Introduction

Iron (Fe) is an essential nutrient for plant growth and consequently for crop productivity. Although abundant in mineral soils (> 6%), Fe is only slightly soluble under aerobic conditions over the physiological pH range and is not bioavailable to plants. Graminaceous plants utilize a chelation strategy to acquire Fe from the soil. The roots of these species release mugineic acid family phytosiderophores (MAs), which bind to and solubilize Fe(III) in the rhizosphere. The resulting Fe(III)–MAs complexes are absorbed by the root cells (Takagi, 1976; Takagi *et al.*, 1984). The levels of both the synthesis and the secretion of MA are markedly increased under Fe-deficient conditions, and tolerance to Fe deficiency in graminaceous plants is closely correlated with the quantity and quality of the MAs secreted. Secretion of MAs in barley was reported to follow a distinct diurnal rhythm (Takagi *et al.*, 1984). A secretion peak occurs just after initial illumination, and ceases within 2–3 h. Specific vesicles have been reported in the root cells of Fe-deficient barley. As they have ribosomes on their cytoplasmic surface, these vesicles may originate from the rough endoplasmic reticulum (rER; Nishizawa and Mori, 1987). These vesicles are localized to the sites of MA synthesis, and are therefore called MAs vesicles (Nishizawa and Mori, 1987; Negishi *et al.*, 2002).

Rice produces and secretes DMA, a representative member of the MAs, as follows: *S*-adenosylmethionine (SAM) → nicotianamine (NA) → keto form → DMA. The genes that participate in this pathway have been identified (Mori, 1999; Kobayashi *et al.*, 2008). In rice, three nicotianamine synthase genes (*OsNAS1*, *OsNAS2*, and *OsNAS3*; Higuchi *et al.*, 2001; Inoue *et al.*, 2003), one nicotianamine aminotransferase gene (*OsNAAT1*; Inoue *et al.*, 2008), and one *DMA* synthase gene (*OsDMAS1*; Bashir *et al.*, 2006) have been isolated, and all of their protein products show enzymatic activity. The enzymatic activity of NAS was markedly decreased at cytoplasmic pH. The optimum pH for the synthesis of NAS is around 9 (Higuchi *et al.*, 1995). The other enzymes involved in the DMA biosynthetic pathway also have similar optimum pH for their activity: about pH 9 for NAAT (Shojima *et al.*, 1990) and about pH 8–9 for DMAS (Bashier *et al.*, 2006). These results suggested that three steps catalyzed by these enzymes may occur in some compartment(s) but not in the cytoplasm of the cell.

In the present study, we produced transgenic rice plants carrying *OsNAS2–sGFP* or *OsNAAT1–sGFP*. We observed sGFP fluorescence in the root pericycle, epidermis, root hairs, and shoots of the OsNAS2–sGFP-transformed rice. In the root cells, OsNAS2:sGFP fusion protein was localized to the rER-derived vesicles. We observed the dynamic movement of OsNAS2:sGFP dots within the cells. These results suggested that at least NA production in rice occurred in the rER-derived vesicles.

Materials and methods

Plant materials and growth conditions

Oryza sativa L. cv. Tsukinohikari was used for transformation. Seeds were surface-sterilized with a 2.5% active chlorite solution for 30 min and rinsed several times with sterile water. Transgenic rice seeds were grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 50 mg/L hygromycin. After germination, the seedlings were transferred to a nutrient solution and kept in a greenhouse under a 14-h, 30°C, light/10-h, 25°C, dark regime under natural light conditions. The nutrient solution contained 2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM Fe(III)-EDTA, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 10 μM H_3BO_3, 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, the plants were transferred to culture medium containing (Fe-sufficient) or lacking (Fe-deficient) Fe.

Plasmid Construction and rice transformation

The *sGFP* ORF was recovered from the CaMV35S–sGFP(S65T)–NOS3' vector (Ishimaru *et al.*, 2005) after digestion with *Sal*I and *Not*I, and inserted into the corresponding sites of pBluescript SK+ (pBS; Toyobo, Japan) to give sGFP/pBS. Then, the *sGFP* ORF was digested with *Xba*I/*Sac*I, and replaced with GUS cDNA from pIG121Hm (Hiei *et al.*, 1994) and the resulting construct was designated as sGFP/pIG121Hm. The 1.5-kb 5'-upstream region and coding sequence of *OsNAS2* was amplified using the following forward and reverse primers: 5'-TCTAGATCGTAGATTATGATTCCAGT-3' and 5'-TCTAGAACATTTTGGTGGTGATGAATAATATTAT-3' using the rice (cv. Nipponbare) genome as a template. The amplified fragment containing the *OsNAS2* promoter region and ORF was subcloned into pCR4Blunt–TOPO (Invitrogen, Carlsbad, CA), and the sequence was verified using an ABI sequencer (Applied Biosystems, Tokyo, Japan). The *OsNAS2* promoter and cDNA sequence was recovered with *Xba*I and inserted into the corresponding site of sGFP/pIG121Hm upstream of *sGFP*. The final product was designated as *OsNAS2*pro–*OsNAS2*–*sGFP*/pIG121. The 1.7-kb 5'-upstream region and coding sequence of *OsNAAT1* were recovered from the plasmids described previously (Inoue *et al.*, 2008).

Agrobacterium tumefaciens (C58) carrying the binary vector was used to transform rice according to the method of Higuchi *et al.* (2001).

Observation of fluorescence

GFP fluorescence was observed by confocal laser scanning microscopy (LSM5 Pascal 510; Zeiss, Oberkochen, Germany) and fluorescence microscopy (Axiovision; Zeiss).

Results and Discussion

We generated transgenic plants expressing *OsNAS2–sGFP* and *OsNAAT1–sGFP* under the control of their own promoters. First, we observed whole-plant localization of the OsNAS2–sGFP fluorescence under Fe-sufficient and Fe-deficient conditions. Under Fe-sufficient conditions, OsNAS2–sGFP fluorescence was observed in the basal part of the roots. No OsNAS2–sGFP fluorescence was observed in root tips. After 3 days under Fe-deficient conditions, OsNAS2–sGFP fluorescence was observed in the root tips in addition to the basal parts. After 7 days under Fe-deficient conditions, strong OsNAS2–sGFP fluorescence was observed throughout the whole root. Strong sGFP fluorescence was observed in the cortex, vascular bundle, epidermis, and root hair. In the shoot, sGFP fluorescence was observed in the phloem parenchyma cells of the leaf blade, the leaf sheath, and lamina joints. In the leaf sheath, sGFP fluorescence was observed in the fundamental parenchyma cells and the phloem parenchyma cells of the large vascular bundle. Under Fe-sufficient conditions, no fluorescence was observed in the shoot. These expression patterns agreed with the results of promoter–GUS analysis described previously (Inoue *et al.*, 2003). In the root cells, sGFP fluorescence was observed as dots, which were scattered throughout the cells. Moreover, we found that the OsNAS2–sGFP dot-like structures moved dynamically in the cells. Our results suggested that DMA biosynthesis may occur in these vesicles and movement of these vesicles may be involved in DMA secretion in the rice roots.

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