Cloning and Characterization of Deoxymugineic Acid Synthase Genes from Graminaceous Plants

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Graminaceous plants have evolved a unique mechanism to acquire iron through the secretion of a family of small molecules, called mugineic acid family phytosiderophores (MAs). All MAs are synthesized from L-Met, sharing the same pathway from L-Met to 2'-deoxymugineic acid (DMA). DMA is synthetized through the reduction of a 3'-keto intermediate by deoxymugineic acid synthase (DMAS). We have isolated DMAS genes from rice (OsDMAS1), barley (HvDMAS1), wheat (TaDMAS1), and maize (ZmDMAS1). Their nucleotide sequences indicate that OsDMAS1 encodes a predicted polypeptide of 318 amino acids, whereas the other three orthologs all encode predicted polypeptides of 314 amino acids and are highly homologous (82–97.5%) to each other. The DMAS proteins belong to the aldo-keto reductase superfamily 4 (AKR4) but do not fall within the existing subfamilies of AKR4 and appear to constitute a new subfamily within the AKR4 group. All of the proteins showed DMA synthesis activity in vitro. Their enzymatic activities were highest at pH 8–9, consistent with the hypothesis that DMA is synthesized in subcellular vesicles. Northern blot analysis revealed that the expression of each of the above DMAS genes is up-regulated under iron-deficient conditions in root tissue, and that of the genes OsDMAS1 and TaDMAS1 is up-regulated in shoot tissue. OsDMAS1 promoter-GUS analysis in iron-sufficient roots showed that its expression is restricted to cells participating in long distance transport and that it is highly up-regulated in the entire root under iron-deficient conditions. In shoot tissue, OsDMAS1 promoter drove expression in vascular bundles specifically under iron-deficient conditions.

Iron is an essential element that is required for various cellular events in plants, including respiration, chlorophyll biosynthesis, and photosynthetic electron transport. Iron is also a component of the Fe-S cluster, which is present in numerous enzymes. Although soil contains abundant iron, it is mainly present as oxidized Fe(III) compounds, which are poorly soluble in neutral to alkaline soils. Therefore, plants have developed sophisticated and tightly regulated mechanisms for acquiring iron from the soil, which can be grouped into two strategies (1). Non-graminaceous plants lower the soil pH by enhancing proton excretion into the rhizosphere, reduce iron to the more soluble ferrous form at the root surface by inducing the expression of ferric-chelate reductase, and transport the resulting ferrous ions across the root plasma membrane. In contrast, graminaceous plants solubilize soil iron by secreting Fe(III) chelators, called mugineic acid family phytosiderophores (MA),4 from their roots (2, 3). The resulting Fe(III)-MA complexes are then reabsorbed into the roots through a specific transporter. The production and secretion of MAs markedly increases in response to iron deficiency, and tolerance to iron deficiency in graminaceous plants is strongly correlated with the quantity and quality of the MAs secreted. For example, rice, wheat, and maize secrete only 2'-deoxymugineic acid (DMA) in relatively low amounts and are thus susceptible to low iron availability. In contrast, barley secretes large amounts of many types of MAs, including MA, 3-hydroxymugineic acid, and 3-epi-hydroxymugineic acid and is therefore more tolerant to low iron availability (4, 5).

The biosynthetic pathway for MAs has been characterized (Fig. 1). MAs are synthesized from L-Met (6). Nicotianamine synthase (NAS) catalyzes the trimerialization of S-adenosyl Met molecules to form nicotianamine (NA) (7, 8), which is then converted into a 3'-keto intermediate by the transfer of an amino group by nicotianamine aminotransferase (NAAT) (9). The subsequent reduction of the 3'-carbon of the keto intermediate produces DMA. DMA is the first MA synthesized in the pathway. The biosynthetic pathway of all MAs is the same from L-Met to DMA, but the subsequent steps differ depending on the plant species or even the cultivar (10). To date, seven types of MAs have been identified (11, 12).

Almost all of the genes involved in the biosynthetic pathway for MAs have been isolated and characterized in our laboratory (8, 13–17). A gene that encodes an Fe(III)-MA transporter, Yellow Stripe1 (YS1), has been isolated from maize (18). The YS1

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4 The abbreviations used are: MA, mugineic acid; DMA, 2'-deoxymugineic acid; DMAS, deoxymugineic acid synthase; AKR, aldo-keto reductase superfamily; NA, nicotianamine; NAS, NA synthase; NAAT, NA aminotransferase; ORF, open reading frame; GUS, β-glucuronidase; HPLC, high pressure liquid chromatography; TAPS, N-[Tris(hydroxymethyl)iminethyl]-3-aminopropane-sulfonic acid.
Cloning of DMAS Genes

FIGURE 1. Biosynthetic pathway of mugineic acid family phytosiderophores. Three molecules of S-adenosyl methionine are combined by NAS to form NA. The amino group of NA is transferred by NAAT, and the resultant 3′-keto intermediate is reduced to DMA by DMAS. The subsequent steps differ with the plant species and cultivar.

mRNA level increases in both roots and shoots, under iron-deficient conditions. YSI-like genes also exist in rice (19). The expression of the genes involved in the biosynthetic pathway of MAs is dramatically enhanced by iron deficiency in barley (8, 13–16, 20), rice (21–23), and maize (24), resulting in increased secretion of MAs. All of the barley iron deficiency-inducible genes that have been isolated are expressed almost exclusively in roots, suggesting that the root-specific expression of these genes is important for the strong tolerance of barley to iron deficiency, whereas many iron deficiency-inducible genes in rice are expressed in both roots and shoots.

However, the isolation of the gene responsible for the conversion of the 3′-keto intermediate to DMA has not been reported previously. Here, we report the cloning of DMAS genes from rice (OsDMAS1), barley (HvDMAS1), wheat (TaDMAS1), and maize (ZmDMAS1). The sequences of proteins in this subfamily are highly conserved among graminaceous plants. Because DMAS belongs to the aldo-keto reductase superfamily 4 (AKR4) but does not fall into the existing subfamilies, we propose a new subfamily for DMAS. The isolation of the DMAS gene from barley, one of the graminaceous plants most tolerant to iron deficiency, is an important step in the production of transgenic rice lines highly tolerant to iron deficiency.

EXPERIMENTAL PROCEDURES

Isolation of DMAS Genes—Analysis of a rice 22-kb custom oligonucleotide DNA microarray containing the sequence data from the rice full-length cDNA project was used to identify OsDMAS1 (AK073738; putative NADPH-dependent oxidoreductase) as a putative member of the aldo-keto reductase superfamily, which is induced under iron-deficient conditions (25). The ORF was amplified using the forward and reverse primers 5′-caccATGAGCCCGCCGGCGCCGC-3′ and 5′-TCATATCTCGCCGTCATAGGTCG-3′, respectively, from a cDNA library prepared from iron-deficient rice roots. HvDMAS1 was identified as unigene number 6858 in the HarvEST database (Version 1.35) because of its strong homology to Oryza sativa NADPH-dependent oxidoreductase (AK073738). The HvDMAS1 ORF was amplified using the forward and reverse primers 5′-caccATGGCCGCGAGGCAGGTCG-3′ and 5′-TCATATCTCGCCGTCATAGGTCG-3′, respectively, from a cDNA library prepared from iron-deficient barley roots. ZmDMAS1 and TaDMAS1 were identified on the basis of homology using BLAST. Forward and reverse primers for ZmDMAS1 were designed with the sequences 5′-caccATGAGCCGCGAGGCAGGTCG-3′ and 5′-TCATATCTCGCCGTCATAGGTCG-3′, respectively, and the ZmDMAS1 ORF was amplified from a cDNA library prepared from iron-deficient maize roots. Forward and reverse primers for TaDMAS1 were designed with the sequences 5′-caccATGAGCCGCGAGGCAGGTCG-3′ and 5′-TCATATCTCGCCGTCATAGGTCG-3′, respectively, and the ORF for TaDMAS1 was amplified from a cDNA pool prepared from iron-deficient wheat roots. The amplified DMAS cDNAs were subcloned into pENTR/D-TOPO (Invitrogen) and sequenced. The resulting plasmids were then digested with XbaI and HindIII, and the excised fragment containing the ORF was amplified with primers to contain an XbaI site at the 5′-end and a HindIII site at the 3′-end. The ORF was amplified using the forward and reverse primers 5′-caccATGAGCCGCGAGGCAGGTCG-3′ and 5′-TCATATCTCGCCGTCATAGGTCG-3′, respectively, from a cDNA library prepared from iron-deficient barley roots. ZmDMAS1 and TaDMAS1 were identified on the basis of homology using BLAST. Forward and reverse primers for ZmDMAS1 were designed with the sequences 5′-caccATGAGCCGCGAGGCAGGTCG-3′ and 5′-TCATATCTCGCCGTCATAGGTCG-3′, respectively, and the ZmDMAS1 ORF was amplified from a cDNA library prepared from iron-deficient maize roots. Forward and reverse primers for TaDMAS1 were designed with the sequences 5′-caccATGAGCCGCGAGGCAGGTCG-3′ and 5′-TCATATCTCGCCGTCATAGGTCG-3′, respectively, and the ORF for TaDMAS1 was amplified from a cDNA pool prepared from iron-deficient wheat roots. The amplified DMAS cDNAs were subcloned into pENTR/D-TOPO (Invitrogen) and sequenced using a Thermo Sequenase cycle sequencing kit (Shimadzu, Kyoto, Japan) and a DNA sequencer (DSQ-2000L; Shimadzu). The amino acid sequences of one representative from each subfamily of the AKR family were aligned, and the phylogenetic relationships were determined as described (26). The phylogenetic relationships were determined as described (27).
cells were induced to produce the recombinant fusion proteins, and the proteines were purified as described (8).

Enzyme Assay—Five μg of HvNAAT-A fusion protein/reaction were centrifuged in an Amicon Ultrafree-MC 30-kDa-cut off filter unit (Millipore) at 6200 × g at 4 °C for 15 min. The flow-through was discarded, and 50 μl of TAPS buffer (50 mM TAPS, 5 mM KCl, 5 mM MgCl₂, 10 mM 2-oxoglutaric acid, 10 μM pyridoxal-5′-phosphate, 150 μM nicotianamine) were added to the filter unit. The solution was mixed several times by pipetting and incubated at 26 °C for 30 min. The filter unit was then placed in a new Eppendorf tube and centrifuged at 6200 × g at 4 °C for 15 min. The flow-through was collected, and NADPH was added to a final concentration of 25 μM. For the chemical control, 4 μl of 0.25 M NaBH₄ were added to each filter unit containing DAMAS, mixed two to three times by pipetting, and incubated at 4 °C for 15 min. The flow-through was collected, and NADPH was added to a final concentration of 25 μM. The protein samples to be assayed (1 μg/reaction) were placed in new filter units and centrifuged at 6200 × g at 4 °C for 1 min. All of the samples were prepared separately, including a chemical control (NaBH₄) and a negative control. Then, 46 μl of flow-through containing the 3′-keto intermediate and NADPH, prepared as described above, were added to each filter unit containing DAMAS, mixed two to three times by pipetting, and incubated at 26 °C for 30 min. All reactions were performed in duplicate.

To perform enzyme assays at pH 7, 8, and 9, NA was first converted to the 3′-keto intermediate as described above, the pH of the flow-through was adjusted to 7 or 8 with Tris-Cl buffer or diluted with TAPS buffer at pH 8 or 9, and the samples were processed as described above. All reactions were performed in triplicate.

Northern Blot Analysis—Seeds of barley (Hordeum vulgare L. cv. Ehimehakadaka number 1), wheat (Triticum aestivum L. cv. Chinese spring), maize (Zea mays cv. Alice), and rice (O. sativa L. cv. Nipponbare) were germinated on wet filter paper and cultured as described (29). For iron deficiency treatments, plants were transferred to culture solution lacking iron. Roots and leaves were harvested after 2 weeks, frozen in liquid nitrogen, and stored at −80 °C until use.

Total RNA was extracted from roots and shoots, and 10 μg/lane were electrophoresed in 1.2% (w/v) agarose gels containing 0.66 M formaldehyde and transferred to Hybond-N membrane (Amersham Biosciences). The HvDMAS1 ORF was labeled with digoxigenin and incubated with the membrane at 68 °C and processed as described (30, 31).

Rice Transformation and Growth Conditions—The 1.3-kb 5′-upstream region of the OsDMAS1 gene was amplified by PCR using genomic DNA as a template. The primers were used to amplify the following primer 5′-gagagaactgaagcccttagacaattttacgtagttgta-3′ and the reverse primer 5′-gagagaacttagggtggaggaggaagttccttccctct-3′, which contain XhoI and Spel restriction sites, respectively. The amplified fragment was fused into the pBluescript II SK vector, and its sequence was confirmed. This 1.3-kb fragment was subcloned upstream of the uidA ORF, which encodes β-glucuronidase (GUS), in the pG121Hm vector (32). An Agrobacterium tumefaciens strain (C58) carrying the above construct was used to transform rice (O. sativa L. cv. Tsukinohikari) as described (21). Six transgenic rice lines carrying the OsDMAS1 promoter-GUS fusion were obtained. T₀ seed were germinated on MS medium containing 50 mg/liters hygromycin B. After 4 weeks, the plants were shifted to iron-sufficient or iron-deficient medium for 2 weeks and analyzed for GUS expression as described (22).

RESULTS

Isolation of DMAS Genes—OsDMAS1 (AK073738: NAD(P)H dependent oxidoreductase) was identified as a putative member of the aldo-keto reductase superfamily, up-regulated under iron-deficient conditions. OsDMAS1 is located on rice chromosome 3 and is composed of four exons and three introns (Fig. 2a). The length of the genomic fragment is 2706 bp, and that of the ORF is 957 bp. The promoter region of the gene contains iron deficiency responsive element 2 (IDE2)-like sequence (23, 33). OsDMAS1 shows 54.7% homology to Papaver somniferum codeinone reductase (AKR4B2–3) and 48 and 50% homology to Medicago sativa and Glycine max chalcone polyketide reductase (AKR4A2 and AKR4A1), respectively. On the basis of sequence homology, DMAS is a member of the AKR4 group. However, it does not fall into any of the previously defined subfamilies as the sequence homologies are below 60%, suggesting that DMAS belongs to a novel subfamily based on amino acid sequence and substrate specificity (Fig. 2b).
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The full-length *HvDMAS1* was identified as unigene number 6858 in the HarvEST data base (Version 1.35) and is homologous to rice NADPH-dependent oxidoreductase. *OsDMAS1* and *HvDMAS* show 86% homology. *TaDMAS1* and *ZmDMAS1* were identified by BLAST on the basis of homology. All four DMAS orthologs were isolated from cDNA libraries prepared from iron-deficient roots. The nucleotide sequences show that *OsDMAS1* encodes a predicted polypeptide of 318 amino acids, and the other three DMAS clones each encode predicted polypeptides of 314 amino acids (Fig. 3). These DMAS sequences from graminaceous plants are highly conserved (82–97.5%). *HvDMAS1* and *TaDMAS1* show the greatest homology (97.5%). All of the DMAS proteins possess the NADPH-binding domain, as do all other AKRs. The active site sequences in these proteins are strictly conserved, although in *ZmDMAS1*, the Lys is replaced with Thr at position 123 (Fig. 3).

**Enzyme Assay**—The DMAS cDNAs from rice, barley, wheat, and maize were expressed in *E. coli*, and the expressed proteins were tested for the ability to convert the 3′-keto intermediate into DMA. As the 3′-keto intermediate could not be chemically synthesized, it was necessary to use an *in vitro* enzyme assay that begins with NA. In this assay, NA is first converted into the 3′-keto intermediate by the action of HvNAAT-1 and is subsequently converted into DMA, which is identified through HPLC (Fig. 4). *In vitro*, all four proteins were able to convert the 3′-keto intermediate into DMA. The highest enzyme activity was observed for *TaDMAS1* followed by *OsDMAS1*, *ZmDMAS1*, and *HvDMAS1*.

The effect of pH on enzyme activity was also examined. Enzyme assays were performed at pH 7, 8, and 9 (Fig. 5). At pH 7, the enzyme activities were 6.5–14.9 times lower than those at pH 8, but the enzyme activities at pH 8 and 9 were comparable. These results strengthen the hypothesis that DMA is synthesized in subcellular vesicles derived from rough endoplasmic reticulum as the DMAS activities were much lower at the neutral pH of the cytoplasm.

**Northern Blot Analysis**—*OsDMAS1* was identified as a gene that is up-regulated under iron-deficient conditions. Its expression pattern was further characterized by Northern blot analysis, which revealed that under iron-deficient conditions, the expression of all of the DMAS genes is up-regulated in root tissue, but only the expression of *OsDMAS1* and *TaDMAS1* is up-regulated in shoot tissue (Fig. 6).

**Spatial Pattern of OsDMAS1 Expression**—To gain a more detailed insight into the physiological roles of the *OsDMAS1* gene, we investigated the localization of its expression in both iron-sufficient and iron-deficient rice plants through the histochemical localization of *OsDMAS1* promoter–GUS transformants. *OsDMAS1* expression was detected in iron-deficient roots and was strongly induced in response to iron deficiency. In the roots of iron-deficient plants, GUS staining derived from *OsDMAS1* promoter activity was observed within the stele, but no staining was observed in epidermal, exodermal, or cortical cells (Fig. 7a). At a higher magnification, subtle staining was detected in the parts of pericycle cells adjacent to the protoxylem and metaxylem (Fig. 7b). Thus, the *OsDMAS1* promoter activity in the roots of iron-deficient plants is localized in cells that participate in long distance transport. Longitudinal sections of iron-deficient roots showed no GUS staining (Fig. 7c).

In roots of iron-deficient plants, the *OsDMAS1* promoter was active in all tissues, including the epidermis, exodermis, cortex, and whole stele (Fig. 7e). In particular, staining was observed in pericycle cells adjacent to the protoxylem and metaxylem (Fig. 7f), as was evident in iron-sufficient roots. Interestingly, GUS activity was detected in cells surrounding the metaxylem I, in both iron-sufficient and iron-deficient roots, in the region from which lateral roots emerge. No GUS expression was detected in leaves of iron-sufficient plants (Fig. 7g), whereas under iron-deficient conditions, GUS activity was detected in phloem, parenchyma, and companion cells (Fig. 7h), suggesting a possible role of DMA in iron homeostasis.

**DISCUSSION**

The biosynthetic pathway for MAs has been characterized (Fig. 1) in extensive physiological and biochemical studies (6, 7).
It was proposed that the 3'-keto intermediate is converted to DMA by the action of the enzyme DMAS (11). All of the genes involved in the DMA biosynthetic pathway have been reported as being isolated, with the exception of DMAS. It was reported (35) that DMAS activity is dependent on NAD(P)H, raising the possibility that DMAS is a member of the AKRs. The AKRs (36) comprise one of the three enzyme superfamilies that encompass NAD(P)(H)-dependent oxidoreductases (27) and catalyze the reduction of aldehydes, ketones, monosaccharides, ketosteroids, and prostaglandins. This superfamily contains more than 120 enzymes, which are divided into 15 families (AKR1-AKR15) (37), nine of which contain multiple subfamilies (27). These proteins possess the (α/β)8 barrel motif, which provides a common scaffold for NAD(P)(H)-dependent catalytic activity, with the substrate...
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AKRs have been well characterized, with three-dimensional structures elucidated for many (26). However, few AKRs from graminaceous crops have been characterized. The isolation and characterization of DMAS genes from rice, barley, wheat, and maize is an important step in the characterization of the AKRs. OsDMAS1 encodes a protein of 318 amino acids that can be considered a model for the structures of other DMASs. Although there is some variation among graminaceous DMASs, the substrate-binding domain is strictly conserved (Fig. 3). The replacement of Lys by Thr at position 123 in ZmDMAS (Fig. 3) has no effect on enzyme activity (Fig. 4). The role of the amino acid at this position in substrate recognition is not well understood (26). The NADPH-binding domain is also conserved, which is a striking feature of the AKRs. An alignment of the sequences of known AKR members revealed that DMASs belong to the AKR4 group and show homology to P. somniferum codeinone reductase (AKR4B2–3) and M. sativa (AKR4A2) and G. max (AKR4A1) chalcone polyketide reductases. However, graminaceous DMASs do not fall within the existing subfamilies, suggesting that a new subfamily can be defined within the AKR4 group.

With the isolation of graminaceous DMAS genes, the genes encoding all of the enzymes involved in the biosynthetic pathway of MA s have been isolated, including S-adenosylmethionine synthetase (SAMS) (38), NAS (8), NAAT (13), IDS2, and IDS3 (16). Moreover, we have proven that 3'-keto acid is an intermediate in the pathway from NA to DMA. All of the DMAS proteins were able to synthesize DMA from the 3'-keto intermediate in vitro (Figs. 4 and 5). TaDMAS1 showed the highest enzymatic activity, and barley secretes the greatest amounts of MA s (4, 5). In rice roots, the amount of NA is higher and the amount of DMA is lower than in barley roots (21), suggesting that HvDMAS1 is more active and utilizes more NA for DMA production. Interestingly, the endogenous NAS activity is also higher in wheat than in barley.

specificity determined by variations in loops on the C-terminal side of the barrel (26). The NADPH-binding domain is conserved, even in proteins with less than 30% homology (26). The majority of known AKRs are monomeric proteins of about 320 amino acids in length, but multimeric forms also exist (27).
DMASs appear more active than NAAT as 5 μg of NAAT protein were used for each reaction as compared with 1 μg of each DMAS, and still, it seems that the 3'-keto intermediate is a limiting factor for TaDMAS1 and the chemical control (Fig. 5). The reduction at the 3'-carbon of the 3'-keto intermediate by NaBH₄ is not thought to be sterically specific, as in the case of enzymatic reduction, and not all of the chemically produced DMA may have the same three-dimensional structure as natural DMA at the 3'-carbon (39). The enzymatic activity of these proteins was severely restricted at cytoplasmic pH, suggesting that the enzyme localizes to some subcellular organelle(s) with high pH. The optimum pH for the synthesis of NAS is 9 (40), whereas for NAAT, it is 8.5 (37) to 9 (35). MAs are thought to be synthesized in root tissue in vesicles derived from rough endoplasmic reticulum (6). These vesicles remain swollen until the onset of MA secretion and become shrunken by the end of secretion (6). The polar transport of these vesicles has been implicated in phytosiderophore secretion in iron-deficient barley roots (20, 41). NAS and NAAT are thought to be localized to these vesicles. The optimum pH for DMAS activity suggests that the protein may localize to these subcellular vesicles where DMA is synthesized and stored until secretion. Although AKRs have been reported to be active at pHs from 6.8 to 11 (37), AKR4 proteins have an optimal pH around 7, as has been determined for H. vulgare aldehyde reductase, P. somniferum codeinone reductase, and Digitalis purpurea aloxe reductase (42–44). More than one gene exists for H. vulgare DMAS, and the genes involved in MA biosynthesis are also expressed in rice and barley roots (21). OsDMAS1 contains cis-acting elements, including an IDE2-like sequence (23, 33) (–1408 to –1434) and evening elements (–262 to –201; –667 to –734; –702 to –752), (AATATCT (41, 46)) suggesting that its expression is regulated by iron deficiency in a diurnal fashion. Like all other genes involved in the MA biosynthetic pathway, DMAS genes are up-regulated under iron-deficient conditions in root tissue. In iron-deficient shoots, DMAS expression was up-regulated in rice and wheat, whereas no expression was detected in barley and maize (Fig. 6). Interestingly, in rice, most of the genes involved in the MA biosynthetic pathway are expressed in both root and shoot tissues, but the expression of these genes is restricted to root tissue in barley, the graminaceous plant most tolerant to iron deficiency. The OsDMAS1 promoter contains IDE2-like elements, suggesting that its expression is regulated by iron deficiency. OsDMAS1 expression was detected in iron-sufficient roots (Fig. 7), consistent with reports of the detection of DMA in iron-sufficient rice and barley roots (21). OsNAS1–2 (22) and OsNAAT15 are also expressed in cells that participate in long distance transport under iron-sufficient conditions. In contrast, HvNAAT-A, which encodes a protein that converts NA into the 3'-keto intermediate, is not expressed in the presence of iron, whereas HvNAAT-B shows a basal level of expression. The expression of both genes increases under iron-deficient conditions (13). DMAS expression was up-regulated under iron-deficient conditions in root tissue (Fig. 6), which is important for the production and secretion of DMA. In rice and barley, DMA was detected in shoots under iron-sufficient conditions and increased under iron-deficient conditions. More DMA was detected in iron-deficient and iron-sufficient rice leaves than in barley (21), although barley secretes higher amounts of MAs (4, 5). No OsDMAS1 promoter–GUS activity was detected in iron-sufficient rice shoots. It is possible that the DMA detected in iron-sufficient rice leaves is translocated from roots in a complex with iron (47). This hypothesis is strengthened by the fact that under iron-sufficient conditions, OsDMAS1 expression was only observed in the portions of roots that are involved in long distance transport. Under iron-deficient conditions, the amount of DMA increases in rice shoots, and the expression of DMAS suggests that DMA is at least partially synthesized in shoot tissue. This DMA is thought to be involved in iron homeostasis and does not participate in the acquisition of iron from the soil.

We are engaged in the development of transgenic rice plants that are tolerant of alkaline soils with low iron availability. Previously, we showed that transgenic rice plants harboring HvNAAT-A and B are tolerant to iron deficiency under alkaline conditions (48). The isolation of the DMAS gene from barley, the graminaceous plant most tolerant to iron deficiency, is an important step in the production of transgenic rice plants highly tolerant to iron deficiency.

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REFERENCES
1. Marschner, H., Römheld, V., and Kissel, M. (1986) J. Plant Nutr. 9, 695–713
2. Takagi, S. (1976) Soil Sci. Plant Nutr. 22, 423–433
3. Takagi, S., Nomoto, K., and Takekoto, S. (1984) J. Plant Nutr. 7, 469–477
4. Römhild, V., and Marschner, H. (1990) Plant Soil 123, 147–153
5. Singh, K., Chino, M., Nishizawa, N. K., Ohata, T., and Mori, S. (1993) in Genetic Aspects of Plant Mineral Nutrition (Randall, R. J., Delhaize, E., Richards, R. A., and Munns, R., eds) pp. 335–339, Kluwer Academic Publishers, Norwell, MA
6. Mori, S., and Nishizawa, N. K. (1987) Plant Cell Physiol. 28, 1081–1092
7. Higuchi, K., Kanazawa, K., Nishizawa, N. K., Chino, M., and Mori, S. (1994) Plant Soil 165, 173–179
8. Higuchi, K., Suzuki, K., Nakashima, H., Yamaguchi, H., Nishizawa, N. K., and Mori, S. (1999) Plant Physiol. 119, 471–480
9. Kanazawa, K., Higuchi, K., Nishizawa, N. K., Fushimi, S., and Mori, S. (1995) J. Exp. Bot. 46, 1241–1244
10. Ma, J. F., Taketa, S., Chang, Y.-C., Takeda, K., and Matsumoto, H. (1999) J. Exp. Bot. 50, 723–726
11. Nomoto, K., Sugiura, Y., and Takagi, S. (1987) in Iron Transport in Microbes, Plants and Animals (Winkelman, G., Van der Helm, D., Nielands, J. B., eds) pp 401–425, VCH Publishers, Weinheim, Germany
12. Ma, J. F., Shinada, T., Matsuda, T., and Nomoto, K. (1995) J. Biol. Chem.
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27. Ogo, Y., Itai, R. N., Nakanishi, H., Inoue, H., Kobayashi, T., Suzuki, M., and Mori, S. (1999) *Plant Physiol.* **121**, 947–956

28. Bradford, M. M. (1976)

29. Kanazawa, K., Higuchi, K., Nishizawa, N. K., Fushiya, S., Chino, M., and Mori, S. (1994) *J. Exp. Bot.* **45**, 1903–1906

30. Engler-Blum, G., Meier, M., Frank, K., and Muller, G. A. (1993) *Anat. Biochem.* **210**, 235–244

31. Yoshihara, T., Kobayashi, T., Goto, F., Masuda, T., Higuchi, K., Nakanishi, H., Nishizawa, N. K., and Mori, S. (2003) *Plant Biotech.* **20**, 33–41

32. Hiei, Y., Ohta, S., Komori, T., and Kumashiro, T. (1994) *Plant J.* **6**, 271–282

33. Kobayashi, T., Nakayama, Y., Itai, R. N., Nakanishi, H., Yoshida, T., Mori, S., and Nishizawa, N. K. (2003) *Plant J.* **36**, 780–793

34. Kawai, S., Takagi, S., and Sato, Y. (1988) *J. Plant Nutr.* **11**, 633–642

35. Shojima, S., Nishizawa, N. K., Fushiya, S., Nozoe, S., Iwafune, T., and Mori, S. (1990) *Plant Physiol.* **93**, 1497–1503

36. Nelson, M. J., and Snell, E. E. (1986) *J. Biol. Chem.* **261**, 15115–15120

37. Yokochi, N., Yoshikane, Y., Trongpanich, Y., Ohnishi, K., and Yagi, T. (2004) *J. Biol. Chem.* **279**, 37373–37384

38. Takizawa, R., Nishizawa, N. K., Nakanishi, H., and Mori, S. (1996) *J. Plant Nutr.* **19**, 1189–1200

39. Ohata, T., Kanazawa, K., Mihashi, S., Nishizawa, N. K., Fushiya, S., Nozoe, S., Chino, M., and Mori, S. (1993) *Soil Sci. Plant Nutr.* **39**, 745–749

40. Higuchi, K., Nishizawa, N. K., Yamaguchi, H., Römhild, V., Marschner, H., and Mori, S. (1995) *J. Exp. Bot.* **46**, 1061–1063

41. Nozoe, T., Itai, R. N., Nagasaka, S., Takahashi, M., Nakanishi, H., Mori, S., and Nishizawa, N. K. (2004) *Soil Sci. Plant Nutr.* **50**, 1125–1131

42. Roncarati, R., Salamini, F., and Bartels, D. (1995) *Plant J.* **7**, 809–822

43. Unterlinner, B., Lenz, R., and Kutchan, T. M. (1999) *Plant J.* **18**, 465–475

44. Gavilà, I., Perez-Bermúdez, P., and Seitz, H. U. (2002) *Eur. J. Biochem.* **269**, 2842–2850

45. Herbig, A., Koch, G., Mock, H. P., Dushkov, D., Czihal, A., Thielmann, J., Stephan, U. W., and Bäumlein, H. (1999) *Eur. J. Biochem.* **265**, 231–239

46. Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H. S., Han, B., Zhu, T., Wang, X., Kreps, J. A., and Kay, S. A. (2000) *Science* **290**, 2110–2113

47. Mori, S., Nishizawa, N. K., Hayashi, H., Chino, M., Yoshimura, E., and Ishihara, J. (1991) *Plant Soil* **130**, 143–156

48. Takahashi, M., Nakanishi, H., Kawasaki, S., Nishizawa, N. K., and Mori, S. (2001) *Nat. Biotechnol.* **19**, 466–469