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In vivo evidence that *Ids3* from *Hordeum vulgare* encodes a dioxygenase that converts 2′-deoxymugineic acid to mugineic acid in transgenic rice

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Abstract. We proposed that an Fe-deficiency-induced gene, *Ids3* (Iron deficiency specific clone no. 3), from barley (*Hordeum vulgare* L.) roots encodes a dioxygenase that catalyzes the hydroxylation step from 2′-deoxymugineic acid (DMA) to mugineic acid (MA). To prove this hypothesis, we introduced the *Ids3* gene into rice (*Oryza sativa* L.), which lacks *Ids3* homologues and secretes DMA, but not MA. Transgenic rice plants, carrying either *Ids3* cDNA or a barley genomic DNA fragment (20 kb) containing *Ids3*, were obtained using *Agrobacterium*-mediated transformation. *Ids3* cDNA under the control of the cauliflower mosaic virus 35S promoter was constitutively expressed in both the roots and the leaves of the transgenic rice, regardless of Fe nutrition status. In contrast, in the roots of transformants carrying a barley genomic fragment, transcripts of *Ids3* were markedly increased in response to Fe deficiency. Slight expression of *Ids3* was also observed in the leaves of the Fe-deficient plants. Western blot analysis confirmed the induction of *Ids3* in response to Fe deficiency in the roots of the transformants carrying a genomic fragment. These expression patterns indicate that the 5′-flanking region of *Ids3* works as a strong Fe-deficiency-inducible promoter in rice, as well as in barley. Both kinds of transgenic rice secreted MA in addition to DMA under Fe-deficient conditions, but wild-type rice secreted only DMA. This is in vivo evidence that IDS3 is the "MA synthase" that converts DMA to MA.

Key words: Dioxygenase – Iron deficiency – Mugineic acid family phytosiderophores – Mugineic acid synthase – *Oryza* (transgenic) – Transgenic rice

Introduction

To solubilize sparingly soluble Fe in the rhizosphere, graminaceous plants secrete mugineic acid family phytosiderophores (MAs), which are natural Fe chelators, from their roots (Takagi 1976). The resulting Fe(III)-MAs complexes are reabsorbed into the root through a specific transporter in the plasma membrane (Takagi 1976; Mihashi and Mori 1989). This mechanism for acquiring Fe(III) with MAs is specific to graminaceous species, and is classified as a Strategy-II mechanism (Marschner et al. 1986). The amount of MAs secreted increases under Fe-deficient conditions (Takagi 1976). To date, seven MAs have been identified, and their biosynthetic pathways have been determined (Fig. 1) (Mori and Nishizawa 1987; Nomoto et al. 1987; Shojima et al. 1990; Ma et al. 1999). Methionine is the primary precursor of MAs (Mori and Nishizawa 1987). It is thought that the methionine is generated in the root itself (Nakanishi et al. 1999), where the methionine cycle is assumed to work vigorously to supply methionine (Ma et al. 1995). A gene involved in this cycle, *IDI1* (Yamaguchi et al. 2000), and a gene closely related to this cycle, an adenine phosphoribosyltransferase gene (Itai et al. 2000), have been isolated from the roots of Fe-deficient barley.

We have cloned and characterized some of the crucial genes involved in the biosynthesis of MAs. Among these, only the expression of *S*-adenosyl-L-methionine synthetase genes was not induced in barley roots by Fe deficiency (Takizawa et al. 1996). The genes encoding nicotianamine synthase and nicotianamine aminotransferase were isolated from roots of Fe-deficient barley through enzyme purification. The expression of both genes and their enzyme activities are strongly induced by Fe deficiency in barley roots (Kanazawa et al. 1994; Higuchi et al. 1996, 1999b; Takahashi et al. 1999). To isolate the genes involved in the Fe-acquisition mechanism of graminaceous species, we also applied an indirect method: "differential hybridization". Seven clones were obtained from a cDNA library prepared from Fe-deficient barley roots. We have reported the sequence characteristics of three cDNAs (*Ids1, Ids2*, and

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Abbreviations: DMA = 2′-deoxymugineic acid; epiHDMA = 3-epihydroxy-2′-deoxymugineic acid; (epi)HMA = 3-(epi)hydroxy-mugineic acid; MA = mugineic acid; MAs = mugineic acid family phytosiderophores

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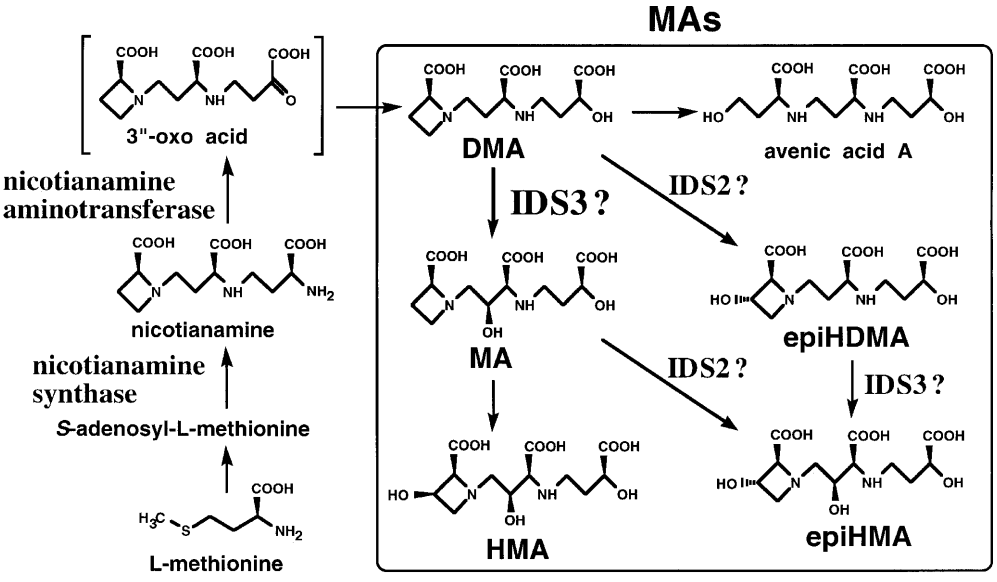

Fig. 1. Biosynthetic pathway of MAs and hypothetical steps that IDS3 and IDS2 catalyze

Ids3) that are expressed in response to Fe deficiency (Okumura et al. 1991, 1992, 1994; Nakanishi et al. 1993, 2000). *Ids1* encodes a plant metallothionein protein; it is homologous to a number of metallothionein genes that have been isolated from other plant species. *Ids2* and *Ids3* are homologous to 2-oxoglutarate-dependent dioxygenases. The expression patterns of the *Ids3* gene in various graminaceous species strongly suggest that *Ids3* encodes an enzyme that catalyzes the hydroxylation steps from 2′-deoxymugineic acid (DMA) to mugineic acid (MA), and from 3-epihydroxy-2′-deoxymugineic acid (epiHDMA) to 3-epihydroxymugineic acid (epiHMA). *Ids2* is thought to encode an enzyme that converts DMA to epiHDMA and MA to epiHMA (Fig. 1) (Nakanishi et al. 2000). In order to confirm the function of *Ids3*, we produced IDS3 protein in *Escherichia coli* and tried to detect enzyme activity in vitro. However, attempts to convert DMA to MA were unsuccessful. Therefore, we tried to prove the function of *Ids3* in vivo, using transgenic rice plants. In this study, we introduced the barley *Ids3* gene into the rice cultivars Nipponbare and Tsukinohikari, which have no *Ids3* homologues and secrete only DMA of the MAs. Consequently, we proved that *Ids3* encodes the "MA synthase" catalyzing the step from DMA to MA.

Materials and methods

Plasmid construction

Plasmid pIGI3c (Fig. 2A) was constructed to constitutively express *Ids3* cDNA as follows: the *Ids3* cDNA sequence containing the entire open reading frame was amplified by the polymerase chain reaction using the forward primer 5′-CGAGAAGCTTCTAGACCCCCTGATTTTACCATGG-3′ and the reverse primer 5′-CGAGGATATCTGCAGACAAGTAAGTGACAACAAATGAGCA-3′, which contain *Xba*I and *Pst*I sites, respectively (underlined). A cDNA pool reverse-transcribed from poly(A)$^+$RNA obtained from roots of Fe-deficient barley was used as the template (Nakanishi et al. 2000). The amplified fragment was digested by

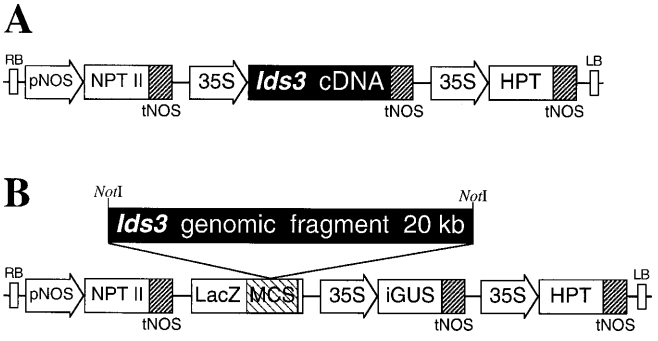

Fig. 2. T-DNA regions of the introduced plasmids pIGI3c (**A**) and pBII3g (**B**). *RB* Right border; *LB* left border; *pNOS* nopaline synthase promoter; *NPTII* neomycin phosphotransferase gene; *tNOS* nopaline synthase terminator; *35S* cauliflower mosaic virus 35S promoter; *HPT* hygromycin phosphotransferase gene; *LacZ* β-galactosidase gene; *MCS* multicloning site; *iGUS* β-glucuronidase gene with an intron

*Xba*I and *Pst*I, and ligated into the same sites of pBluescriptII SK(+). The nopaline synthase terminator (tNOS) fragment was excised from pBlue-sGFP(S65T)-NOS SK (a gift from Dr. Y. Niwa, University of Shizuoka; Chiu et al. 1996) using *Pst*I and *Sal*I, and was fused downstream from the *Ids3* cDNA at the *Pst*I and *Sal*I sites. The resulting *Ids3* cDNA-tNOS fragment was excised using *Xba*I and *Sal*I, and was replaced with the β-glucuronidase gene-tNOS fragment of pIG121Hm (Hiei et al. 1994) to generate pIGI3c.

Plasmid pBII3g (Fig. 2B) was constructed as follows: an approximately 20-kb barley genomic fragment containing the *Ids3* gene was obtained from a commercially available λFIXII genomic library (StrataGene; variety Igri) (Nakanishi et al. 2000). To transform rice, this fragment was excised using *Not*I and ligated into the *Not*I site of pBIGRZ1 (Akiyama et al. 1997), which is capable of holding a large insert.

Rice transformation

pIGI3c was introduced into rice (*Oryza sativa* L.) cultivar Nipponbare, and pBII3g was introduced into cultivar Tsukinohikari. The method described by Hiei et al. (1994) was used for *Agrobacterium*-mediated rice transformation with some modifica-

tion. Four-week-old calli developed from mature seeds were immersed in the *Agrobacterium* suspension at a density of about 3×10^8 cells/ml for 30 s. After co-cultivation, selection with hygromycin was performed using 10 mg/l hygromycin B for 1 week and 50 mg/l hygromycin B for 3 weeks. Then, calli were cultured on MS medium (Murashige and Skoog 1962) containing 30 g/l sucrose, 30 g/l sorbitol, 2 g/l casamino acid, 5 mM 2-morpholinoethanesulphonic acid, 2 mg/l α-naphthaleneacetic acid, 1 mg/l kinetin, 250 mg/l Claforan, 50 mg/l hygromycin B, and 0.4% gellan gum for 3 weeks, and then on MS medium containing 30 g/l sucrose, 250 mg/l Claforan, 50 mg/l hygromycin B, and 0.8% agar at 28 °C until regeneration, under a 16 h light and 8 h dark regime. Regenerated plantlets (R_0 plants) were transferred to soil and grown under the conditions described below.

Plant materials and culture conditions

Transgenic and wild-type plants were grown in a controlled environment with a 30 °C/14 h light −25 °C/10 h dark cycle.

For analysis of R_1 plants, selfed transgenic seeds (R_1) were germinated on MS medium containing 50 mg/l hygromycin B after seed sterilization. Wild-type seeds (cultivar Nipponbare for the analysis of transgenic plants with introduced pIGI3c and cultivar Tsukinohikari for plants with introduced pBII3g) were germinated on MS medium without hygromycin, at the same time. The cultivar Nipponbare and cultivar Tsukinohikari seedlings were transplanted to hydroponic culture after 17 or 38 d incubation, respectively. The composition of the nutrient solution was as follows: 0.35 mM $(NH_4)_2SO_4$, 0.18 mM Na_2HPO_4, 0.27 mM K_2SO_4, 0.36 mM $CaCl_2$, 0.46 mM $MgSO_4$, 18 μM H_3BO_3, 4.6 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 1.0 μM Na_2MoO_4, and 45 μM Fe(III)-EDTA for cultivar Nipponbare, and 0.70 mM K_2SO_4, 0.10 mM KCl, 0.10 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 0.50 mM $MgSO_4$, 10 μM H_3BO_3, 0.50 μM $MnSO_4$, 0.50 μM $ZnSO_4$, 0.20 μM $CuSO_4$, 0.010 μM $(NH_4)_6Mo_7O_{24}$, and 100 μM Fe(III)-EDTA for cultivar Tsukinohikari. The pH of the culture solution was adjusted to 5.5 with 1 M NaOH or 1 M HCl daily. Culture solutions were renewed weekly. Iron-deficiency treatment was started on the 26th day of the hydroponic culture for cultivar Nipponbare and on the 52nd day for cultivar Tsukinohikari. Iron-deficiency treatment consisted of omitting Fe(III)-EDTA from the culture solution. Root washings of cultivars Nipponbare and Tsukinohikari were collected on the fifth and eighth days, respectively, after the start of Fe-deficiency treatment as described below. The roots and the leaves were subsequently harvested and stored at –80 °C until used for DNA, RNA, and protein extraction.

Southern blot analysis

Genomic DNA was isolated from the leaves of transformants and wild-type plants by the method of Murray and Thompson (1980). DNA (20 μg) from each sample was digested with the restriction enzymes *Sal*I for cultivar Nipponbare and *Xba*I for cultivar Tsukinohikari, electrophoretically separated in 0.8% (w/v) agarose gels, and transferred to Hybond-N^+ membranes (Amersham). The 1.3-kb fragment that covers the entire *Ids3* cDNA was excised from pBluescriptII SK(+) containing the *Ids3* cDNA fragment, and used as a probe after ^{32}P-labeling. The hybridizations were performed overnight at 65 °C in Church phosphate buffer following the method of Church and Gilbert (1984). After hybridization, the membranes were washed twice with Church hybridization wash buffer for 10 min at 42 °C, and once with 5×SSPE (0.05 M NaH_2PO_4, 0.75 M NaCl, 5 mM EDTA, pH 7.4), 0.1% (w/v) SDS for 10 min at 65 °C. Autoradiographic images of the membranes were obtained using imaging plates and the BAS-2000 system (Fuji Film, Tokyo).

Northern blot analysis

Total RNA was extracted from the roots and leaves of transformants and wild-type plants by the method of Prescott and Martin (1987). Total RNA (20 μg each) was electrophoretically separated in 1.2% (w/v) agarose gels containing 0.66 M formaldehyde and transferred to Hybond-N^+ membranes (Amersham). The hybridizations, washes, and detection were performed under the same conditions as used for Southern hybridization.

Immunoblot analysis

Crude protein was extracted from the roots of transformants and wild-type plants by the trichloroacetic acid and acetone method, as described by Higuchi et al. (1999a). The extracts (20 μg protein each) were applied to SDS-PAGE gels (12.5% (w/v) polyacrylamide), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) by electroblotting. Western analysis was performed using rabbit anti-IDS3 antibody (Nakanishi et al. 2000) with secondary antibody consisting of goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate (BioRad, Tokyo) stained with diaminobenzidine.

Root-washing collection and HPLC analysis of MAs

All plant roots were soaked in distilled water before illuminating, and then root washings were collected for 4 h. Collection was carried out twice, changing the distilled water 2 h after starting. Root washings of 3–16 plants from each line of Fe-deficient or Fe-sufficient plants were collected together. The cationic fraction of the root washings was prepared as 2 M NH_4OH eluates from Amberlite IR(H^+) 120. Condensed and microfiltered samples were subjected to HPLC analysis, as described previously (Mori and Nishizawa 1987). The pH of the Li-citrate buffer was lowered to 2.70 for better separation of MAs.

Results

Transgenic rice plants harboring the Ids3 gene

Two kinds of transgenic rice plant were generated for *Ids3* expression by *Agrobacterium*-mediated transformation. First, plasmid pIGI3c (Fig. 2A), which carries *Ids3* cDNA downstream from the cauliflower mosaic virus 35S promoter, was introduced into rice cultivar Nipponbare. Thirteen independent lines of transformants (R_0 generation) were obtained. Six had an *Ids3* insertion in their genome and four of the six expressed *Ids3* mRNA, as confirmed by Southern and Northern analysis (data not shown). Lines 2a, 5, and 9, which expressed large amounts of *Ids3* mRNA, were used for further analysis in the R_1 generation. Next, plasmid pBII3g (Fig. 2B), which carries a barley genome fragment (20 kb) containing the *Ids3* gene and its 5′-flanking region of about 10 kb, was introduced into rice cultivar Tsukinohikari. Sixty lines of R_0 plants were obtained. Of these, lines 1 and 36 were used for further R_1 generation analysis.

Insertion of Ids3 into R_1 transgenic plants

Genomic Southern hybridization was conducted to confirm integration of the *Ids3* gene in the genome of

R_1 plants (Fig. 3). All transgenic lines with introduced pIGI3c showed a single band at around 10 kb (Fig. 3A). Since pIGI3c has the sole *Sal*I site downstream from the *Ids3* cDNA, the slight differences in the band positions of the three lines suggested that they probably had independent insertions. Their band patterns were identical to the patterns of the corresponding R_0 plants (data not shown), showing that the *Ids3* transgene was correctly inherited. Transgenic plants with introduced pBII3g also showed bands (Fig. 3B). The *Ids3* genomic sequence has *Xba*I sites that would result in excision of a 4.8-kb fragment containing the latter half of the *Ids3* coding region by *Xba*I digestion. Thus, hybridization should detect a band at 4.8 kb and a band at a position dependent on the integration pattern. Transgenic line 1 was thought to have one insert, because only one band was detected other than the band at 4.8 kb, while the high intensity of the band at 4.8 kb and two other bands at distinct positions suggested that line 36 had at least two inserts. The wild-type rice cultivars Nipponbare and Tsukinohikari showed no bands.

Expression of Ids3 in R_1 transgenic plants

Transgenic (R_1 generation) and wild-type plants were hydroponically cultured and Fe deficiency was imposed. Patterns of *Ids3* expression were investigated by both Northern and Western blot analyses. Expression of *Ids3* in the transformants introduced with pIGI3c was detected by Northern analysis (Fig. 4A). All transgenic lines showed constitutive expression of *Ids3* in their roots and leaves irrespective of Fe status. On the other hand, the wild-type rice cultivar Nipponbare showed no *Ids3* expression.

As for the plants with introduced pBII3g, strong induction of *Ids3* expression in response to Fe deficiency was observed by Northern analysis (Fig. 4B). Transcripts of *Ids3* were markedly increased in roots in Fe-deficient transgenic lines 1 and 36, while they were rare in Fe-sufficient roots. In line 36, especially high amounts of mRNA accumulated in the roots of Fe-deficient plants. Furthermore, unlike the expression pattern in barley, *Ids3* was also slightly expressed in the leaves of Fe-deficient line 36. The wild-type rice cultivar Tsukinohikari showed no *Ids3* expression. The expression of IDS3 protein in the roots of transformants with introduced pBII3g was examined by Western blot analysis (Fig. 5). Bands corresponding to IDS3 were detected in roots of Fe-deficient transformant lines 1 and 36. In accordance with the large accumulation of *Ids3* transcripts, there was a large amount of IDS3 in line 36. The wild-type rice cultivar Tsukinohikari showed no IDS3 expression.

Pattern of MA secretion from R_1 transgenic plants

Root washings collected from roots of Fe-deficient and Fe-sufficient plants were analyzed by HPLC. Figure 6 shows representative HPLC profiles of root washings from Fe-deficient plants. Wild-type rice plants of cultivars Nipponbare and Tsukinohikari secreted only DMA (Fig. 6A, C). In contrast, transformant line 2a, with introduced pIGI3c, secreted DMA and a small, but distinct, amount of MA (Fig. 6B). Transformant line 36 with introduced pBII3g secreted a large amount of MA

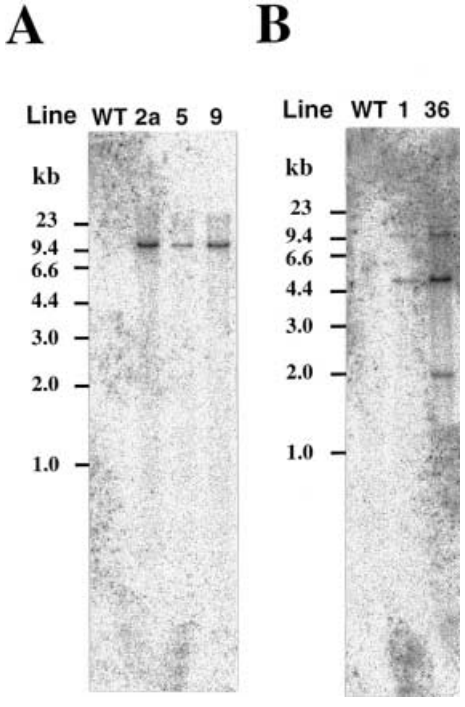

Fig. 3. Southern blot analysis of R_1 plants with introduced pIGI3c (**A**) and pBII3g (**B**). Genomic DNA (20 μg each) was digested with *Sal*I (**A**) and *Xba*I (**B**). Hybridizations were performed with a full-length *Ids3* cDNA probe. *WT* Wild-type rice cultivar Nipponbare for **A** and cultivar Tsukinohikari for **B**

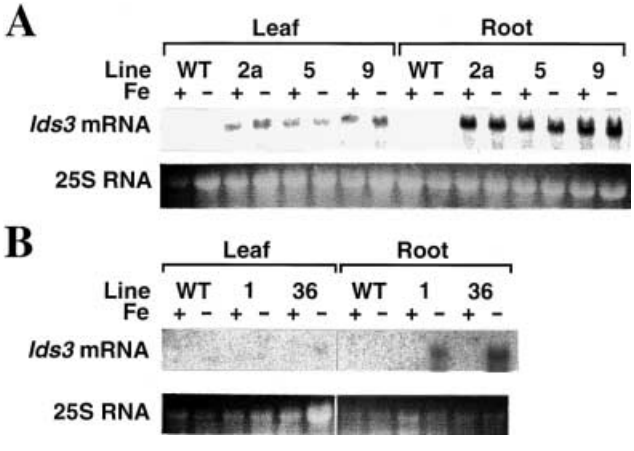

Fig. 4. Northern blot analysis of R_1 plants with introduced pIGI3c (**A**) and pBII3g (**B**). Each lane contains 20 μg total RNA extracted from the roots or leaves of Fe-sufficient (+) or Fe-deficient (−) transformants or wild-type plants. Hybridizations were performed with a full-length *Ids3* cDNA probe. Equal loadings of total RNA were checked by ethidium bromide staining of 25S RNA. *WT* Wild-type rice cultivar Nipponbare for **A** and cultivar Tsukinohikari for **B**

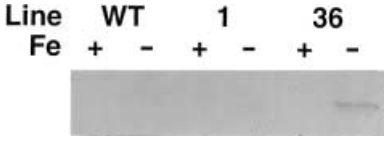

Fig. 5. Western blot analysis of R_1 plants with introduced pBII3g. Each lane contains crude protein (20 μg) extracted from the roots of Fe-sufficient (+) or Fe-deficient (–) transformants or wild-type plants. The IDS3 protein was detected using anti-IDS3 antibody. *WT* Wild-type rice cultivar Tsukinohikari

in addition to DMA (Fig. 6D). These detected MAs were identified by the simultaneous injection of root-washing samples and chemically synthesized MA and DMA standards (Fig. 7). The peaks with retention times of 21.9 and 24.9 min detected in the chromatogram of the root washings from Fe-deficient transformant line 36 with introduced pBII3g (Fig. 7A) were identified as corresponding to MA (Fig. 7B) and DMA (Fig. 7C), respectively. Furthermore, since these peaks disappeared in the analysis using the reagent solution without hypochlorite, which is added to detect secondary amines (Fig. 7D), it was confirmed that these peaks actually correspond to MAs. Peaks detected in the samples from cultivar Nipponbare were identified in the same way (data not shown).

Tables 1 and 2 summarize the MAs detected in each root washing. Wild-type rice cultivar Nipponbare secreted DMA (Table 1). On the other hand, all the transgenic lines with introduced pIGI3c secreted MA in addition to DMA. In the cultivar Nipponbare, root washings contained very small amounts of MAs, which were detected only in Fe-deficient plants in both wild-type and transgenic lines. Cultivar Tsukinohikari (Table 2) secreted about 10 times more MAs than cultivar Nipponbare under Fe-deficient conditions. Wild-type rice cultivar Tsukinohikari secreted DMA under both Fe-sufficient and Fe-deficient conditions, although much more was secreted under Fe-deficient conditions. Transformants with introduced pBII3g secreted a large amount of MA in addition to DMA, and secreted far more MA and DMA under Fe-deficient conditions.

Discussion

The biosynthetic pathways of MAs have been investigated extensively. All MAs share the same pathway from methionine to DMA; DMA is then converted to other MAs. The kind of MA secreted depends on the plant species (Mori and Nishizawa 1987; Mori et al. 1990; Ma et al. 1999). These differences in the capacity for MA biosynthesis among graminaceous species are thought to depend on their possession and expression of the genes involved in the conversion, mainly hydroxylation, of MAs. Since wheat (cultivar Chinese Spring) secretes only DMA and barley (cultivar Betzes) secretes mainly

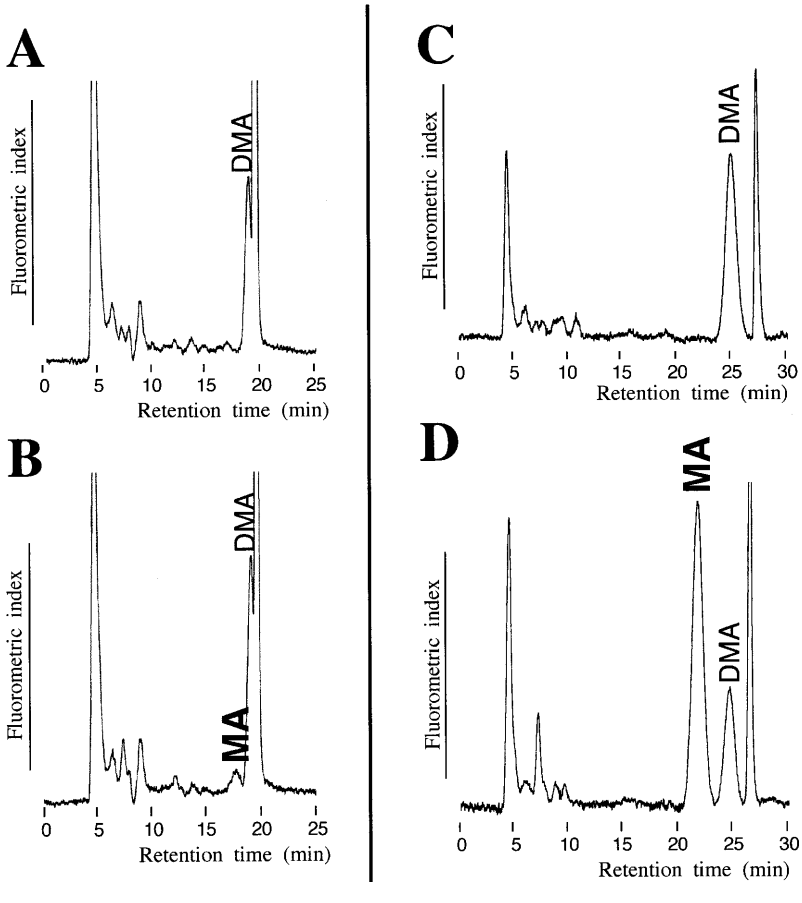

Fig. 6A–D. High-performance liquid chromatographic profiles of root washings collected from Fe-deficient wild-type and R_1 transgenic plants. **A** Wild-type rice cultivar Nipponbare. **B** Transgenic line 2a with introduced pIGI3c. **C** Wild-type rice cultivar Tsukinohikari. **D** Transgenic line 36 with introduced pBII3g. Letters specify the peaks corresponding to DMA and MA, and it was confirmed that all the other peaks were impurities. The pH of the Li-citrate buffer was 2.70

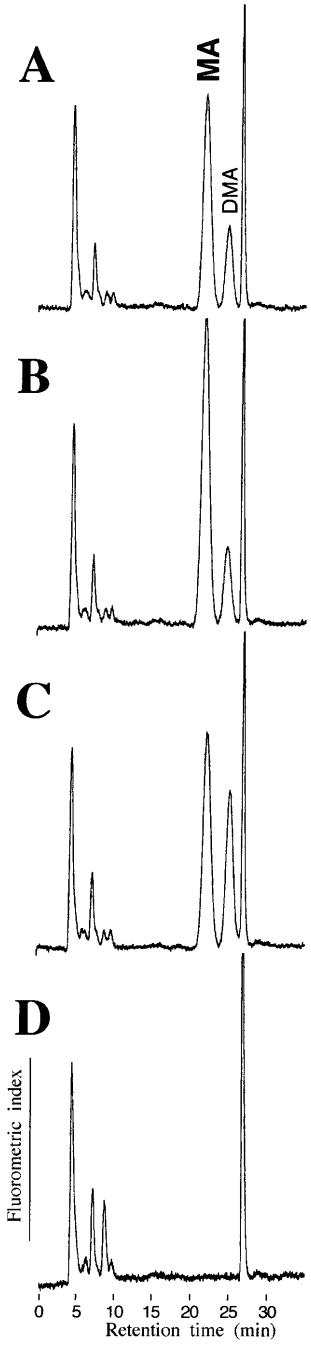

Fig. 7A–D. Identification of the HPLC peaks corresponding to MA and DMA. **A** The root washings from Fe-deficient transformant line 36 with introduced pBII3g. **B** Chemically synthesized MA standard was simultaneously injected with the sample shown in **A**. **C** Chemically synthesized DMA standard was simultaneously injected with the sample shown in **A**. **D** the sample shown in **A** was analyzed without hypochlorite in the reagent solution. The peaks corresponding to MA and DMA were identified by the retention times of the standards and their disappearance when analyzed without hypochlorite in the reagent solution. The pH of the Li-citrate buffer was 2.70

epiHMA in addition to DMA and MA, the chromosome locations of the genes coding these hydroxylation enzymes were examined using wheat (cultivar Chinese Spring)-barley (cultivar Betzes) addition lines. The gene for hydroxylation at the C-2′ position of MAs, namely conversion from DMA to MA and from epiHDMA to epiHMA, is located on the long arm of barley chromosome 4H. The gene for hydroxylation at the C-3 position (DMA to epiHDMA and MA to epiHMA) is located on the long arm of barley chromosome 7H (Mori and Nishizawa 1989; Ma et al. 1999). Similarly, since rye secretes mainly HMA in addition to DMA and MA, rye chromosome 5R was identified as carrying the genes for conversion from DMA to MA and MA to HMA using wheat (cultivar Chinese Spring)-rye (cultivar Imperial) addition lines (Mori et al. 1990).

When we isolated the *Ids3* gene from Fe-deficient barley roots, its high homology to 2-oxoglutarate-dependent dioxygenases suggested its possible involvement in the hydroxylation of MAs (Nakanishi et al. 1993). This possibility seemed much more likely when *Ids3* expression patterns in graminaceous species were compared; the species that express *Ids3* are also the species possessing DMA-hydroxylating ability (Nakanishi et al., 2000). This, along with the existence of the *Ids3* gene on the long arm of barley chromosome 4H (Nakanishi et al. 2000), strongly suggests that the *Ids3* gene product hydroxylates DMA and epiHDMA, to produce MA and epiHMA respectively (Fig. 1).

Here, we have demonstrated that IDS3 hydroxylates the C-2′ position of MAs by showing the production of MA from DMA, using transgenic rice plants. We generated rice transformants, carrying either *Ids3* cDNA or a fragment of the barley genome containing *Ids3*, by introducing pIGI3c or pBII3g, respectively (Fig. 2). The integration and expression of *Ids3* were confirmed by Southern, Northern, and Western analyses (Figs. 3–5). Transgenic plants expressing *Ids3* clearly secreted MA in addition to DMA, while wild-type plants secreted only DMA and not MA (Fig. 6, Tables 1 and 2). This is clear evidence that IDS3 converts DMA to MA in transgenic rice plants; in other words, IDS3 has been shown to be the "MA synthase". To our knowledge, this is the first time that the function of a dioxygenase gene has been shown directly in vivo. An in vitro demonstration of IDS3-mediated DMA hydroxylation is needed to exclude the remaining possibility that IDS3 only assists regulation or activity of the real, still unknown, "MA synthase".

Transformants with introduced pBII3g secreted large amounts of MA. This is due to the combined effects of their secretion of large amounts of DMA and their high conversion rate from DMA to MA. Especially, Fe-deficient transformant line 36 secreted a large amount of MA, and secreted more MA than DMA (Fig. 6D, Table 2). This is consistent with the strong induction of *Ids3* expression by Fe-deficiency treatment, especially in line 36, shown by Northern and Western blot analysis (Figs. 4B, 5). This high-level expression of barley *Ids3* in line 36 seems to be due to the multiple copies of the inserted gene (Fig. 3B).

In barley, *Ids3* expression is dramatically induced in Fe-deficient roots and is strictly regulated transcriptionally (Nakanishi et al. 1993, 2000). The expression of the *Ids3* gene in transformants with an introduced genomic fragment containing *Ids3* also responded to Fe deficiency (Figs. 4B, 5). This indicates that other factors required

Table 1. The amounts and types of MAs secreted from R_1 plants with introduced pIGI3c. Root washings from sixteen plants of each line were collected and analyzed. *WT* Wild-type rice cultivar Nipponbare, *n.d.* not detected

	MA secretion (nmol/plant)							
Lines	WT		2a		5		9	
Fe	+	–	+	–	+	–	+	–
DMA	n.d.	0.33	n.d.	0.42	n.d.	0.13	n.d.	0.11
MA	n.d.	n.d.	n.d.	0.03	n.d.	0.04	n.d.	0.03

Table 2. The amounts and types of MAs secreted from R_1 plants with introduced pBII3g. Root washings from three to five plants of each line were collected and analyzed. *WT* Wild-type rice cultivar Tsukinohikari, *n.d.* not detected

	MA secretion (nmol/plant)					
Lines	WT		1		36	
Fe	+	–	+	–	+	–
DMA	2.38	5.65	1.23	3.80	0.27	6.62
MA	n.d.	n.d.	0.54	1.64	0.49	9.36

for Fe deficiency, which regulate expression of the *Ids3* gene, are present in rice and capable of regulating the gene. This also indicates that the *Ids3* 5′-flanking region has the ability to induce strong expression of the gene under Fe-deficient conditions in the roots of transgenic rice plants. Thus, the *Ids3* 5′-flanking region works as a strong Fe-deficient-inducible promoter in the roots of other graminaceous plants. Although the *Ids3* 5′-flanking region has no known *cis*-element-like sequences, it surely contains *cis*-elements that are responsible for the Fe-deficiency-specific gene expression. Interestingly, Fe-deficient transformant line 36 with introduced pBII3g also expressed *Ids3* slightly in leaves (Fig. 4B). Rice may lack the ability to drive root-specific expression under control of the *Ids3* 5′-flanking region.

Barley is highly tolerant of Fe deficiency. This is thought to be due to its high production of MAs and the kinds of MAs that it secretes (Takagi et al. 1984). Recently, von Wirén et al. (2000) showed that more-hydroxylated MAs have greater Fe(III)-complex stability at slightly acid pH. Therefore, hydroxylation steps should play an important role in Fe acquisition from the rhizosphere. Barley vigorously hydroxylates DMA and secretes mainly epiHMA among MAs (Mori and Nishizawa 1987, 1989; Nakanishi et al. 1993; Ma et al. 1999). Thus, *Ids3* surely contributes to Fe-deficiency tolerance in barley. In addition, the hydroxylation of DMA may promote DMA synthesis from its precursor by equilibrium shifting. We found enhanced MA secretion from transformant line 36 with introduced pBII3g compared to wild-type plants (Table 2). Transgenic rice plants with introduced pBII3g may have enhanced tolerance to Fe deficiency.

In this study, we demonstrated that IDS3 converts DMA to MA in vivo. Demonstration that IDS3 catalyzes epiHDMA to epiHMA might be more difficult, because epiHDMA is not abundant in graminaceous plants, and barley predominantly synthesizes epiHMA by another pathway via MA.

We have isolated another dioxygenase gene, *Ids2*, from barley (Okumura et al. 1994). *Ids2* shows high similarity to *Ids3* and exists on the long arm of barley chromosome 7H, suggesting that IDS2 hydroxylates DMA to epiHDMA and MA to epiHMA (Fig. 1) (Nakanishi et al. 2000). If we introduce *Ids2* into rice, the transgenic rice will probably produce epiHDMA in addition to DMA.

As mentioned above, in vitro demonstration of the enzymatic function of IDS3 and IDS2 are the next steps to be performed. The main reason that the enzyme activity of IDS3 has not been detected seems to be related to the action of Fe^{2+} (Nakanishi et al. 2000). Dioxygenase needs Fe^{2+} for its activity, while Fe^{3+} may either inactivate the enzyme or consume the substrate MAs, under oxidative conditions. Improvement of the reaction conditions will surely lead to in-vitro hydroxylation of MAs by IDS3 and IDS2.

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