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Coordinated Expression of Cytosolic and Chloroplastic Glutamine Synthetase During Reproductive Stage and Its Impact in *GS1* RNAi Transgenic Rice

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Abstract: To understand the reallocation of organic nitrogen from leaf to the flower head of rice, the role of glutamine synthetase (GS) was investigated by characterizing *GS1* RNAi transgenic rice, which revealed a significant reduction in panicle number and number of seeds per panicle. We observed the expression of GS isotypes at transcriptional and protein levels in flag leaves, leaf sheaths and panicles at three different flower development stages. The mRNA expression of *GS1;1* was clearly suppressed in flag leaves, especially at the flowering stage. GS1 protein was barely detectable in flag leaves until the flowering stage, while GS1 protein was compromised in the leaf sheath and panicle, with transient expression of GS2 protein at the flowering stage. The glutamine level in transgenic plants was significantly reduced in both flag leaves and panicles, but ammonium was highly accumulated. The level of other amino acids, including aspartate and asparagine, tended to be higher in RNAi transgenic plants than the wild type plants during the reproductive stage. In addition, accumulation of toxic ammonium in panicles with low glutamine level might have caused low seed-setting in the transgenic rice. These results indicated that nitrogen reallocation was critical for panicle development, and that multiple GS isotypes functioned cooperatively to complete the rice life cycle when leaf nitrogen was remobilized to the developing reproductive organs.

Key words: ammonium; grain yield; RNA interference; panicle development; nitrogen reallocation; rice; glutamine synthase; flowering stage

Rice is the most important cereal grain and staple food for half of the world's human population. In rice cultivation, nitrogen availability is a critical factor that can limit plant growth and development. To meet the demand for this crop, improving nitrogen utilization by rice plants is important. The life cycle of rice is divided into three distinct growth phases comprising vegetative, reproductive and maturation stages. The reproductive stage has a significant impact on rice grain productivity, because grain yield is primarily associated with the number of panicles and the number of grains per panicle (Miller et al, 1991; Gravois and Helms, 1992; Xu et al, 2015). Thus, it is beneficial to understand the reallocation of nitrogen during panicle development stage.

The transition to the reproductive stage in rice is accompanied by metabolic changes that support the growth of young reproductive tissues. A significant portion of reserved protein in leaves is catabolized into amino acids and ammonium, and then reallocated to the developing panicles (Mae and Ohira, 1981). In rice, glutamate is a major free amino acid in both young and mature leaf blades (Kamachi et al, 1991), whereas glutamine is a main component in the

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transport of amino acids from mature leaves (Hayashi and Chino, 1990; Tobin and Yamaya, 2001). The physiological and molecular markers of nitrogen remobilization have been extensively studied in senescing leaves of cereal crops, and the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle have been shown to be the major process in nitrogen transport and reassimilation into amino acids (Lea and Miflin, 2003; Lea et al, 2006).

GS catalyzes the ATP-dependent addition of ammonium (NH_4^+) to glutamate, so as to form glutamine (Bernard and Habash, 2009; Postles et al, 2016). Plants have two isoforms of GS, designated cytosolic GS1 and chloroplastic GS2. Molecular analysis of GS sequences from many plant species has shown that cytosolic *GS1* genes belong to a small multi-gene family, but chloroplastic GS2 is generally encoded by a single gene (Swarbreck et al, 2011). Cytosolic GSs, which are differentially expressed in roots and shoots, are involved in both primary nitrogen assimilation and the reassimilation of nitrogen released from NH_4^+-evolving processes, such as transamination of amino acid and phenylpropanoid (Cantón et al, 2005; Tabuchi et al, 2007). In contrast, major function of GS2 is to assimilate NH_4^+ derived from nitrite reduction and photorespiration in leaves (Blackwell et al, 1987; Wallsgrove et al, 1987). The complexity of GSs suggests the existence of multiple specialized pathways of glutamine biosynthesis that are regulated spatially and temporally.

GS1 is expressed in specialized tissues, involved in the generation and transport of reduced nitrogen. Immunolocalization studies have shown that cytosolic GS is specific to the vascular bundles of rice leaves (Kamachi et al, 1992). In senescing leaf blades of rice, GS1 protein is mainly localized in companion cells and parenchyma cells of vascular bundles (Sakurai et al, 1996). This strongly suggests that GS1 is involved in the synthesis of glutamine, which is a major form of nitrogen exported via the phloem from senescing rice leaves (Hayashi and Chino, 1990). GS1 protein has also been detected in young leaves of rice (Yamaya et al, 1992). In immature vascular bundles of unexpanded leaf blades of rice, GS1 mRNA is mainly detected in xylem parenchyma cells, mestome-sheath cells and sclerenchyma cells (Sakurai et al, 2008). During the reproductive transition, strong signals for GS1 are detected in phloem cells of vascular bundles in relatively older rice leaves, but not in younger leaves (Sakurai et al, 1996). Mae and Ohira (1981) reported that about 80% of the total nitrogen content in the panicle is remobilized through the phloem from senescing organs in rice. This indicates that developmentally-regulated expression of GS1 in rice leaves functions in the export of leaf nitrogen from mature leaves to panicles. Despite the significant progress on the understanding of the role of GS in vegetative tissues, their expressions and functions in reproductive organs are not fully understood.

Cytosolic *GS* genes are overexpressed in various plants, although the effects are variable and dependent on the plant species (Fuentes et al, 2001; Oliveira et al, 2002; Jing et al, 2004; Habash et al, 2006). Improved growth was reported in wheat, maize and rice (Habash et al, 2006; Martin et al, 2006), but some transgenic plants expressing the *GS1* gene exhibit no difference or show compromised growth and phenotype compared with the wild-type (WT) plants (Cai et al, 2009). Studies on maize and *Arabidopsis* mutants deficient in leaf cytosolic GS have also shown various results. *gln1-3* and/or *gln1-4* maize mutants show a reduction in kernel yield and the accumulation of large amounts of amino acids and NH_4^+ in source leaves below the ear as a consequence of dysfunctional nitrogen export (Martin et al, 2006). Growth of *gln1;1* single and *gln1;1&gln1;2* double mutants of *Arabidopsis* is significantly impaired compared with that of the WT (Guan et al, 2016).

In rice, there are one chloroplastic *GS2* and three cytosolic *GS1*, designated *OsGS1;1*, *OsGS1;2* and *OsGS1;3* (Ishiyama et al, 2004; Tabuchi et al, 2005; Tabuchi et al, 2007). *OsGS1;1* knockout rice mutant displays a retarded growth rate, decreased spikelet number and reduced fertility (Tabuchi et al, 2005). The *OsGS1;2* mutant also shows reduction in tiller and panicle numbers (Funayama et al, 2013). In this study, we generated *GS1;1* RNAi transgenic rice and studied the reproductive stages of pre-flowering (7 d before flowering), flowering and post-flowering (7 d after flowering) to elucidate the impacts of cytosolic GS1 suppression underlying GS expression, NH_4^+ content, amino acid levels and rice phenotypes.

MATERIALS AND METHODS

Plant materials and genetic transformation

Full-length rice *GS1;1* cDNA (accession No. AK104987) was amplified by PCR using primers harboring the *att*B recombination sequence (Supplemental Table 1).