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Identification and localisation of the rice nicotianamine aminotransferase gene *OsNAAT1* expression suggests the site of phytosiderophore synthesis in rice

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Abstract Rice plants (*Oryza sativa* L.) take up iron using iron-chelating compounds known as mugineic acid family phytosiderophores (MAs). In the biosynthetic pathway of MAs, nicotianamine aminotransferase (NAAT) catalyses the key step from nicotianamine to the 3″-keto form. In the present study, we identified six rice *NAAT* genes (*OsNAAT1–6*) by screening a cDNA library made from Fe-deficient rice roots and by searching databases. Among the NAAT homologues, OsNAAT1 belongs to a subgroup containing barley functional NAAT (HvNAAT-A and HvNAAT-B) as well as a maize homologue cloned by cDNA library screening (ZmNAAT1). Northern blot and RT-PCR analysis showed that *OsNAAT1*, but not *OsNAAT2–6*, was strongly up-regulated by Fe deficiency, both in roots and shoots. The OsNAAT1 protein had NAAT enzyme activity in vitro, confirming that the *OsNAAT1* gene encodes functional NAAT. Promoter–*GUS* analysis revealed that *OsNAAT1* was expressed in companion and pericycle cells adjacent to the protoxylem of Fe-sufficient roots. In addition, expression was induced in all cells of Fe-deficient roots, with particularly strong GUS activity evident in the companion and pericycle cells. *OsNAAT1* expression was also observed in the companion cells of Fe-sufficient shoots, and was clearly induced in all the cells of Fe-deficient leaves. These expression patterns highly resemble those of *OsNAS1*, *OsNAS2* and *OsDMAS1*, the genes responsible for MAs biosynthesis for Fe acquisition. These findings strongly suggest that rice synthesises MAs in whole Fe-deficient roots to acquire Fe from the rhizosphere, and also in phloem cells to maintain metal homeostasis facilitated by MAs-mediated long-distance transport.

Keywords Deoxymugineic acid · Iron deficiency · Long-distance transport · Mugineic acid family phytosiderophores · Nicotianamine aminotransferase · *Oryza sativa*

Abbreviations

DMA	2′-Deoxymugineic acid
GUS	β-Glucuronidase
MAs	Mugineic acid family phytosiderophores
NA	Nicotianamine

Introduction

Graminaceous plants take up Fe using a unique mechanism known as Strategy II, secreting Fe-chelating compounds into the rhizosphere when the plants sense Fe deficiency (Takagi 1976; Römheld and Marschner 1986). The chelating compounds, which have six coordination sites (three –COOH, two –NH and one –OH) that bind to Fe, are termed mugineic acid family phytosiderophores (MAs). Although Fe is

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mainly present as oxidised Fe(III) compounds, poorly soluble in neutral to alkaline soil, chelation of MAs to Fe(III) dramatically increases the solubility of Fe(III) in the rhizosphere, making graminaceous plants capable of taking up Fe as Fe(III)–MAs complexes. The biosynthesis and secretion of MAs markedly increase in roots in response to Fe deficiency (Takagi 1976; Takagi et al. 1984). The tolerance to Fe deficiency among graminaceous plants is thought to be dependent on the amount and kinds of MAs that they secrete. Rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L.) and maize (*Zea mays* L.) secrete only small amounts of 2′-deoxymugineic acid (DMA) among the possible MAs, and thus are susceptible to low-Fe availability. In contrast, barley (*Hordeum vulgare* L.) secretes large amounts of MAs, including mugineic acid and 3-epihydroxymugineic acid, in addition to DMA, under Fe deficiency; therefore, it is more tolerant to Fe deficiency than other graminaceous plants (Mori and Nishizawa 1987; Lytle and Jolley 1991; Nakanishi et al. 1993; Kanazawa et al. 1994; Higuchi et al. 1996; Ma et al. 1999).

The biosynthetic pathway of MAs in graminaceous plants has been identified through extensive biochemical and physiological studies (Mori and Nishizawa 1987; Kawai et al. 1988, Shojima et al. 1990; Ma and Nomoto 1993; Ma et al. 1999). Methionine is the precursor of MAs (Mori and Nishizawa 1987) and is adenosylated by *S*-adenosylmethionine (SAM) synthetase (Takizawa et al. 1996). Nicotianamine synthase (NAS) catalyses the trimerisation of SAM to nicotianamine (NA) (Higuchi et al. 1994). Nicotianamine aminotransferase (NAAT) catalyses the amino transfer of NA to produce the 3′′-keto intermediate (Shojima et al. 1990; Kanazawa et al. 1995) and is a key enzyme in the biosynthetic pathway of MAs; this is the first step specific to graminaceous plants. Deoxymugineic acid synthase (DMAS) reduces the 3′′-keto form to DMA (Bashir et al. 2006). All the MAs share their biosynthetic pathway from methionine to DMA, which is then hydroxylated to form other MAs in barley by IDS2 and IDS3 dioxygenases (Nakanishi et al. 1993, 2000; Kobayashi et al. 2001).

The genes involved in MAs biosynthesis have been cloned from graminaceous plants. *NAS* genes were first isolated from barley (*HvNAS1–7*) through enzyme purification from Fe-deficient barley roots (Higuchi et al. 1999). Subsequently, *NAS* genes were again isolated from barley (*NASHOR1* and *NASHOR2*; Herbik et al. 1999), as well as from rice (*OsNAS1–3*; Higuchi et al. 2001) and maize (*ZmNAS1–3*; Mizuno et al. 2003). Two barley *NAAT* genes, *HvNAAT-A* and *HvNAAT-B*, were also cloned through enzyme purification from Fe-deficient barley roots (Takahashi et al. 1999). Recently, *DMAS* genes were also cloned from rice (*OsDMAS1*), barley (*HvDMAS1*), wheat (*TaDMAS1*) and maize (*ZmDMAS1*), through identification of an Fe deficiency-inducible aldo–keto reductase gene and establishment of an enzyme activity assay (Bashir et al. 2006).

Graminaceous plants take up Fe(III)–MAs complexes through specific transporters. The gene encoding the transporter was first cloned from the maize *yellow stripe1* (*ys1*) mutant (Curie et al. 2001), which is defective in Fe(III)–MAs uptake (von Wirén et al. 1994). YS1 transports not only metal–MAs complexes but also metal–NA complexes (Schaaf et al. 2004). In rice, among 18 putative YS1 homologues (*OsYSLs*), *OsYSL2* is strongly expressed in Fe-deficient leaves, and its encoding protein transports Fe(II)–NA and Mn(II)–NA complexes (Koike et al. 2004). In barley, HvYS1 is also up-regulated in root epidermal cells in response to Fe deficiency and transports the Fe(III)–MAs complex (Murata et al. 2006). In non-graminaceous plants, YSL transporters are considered to play important roles in metal homeostasis by transporting metal–NA complexes because non-graminaceous plants synthesise NA but not MAs (Colangelo and Guerinot 2006).

Rice is one of the most important crop species in the world, and its low tolerance to Fe deficiency in calcareous and marginal soils restricts the world food supply. Thus, molecular characterisation of rice genes involved in the MAs-based Fe uptake system is of special importance. In the present report, six rice *NAAT* genes, *OsNAAT1–6*, were identified to determine the uncharacterised key step in DMA biosynthesis in rice. Among the rice *NAAT* genes, *OsNAAT1* was found to be strongly induced under Fe deficiency. By using in vitro enzyme activity assay system, we demonstrated that *OsNAAT1* encodes a functional protein with NAAT activity. *OsNAAT1* promoter–*GUS* analysis strongly suggested that DMA is synthesised in all root cells for Fe acquisition from the rhizosphere, and also plays a role in long-distance transport of Fe in rice. The cloning and characterisation of the *OsNAAT1* gene should serve as an important step in uncovering the role of DMA in rice Fe nutrition.

Materials and methods

Plant materials

Wild-type and transgenic rice seeds were germinated on Murashige and Skoog (MS) medium and transferred into a nutrient solution in a glass house with 30°C light/25°C dark periods under natural light conditions. The composition of the nutrient solution was 2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 10 μM H_3BO_3, 0.1 mM Fe(III)–EDTA, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$ and 0.01 μM $(NH_4)_6Mo_7O_{24}$. The pH of the culture solution was adjusted daily to 5.3 with 1 N HCl. When the fifth leaves appeared, plants were cultured without Fe. Control plants were cultured continuously in the

standard culture solution. Shoots and roots were harvested 2 weeks after transplanting for Northern blot and histochemical analyses, or 1 week after transplanting for quantitative real-time PCR (RT-PCR) analysis.

Cloning *OsNAAT* and *ZmNAAT* genes

To clone rice NAAT homologues, a cDNA library was synthesised using poly(A) + RNA extracted from Fe-deficient rice roots (Higuchi et al. 2001). In 2002, putative homologues of *HvNAAT-A* and *HvNAAT-B* genes were searched in rice databases, predicting the presence of four rice *NAAT* genes, which we designated as *OsNAAT1–4*. Specific primers were designed: OsNAAT1-F: 5′-TAAGAGGATAATTGATTTGCTTAC-3′, OsNAAT1-R: 5′-CTGATCATTCCAATCCTAGTACAAT-3′, OsNAAT2-F: 5′-CAACAAAATTCTCGATCAATTAAG-3′, OsNAAT2-R: 5′-ATGAAATATCTCAACACCTTTGTGC-3′, OsNAAT3-F: 5′-CACCAATGCCCTTGGGGTGGTGAA-3′, OsNAAT3-R: 5′-CTGAAAGCCTGAAACTATTCACGAG-3′, OsNAAT4-F: 5′-CCGAGCTATTGCAGAGTACCTATC-3′, OsNAAT4-R: 5′-GGAGTGCTTCCATAAACAAGGTGA-3′. These primers were used to successfully amplify probes of the corresponding genes from the cDNA library. Approximately 400,000 colonies of the cDNA library were screened using colony hybridisation. Isolated cDNA clones were sequenced using a Thermo Sequenase Cycle Sequencing kit (Shimadzu, Kyoto, Japan) and a DNA sequencer (DSQ-2000L; Shimadzu). To clone maize NAAT homologues, a cDNA library was synthesised using poly(A) + RNA extracted from Fe-deficient maize roots (Mizuno et al. 2003). Based on *HvNAAT-A* sequence, a primer pair was designed: forward, 5′-GCCGTAGCAGAGCACTTGTCACAG and reverse, 5′-GATGACCATCGCGGTGGTGTTCTT. An amplified fragment using these primers and the maize cDNA library as a template was used for colony hybridisation.

Northern blot analysis

Total RNA was isolated from rice shoots and roots and was subjected to Northern blot analysis, as described by Higuchi et al. (1999), using the same probes as those used in colony hybridisation. No obvious cross-hybridisation was observed, confirming the specific nature of the probes.

Quantitative RT-PCR analysis

Total RNA was extracted from rice leaves and roots using a RNeasy Plant Kit (QIAGEN, Tokyo, Japan), and was treated with RNase-free DNase I (TaKaRa, Japan) to remove contaminating genomic DNA. First-strand cDNA was synthesised using ReverTra Ace reverce transcriptase (TOYOBO, Japan) by priming with oligo-d$(T)_{17}$. The NAAT fragments were amplified by PCR in a SmartCycler (TaKaRa) with SYBR Green I and ExTaq^{TM} Real-Time-PCR version (TaKaRa). Gene-specific primers used for PCR were as follows: *OsNAAT1* forward, 5′-TAAGAGGATAATTGATTTGCTTAC, *OsNAAT1* reverse, 5′-CTGATCATTCCAATCCTAGTACAAT, *OsNAAT2* forward, 5′-CAACAAAATTCTCGATCAATTAAG, *OsNAAT2* reverse, 5′-ATGAAATATCTCAACACCTTTGTGC, *OsNAAT3* forward, 5′-CACCAATGCCCTTGGGGTGGTGAA, *OsNAAT3* reverse, 5′-CACGAGCTAGCTGGCTTCCTTGA, *OsNAAT4* forward, 5′-CCGAGCTATTGCAGAGTACCTATC, *OsNAAT4* reverse, 5′-GGAGTGCTTCCATAAACAAGGTGA, *OsNAAT5* forward, 5′-GAAACTGGATCTGTCCTGCC, *OsNAAT5* reverse, 5′-TTCGGCTTGCTATGTCGCGA, *OsNAAT6* forward, 5′-CACTTCTGTTCGGTGTTGAA, and *OsNAAT6* reverse, 5′-TAGCTTTACTTGAACTGCTC. The primers used for internal control in RT-PCR were *Actin* forward, 5′-CGCCAYACNGGTGYTATGGTTGG, and *Actin* reverse, 5′-ACACGGAGCTCATTGTAGAA.

Protein expression and purification

To subclone *OsNAAT1* into pMAL-c2 (New England Biolabs, Ipswich, MA, USA), the ORF sequence was amplified using primers 5′-gagagaagatctATGGCACCGACGACGGCGGCGGCGG-3′ and 5′-gagagatctagaCTAGATATAATTTAAAGGGTTTTTC-3′, which contain *Eco*RI and *Bam*HI restriction sites, respectively. The amplified fragments were cloned into pBluescript II SK+ (Stratagene, La Jolla, CA, USA). The verified fragment was excised with *Eco*RI and *Bam*HI, and was subcloned into pMAL-c2. The resultant fusion plasmid was introduced into *Escherichia coli* strain XL1-Blue to produce the OsNAAT1-MBP fusion protein. The protein was purified as described by Higuchi et al. (1999).

NAAT enzyme assay

The NAAT enzyme assay was carried out according to the methods of Ohata et al. (1993) and Kanazawa et al. (1994). Five micrograms of OsNAAT1 fusion protein was centrifuged in an Amicon Ultrafree-MC 30-kDa cutoff filter unit (Millipore, Billerica, MA, USA) at 6,200 × *g* and 4°C for 15 min. The flow-through was discarded, and 50 µl of *N*-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid buffer (50 mM TAPS, 5 mM KCl, 5 mM

$MgCl_2$, 10 mM 2-oxoglutaric acid, 10 μM pyridoxal 5′-phosphate (PLP), 150 μM nicotianamine (Hasegawak-oryo)) 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 tube and centrifuged at 6,200 × *g* and 4°C for 15 min. The flow-through was collected and 4 μl of 0.25 M $NaBH_4$ were added to allow the reduction for 1 min at room temperature. Then, 50 μl of each sample were analysed by HPLC using DMA as a standard (Mori and Nishizawa 1987; Takahashi et al. 1999). All reactions were performed in duplicate.

Rice transformation and histochemical analysis

The 1.7-kb 5′-upstream region of the *OsNAAT1* gene was amplified by PCR using genomic DNA as a template. The primers used were the forward primer 5′-ctctctaagcttCT-TAATGGCACAGAGGGAAAAACCT-3′ and the reverse primer 5′-ctctcttctagaGGCCGTGCTCTGTTTTTTGTTG GT-3′, which contain *Xba*I and *Hind*III restriction sites, respectively. The amplified and verified fragment was excised by *Xba*I and *Hind*III and was subcloned upstream of the *uidA* ORF, which encodes *β*-glucuronidase (GUS), in the pIG121Hm vector (Hiei et al. 1994). An *Agrobacterium tumefaciens* strain (C58) carrying the above construct was used to transform rice (*Oryza sativa* L. cv. Tsukinohikari) as described previously (Higuchi et al. 2001). T_1 seeds were germinated and cultured as described above, and subjected to GUS expression analysis as described by Inoue et al. (2003).

Results

Cloning of OsNAAT1

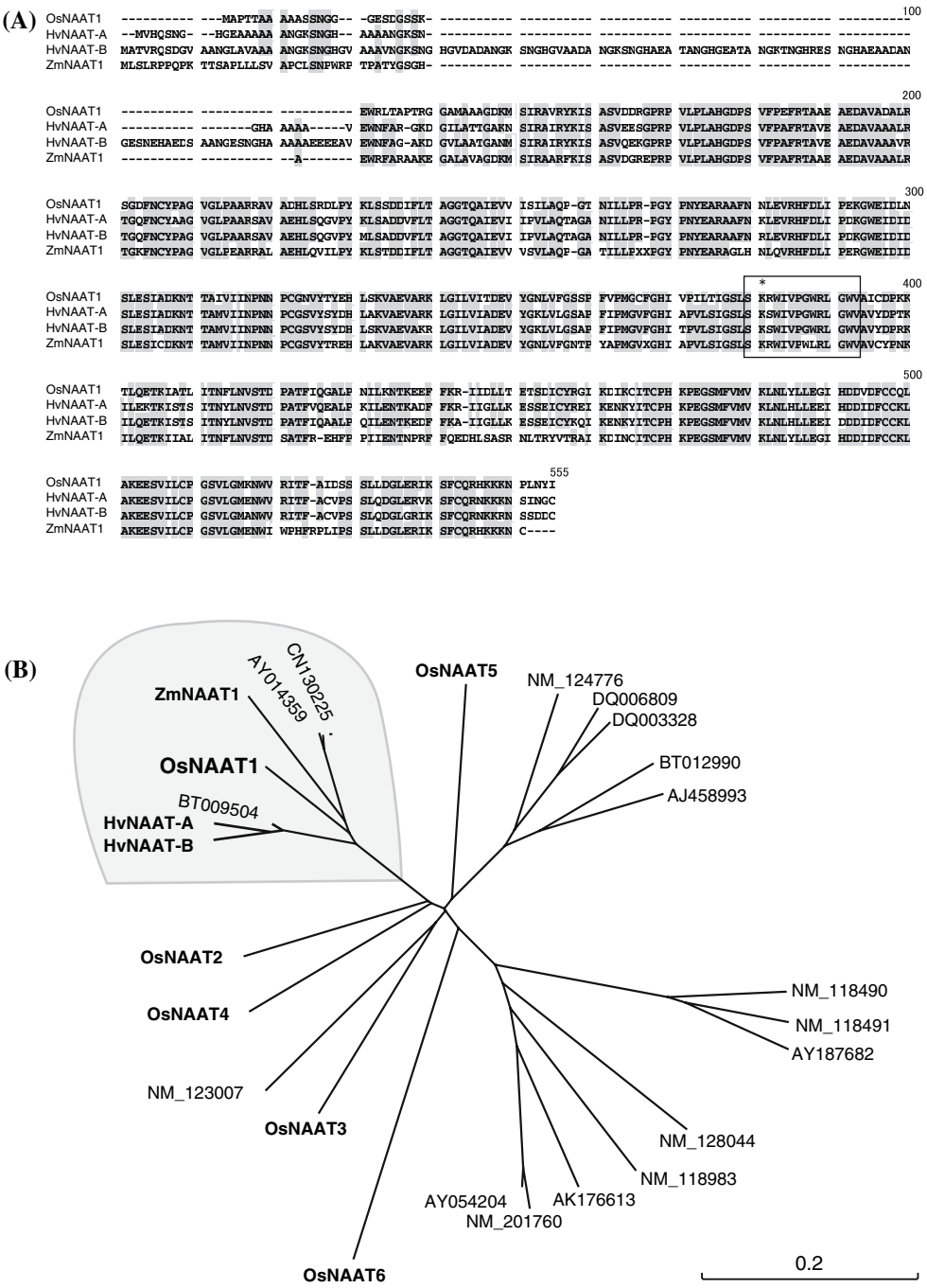
To clone *OsNAAT* genes, *HvNAAT* homologues in a cDNA library made from Fe-deficient rice roots were screened (Higuchi et al. 2001). Sequence analysis of the obtained clones revealed the presence of four distinct cDNA clones, designated as *OsNAAT1* (Accession No. AB206814), *OsNAAT2* (AK060537), *OsNAAT3* (Os02g0302400) and *OsNAAT4* (AK107186). A database search identified the presence of two more cDNA-encoding putative NAAT, designated as *OsNAAT5* (Os11g0552000) and *OsNAAT6* (AK060557). Among these, OsNAAT1 is the most homologous to HvNAAT-A and HvNAAT-B (Fig. 1). The *OsNAAT1* is 1423 nucleotides in length, located on rice chromosome 3, and encodes a predicted 444 polypeptides. Searching for conserved domains revealed that OsNAAT1 has PLP-dependent aminotransferase class I and II domains (pfam00155.12). The *OsNAAT1* gene contains an Fe deficiency-responsive element 1 (IDE1)-like sequence at 290–273 bases upstream from the putative translation start site (Kobayashi et al. 2005). We also screened a maize cDNA library made from Fe-deficient roots to isolate *HvNAAT* homologues. One positive clone putatively encoding a close homologue of *HvNAAT* was identified, which we designated as *ZmNAAT1* (Fig. 1a).

To compare the amino acid sequences of NAAT homologues, we used CLUSTAL W, which found 17 additional homologous proteins from graminaceous and non-graminaceous plants (Fig. 1b). Among the NAAT homologues, HvNAAT-A, HvNAAT-B, OsNAAT1 and ZmNAAT1 form a distinct subgroup. This subgroup also contains putative NAAT from wheat (BT009504), maize (AY014359) and sorghum (CN130225) but contained no homologous sequences from non-graminaceous species. OsNAAT1 is 76% homologous to HvNAAT-A, 74% to HvNAAT-B, and 75% to ZmNAAT1. In contrast, OsNAAT1 displays lower homology to OsNAAT2 (61%), OsNAAT3 (56%), OsNAAT4 (61%), OsNAAT5 (63%), OsNAAT6 (46%) and an *Arabidopsis* putative tyrosine aminotransferase, NM_124776 (55%).

Expression pattern of OsNAATs

To further estimate possible candidates of *OsNAAT* genes related to MAs-based Fe uptake, expression pattern of

Fig. 1 Sequence characteristics of OsNAAT and other NAAT homologues. (**a**) The deduced amino acid sequence of OsNAAT1 is aligned with HvNAAT-A, HvNAAT-B and ZmNAAT1. The shaded areas represent identical residues in at least three of the four proteins. The asterisk and box indicate the lysine residue of putative pyridoxal phosphate binding site and the neighbouring consensus sequence, respectively. (**b**) Unrooted phylogenic tree for the deduced amino acid sequence of NAAT homologues. BT009504, *Triticum aestivum* clone wr1.pk0085.h9:fis, full insert mRNA sequence; AY014359, *Zea mays* PCO115235 mRNA sequence; CN130225, *Sorghum bicolor* cDNA clone RHOH1_40_C05_A002 3′, mRNA sequence; NM_124776, *Arabidopsis thaliana* aminotransferase, putative (At5g53970) mRNA; DQ006809, *Medicago truncatula* tyrosine aminotransferase mRNA; DQ003328, *Glycine max* tyrosine aminotransferase mRNA; BT012990, *Lycopersicon esculentum* clone 114210R, mRNA sequence; AJ458993, *Coleus blumei* mRNA for tyrosine aminotransferase (tat gene); NM_123007, *Arabidopsis thaliana* aminotransferase-related (At5g36160) mRNA; AY054204, *Arabidopsis thaliana* At2g20610/F23N11.7 mRNA; NM_201760, *Arabidopsis thaliana* SUR1 (SUPERROOT 1); transaminase (SUR1) mRNA; AK176613, *Arabidopsis thaliana* mRNA for tyrosine transaminase-like protein; NM_118983, *Arabidopsis thaliana* aminotransferase-related (At4g28410) mRNA; NM_128044; *Arabidopsis thaliana* TAT3 (TYROSINE AMINOTRANSFERASE 3); transaminase (TAT3) mRNA; NM_118490, *Arabidopsis thaliana* aminotransferase class I and II family protein (At4g23590) mRNA; NM_118491, *Arabidopsis thaliana* CORI3 (CORONATINE INDUCED 1, JASMONIC ACID RESPONSIVE 2); transaminase (CORI3) mRNA; AY187682, *Brassica oleracea* cystine lyase BOCL-3 mRNA



OsNAAT genes in response to Fe deficiency was analysed. Northern blot analysis of *OsNAAT1–4* genes was performed using rice plants under Fe-sufficient or Fe-deficient conditions (Fig 2a). *OsNAAT1* was weakly expressed in roots of Fe-sufficient plants and was strongly induced in both roots and shoots of Fe-deficient plants. *OsNAAT2* was constitutively expressed in both roots and shoots at low levels. *OsNAAT3* was constitutively expressed in the shoots of both Fe-sufficient and Fe-deficient plants, and *OsNAAT4* was expressed in the roots of both Fe-sufficient and Fe-deficient plants. Quantitative RT-PCR analysis was further conducted to detect the expression of *OsNAAT1–6* genes (Tables 1 and 2). Under Fe deficiency, *OsNAAT1* expression was induced to 18.2- and 8.0-fold in roots and leaves, respectively. In contrast, transcript abundance of the other *OsNAAT* genes (*OsNAAT2*, *OsNAAT3*, *OsNAAT4*, *OsNAAT5* and *OsNAAT6*) showed no clear change in response to Fe deficiency both in roots and leaves (Table 1). Transcript

Table 1 Quantitative RT-PCR analysis of *OsNAAT1–6*

Rice plants were grown hydroponically under Fe sufficiency or deficiency for 1 week. Transcript abundance was normalised against rice *Actin* transcript level, and was expressed as a relative ratio with Fe-sufficient roots. Values represent the mean ± SD ($n = 3$–6)

	Relative transcripts			
	+Fe Root	−Fe Root	+Fe Leaf	−Fe Leaf
OsNAAT1	1.00 ± 0.19	18.2 ± 3.59	1.88 ± 1.04	15.0 ± 2.90
OsNAAT2	1.00 ± 0.10	0.80 ± 0.09	1.66 ± 0.41	0.92 ± 0.14
OsNAAT3	1.00 ± 0.66	1.77 ± 0.74	64.0 ± 18.0	31.2 ± 6.22
OsNAAT4	1.00 ± 0.32	1.90 ± 0.70	0.89 ± 0.23	0.61 ± 0.29
OsNAAT5	1.00 ± 0.19	1.30 ± 0.41	0.56 ± 0.08	0.57 ± 0.10
OsNAAT6	1.00 ± 0.58	0.40 ± 0.26	0.66 ± 0.36	1.20 ± 0.62

abundance of *OsNAAT1* reached up to more than 10^7 copies per 1 μg of total RNA in Fe-deficient roots and leaves (Table 2). Expression of *OsNAAT1-6* was also monitored by microarray analyses, which confirmed the Northern and RT-PCR results and also suggested a dominance of *OsNAAT1* transcripts among the *OsNAAT1-6* in Fe-deficient roots (data not shown).

A Northern blot analysis of *OsNAAT1* was then performed using the green old leaves and yellow young leaves (Fig. 2b). Transcripts of *OsNAAT1* were more abundant in the yellow young leaves than in green old leaves. These expression patterns of *OsNAAT1* are similar to those of *OsNAS1*, *OsNAS2* and *OsDMAS1* (Inoue et al. 2003; Bashir et al. 2006). We also examined the expression of *ZmNAAT1* in response to Fe deficiency by Northern blot analysis. *ZmNAAT1* expression was strongly induced under Fe deficiency in roots, but not in leaves (data not shown), resembling the expression pattern of *HvNAAT-A* and *HvNAAT-B* (Takahashi et al. 1999).

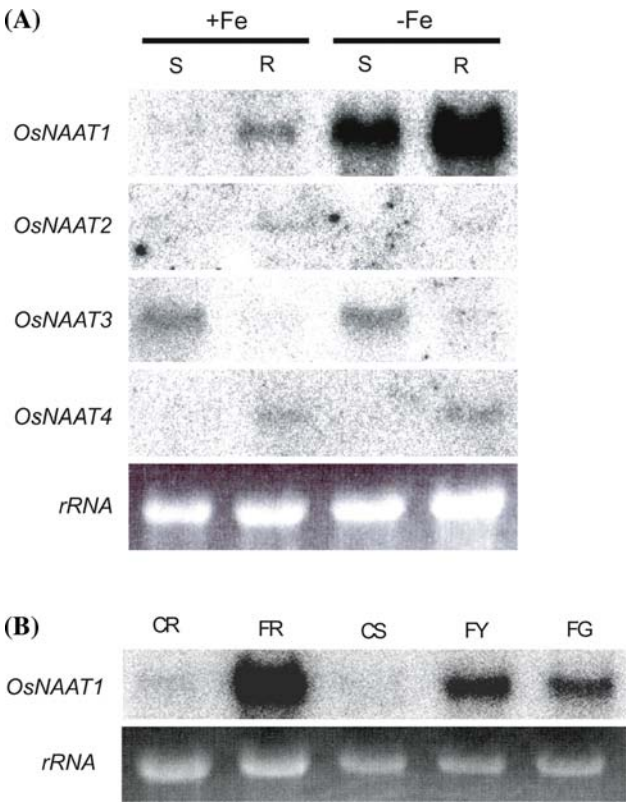


Fig. 2 Northern blot analysis of *OsNAAT*. Rice plants were grown hydroponically under Fe sufficiency or deficiency for 2 weeks. (**a**) +Fe, Fe sufficiency; −Fe, Fe deficiency; S, shoots; R, roots. (**b**) CR, Fe-sufficient roots; FR, Fe-deficient roots; CS, Fe-sufficient shoots; FY, Fe-deficient chlorotic young leaves; FG, Fe-deficient green old leaves

OsNAAT1 has nicotianamine aminotransferase activity

From the sequence comparison and expression patterns of OsNAATs, OsNAAT1 was thought to play a dominant role in rice DMA biosynthesis for Fe acquisition. Therefore, the enzyme activity of OsNAAT1 was examined using a fusion protein to maltose-binding protein (MBP). Performance of the in vitro reaction with recombinant OsNAAT1–MBP was identified by DMA detection using HPLC (Fig. 3). The HPLC signal of the OsNAAT1–MBP reaction demonstrated the generation of DMA, showing identical retention time to that of standard DMA. In contrast, the signal of the MBP reaction showed no peak in the corresponding retention time. Therefore, the OsNAAT1 protein was confirmed to possess NAAT enzyme activity.

Table 2 Quantitfication of *OsNAAT1* transcripts shown in Table 1, expressed as number of copies of transcripts per 1 μg of total RNA

	Transcripts ($\times 10^6$ copies μg^{-1} RNA)			
	+Fe Root	−Fe Root	+Fe Leaf	−Fe Leaf
OsNAAT1	2.63 ± 0.50	47.8 ± 9.42	4.93 ± 2.74	39.5 ± 7.62

Spatial pattern of OsNAAT1 expression

To gain a more detailed insight into the physiological roles of the *OsNAAT1* gene, the localisation of its expression in both Fe-sufficient and Fe-deficient rice plants was investigated through histochemical localisation of the *OsNAAT1* promoter–*GUS* transformants. GUS activity expressed by

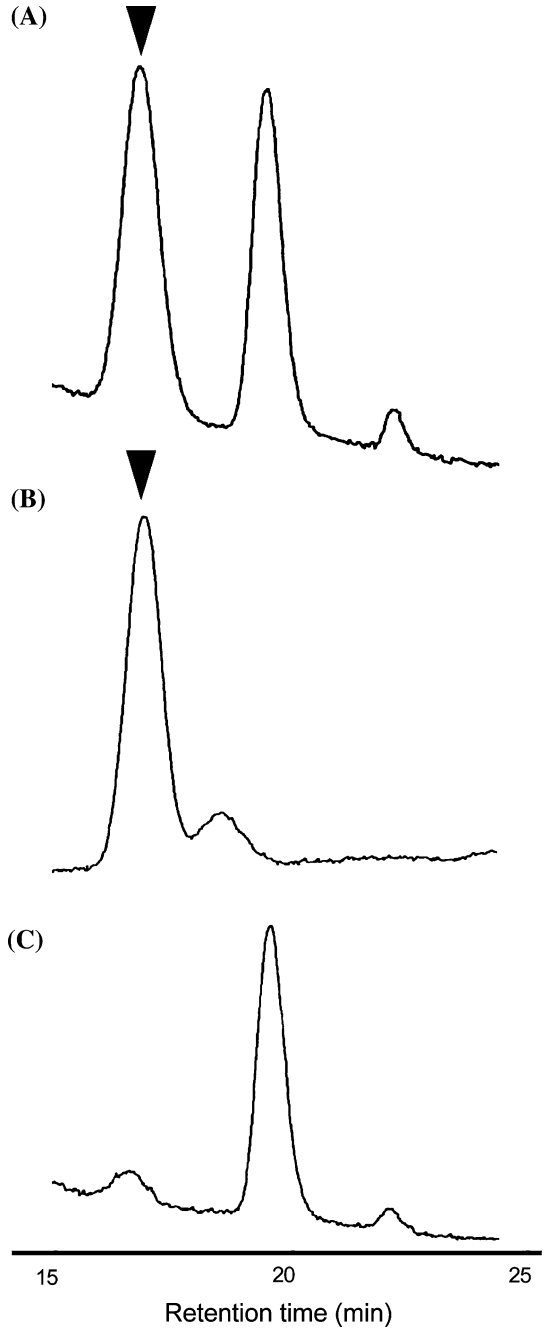


Fig. 3 HPLC profile of enzymatic activity of the recombinant OsNAAT1 protein. Enzymatic activity was determined through detection of DMA by HPLC, followed by the chemical reduction of a 3′′-keto acid generated by NAAT. (**a**) OsNAAT1-MBP, (**b**) DMA standard, (**c**) free MBP. The peaks corresponding to DMA are indicated by arrowheads

the *OsNAAT1* promoter was histochemically detected by blue colour staining (Fig. 4).

In the roots of Fe-sufficient plants, GUS staining was mainly detected within the stele, and was also observed in some part of the epidermal and exodermal cells (Fig. 4a, c). At higher magnification, the staining was detected in part of the pericycle cells adjacent to the protoxylem and metaxylem, as well as in the protophloem cells along the vascular bundles in Fe-sufficient roots (Fig. 4b). Longitudinal sections of Fe-sufficient roots showed that staining was mainly detected in the stele (Fig. 4g). In roots of Fe-deficient plants, the promoter activity of *OsNAAT1* was much stronger and was detected in all tissues, including the epidermis, exodermis, cortex and whole stele (Fig. 4d–f, h). Strong staining was obvious in pericycle cells adjacent to the protoxylem (Fig. 4e), and was also evident in Fe-sufficient roots. Under both Fe sufficiency and Fe deficiency, staining was detected in cells surrounding the metaxylem I, which is typically the region from which lateral roots emerge (data not shown).

In leaves, GUS staining under Fe sufficiency was slight and was only detected in phloem companion cells (Fig. 4 i). In contrast, strong staining was detected in all vascular bundles and mesophyll cells in chlorotic young leaves of Fe-deficient plants (Fig. 4j). GUS staining was especially prominent in the vascular bundles, both in xylem and phloem cells. In older green leaves of Fe-deficient plants, GUS staining was detected in vascular bundles but not in mesophyll cells (Fig. 4k). These spatial patterns of *OsNAAT1* expression were highly consistent with results from the Northern blot and RT-PCR analyses (Fig. 2; Tables 1 and 2).

Discussion

OsNAAT1 encodes a functional nicotianamine aminotransferase involved in the biosynthesis of DMA in rice

The rice *OsNAAT1* gene, which encodes a key enzyme in the biosynthesis of MAs, was isolated for the first time. The OsNAAT1 protein possesses NAAT activity in vitro (Fig. 3), and the gene expression was strongly upregulated in Fe-deficient roots and shoots (Figs. 2 and 4; Tables 1 and 2). In contrast, the expression of *OsNAAT2–6* was not induced in response to Fe deficiency (Fig. 2a; Table 1). Furthermore, *OsNAAT1* belongs to a distinct subgroup containing *HvNAAT-A* and *HvNAAT-B*, the barley functional *NAAT* genes responsible for biosynthesis of MAs (Takahashi et al. 1999, 2001; Fig. 1). A close homologue of *HvNAAT* from maize, *ZmNAAT1*, was also cloned. ZmNAAT1 also belongs to the same subgroup with *HvNAAT-A* and *HvNAAT-B* (Fig. 1b) and exhibits Fe deficiency-induced expression. These results suggest that OsNAAT1 plays a major role in DMA biosynthesis for rice Strategy II Fe acquisition. Characterisation of transgenic rice lines with altered *OsNAAT1* expression would further clarify the importance of OsNAAT1 in DMA biosynthesis.

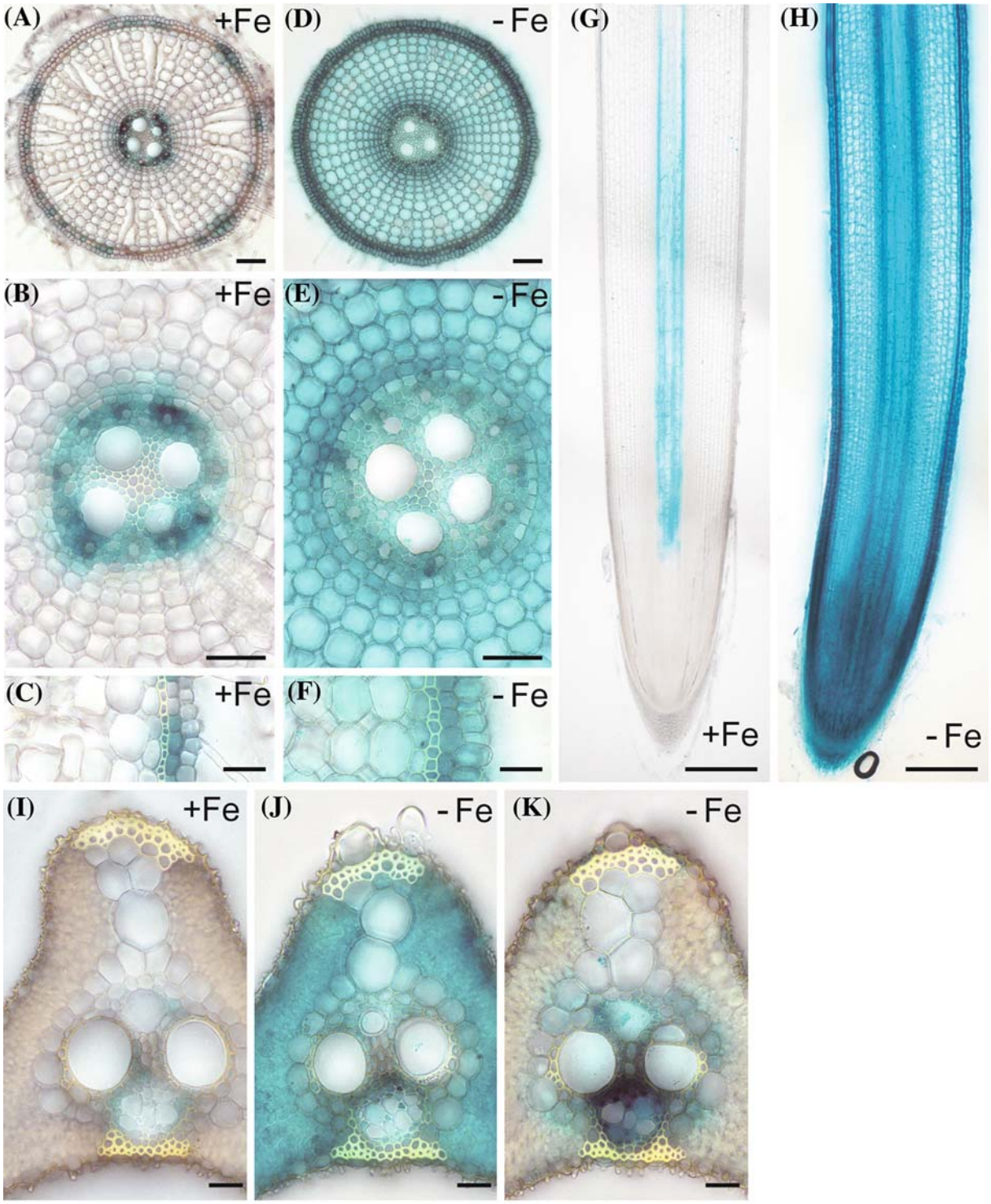


Fig. 4 Histochemical localisation of the *OsNAAT1* promoter–*GUS* expression in transgenic rice plants grown under Fe sufficiency (**a**–**c**, **g**, **i**) or Fe deficiency (**d**–**f**, **h**, **j**, **k**). (**a**, **d**) Root transverse sections. (**b**, **e**) Enlarged part of the stele. (**c**, **f**) Enlarged part of the epidermis and exodermis. (**g**, **h**) Root longitudinal section. (**i**) Fe-sufficient leaf. (**j**) Chlorotic young leaf of Fe-deficient plants. (**k**) Green old leaf of Fe-deficient plants. Scale bars = 100 μm for (**a**, **d**); 50 μm for (**b**, **e**, **i**–**k**); 25 μm for (**c**, **f**); and 500 μm for (**g**, **h**)

DMA is suggested to be biosynthesised in all root cells under Fe-deficient conditions

To clarify the role of DMA biosynthesis and secretion in Fe homeostasis, expression patterns of the genes involved in DMA biosynthesis need to be spatially determined. To this end, promoter–*GUS* transgenic rice plants were previously produced and analysed to clarify localisation regarding the expression of the *OsNAS1–3* and *OsDMAS1* genes (Inoue et al. 2003; Bashir et al. 2006). Thus, the present finding on the expression pattern of *OsNAAT1* in comparison to previous data on *OsNAS* and *OsDMAS* expression now enables estimation of the site of DMA production in rice plants. *OsNAS1*, *OsNAS2* and *OsDMAS1* are expressed in all root cells under Fe-deficient conditions (Inoue et al. 2003; Bashir et al. 2006). The localisation of *OsNAAT1* expression closely resembled these previously identified patterns (Fig. 4), strongly suggesting that DMA is synthesised in all root cells under Fe-deficient conditions. To meet the increased demand for methionine required for the synthesis of DMA, the methionine cycle is highly active in Fe-deficient wheat roots (Ma et al. 1995). Microarray analysis and Northern blot analysis revealed that expression of the genes required for all the predicted steps in the methionine cycle are strongly induced in response to Fe deficiency in roots of barley and rice (Negishi et al. 2002; Kobayashi et al. 2005; Suzuki et al. 2006). Some of the genes participating in the methionine cycle and biosynthesis of MAs possess similar sequences to Fe deficiency-responsive *cis*-acting elements, IDE1 and IDE2, in their upstream regions (Kobayashi et al. 2003, 2005). Furthermore, IDE1 and IDE2 confer similar expression patterns to *OsNAS1*, *OsNAS2*, *OsDMAS1* and *OsNAAT1* promoters in rice (Kobayashi et al. 2004). More recently, the genes participating in DMA biosynthesis in rice, including *OsNAS1*, *OsNAS2*, *OsDMAS1* and *OsNAAT1*, have been found to be under the regulation of an Fe deficiency-inducible bHLH transcription factor, OsIRO2 (Ogo et al. 2006, 2007). Thus, the genes involved in DMA biosynthesis are thought to be co-ordinately regulated in an Fe deficiency-inducible fashion.

These results raise the possibility that a series of the genes involved in the methionine cycle and DMA biosynthesis may be expressed in the same cells, suggesting that the biosynthesis from methionine to DMA takes place in all root cells.

In germinating seeds, *OsNAAT1* is expressed in the vascular bundle of the scutellum, the coleoptile, the coleorhiza, and the base of the seminal root (Nozoye et al. 2007). This observation, along with the expression of other genes participating in the biosynthesis of DMA (*OsNAS1–3* and *OsDMAS1*) and Fe transport (*OsYSL2* and *OsIRT1*), suggest that DMA and NA are produced and involved in Fe transport during seed germination (Nozoye et al. 2007).

DMA is involved in long-distance transport of Fe via the phloem

Previously, we proposed that NA is involved in the long-distance transport of Fe in rice (Inoue et al. 2003; Koike et al. 2004). Three *OsNAS* genes and an Fe(II)–NA complex transporter gene, *OsYSL2*, are expressed in phloem companion cells in rice. In the present report, we propose that DMA, in addition to NA, is also involved in Fe transport via the phloem. *OsNAAT1* is expressed in the phloem companion cells of Fe-deficient leaves (Fig. 4), where *OsNAS1–3* and *OsDMAS1* are also expressed (Inoue et al. 2003; Bashir et al. 2006). These results support the possibility that DMA is synthesised in the phloem companion cells, and may be involved in Fe transport. This is further supported by the findings that a large amount of DMA is detected in phloem sap from leaves of Fe-sufficient rice plants (Mori et al. 1991) and a large amount of DMA is detected in both Fe-sufficient and Fe-deficient rice leaves (Higuchi et al. 2001). Since a computer simulation predicts that the Fe(III)–DMA complex appears to be slightly less stable at a higher pH, near to that in phloem (pH 7.8–8.0) (von Wirén et al. 1999), unknown factors are likely to participate in Fe–DMA transport via the phloem.

We attempted to develop Fe-deficiency tolerance in rice by introducing genes for MAs biosynthesis to increase the secretion of MAs under Fe-deficient conditions (Takahashi et al. 2001). Other strategies, applying a reconstructed ferric chelate reductase, Refre1-372, or overexpression of a transcription factor, OsIRO2, also lead to enhanced tolerance to Fe deficiency (Ishimaru et al. 2007; Ogo et al. 2007). A future strategy to produce Fe-deficiency tolerant plants could be realised through facilitating Fe translocation to increase Fe availability by a combined manipulation of the genes involved in DMA biosynthesis and the *YSL* transporter genes.

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