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Molecular Basis on Nitrogen Utilization in Rice

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Summary

Rice (*Oryza sativa* L.) is the major provision for half of the world population and is the important model crop in terms of synteny. Nitrogen is a massive prerequisite element for rice during its life span. During evolutionary processes, rice has acquired strategic systems of nitrogen metabolism for the survival, i.e., the highly efficient ammonium assimilation in roots and nitrogen remobilization (nitrogen recycling). In our laboratory, research is underway to elucidate molecular mechanisms, cellular functions and the communication mechanisms in nitrogen metabolisms, especially ammonium assimilation in roots and nitrogen recycling, in rice. In this article, aim and overview of our research projects, and some recent research topics are shown.

Key words: ammonium assimilation, glutamate synthase, glutamine synthetase, nitrogen remobilization, nitrogen sensing, rice

Abbreviations: ACR, ACT domain repeat protein; AMT, ammonium transporter; GLND, PII-uridylyltransferase/uridylyl-removing enzyme; GOGAT, glutamate synthase; GS, glutamine synthetase; sGFP, synthetic green fluorescent protein.

1. Aim and overview of our research projects

Nitrogen (N) is a massive prerequisite element for the plant during its life span and available N in the soil frequently limits growth, development and productivity of the plant. In hypoxic or anaerobic paddy fields, ammonium is

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the major form of available inorganic N and is preferentially taken up and used by rice (Yamaya and Oaks, 2004). The process of N recycling, N remobilization, is also very important in determining both productivity and quality of rice (Tobin and Yamaya, 2001). Rice plants are consisted of multiple and functionally different cells. Each cell type possesses distinct function in metabolisms, but whole process for plant growth and reproduction proceeds as an integrated manner of each cell function. Molecular aspects for metabolic compartmentation and signal transduction among cells and organs are, however, poorly understood.

In this lab, we intend to contribute the cellular functions and the communication mechanisms in the primary N metabolism in rice. Research is underway to analyze molecular mechanisms of N use efficiency in rice plants from several directions. These involve 1) cell-specific distribution of multiple forms of ammonium transporters (AMTs: OsAMT1;1-OsAMT1;3, OsAMT2;1-OsAMT2;3 and OsAMT3;1-OsAMT3;3; Suenaga *et al.*, 2003), glutamine synthetases (GSs: cytosolic OsGS1;1-OsGS1;3 and plastidic OsGS2, EC 6.3.1.2; Ishiyama *et al.*, 2004; Tabuchi *et al.*, 2005), glutamate synthases [GOGATs: OsNADH-GOGAT1 and OsNADH-GOGAT2, EC 1.4.1.14; Goto *et al.*, 1998; Tabuchi *et al.*, 2007; OsFerredoxin(Fd)-GOGAT, EC 1.4.7.1; Hayakawa *et al.*, 2003], glutamate dehydrogenases (GDHs: OsGDH1-OsGDH3, EC 1.4.1.2; Abiko *et al.*, 2005), and asparagine synthetase (AS: OsAS, EC 6.3.5.4; Nakano *et al.*, 2000), 2) molecular mechanisms for glutamine (Gln) sensing and signaling to regulate *OsNADH-GOGAT1* gene and other related genes (Sugiyama *et al.*, 2004; Hayakawa *et al.*, 2006; Kudo *et al.*, 2008), 3) 2-oxoglutarate-generation system to provide the carbon skeleton for the reaction of OsNADH-GOGATs (Abiko *et al.*, 2005), and 4) finding genes that coordinately regulate agronomic traits, such as panicle weight and panicle number, and these biochemical traits (Obara *et al.*, 2001; 2003; 2004; Yamaya *et al.*, 2002). To conduct these researches, analytical methods of molecular physiology, gene manipulation, immuno-localization, gene knockout, quantitative trait loci (QTL) analysis, and others are used together with the increasing information in-silico.

Until the late 21th century, human populations in the world will be predicted to amount to more than 90 hundred million people and severe food deprivation has been feared to date. Findings of our projects are important not only for plant science but also for fundamental solving of food deprivation in the world. Furthermore, in terms of synteny, findings of our projects in a model crop, rice, will be expected to be applicable to study in other important crops, i.e., barley, wheat, and corn.

2. Molecular entities involved in ammonium assimilation in rice roots

The NH_3 molecule is a weak base which rapidly protonates to form the NH_4^+

ion with a dissociation constant of $10^{-9.25}$ (Kleiner, 1981). According to the equation proposed by Freney *et al.* (1985), 99.4% of the total ammonia is in the protonated form in water at pH 7.0 and 25°C, and the proportion of the ionic form increases as a function of decreasing pH. Thus, the protonated form, NH_4^+, is the major molecular species in paddy fields. In terms of fertilizer efficiency, NH_4^+ is superior to NO_3^- in paddy fields (Yoshida *et al.*, 1981). However, an excess supply of NH_4^+ causes irreversible damage to plant root growth (Britto *et al.*, 2001 ; Kronzucker *et al.*, 2001). In rice roots, the highly efficient NH_4^+ assimilation system for usage and scavenge could be occurred.

Surface parts of rice roots are composed of epidermis, exodermis and sclerenchyma cells (Fig. 1A). In exodermis cells of rice roots following supply of NH_4^+ ions, the *OsAMT1;2* transcript was inducibly expressed within a few hours (Sonoda *et al.*, 2003a), suggesting that OsAMT1;2 mainly acts in NH_4^+ uptake from NH_4^+-enriched soil. It is now well established the GS/GOGAT cycle is the only route for the primary assimilation of NH_4^+ in plants grown under normal conditions (Ireland and Lea, 1999). We showed that in epidermis and exodermis cells of rice roots after application of NH_4^+ ions, up-regulated expression of *OsGS1;2* transcript, encoding cytosolic GS with the high-affinity to NH_4^+, (Ishiyama *et al.*, 2004) and marked accumulation of plastidic OsNADH-GOGAT1 protein were occurred within a few hours (Yamaya *et al.*, 1995; Ishiyama *et al.*, 1998; 2003; Hayakawa *et al.*, 1999; Tabuchi *et al.*, 2007). The real-time ^{13}N-translocation analysis for NH_4^+ transport in rice using the positron emitting tracer imaging system (PETIS) showed that ^{13}N signals taken up from roots were translocated via xylem to the newest leaf within a short time (Kiyomiya *et al.*, 2001). This translocation was completely inhibited by methionine sulfoximine, an inhibitor of GS activity. Taken together, these findings suggest that in surface cells of rice roots, NH_4^+ ions taken up by OsAMT1;2 could be rapidly assimilated to Gln through an OsGS1;2 reaction coupled with an OsNADH-GOGAT1 reaction (Sonoda *et al.*, 2003a ; Tabuchi *et al.*, 2007) (Fig. 1B). We also suggested that mitochondrial NAD-isocitrate dehydrogenase (OsIDH) was a good candidate to generate the 2-oxoglutarate required for an NH_4^+-induced OsNADH-GOGAT1 reaction in rice roots (Abiko *et al.*, 2005).

Gln and glutamate (Glu) produced by the OsGS1;2/OsNADH-GOGAT1 reactions in epidermis and exodermis cells and subsequently assimilated amino acids could be transported to the central vascular cylinder and then translocated via the xylem to shoots (Fig. 1A, B). The major N forms in xylem sap of rice plants are Gln and asparagine (Asn), which is produced from Gln by asparagine synthetase, (Fukumorita and Chino, 1982 ; Ireland and Lea, 1999), suggesting Gln may be a major transport N form within roots. By the way, apoplastic barriers, exodermis and endodermis with Casparian bands and lignified sclerenchyma cells, exist in rice roots (Steudle, 2000 ; Ranathunge *et al.*, 2003 ; Yamaji and Ma,

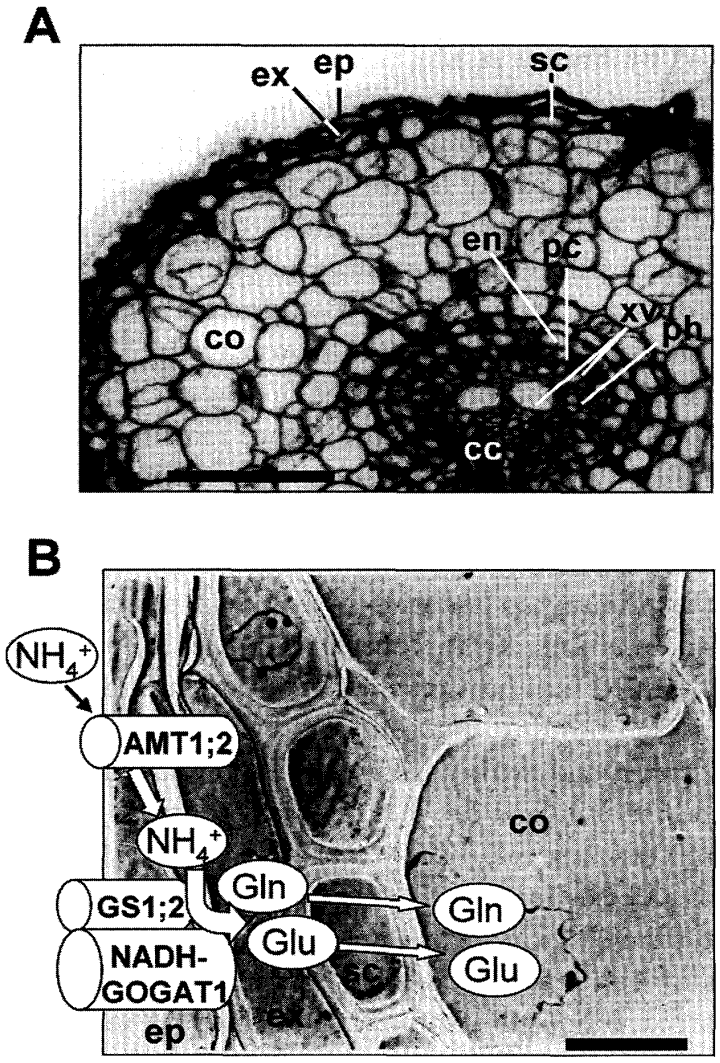

FIG. 1. Ammonium uptake and assimilation in rice roots. (A) Rice root morphology in cross section. Scale bar: 100 μm. (Modified from Hayakawa *et al.*, 1999) (B) A proposed model of ammonium uptake and assimilation in surface parts of rice roots. Scale bar: 5 μm. AMT1;2, ammonium transporter1;2; GS1;2, cytosolic glutamine synthetase1;2; NADH-GOGAT1, NADH-glutamate synthase1. cc, central cylinder; co, cortex; en, endodermis; ep, epidermis; ex, exodermis; ph, phloem; xv, xylem vessel element; pc, pericycle; sc, sclerenchyma cell. (Modified from Tabuchi *et al.*, 2007)

2007). Passage of solutes across these cells could largely depend on cell-to-cell pathways, symplastic path mediated by plasmodesmata and transcellular path crossing plasma membranes (Steudle, 2000; Yamaji and Ma, 2007). These findings imply that the necessity of transport systems of amino acids, especially Gln, on cell-to-cell pathways within exodermis/sclerenchyma cells at surface parts and in endodermis cells at outer boundary of central vascular cylinders of rice roots; however, molecular entities and the mechanisms involved in these amino acid transport systems remain to be elucidated. We are now analyzing a candi-

date of the amino acid transporter involved in amino acid transport on cell-to-cell pathways within exodermis/sclerenchyma cells (T. Hayakawa and T. Yamaya, unpublished data).

3. Key enzymes involved in nitrogen recycling in rice

In rice, more than half of the N in both young leaves and grains is remobilized from senescing leaves via the phloem, with the remainder being transported from the roots via the xylem (Mae and Ohira, 1981) (Fig. 2). In both the xylem (Fukumorita and Chino, 1982) and phloem (Hayashi and Chino, 1990) of rice, Gln is one of the major forms of remobilized N. Metaxylem-parenchyma cells in young rice leaf blades are considered to be active in the absorption of solutes from the vessel element, since they contain abundant mitochondria and endoplasmic reticulum (Chonan *et al.*, 1981). Companion cells are important in the regulation of phloem loading (Van Bel, 1993). The parenchyma cells of metaxylem and metaphloem and mestome-sheath cells are interconnected by numerous plasmodesmata (Chonan *et al.*, 1981). Solutes transported from the phloem and xylem

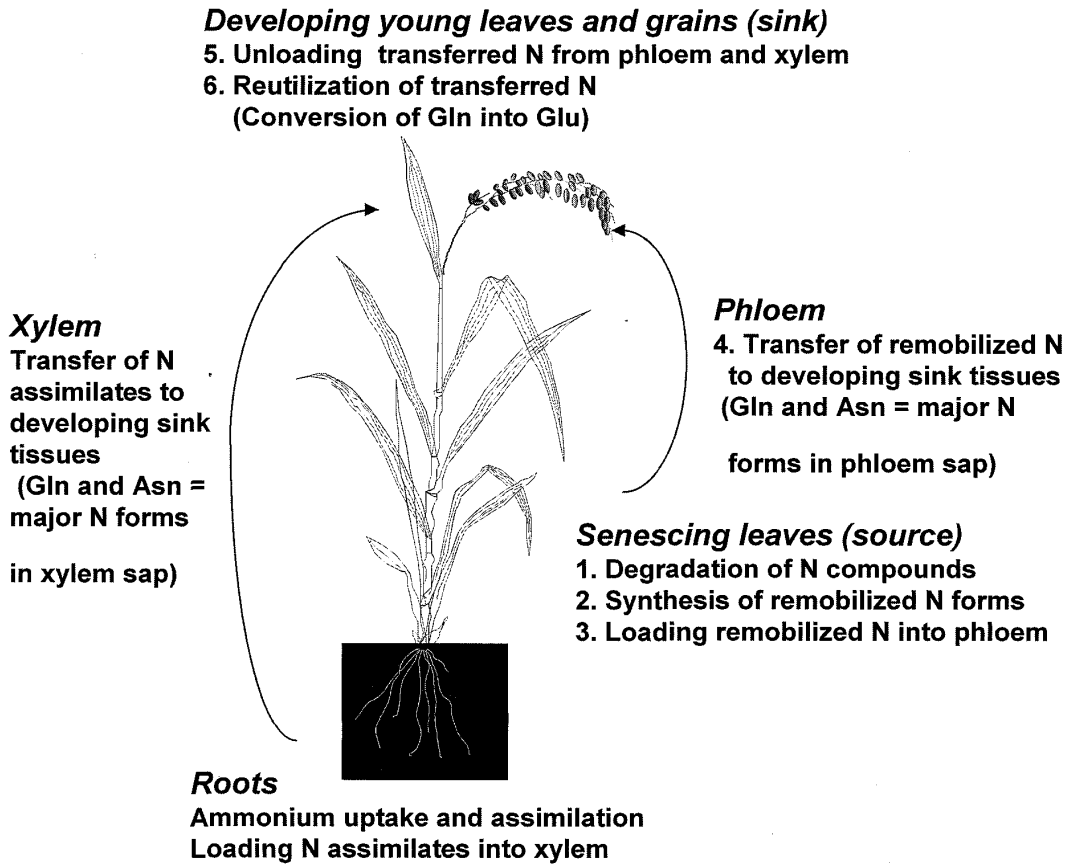

FIG. 2. Nitrogen recycling (nitrogen remobilization) in rice. (Modified from Tobin and Yamaya, 2001)

enter the developing endosperm via phloem- and xylem-parenchyma cells in the dorsal vascular bundle, nucellar projection, and/or nucellar epidermis (Matsuda *et al.*, 1979 ; Oparka and Gates 1981a ; b ; 1982).

In rice leaf blades during senescing process, a content of cytosolic GS1 protein was apparently constant, while those of soluble proteins and chloroplastic proteins were dramatically decreased (Kamachi *et al.*, 1991). Cytosolic GS1 protein specifically accumulated in metaphloem- and metaxylem-parenchyma cells and phloem companion cells of vascular bundles of rice leaf blades (Fig. 3A ; Sakurai *et al.*, 1996 ; 2001). We have therefore considered that the major role of cytosolic GS1 is synthesis of remobilized Gln in the senescent rice leaf blades (Kamachi *et al.*, 1991 ; Sakurai *et al.*, 1996). The *OsGS1;1* gene encodes the most abundant isoenzyme of cytosolic GS1 in rice leaf blades (Ishiyama *et al.*, 2004 ; Tabuchi *et al.*, 2005). Tabuchi *et al.* (2005) recently analyzed rice knockout mutants caused by insertion of endogenous retrotransposon *Tos17* into the *OsGS1;1* gene. The GS1 protein and its activity in the leaf blades were barely detectable in homozygously inserted *OsGS1;1* knockout rice mutants. These *OsGS1;1* knockout rice mutants showed severe retardation in growth rate and grain filling. Reintroduction of *OsGS1;1* cDNA under the control of its own promoter into the mutants successfully complemented these phenotypes. These results indicate that OsGS1;1 could be responsible for synthesis of remobilized Gln in the senescent rice leaf blades (Tabuchi *et al.*, 2005 ; 2007).

OsNADH-GOGAT1 protein was abundant in young unexpanded rice leaf blades at the early stage of development and in young grains at the early stage of ripening (Yamaya *et al.*, 1992 ; Hayakawa *et al.*, 1993). In young unexpanded rice leaf blades, OsNADH-GOGAT1 protein was specifically accumulated in vascular-parenchyma and mestome-sheath cells (Fig. 3B ; Hayakawa *et al.*, 1994). In young rice grains, the protein was specifically detected in vascular-parenchyma cells of dorsal and lateral vascular bundles, and in nucellar projections, nucellar epidermis, and aleurone cells (Fig. 3C ; Hayakawa *et al.*, 1994). The same results were obtained using transgenic rice expressing a β-glucuronidase (GUS) reporter gene under the control of an *OsNADH-GOGAT1* promoter (Kojima *et al.*, 2000). Since these cells expressing the *OsNADH-GOGAT1* gene are important for cell-to-cell transfer of solutes (Matsuda *et al.*, 1979 ; Chonan *et al.*, 1981 ; Oparka and Gates, 1981a ; b ; 1982), it has been suggested that in these young developing organs, OsNADH-GOGAT1 regenerates Glu from Gln transferred from source organs or roots via phloem or xylem, respectively (Tobin and Yamaya, 2001 ; Yamaya *et al.*, 2002). Analysis using transgenic rice over-expressing or co-suppressing of OsNADH-GOGAT1 under the control of its own promoter was performed (Yamaya *et al.*, 2002). Plant height and the number of spikelets on the main stem were identical among these transformants and wild-type plants. However, the mean value of one spikelet weight of over-expressed lines was higher

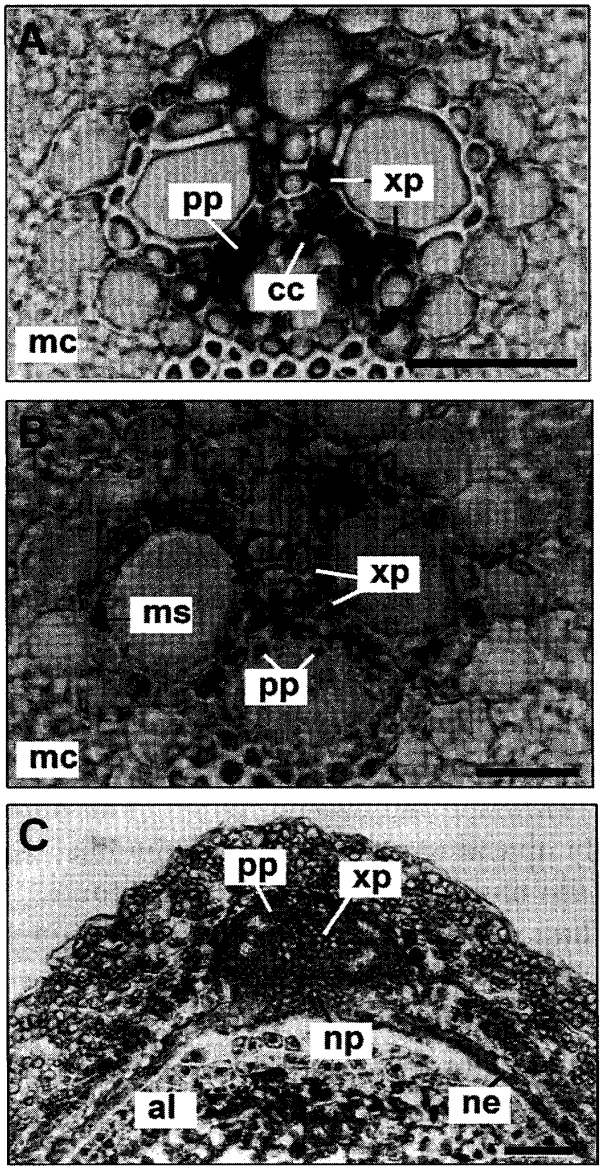

FIG. 3. Cellular distribution of cytosolic GS1 protein in senescing leaf blades and NADH-GOGAT1 protein in young leaf blades and grains in rice. (A) A cross section of the senescing rice leaf blade after immunostaining with anti-OsGS1 synthetic peptide antibody. Close-up view of the large vascular bundle is shown. Scale bar: 30 μm. (Modified from Sakurai *et al.*, 1996) (B) A cross section of the young unexpanded rice leaf blade after immunostaining with anti-OsNADH-GOGAT antibody. Close-up view of the large vascular bundle is shown. Scale bar: 25 μm. (Modified from Hayakawa *et al.*, 1994) (C) A cross section of the young rice grain after immunostaining with anti-OsNADH-GOGAT antibody. Close-up view of the dorsal vascular bundle is shown. Scale bar: 50 μm. (Modified from Hayakawa *et al.*, 1994) al, aleurone layer; cc, phloem-companion cell; mc, mesophyll cell; ms, mestome-sheath cell; ne, nucellar epidermis; np, nucellar projection; pp, phloem-parenchyma cell; xp, xylem-parenchyma cell.

than that of the wild-type, while co-suppressed lines showed a much lower one-spikelet weight. These results suggest that OsNADH-GOGAT1 is indeed a key enzyme for N-reutilization (Yamaya *et al.*, 2002; Tabuchi *et al.*, 2007).

4. Candidates for Gln sensor in the Gln signal transduction system in rice

In plants, little is known about molecular mechanisms for N-sensing and signaling systems (Coruzzi and Bush, 2001; Foyer *et al.*, 2003). Gln changes transcript accumulation of some key N-assimilatory genes in up- or down-regulated manners; for example, *OsNADH-GOGAT1* (Hirose *et al.*, 1997; Hirose and Yamaya, 1999) and *OsAMT1;1-OsAMT1;3* (Sonoda *et al.*, 2003b) in rice. Systems for Gln-sensing and signaling to modulate transcript expression and/or stability of crucial N-assimilatory genes in plants have been proposed (Hirose *et al.*, 1997; Coruzzi and Bush, 2001; Foyer *et al.*, 2003; Sonoda *et al.*, 2003b); however, none of the molecular entities and mechanisms of Gln-signal transduction are known to date.

The ACT domain [Protein Family Database (Pfam) accession number PF01842] was named after three types of bacterial enzyme, i.e., aspartate kinase (EC 2.7.2.4), chorismate mutase (EC 5.4.99.5) and prephenate dehydrogenase (TYRA, EC 1.3.1.12) (Aravind and Koonin, 1999). This domain has mostly been found in functionally diverse enzymes and transcriptional regulators involved in amino acid and purine metabolism (Aravind and Koonin, 1999; Chipman and Shaanan, 2001). These domains are thought to serve primarily as amino acid-binding sites. Two ACT domains have been identified in the bacterial Gln sensor GLND (EC 2.7.7.59; Arcondéguy *et al.*, 2001) at its C-terminal (Chipman and Shaanan, 2001) (Fig. 4). Site-directed mutagenesis analysis of *Rhodospirillum rubrum* GLND deduced that these two ACT domains could be essential for Gln binding (Zhang *et al.*, 2005).

In *Arabidopsis* and rice, novel gene families were discovered, encoding proteins associated with four copies of the ACT domain in the entire polypeptide chains [ACT domain repeat protein (ACR); encoded by *AtACR1-AtACR8* and *OsACR1-OsACR9*, respectively] (Hsieh and Goodman, 2002; Liu, 2006) (Fig. 4). Since the amino acid sequences of the ACT domains of *Arabidopsis* and rice ACRs are similar to those of bacterial GLND, especially in the region corresponding to the putative ligand-binding site, these proteins have been proposed as candidate proteins for amino acid sensing (Hsieh and Goodman, 2002; Liu, 2006). However, the physiological functions of these gene products remain to be characterized. We isolated six ACR-expressing genes in rice (*OsACR1*, and *OsACR5* to *OsACR9*) (Hayakawa *et al.*, 2006). Expression of *OsACR7* was the most abundant in the six *ACR* genes in rice. Gene products of *OsACR7* were most abundant in young developing leaf blades of rice and OsACR7 protein was

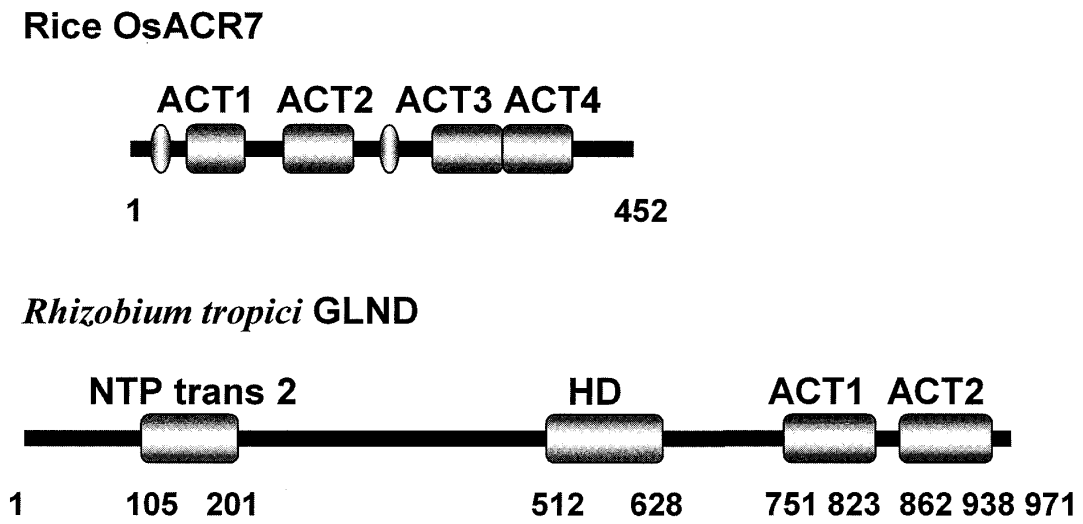

FIG. 4. Diagrammatic representation of structures of rice OsACR7 (accession no. AB117750) and *Rhizobium tropici* GLND (U47030) proteins. ACT, ACT domain; NTP trans 2, nucleotide transferase domain; HD, nucleotide hydrolase domain. Rice ACR proteins (OsACR1, and OsACR5-OsACR9) contain four ACT domains and two distinct highly conserved regions (ovals).

specifically localized in the nucleus of the phloem- and xylem-parenchyma cells in the vascular bundles. In these cells, considerable influx of remobilized Gln occurs (Mae and Ohira, 1981; Fukumorita and Chino, 1982; Hayashi and Chino, 1990) and Gln-responsive *OsNADH-GOGAT1* is transiently expressed (Hayakawa *et al.*, 1994; Kojima *et al.*, 2000; Tabuchi *et al.*, 2007). Yeast two-hybrid screen identified a putative chaperone (OsHSP18.0-CII), as an interactive protein with the OsACR7. Transient expression analysis of OsHSP18.0-CII fused with a synthetic green fluorescent protein (sGFP) in cultured rice cells, followed by co-immunoprecipitation, suggests that the nuclear OsACR7 indeed interacted with nucleocytoplasmic OsHSP18.0-CII in vivo. Furthermore, recombinant OsACR7 protein was able to bind with Gln-agarose in vitro. These findings suggest that nuclear protein OsACR7 is a potential candidate for Gln sensor in rice leaves.

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