enlarged parts of vascular tissues shown in (C) and (D). Arrowheads: phloem cells. (G–I) Transverse sections of Fe-deficient leaf blades. (I) An enlarged part of the phloem cells in (H). Arrowheads: phloem companion cells. (J, K) Transverse sections of the basal parts of Fe-deficient (J) or Fe-sufficient (K) leaf sheaths. (L–V) Flowers and maturing seeds. (L, S–U) Before anthesis. (M–R, V) After anthesis. Just after fertilization (M), and 3 d (N), 5 d (O), 10 d (P), 15 d (Q) and 30 d (R) after fertilization. (S) An enlarged part of the anther. (T) Pollen sac. (U) Pollen. (V) An enlarged part of the embryo 30 d after fertilization. (W–Z) Germinating seeds. Just before sowing (W), and 1 d (X), 2 d (Y) and 3 d (Z) after sowing. Abbreviations: AL, aleurone layer; An, anther; Co, coleorhiza; DV, dorsal vascular bundle; Et, epithelium; Le, lemma; LP, leaf primodium; LR, lateral root; Ov, ovary; Pa, palea; Sc, scutellum; SR, seminal root; VV, ventral vascular bundle. Scale bars = 50 µm for (C–F); 100 µm for (G, J, K); and 10 µm for (H, I).

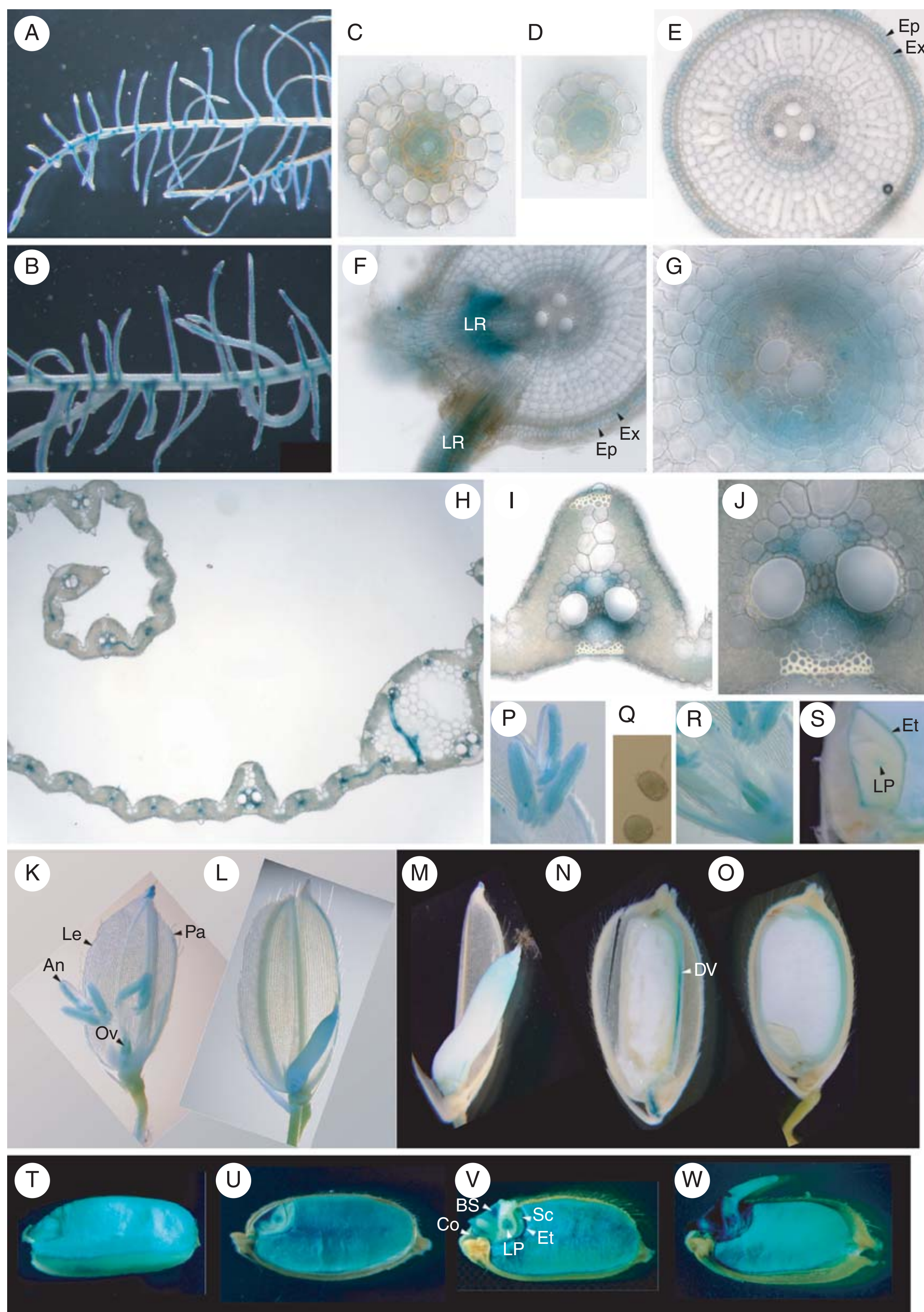

Fig. 2. Cellular localization of *IDEF2* expression as observed by histochemical staining of *IDEF2* promoter-*GUS* expression in transgenic rice plants. (A) Fe-sufficient roots. (B) Fe-deficient roots. (C, D) Transverse sections of Fe-sufficient secondary roots. (E–G) Transverse sections of Fe-deficient main roots. (G) Enlarged parts of vascular tissues. (H–J) Transverse sections of Fe-deficient leaf blades. (J) An enlarged part of the phloem cells in (I). (K–S) Flowers and maturing seeds. (K, P–R) Before anthesis. (P) An enlarged part of the anther. (Q) Pollen. (R) Ovary. (L–O, S) After anthesis: 3 d (L), 5 d (M), 10 d (N) and 30 d (O) after fertilization. (S) An enlarged part of the embryo 30 d after fertilization. (T–W) Germinating seeds. Just before sowing (T), and 1 d (U), 2 d (V) and 3 d (W) after sowing. Abbreviations: An, anther; BS, bud scale; Co, coleorhiza; DV, dorsal vascular bundle; Ep, epidermis; Et, epithelium; Ex, exodermis; Le, lemma; LP, leaf primodium; LR, lateral root; Ov, ovary; Pa, palea; Sc, scutellum.

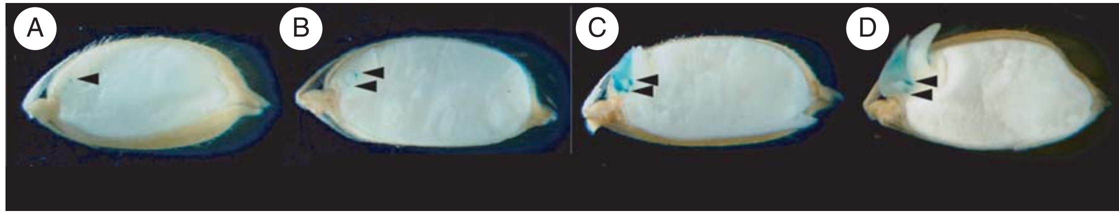

FIG. 3. Cellular localization of *IDS2* promoter-driven expression in germinating seeds as observed by histochemical staining of *IDS2* promoter-*GUS* expression in transgenic rice plants. Just before sowing (A), and 1 d (B), 2 d (C) and 3 d (D) after sowing. Arrowheads: outer layer of the basal seminal root.

seeds of *IDS2* promoter-*GUS* transformants was carried out (Fig. 3). In contrast to *IDEF1* and *IDEF2*, expression driven by the *IDS2* promoter was absent in the endosperm (Fig. 3A–D). In embryos, *IDS2* promoter activity was dominant in the outer layer of the basal seminal root and was also detected in the bud scales and coleorhiza.

IDEF1-mediated gene regulation under Fe-rich conditions

IDEF1 positively regulates various Fe deficiency-responsive genes in Fe-deficient roots and leaves (Kobayashi *et al.*, 2007, 2009). To identify IDEF1 function under conditions of Fe sufficiency, NT or transgenic rice plants with induced or repressed *IDEF1* expression were grown hydroponically and supplied with extra Fe fertilizer (Tetsuriki-TypeX; Matsuyama *et al.*, 2008) for 1 d to ensure Fe availability. Roots grown under this Fe-rich condition were subjected to quantitative RT-PCR analysis (Fig. 4). Transgenic lines with induced *IDEF1* expression driven by the *IDS2* promoter (*I2*pro-*IDEF1*; Kobayashi *et al.*, 2007) had *IDEF1* levels about seven times higher than that in NT, even under Fe-rich conditions (Fig. 4A), confirming that the *IDS2* promoter drives substantial basal expression in Fe-replete roots. *IDEF1* expression levels in *IDEF1*-knockdown lines (*IDEF1*-RNAi; Kobayashi *et al.*, 2009) were strongly repressed to 0·09 and 0·06 times that in NT in lines 2 and 5, respectively (Fig. 4A). Expression levels of several *IDEF1* target genes during early Fe deficiency (Kobayashi *et al.*, 2009) were quantified in these plants (Fig. 4B). *OsIRO2*, *OsYSL2*, *OsNAS2* and *OsYSL15* exhibited higher expression in the roots of the *I2*pro-*IDEF1* line than in NT roots. Expression levels of these genes were similar in NT and *IDEF1*-RNAi line 2, whereas expression levels of *OsYSL2* and *OsNAS2* were repressed in *IDEF1*-RNAi line 5.

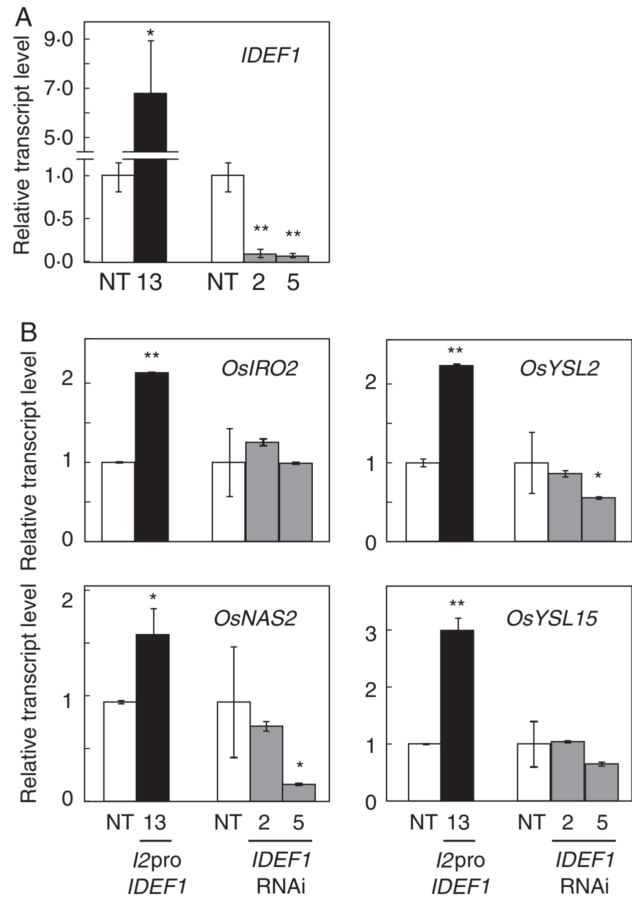

FIG. 4. Quantitative RT-PCR analysis of *IDEF1* (A) and its typical target genes *OsIRO2*, *OsYSL2*, *OsNAS2* and *OsYSL15* (B) under Fe-rich conditions in hydroponic culture. *I2*pro-*IDEF1* transformants (line 13, T_3) or *IDEF1*-RNAi transformants (line 2, T_3; line 5, T_2) were individually cultured with non-transformants (NT) and supplied with 15·8 mg L^{-1} Tetsuriki-TypeX fertilizer for 24 h. Transcript abundances in the roots were quantified and are expressed as ratios relative to NT levels (mean ± s.d.; $n = 3–6$). Significant differences from NT, analysed using a *t*-test (*$P < 0·05$; **$P < 0·01$), are shown.

IDEF2-mediated gene regulation under Fe-rich conditions and during an early stage of Fe deficiency

The target genes of IDEF2 were previously identified during a 7-d Fe deficiency treatment (Ogo *et al.*, 2008). In the present study, expression of these IDEF2 target genes under Fe-rich or early Fe-deficient conditions was analysed. *IDEF2* knockdown lines (*IDEF2*-RNAi; Ogo *et al.*, 2008) or NT plants were hydroponically grown with supplemented Tetsuriki-TypeX fertilizer or transplanted to Fe-free solution for 1 d, and the roots were subjected to quantitative RT-PCR (Fig. 5). *IDEF2* expression was effectively repressed to 0·13–0·21 times that in NT in *IDEF2*-RNAi lines 22 and 23 under both Fe-rich and early Fe-deficient conditions (Fig. 5A). Among the typical IDEF2 target genes under prolonged Fe deficiency, *OsYSL2*, *AK099523* (function unknown) and *AK103890* (ubiquitin gene) showed repressed expression in *IDEF2*-RNAi lines under both Fe-rich and early Fe-deficient conditions (Fig. 5B). Expression of *AK065090* (heme peroxidase gene) was repressed in *IDEF2*-RNAi lines under Fe-deficient conditions but not in Fe-rich conditions (Fig. 5B). Although these four genes are induced after 7 d of Fe deficiency (Ogo *et al.*, 2008), only *OsYSL2* showed induction in NT roots

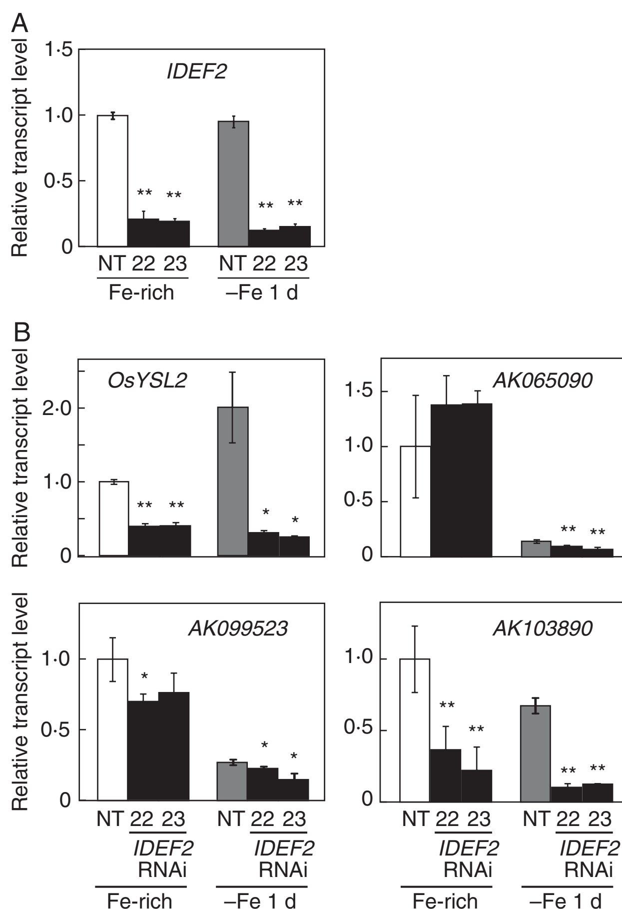

FIG. 5. Quantitative RT-PCR analysis of *IDEF2* (A) and its typical target genes *OsYSL2*, *AK065090*, *AK099523* and *AK103890* (B) under Fe-rich and early Fe-deficient conditions in hydroponic culture. *IDEF2*-RNAi transformants (lines 22 and 23, T_1) and NT were either supplied with 15·8 mg L^{-1} Tetsuriki-TypeX fertilizer (Fe-rich) or cultured in Fe-free solution (−Fe 1 d) for 24 h. Transcript abundances in roots were quantified and are expressed as ratios relative to NT levels in Fe-rich conditions (mean ± s.d.; $n = 3–6$). Significant differences from NT, analysed using a *t*-test (*$P < 0·05$; **$P < 0·01$), are shown.

after 1 d of Fe deficiency; expression of *AK065090*, *AK099523* and *AK103890* was repressed (Fig. 5B).

DISCUSSION

IDEF1 *and* IDEF2 *are expressed throughout the life cycle*

Plants manage to maintain Fe homeostasis under conditions of low Fe availability through the induction of Fe uptake- and utilization-related genes, although this Fe deficiency response has been investigated almost exclusively in vegetative tissues, predominantly roots (Kobayashi and Nishizawa, 2008). Recent studies have revealed that IDEF1 and IDEF2 play crucial roles in the regulation of Fe deficiency-responsive genes in rice roots and leaves (Kobayashi *et al.*, 2007, 2009; Ogo *et al.*, 2008). The present study utilized the *IDEF1* and *IDEF2* promoters containing 2·0 and 3·5 kb, respectively, from the translation start site for promoter-*GUS* analysis, because these promoter fragments contain the whole 5′-unstranslated regions and about 2·0 kb of the upstream regions from the transcription start sites. Constitutive expression derived from both the *IDEF1* and the *IDEF2* promoters was observed not only in vegetative tissues but also in reproductive tissues (Figs 1 and 2), consistent with previous Northern blot and microarray analyses (Kobayashi *et al.*, 2007; Ogo *et al.*, 2008; K. Usuda *et al.*, University of Tokyo, Tokyo, unpubl. res.). This suggests that these promoter fragments contain all the essential elements for expression. Notably, the dominant Fe deficiency-responsive genes involved in Fe uptake and/or utilization are also expressed in reproductive tissues grown in Fe-replete soil or media (Table S2, available online; Koike *et al.*, 2004; Nozoye *et al.*, 2007; Inoue *et al.*, 2009; Ogo *et al.*, 2009). The presence of *IDEF1* and *IDEF2* expression in reproductive tissues suggests the regulation of their target genes, including those related to Fe homeostasis, in these tissues.

IDEF1 belongs to the ABI3/VP1 family of transcription factors, which regulate seed-specific gene expression through recognition of RY elements (CATGCA; Suzuki *et al.*, 1997; Suzuki and McCarty, 2008). IDEF1 is an exceptional member in this family in that it is able to efficiently recognize a shorter sequence (CATGC) and, thus, the Fe deficiency-responsive IDE1 element (Kobayashi *et al.*, 2007). However, IDEF1 also recognizes RY elements and regulates late embryogenesis-abundant genes in Fe-deficient roots and leaves (Kobayashi *et al.*, 2009). Therefore, IDEF1 might have a dual function in reproductive tissues: CATGC-mediated regulation of Fe utilization-related genes and RY element-mediated regulation of seed maturation-related genes.

Overlapping expression of IDEF1 *and* IDEF2 *with their target genes is achieved in restricted cell types*

Previous promoter-*GUS* analyses revealed that expression patterns of the dominant Fe deficiency-responsive genes involved in MA-based Fe acquisition, including *OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1* and *OsYSL15*, are very similar in roots and leaves (Table S2; Inoue *et al.*, 2003, 2008, 2009; Bashir *et al.*, 2006; Ishimaru *et al.*, 2006). Expression of these genes is observed mainly in phloem tissues of Fe-sufficient roots and leaves and is strongly induced throughout all tissues in the roots and leaves in response to Fe deficiency. Expression of these genes is positively regulated by the Fe deficiency-inducible transcription factor OsIRO2 (Ogo *et al.*, 2007). The expression pattern of *OsIRO2* is also highly similar to that of these Fe uptake/utilization-related genes (Y. Ogo *et al.*, unpubl. res.), suggesting that OsIRO2 directly regulates these target genes in the same cells in which it is expressed.

In contrast to these synchronous expression patterns, the present results revealed that the regions of *IDEF1* and *IDEF2* expression in roots and leaves are more restricted than their target genes (Figs 1 and 2; Table S2). The predominant expression of *IDEF1* and *IDEF2* in the basal parts of the lateral roots and phloem cells (Figs 1A–F, 2A–G) is consistent with the preferred expression of the Fe utilization-related genes *OsYSL2*, *OsNAS1*, *OsNAS2*, *OsDMAS1*, *OsYSL15* and *OsIRT1* in these cells (Inoue *et al.*, 2003, 2009; Koike *et al.*, 2004; Bashir *et al.*, 2006; Ishimaru *et al.*, 2006). This observation suggests that IDEF1 and IDEF2 induce Fe deficiency-responsive genes in these cells and that this may play an important role in Fe utilization. However, lack of substantial expression of *IDEF1* in the epidermis/exodermis and cortex of the main roots

(Fig. 1C–F) is not consistent with induction of its main target genes throughout the Fe-deficient roots (Table S2; Inoue *et al.*, 2003, 2009; Bashir *et al.*, 2006; Ishimaru *et al.*, 2006). In addition, *IDEF1* expression in leaves is stronger in mesophyll cells than in vascular cells (Fig. 1G–I), opposite to the predominant expression of Fe utilization-related genes in vascular tissues (Table S2; Inoue *et al.*, 2003, 2009; Koike *et al.*, 2004; Bashir *et al.*, 2006). If shoot-borne long-distance signal which regulates Fe deficiency-induced genes in roots might exist, as suggested by Enomoto *et al.* (2007, 2009), the predominant expression of *IDEF1* in root vascular tissues might be favourable for receiving such signals. However, induction of Fe uptake-related genes in the epidermis/exodermis of the main roots, where *IDEF1* expression is absent, would require the radial movement of IDEF1 proteins and/or related factors from vascular cells. Horizontal signal transfer has also been suggested in *Arabidopsis* sulfur deficiency response from the vasculature-expressed key transcription factor SULFUR LIMITATION 1 (SLIM1) toward its epidermis-expressed target gene *SULTR1;2* (Maruyama-Nakashita *et al.*, 2006). Radial movement of transcription factors was reported for SHOOT-ROOT (SHR) from *Arabidopsis* stele to adjacent cells in root cell patterning (Nakajima *et al.*, 2001). Alternatively, Fe deficiency-induced expression in the root epidermis/exodermis might be mediated by a distinct mechanism from IDEF1-dependent transactivation in vascular cells.

Expression of *IDEF1* and *IDEF2* in reproductive organs tended to be more widely distributed than their target genes in vegetative organs (Table S2). In germinating seeds, the expression patterns of *IDEF1*, *IDEF2* and *OsNAS1* were similar; all were expressed in the embryo and endosperm (Figs 1W–Z, 2T–W, Table S2; Nozoye *et al.*, 2007). In contrast, other Fe utilization-related genes were not expressed in the endosperm (Table S2; Nozoye *et al.*, 2007; Inoue *et al.*, 2009). In addition, expression of the heterologously introduced barley *IDS2* gene promoter in germinating seeds was restricted to the embryo and was absent in the endosperm (Fig. 3), even though the *IDS2* promoter contains a functional IDE1 and IDE2 set (Kobayashi *et al.*, 2003, 2004). These observations indicate that the expression of IDEF1 and IDEF2 is not necessarily sufficient for the induction of the vegetative target genes in germinating seeds, suggesting the presence of unknown factors mediating IDEF-based gene expression in seeds. Also, the target genes of IDEF1 and IDEF2 might not be identical between reproductive and vegetative organs.

In floral tissues, *IDEF1* expression was especially high in pollen (Fig. 1T, U). *IDEF2* was also expressed in pollen but to a lesser extent (Fig. 2P, Q). Interestingly, an Fe(III)-DMA transporter gene, *OsYSL18*, is expressed in pollen grains (Aoyama *et al.*, 2009). Although the expression of *OsYSL18* is neither induced under Fe deficiency nor regulated by IDEF1 or IDEF2 in vegetative tissues (Aoyama *et al.*, 2009; T. Kobayashi *et al.* and Y. Ogo *et al.*, unpubl. res.), IDEF1 and IDEF2 might regulate *OsYSL18* or other genes involved in pollen Fe acquisition.

IDEF1 and IDEF2 regulate gene expression in both Fe-sufficient and Fe-deficient roots

The constitutive expression of *IDEF1* and *IDEF2* suggests a role in sensing Fe deficiency signals and triggering a transcriptional cascade. In this respect, it is important to know whether IDEF-mediated transactivation occurs even under conditions of Fe sufficiency. Recent analysis suggested that IDEF1 regulates the expression of its target genes even when grown in standard hydroponic culture, similar to early Fe-deficiency stages (Kobayashi *et al.*, 2009). However, it was not clear whether this transactivation is due to the basal activity of IDEF1 under Fe sufficiency or, alternatively, due to a response to physiological Fe deficiency that might occur even in standard 'Fe-sufficient' culture. To discriminate among these possibilities, IDEF-mediated transactivation under Fe-rich conditions was confirmed (Figs 4 and 5). By using Tetsuriki fertilizer (Matsuyama *et al.*, 2008), the basal expression of sensitive Fe deficiency-inducible genes was repressed to lower levels than in standard 'Fe-sufficient' conditions (data not shown). Even under these 'Fe-rich' conditions, the *I2*pro-*IDEF1* transformants induced substantially higher *IDEF1* expression (Fig. 4A), indicating the presence of basal activity of the *IDS2* promoter that is possibly conferred by IDE1 and IDE2, and thus IDEF1 and IDEF2. Furthermore, the transcript levels of *IDEF1* were positively correlated with those of their target genes as under early Fe deficiency (Fig. 4B). Similarly, the gene expression patterns of *IDEF2* knockdown lines (Fig. 5) indicated that the expression of many IDEF2 target genes on day 7 of Fe deficiency was also regulated by IDEF2 under Fe-rich conditions as well as during early Fe deficiency. In contrast to IDEF1, IDEF2 appears to cause no remarkable alteration in the gene regulation patterns between early and subsequent stages of Fe deficiency (Fig. 5; Ogo *et al.*, 2008). These results indicate that IDEF1 and IDEF2 function to activate the expression of Fe homeostasis-related genes even under Fe sufficiency. Thus, IDEF1 and IDEF2 may already be present before the onset of Fe deficiency in their active forms. At present, other factors that mediate the IDEF functions are not known.

In contrast to observations in rice, constitutive IDEF1 overexpression in tobacco resulted in IDE1-mediated transactivation in Fe-deficient roots but not in Fe-sufficient roots (Kobayashi *et al.*, 2007), suggesting the requirement of Fe-deficient conditions for IDEF1 function in tobacco plants. Nevertheless, the lack of information on IDEF1 orthologues in tobacco, as well as the distinct tissue-specific response of IDE1 between rice and tobacco (Kobayashi *et al.*, 2003, 2004, 2007), makes it difficult to explain the IDEF1 function from the heterologous tobacco system. Future identification of IDEF-interacting factors, as well as the nature of the Fe deficiency signal, will be required to understand the gene regulation mechanisms mediated by IDEF1 and IDEF2.

In summary, the constitutive expression of *IDEF1* and *IDEF2* during the rice life cycle and generally conserved gene regulation mediated by IDEF1 and IDEF2 between Fe sufficiency and deficiency suggest complicated interactions with unknown factors in sensing and transmitting Fe deficiency signals to facilitate Fe utilization-related genes.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following tables. Table S1: Primers used for quantitative RT-PCR. Table S2: Summary of the expression patterns of *IDEF1*, *IDEF2* and

Fe deficiency-inducible genes involved in Fe uptake and/or utilization.

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