Evidence supporting distinct functions of three cytosolic glutamine synthetases and two NADH-glutamate synthases in rice

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Abstract

The functions of the three isoenzymes of cytosolic glutamine synthetase (GS1;1, GS1;2, and GS1;3) and two NADH-glutamate synthases (NADH-GOGAT1 and NADH-GOGAT2) in rice (Oryza sativa L.) were characterized using a reverse genetics approach and spatial expression of the corresponding genes. OsGS1;2 and OsNADH-GOGAT1 were mainly expressed in surface cells of rice roots in an NH4+-dependent manner. Disruption of either gene by the insertion of endogenous retrotransposon Tos17 caused reduction in active tiller number and hence panicle number at harvest. Re-introduction of OsGS1;2 cDNA under the control of its own promoter into the knockout mutants successfully restored panicle number to wild-type levels. These results indicate that GS1;2 and NADH-GOGAT1 are important in the primary assimilation of NH4+ taken up by rice roots. OsGS1;1 and OsNADH-GOGAT2 were mainly expressed in vascular tissues of mature leaf blades. OsGS1;1 mutants showed severe reduction in growth rate and grain filling, whereas OsNADH-GOGAT2 mutants had marked reduction in spikelet number per panicle. Complementation of phenotypes seen in the OsGS1;1 mutant was successfully observed when OsGS1;1 was re-introduced. Thus, these two enzymes could be important in remobilization of nitrogen during natural senescence. Metabolite profiling data showed a crucial role of GS1;1 in coordinating metabolic balance in rice. Expression of OsGS1:3 was spikelet-specific, indicating that it is probably important in grain ripening and/or germination. Thus, these isoenzymes seem to possess distinct and non-overlapping functions and none was able to compensate for the individual function of another.

Key words: Glutamate synthase, glutamine synthetase, metabolic balance, nitrogen utilization, physiological function, rice.

Introduction

Inorganic nitrogen is quantitatively the most essential nutrient for plants and a major limiting factor for their growth and productivity. Although most plants grown in oxidative upland fields utilize NO3− as an inorganic nitrogen source (Andrews et al., 2013), rice (Oryza sativa L.) plants grown in a reductive paddy soil prefer NH4+ (Tobin and Yamaya, 2001). The NH4+ is taken up by rice roots via NH4+ transporters (Sonoda et al., 2003) and then assimilated into the amide residue of glutamine by the coupled reactions of glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT). Since the discovery of the GS/GOGAT cycle by Lea and Miflin (1974), it is now well established that this cycle is the only route for the primary assimilation of NH4+ in plants grown under normal conditions (Ireland and Lea, 1999; Lea and Miflin, 2003). The major forms of nitrogen in the xylem sap of rice are glutamine and asparagine (Fukumorita and Chino, 1982). The positron emitting tracer imaging system showed that the signals of 13N taken up by the roots were detected in the basal...
part of shoots within a short period, but the translocation of $^{13}$N was completely inhibited by methionine sulfoximine, an inhibitor of GS (Kiyomiya et al., 2001). These results suggest that the major part of NH$_4^+$ taken up by rice roots is assimilated within the roots.

In japonica rice, approximately 80% of the total nitrogen in the panicle arises from remobilization through the phloem from naturally senescing organs (Mae and Ohira, 1981). Thus, this process is very important in determining the productivity of rice. The major forms of nitrogen in the phloem sap are glutamine and asparagine (Hayashi and Chino, 1990). As asparagine is synthesized by the transfer of the amide group from glutamine (Lea et al., 2007), the synthesis of glutamine in senescing organs is essential for this nitrogen recycling. The slight basic solution of the phloem sap (Nishiyama et al., 2012) makes it possible to solubilize these amides at concentrations as high as 30 mM or more. In sink organs, such as developing leaves and grains, the remobilized glutamine is re-utilized for many biosynthetic reactions, via GS/GOGAT reactions (Lea and Miflin, 2003), which are responsible for the metabolism of glutamine in rice (Tobin and Yamaya, 2001).

It is well known from work in barley mutants that GS2 and Fd-GOGAT in chloroplasts are responsible for the assimilation of NH$_4^+$ released during photorespiration (Wallsgrove et al., 1987; Kendall et al., 1986). However, mutants are able to grow normally under non-photorespiratory conditions. This suggests that other species of GS and GOGAT are apparently important in the normal growth and development of plants (Yamaya and Oaks, 2004). A small gene family has been identified that encodes cytosolic GS (GS1) in various plants (Goodall et al., 2013; Ishiyama et al., 2004; Martin et al., 2006; Swarbreck et al., 2011), including rice in which OsGS1;1, OsGS1;2, and OsGS1;3 were identified (Tabuchi et al., 2005). There is also a small gene family that encodes NADH-GOGAT (EC 1.4.1.14), i.e. OsNADH-GOGAT1 and OsNADH-GOGAT2, in rice (Tamura et al., 2010). This is unlikely to be the case in Arabidopsis thaliana, which possesses only a single gene for NADH-GOGAT (The Arabidopsis Genome Initiative, 2000).

Reverse genetic approaches are powerful for elucidating individual gene function. In rice, knockout mutants lacking OsGS1;1 (Tabuchi et al., 2005), OsGS1;2 (Funayama et al., 2013), OsGS1;3 (Nakamura et al., unpublished results), OsNADH-GOGAT1 (Tamura et al., 2010), and OsNADH-GOGAT2 (Tamura et al., 2011) have been successively isolated and characterized. Rice is the only example where knockout mutants lacking each individual gene for GS1 and NADH-GOGAT isoenzymes have been characterized.

In this review, distinct functions of each GS1 and NADH-GOGAT isoenzyme in rice plants are discussed. In the course of profiling of metabolites in a GS1;1 mutant, we found that GS1;1 is important in coordinating the global metabolic network in rice. This point is also discussed in this review.

The primary assimilation of NH$_4^+$ taken up by rice roots

Knockout mutants caused by homozygous insertion of an endogenous retrotransposon Tos17 into OsGS1;2 or OsNADH-GOGAT1 genes (Fig. 1) were characterized phenotypically. Recent observations strongly suggest that both GS1;2 (Funayama et al., 2013) and NADH-GOGAT1 (Tamura et al., 2010) are important in the primary assimilation of NH$_4^+$ taken up by rice roots (Fig. 2). Both mutants showed severe reduction in active tiller number and hence panicle number, when those mutants were grown in paddy fields with normal fertilizer until harvest. Other yield

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**Fig. 1.** Isolation of knockout mutants lacking either OsGS1 or OsNADH-GOGAT in rice. Diagram of the insertion position of the retrotransposon Tos17 (arrowhead) in OsGS1;1 (A), OsGS1;2 (B), OsGS1;3 (C), OsNADH-GOGAT1 (D), and OsNADH-GOGAT2 (E). Exons are indicated as boxed regions, whereas lines represent introns and 5'- and 3'-untranscribed regions. The open boxes correspond to untranslated regions.
components, such as spikelet number per panicle, 1000-spikelet weight, and proportion of well ripened-grains, were nearly identical between both mutants and wild-type plants. This phenotype is quite similar to N deficiency symptoms in rice (Mae, 1997). It has been suggested that the development of tillers is inhibited by a high concentration of a phytohormone, strigolactone (Luo et al., 2012; Seto et al., 2012). It was therefore hypothesized that the loss of GS1;2 may increase the concentration of strigolactone in the GS1;2 mutants. However, our preliminary analysis of strigolactone in roots of this mutant suggested that, unlike Pi deficiency (Seto et al., 2012), there were apparently no big differences in content of this phytohormone between the mutants and wild type (Ohashi et al., unpublished data). The GS1;2 mutants at the seedling stage showed that there were marked reductions in the content of glutamine, glutamate, asparagine, and aspartate, but a remarkable increase in free NH₄⁺ ions in the roots when compared with those in the roots of wild-type plants. In xylem sap, concentrations of these amino acids and free NH₄⁺ ions behaved in a similar fashion (Funayama et al., 2013). Contents of amino acids and free NH₄⁺ ions in roots of NADH-GOGAT1 mutants also fluctuated similarly to the GS1;2 mutants (Tamura et al., 2010). Re-introduction of OsGS1;2 cDNA under the control of its own promoter into the GS1;2 mutants successfully restored active tiller number, contents of amino acid and free NH₄⁺ ions in the roots and in the xylem sap. The expression of OsGS1;1 and of other genes involved in nitrogen metabolism is the same in the GS1;2 mutants as it is in the wild type, hence there is no indication that these compensate for the loss of GS1;2 in the mutants. Similarly, expression of NADH-GOGAT2 and Fd-GOGAT genes in the NADH-GOGAT1 mutant was identical with that in wild type, suggesting that these GOGATs are not able to compensate for NADH-GOGAT1 function.

Abundant expression of OsGS1;2 was detected in the surface cell layers of rice roots, i.e. in the epidermis and exodermis cells, following the supply of NH₄⁺ ions as a nutrient (Ishiyama et al., 2004; Tabuchi et al., 2007). Accumulation of OsNADH-GOGAT1 transcript was also detected in the two cell layers of the root surface in an NH₄⁺-dependent manner (Ishiyama et al., 2003), similar to the expression of OsGS1;2. In rice, there is the Casparian strip between the second and third cell layers of the root surface (Morita et al., 1996) in addition to the Casparian strip in the endodermis. This additional Casparian strip probably protects radial oxygen loss from the roots of rice plants grown in paddy fields (Watanabe et al., 2013). As discussed in our previous review on localization studies, GS1;2 and NADH-GOGAT1, both located in cells outside the additional Casparian strip, are responsible for the assimilation of NH₄⁺ within these cell types, and the resulting glutamine and/or glutamate could be transported through the Casparian strip into the cortex and central cylinder (Tabuchi et al., 2007). It is likely that the occurrence of GS1;2 and NADH-GOGAT1 in the outer two cell layers of the root surface could protect these cells from potential toxicity of NH₄⁺ (Hachiya et al., 2012; Kronzucker et al., 2001), even in rice plants. Knockout of either gene caused severe reductive deficiency of NH₄⁺ in the roots and showed the N-deficiency-like phenotype. Free NH₄⁺ unassimilated within the roots is probably transported to the aerial parts where other types of GS/GOGAT are operating.

There is little progress on understanding the regulation of OsGS1;2 and OsNADH-GOGAT1 in response to NH₄⁺/glutamine status in rice, since our previous discussion (Tabuchi et al., 2007). On the other hand, some of the genes for adenosine phosphate-isopentenytransferase, an enzyme that synthesizes cytokinins that are involved in metabolic and signalling processes closely related to nitrogen availability, were found to be upregulated by glutamine or a related metabolite

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**Fig. 2.** Schematic model for the primary assimilation of NH₄⁺ by GS1;2 and NADH-GOGAT1 in rice roots. NADH-GOGAT1 is apparently important in the reutilization of glutamine in sink organs transported from roots and senescing organs. Abbreviations: Gln, glutamine; Glu, glutamate; and 2-OG, 2-oxoglutarate.
in rice (Kamada-Nobusada et al., 2013), as has been found for OsGS1;2 and OsNADH-GOGAT1 (Tabuchi et al., 2007).

Remobilization and reutilization of nitrogen during natural senescence

Expression of both OsGS1;1 and OsNADH-GOGAT2 has been mainly detected in mature leaves of rice plants (Tabuchi et al., 2005). Disruption of OsGS1;1 caused very severe reduction of growth rate and grain filling (Tabuchi et al., 2005). At the vegetative stage of the GS1;1 mutants grown with a normal supply of nitrogen, the plant height and leaf-blade elongation were strongly reduced. At the harvest stage, ripening was retarded, panicle size was very small, and filled spikelet number was severely reduced. This severe phenotype was successfully complemented by the re-introduction of OsGS1;1 cDNA under the control of its own promoter (Tabuchi et al., 2005). During natural senescence of rice leaf blades, total chlorophyll and soluble protein contents decline continuously (Kamachi et al., 1991). A large proportion of nitrogen is remobilized, in the form of glutamine and asparagine, through the phloem (Hayashi and Chino, 1990) from senescing organs to sink organs (Mae and Ohira, 1981). For example, approximately 80% of the total nitrogen in the panicle arises from remobilization (Fig. 3). GS1 protein was detected in phloem companion cells and phloem parenchyma cells in the vascular bundle of mature leaf blades of rice (Sakurai et al., 1996). Because OsGS1;1 transcript was the major isogene product in mature leaf blade (Tabuchi et al., 2007), the GS1 protein detected in phloem cells could be the translation product of OsGS1;1 mRNA. Thus, GS1;1 should have a central function to generate glutamine, as the primary form of remobilized nitrogen, in senescing leaves for long-distance transport. Disruption of OsNADH-GOGAT2 also caused marked reduction in spikelet number, and hence productivity of rice (Tamura et al., 2011). Expression of an OsNADH-GOGAT2 promoter::GUS gene was identical to the localization of GS1;1 protein in the fully expanded leaf blade (Tamura et al., 2011). These results strongly suggest that NADH-GOGAT2 provides glutamate for the reaction of GS1;1 in vascular tissues of rice leaves during senescence. As large amounts of free glutamate were detected in the mature leaf blade of rice (Kamachi et al., 1991), the phenotype of NADH-GOGAT2 mutants was probably not severe.

Fig. 3. Origin of nitrogen in spikelets from various organs and soils in rice during natural senescence. The results using 15N-pulse-chase labelling studies, obtained by Mae and Ohira (1981), were drawn in this figure by permission of Oxford University Press (Japanese Society of Plant Physiologist).

Fig. 4. Schematic model of the synthesis of glutamine by GS1;1 and NADH-GOGAT2 for the remobilization of nitrogen from senescing organs to sink organs via phloem. Abbreviations: Gln, glutamine; Glu, glutamate; and 2-OG, 2-oxoglutarate.
when compared with that of GS1;1 mutants. These results all indicate that GS1;1 and NADH-GOGAT2 are important in the process of nitrogen remobilization accompanying the generation of glutamine from catabolically degraded proteins, nucleic acids, and chlorophyll in senescing organs (Fig. 4). Although transcripts of GS1;2, GS2, NADH-GOGAT1, and Fd-GOGAT were all detected in mature leaves, our results indicate that these enzymes are not able to compensate for the

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**Fig. 5.** Metabolite changes in the primary and secondary metabolic pathways in leaf blade (A) and roots (B) of OsGS1;1 knockout mutant of rice. Fold changes in the metabolite level are represented by circle size and colour scale on the metabolite map. Abbreviations: AA, ascorbate; GABA, \(\gamma\)-aminobutyrate; dHAA, dehydroascorbate; 1,3-DAP, 1,3-diaminopropane; CA, \(\beta\)-cyanoalanine; Put, putrescine; and Spd, spermidine. Amino acids in three letter code and organic acids in the tricarboxylic acid (TCA) cycle are not listed here. The figure was modified from the work by Kusano Tabuchi M, Fukushima A, Funayama K, Diaz C, Kobayashi M, Hayashi N, Tsuchiya YN, Takahashi H, Kamata A, Yamaya T, Saito K. 2011. Metabolomics data reveal a crucial role of cytosolic glutamine synthase 1;1 in coordinating metabolic balance in rice. *The Plant Journal* 66, 456–466; with permission.
function of GS1;1 and NADH-GOGAT2. Because NADH-GOGAT1 protein was detected in vascular tissues of developing leaves and spikelets (Hayakawa et al., 1994), re-utilization of glutamine transported via the phloem could be catalysed by this enzyme. Reduction in the size of sink tissues (panicle number per plants) in the mutants could be the result of the lack of this reaction (Tamura et al., 2010).

A crucial role for GS1;1 in coordinating metabolic balance in rice

In general, the metabolism of carbon and nitrogen in plants is well balanced under normal or suboptimal growth conditions (Reich et al., 2006). GS and GOGAT are the crucial steps for nitrogen metabolism. GS catalyses the assimilation of inorganic NH$_4^+$ to generate the organic product glutamine, whereas GOGAT is the contact point between nitrogen (glutamine) and carbon (2-oxoglutarate) metabolites. The two reactions together provide the only route in plants for assimilation of ammonium into organic products (Ireland and Lea, 1999). When a single gene among multiple GS1 or NADH-GOGAT genes was mutated, rice plants seem to maintain the capacity to reproduce fully ripened seeds, although significant reduction of panicle or spikelet number was observed in knockout mutants of OsGS1;2 (Funayama et al., 2013), OsNADH-GOGAT1 (Tamura et al., 2010), or OsNADH-GOGAT2 (Tamura et al., 2011). On the other hand, a knockout mutant for OsGS1;1 was nearly lethal and no production of the next generation was observed as long as inorganic NH$_4^+$ was supplied (Tabuchi et al., 2005). This severe phenotype could be a good way to learn the coordination of metabolic balance in rice. For understanding the global coordination of carbon and nitrogen metabolism, metabolite profiling is a powerful tool. This relatively new technology has been applied to understand metabolic networks even with a silent phenotype (Weckwerth et al., 2004) and in transgenic plants (Mattoo et al., 2006). Because the GS reaction is the first step for inorganic nitrogen to enter into an organic nitrogen form in plants, GS1;1 knockout mutants provide a valuable resource for investigating whole-plant metabolic regulation. Even though flux in primary metabolism is complicated, metabolite profiling as well as metabolite-to-metabolite correlation analysis between the GS1;1 knockout mutant and wild-type rice provide us with a global picture of whole metabolic processes. According to our previous findings (Kusano et al., 2011), the metabolite profiles of the GS1:1 knockout mutants showed (i) an imbalance in metabolites of sugars, amino acids, and organic acids in the TCA cycle, and (ii) over-accumulation of secondary metabolites with nitrogen atom(s) in their chemical structures, particularly in the roots under continuous supply of NH$_4^+$ nutrition (Fig. 5). The occurrence of mutant-specific networks between tryptamine and other primary metabolites was observed from the correlation analysis. These results clearly show that GS1:1 performs a crucial function in coordinating the global metabolic network in rice grown in the presence of NH$_4^+$ nutrition (Kusano et al., 2011). It is interesting to note that the photorespiratory metabolites, such as serine, showed little change in the GS1;1 knockout mutants (Fig. 5A). This is probably caused by the fact that GS2 activity in the leaves of GS1;1 mutants was at the same level as in the wild type (Tachuchi et al., 2005). On the other hand, rice mutants lacking OsGS1;2 showed metabolite levels very similar to those in the wild-type plants (Kusano et al., unpublished results). These contrasting results from metabolite profiling further indicate that there are distinct and separate functions for GS1;1 and GS1;2 in rice.

Conclusions

Approaches using reverse genetics and metabolite profiling clearly provide evidence that GS1;2 and NADH-GOGAT1 are important in the primary assimilation of NH$_4^+$ taken up by rice roots, whereas GS1;1 and NADH-GOGAT2 are important in the remobilization of nitrogen in senescing organs of rice plants. The two GS and two NADH-GOGAT isoenzymes apparently possess a distinct function and none of the isoenzymes is able to compensate for the specific function of another. This is the first opportunity to characterize the majority of the multiple GS1 isoenzymes and all NADH-GOGAT isoenzymes in plants. From the approach of metabolite profiling, GS1;1 in rice possesses a crucial role in coordinating metabolic balance. There is only one further GS1 isoenzyme, GS1;3, remaining to be characterized in rice. The OsGS1;3 is specifically expressed in the spikelet (Tabuchi et al., 2007), similar to TaGSe in wheat (Swarbeck et al., 2011), ZmGhi1-2 in maize (Martin et al., 2006), and HvGS1_3 in barley (Goodall et al., 2013). Several lines of knockout mutants lacking GS1;3 have already been obtained, and the mutants apparently exhibited a reduced rate of natural senescence in the paddy field, and also showed a relatively slow rate of germination (Nakamura et al., unpublished results). However, there has been no strong evidence supporting its function from these phenotypic characterizations. This isoenzyme probably operates in the maturation of the spikelet in relation to the accumulation of storage protein, for example, or in the germination process. GS1;3 is probably associated with NADH-GOGAT1, because its gene expression was observed in the spikelets (Tabuchi et al. 2007). Metabolomics data, together with the reverse genetics approach, may give an answer for our last question in the near future.

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